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 reuptake 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, N,N-methylamphetamine 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 IC50 values were in line with previous results from radiometric assays and within estimated human brain concentrations. However, phenethylamines showed higher IC50 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,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-fluoro-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 (Milano 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 (EMDDA, 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 extra-cellular brain levels of monoamines, including dopamine, nor-epinephrine 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), nor-epinephrine 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, hypertonse 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 (Mugie et al., 2012).

Cocaine, amphetamine and MDMA are known inhibitors of mono-amine 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 (Eshelman 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 up-take 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 3,4-ampetamine hydrochloride salts (purity > 98.5%) were obtained from Spruyt Hillen (Usselstein, the Netherlands). 3,4-Methylendioxyn-N-methylamphetamyline (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-methoxyphynyl)-2-(ethylamino)cyclohexanone (MXX), 2-(4-bromo-2,5-dimethoxyphenyl)-N-(2-methoxynphyl)methyl)ethanamine (25B-NBOMe), 2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxynphyl)methyl]ethanamine (25I-NBOMe), (RS)-1-phenyl-2-(1-pyrrolidinyl)-1-pentanone (α-PVP) and 5-(2-aminoisopropyl)benzofuran (5-APB) hydrochloride salts (purity > 98.5%) were obtained from Lipomed (Weil am Rhein, Germany). Poli-γ-lysine hydrobromide (PLL) and fluoroxetine 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 H2O (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

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cultured in T75 flasks (Thermo Fisher Scientific, Massachusetts, USA) at 37 °C and 5% CO2. 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% CO2/95% 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, α-PVP, PMMA, 5-APB, 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

![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).](image-url)
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 490/515 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 experiments) 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 Axiostar 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, GmbBH, Gräfelfing, Germany). Fluorescence was measured every 3 min at 430/515 nm excitation/emission wavelength using an Image Senicam 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 μM). 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 incubation with drugs (t = 0). Subsequently, HBSS was replaced by 500 μL HBSS without (control experiments) or with MDMA (1 μM). Fluorescence was continuously measured in single cells and in areas without cells for 30 min following exposure.

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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 ± 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 ($\Delta FU$) 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). $\Delta FU$ 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 fluorescent at $t = 0$. A possible change in background fluorescence was also analyzed. Data is expressed as mean ± 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 extra-cellular fluorescent substrate concentration and slowed down the increase in FU. As illustrated in Fig. 3, $\Delta FU$ at 6 min after addition of HBSS ($t = 6$) was increased by 18% ± 0.3 ($n = 204$ wells, $N = 27$ plates), 20% ± 0.2 ($n = 167$ wells, $N = 22$ plates) and 28% ± 0.3 ($n = 182$ wells, $N = 23$ plates), respectively for hDAT, hNET and hSERT. $\Delta FU$ at 12 and 30 min after addition of HBSS ($t = 12$ and $t = 30$) was 29% ± 0.4 and 54% ± 0.6 (hDAT), 34% ± 0.4 and 62% ± 0.6 (hNET), and 55% ± 0.5 and 109% ± 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 $t = 0$ 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 hallucinogenic 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 re-uptake 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

![Graph showing kinetic uptake curves of monoamine transporters.](https://www.toxsci.org/content/45/1/60/F1)

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 $\Delta FU$ was calculated are depicted by $t = 6, 12$ and $t = 30$. The fluorescence following drug exposure ($t = 12$) was compared to the fluorescence prior to 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. 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 ± 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).
comparable to the exposure duration generally used in radiometric assays, we also calculated IC50 values following 6 and 30 min exposure ($t = 6$ and $t = 30$). For most substances, IC50 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 IC50 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 IC50 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.

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 IC50 values can be found in Table 1.
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,

| Group       | Drug              | **IC$_{50}$ (µM) [95% CI]** |
|-------------|-------------------|-------------------------------|
| 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]                 |               |

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.

**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).
porter 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-HT2A 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 IC50 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 used to counteract the effects of these substances. 2C-B and 25B-NBOMe are potent 5-HT2A receptor agonists (nM range), which is currently considered as their main mechanism of action (Kyriakou et al., 2015).

| Group | Drug | Serum concentration (µM) | Brain partitioning factor (BPF) | Estimated brain concentration (nM) | IC50 (µM) |
|-------|------|--------------------------|-------------------------------|-----------------------------------|-----------|
| Stimulant | Cocaine | 0.2 - 0.4 x | 2.4 - 5.5 1.2 | 0.9 - 5.5 | 4.3/1.9/1.6 |
| | α-PVP | 0.1 - 4 x | 2.4 - 5.5 1.2 | 0.9 - 5.5 | 4.3/1.9/1.6 |
| | Amphetamine | 0.1 - 8 4,5 | 8.5 - 12 3,4,5 | 0.9 - 96 | 7.5/1.0/300 |
| | 4-FA | 0.1 - 3 3 | 3 1.5 | 0.3 - 9 | 21/8/205 |
| | PMMA | 0.1 - 4 | 4 - 5.4 9 | 0.4 - 22 | 83/7/180 |
| | MDMA | 0.4 - 2 | 2 - 4 | 2 - 10 | 43/4/121 |
| | 5-APB | 0.03 - 1 1,2 | 1,2 | > 0.03 - 1 | 7.7/1.6/32 |
| | 2C-B | 0.006 - 1.3 x, 7 | 1.0 | 0.04 - 9 | 240/166/54 |
| | 25I-NBOMe | 0.0006 - 0.007 1,2 | 1,2 | 0.004 - 0.05 | 75/19/4.3 |
| | 25B-NBOMe | 0.0004 - 0.003 1,2 | 1,2 | > 0.0004 - 0.003 | 137/164/9 |
| | MXE | 0.4 - 2 | 2.4 12 | 1 - 5 | 33/20/2.4 |
| SSRI | Fluoxetine | 0.2 - 0.4 y | 20 - 23 11,14 | 4 - 9 | 136/7/90.3 |

References for serum concentrations: 1 Javaid et al., 1978; 2 Cone, 1995; 3 Jeffcoat et al., 1989; 4 Ivenschmid et al., 1992; 5 Jenkins et al., 2002; 6 Lee et al., 2000; 7 Angrist et al., 1987; 8 Röhrich et al., 2012; 9 Eiden et al., 2013; 10 Wright and Harris, 2016; 11 de la Torre et al., 2000; 12 Vevelstad et al., 2012; 13 Elliot and Evans, 2014; 14 Adamowicz et al., 2014; 15 Adamowicz et al., 2016; 16 Ho et al., 2013; 17 Poklis et al., 2014; 18 Otsuka et al., 1988; 19 Rambourg Schepens, 1996; 20 Johansen and Hansen, 2012; 21 Holmgren et al., 2008. References for BPF calculation: 1 Brajkovic et al., 2016; 2 Bystrowska et al., 2012; 3 Laskowski et al., 2015; 4 Poklis et al., 2013; 5 Wood et al., 2012; 6 Shields et al., 2012; 7 Orsulak et al., 1988; 8 Ramberg Schepens, 1996; 9 Johansen and Hansen, 2012; 10 Shiue et al., 1993.
Table 3
Inhibition of monoamine transporter uptake (IC50, 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, 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 IC50(µM) | hDAT | Literature | hNET | Literature | hSERT | Literature |
|------------|------------|-------------------|------|------------|------|------------|-------|------------|
|            |            |                   |      |            |      |            |       |            |
| Stimulant  | Cocaine    | 1.3               | 0.5±0.9, 0.4±0.1, 0.2±0.08 | 1.9  | 0.3±0.3, 0.2±0.3, 0.3±0.3, 0.5±0.5 | 1.6  | 0.5±0.4, 2±0.4 | 1.2   |            |
|            | α-PVP      | 0.1               | 0.01±0.01, 0.1±0.01 | 0.1  | 0.01±0.01, 0.02±0.02 | >300 | >10±, >100 |       |            |
| Entactogen | Amphetamine| 7.5               | 0.2±0.1±, 1.5±, 1.3, 1.3± | 1    | 0.2±0.1±, 1.5±, 0.1±, 0.1± | >300 | 3.8±3.6±, 110± | >10±, 45± |            |
| Halogenoid | 4-FA       | 21                | 0.8±, 0.8±, 10±, 7.7± | 1.8  | 0.4±, 0.4±, 10±, 0.2± | 205  | 2.4±, 6.8±, 9.8±, 19± |       |            |
|            | PMMA       | 83                | 1.2± | 49±        | 7.4  | 1.2±, 1.2± | 18±  | 1.8±       |       |            |
|            | MDMA       | 43                | 0.5±, 1.4±, 0.2±, 10±, 17±, 31±, 17± | 4.4  | 2.1±, 0.7±, 0.0±, 12±, 0.5±, 0.4±, 0.36± | 121  | 1.4±, 0.7±, 0.1±, 0.88±, 1.4±, 2.0±, 2.4± |       |            |
|            | 5-APB      | 7.7               | 6.1± | 1.6±       | 0.2± | 32          | 54   | 18±        |       |            |
|            | 2C-B       | 240               | 231± | 166        | 44±  | 54          | 18±  | 18±        |       |            |
|            | 25I-NBOMe  | 75                | 65±  | 19         | 10±  | 4.3         | 6.8± | 3±         |       |            |
|            | 25B-NBOMe  | 137               | 117± | 16         | 6.7± | 4.9         | 7.1± |            |       |            |
|            | MXE        | 33                | -    | 20         | -    | 2.4         | -    |            |       |            |
| SSRI       | Fluoxetine | 136               | 5±, 8±, 15±, 210± | 7.9  | 0.8±, 0.5±, 0.2± | 0.3  | 0.06±, 0.02±, 0.01±, 0.05± |       |            |

References: aMarona-Lewicka et al., 1995; bHyttel, 1982; cCozzi et al., 1999; dMartel and Keating, 2003; eMeltzer et al., 2006; fNagai et al., 2007; gJørgensen et al., 2008; hYoon et al., 2009; iBarker, 2004; jOz et al., 2010; kTsuruda et al., 2010; lJørgensen et al., 2008; mVoorn et al., 2009; nJohansen et al., 2013; oBaumann et al., 2018; pRosenauer et al., 2013; qSimmler et al., 2013; rSimmler et al., 2014; sMarusch et al., 2014; tRickli et al., 2015a; uRickli et al., 2015b; vRickli et al., 2015c.

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 IC50 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 IC50 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 IC50 data measured using the fluorescence-based and radiometric-based assays, especially with exposure to phenylethylamines. Therefore, a drug of the phenylethylamine class (MDMA) and a non-phenylethylamine 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 IC50 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 (IC50 ± 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 IC50 values of phenethylamines on hSERT inhibition measured using the fluorescent substrate and radioactively labelled monooamines. Even though our fluorescence measurements are closer to physiological conditions, which of both methods derives correct IC50 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 psychoactive substances in biological material - a three-year review of casework in 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 o-amphetamine in normal subjects. Biol. Psychiatry 22, 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., Cozzu, N.V., Schindler, C.W., 2013. Powerful cocaine-like actions of 3,4-Methylenedioxyxypyrvalone (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 fluorescence-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.

Bruzgovic, K., Babic, G., Stonic, J.J., Tomasevic, G., Rancic, D., Kilibarda, V., 2016. Fatal cocaine intoxication in a body packer. Vojnosanit. Pregl. 73, 193–198. http://dx.doi.org/10.2298/VSP141105022B.

Butterfield, M., Riguetti, C., Frenkel, O., Nagdev, A., 2015. Stimulant-related Takotsubo cardiomyopathy. Am. J. Emerg. Med. 33, 476–476.e3. http://dx.doi.org/10.1016/j.ajem.2014.10.007.

Bystron, B., Adamczyk, P., Moniczewski, A., Ziemkowska, M., Fuxe, K., Filip, M., 2012. LC/MS/MS evaluation of cocaine and its metabolites in different brain areas, peripheral organs and plasma in cocaine self-administered rats. Pharmacol. Rep. 64, 1337–1349. http://dx.doi.org/10.1016/j.slephar.2012.07.0931-3.

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.

Cozzu, N.V., Sieve, M.K., Shulgin, A.T., Jacobisi, P., Rhuilo, A.E., 1999. Inhibition of plasma membrane monoamine transporters by β-ketomonoamines. 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-pyrrolidinovalerophenone. Clin. Toxicol. (Phila.) 51, 899–903. http://dx.doi.org/10.1080/15563605.2013.847187.

Elving, B., Björnholm, 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/cdr/trends-developments/2015 (accessed on 5.6.2016).

Eshelman, A.J., Wolfram, K.M., Hatfield, M.G., Johnson, R.A., Murphy, K.V., Janowsky, A., 2013. Substituted methcathinones differ in transporter and receptor mechanisms. 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.droggabenalz.de/22196/13/Eurobarometer_401%20Young%20people%20and%20drugs%20%20full%20report.pdf (accessed 5.6.2015).

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., Millauer, A.F., Holy, M., Stettler, T., de Saizieu, A., Braitberg, G., 2006. A nonradioactive high-throughput/high-content assay for measurement of the human serotonin reuptake transporter in vitro. J. Biomol. Screen. 11, 459–469. http://dx.doi.org/10.1089/jbms.2006.11.459.

Hasegawa, K., Suzuki, P., Wurita, A., Yamagishi, I., Nozawa, H., Takahashi, K., Watanabe, K., 2014. Postmortem distribution of α-pyroiodoxvalerophenone 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/s16800-014-9227-8.

Haunsø, A., Buchanan, D., 2007. Pharmacological characterization of a fluorescent uptake assay for the noradrenaline transporter. J. Biomol. Screen. 12, 378–384. http://dx.doi.org/10.1089/jbms.2006.12.378.

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/s12248-006-0040-1.

Henry, M.E., Schmidt, M.E., Hennen, J., Villafuerte, R.A., Butman, M.E., Tran, P., Kerner, N. et al. 2015. The pre-clinical evaluation of a fluorescent uptake assay for the dopamine transporter. Toxicol. in Vitro 45 (2017) 60–71.

Conflict of interest

The authors do not have any conflict of interest.

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

Rudnick, A., Liethi, D., Reinsic, J., Buchy, D., Hoener, M.C., Liechti, M.E., 2015b. Receptor interaction profiles of novel N-2-methoxybenzyl (NB0Me) amines and pyrvalorenone cathinones (2e drugs). Neuropharmacology 99, 546–553. http://dx.doi.org/10.1016/j.neuropharm.2015.08.034.

Rohanová, M., Pálenícek, T., Balíková, M., 2008. Disposition of 4-bromo-2,5-dimethoxyamphetamine (MDMA) metabolite amphetamine in brain and other tissues in rats after intravenous administration. J. Pharmacol. Exp. Ther. 292, 1042–1047. http://dx.doi.org/10.1171/journal.pone.002060.

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

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

Rosenauer, R., Lu, A., Hely, M., Freismuth, M., Schmid, R., Sitté, 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/cn300176d.

Rudnick, G., Wall, S.C., 1992. The molecular mechanism of action of MDMA: serotonin transporters are targets for MDMA-induced serotonin depletion. Proc. Natl. Acad. Sci. U. S. A. 89, 1812–1817.

Saldana, S.N., Barker, E.L., 2004. Temperature and 3,4-methylenedioxymethamphetamine alter human serotonin transporter-mediated dopamine uptake. Neurosci. Lett. 354, 209–215. http://dx.doi.org/10.1016/j.neulet.2004.04.026.

Schwarz, W.J., Blakely, R.D., DeFelice, L.J., 2003. Binding and transport in nor-epinephrine transporters: real-time, spatially resolved analysis in single cells using a fluorescent substrate. J. Biol. Chem. 278, 9764–9777. 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., Pucharewicz, 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/10.1080/15563650.2012.683437.

Shiue, C.Y., Shiue, G.G., Rysavy, J.A., Pleus, R.C., Huang, H., Bai, L.Q., Cornish, K.G., Sonderland, 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. Nud. 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/10.1016/7854.2016.20.

Simmler, L.D., Buser, T., Donzelli, M., Schrann, Y., Dieu, L.H., Huwyler, J., Chabou, 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.1476-5381.2012.02415.x.

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

Sundgren, H., Dager, S.R., 2001. Magnetization transfer of fluoxetine in the human brain using magnetic resonance spectroscopy. Acta Radiol. 42, 279–286.

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/nrneuromol.

Tsuuda, R.P., 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 norpinephrine 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?fl=1&lng=en (accessed on 5.6.2016).

Vervico, C.D., Miller, G.M., Madras, B.K., 2007. MDMA (Ecstasy) and human dopamine, noradrenaline, and serotonin transporters: implications for MDMA-induced neurotoxicity and treatment. Psychopharmacology 189, 489–503. http://dx.doi.org/10.1007/s00213-007-0717-4.

Vevelstad, M., Oestad, E.L., Middelkoop, G., Hasvold, I., Lilleg, P., Delawski, G.J.M., Eggcn, T., Marland, J., Arrested, 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., Talcg, 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., Hoenderbrink, L., Niesink, R.J.M., Croes, E.A., 2017. Acute toxic effects related to 4-fluoromephedone. Lanexet 389, 600.

Wood, D.M., Davies, S., Pucharewicz, M., Jhonston, 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-pyrrolidinophene (a-PVP). J. Analy. 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.