Toxicology 422 (2019) 69–75

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Differential effects of psychoactive substances on human wildtype and polymorphic T356M dopamine transporters (DAT)

Anne Zwartsen[a,b,1], Carlijn H.C. Litjens[c,1], Laura Hondebrink[b], Jeroen J.M.W. van den Heuvel[c], Rick Greupink[c], Frans G.M. Russel[c], Dylan W. de Lange[b], Juliette Legler[a], Jan B. Koenderink[c], Remco H.S. Westerink[a,*]

[a] Neurotoxicology Research Group, Division Toxicology, Institute for Risk Assessment Sciences (IRAS), Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

[b] Dutch Poisons Information Center (DPIC), University Medical Center Utrecht, Utrecht University, P.O. Box 85.500, NL-3508, GA, Utrecht, the Netherlands

[c] Department of Pharmacology and Toxicology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, 6525 GA, Nijmegen, the Netherlands

ARTICLE INFO

Keywords:
Dopamine reuptake transporter
DAT polymorphism
Designer drugs

ABSTRACT

Many psychoactive substances affect the human dopamine (DA) reuptake transporter (hDAT). Polymorphisms in the encoding gene could affect the functionality of the transporter and consequently alter effects of psychotropic and recreational drugs. Recently, a T356 M single nucleotide polymorphism in the human *SLC6A3* gene was described, which resulted in functional impairments of DA uptake. Therefore, we investigated the effects of 10 psychoactive substances (0.01–1000 μM)) on DA uptake in human embryonic kidney (HEK) 293 cells transiently overexpressing wildtype (WT) or T356 M hDAT.

Our data shows that T356 M hDAT has a 3 times lower V_{max} and a 3 times higher K_m compared to WT hDAT. Additionally, all psychoactive substances inhibited DA uptake by T356 M and WT hDAT. The DA reuptake inhibitors (methylphenidate, cocaine, and bupropion) inhibited DA uptake by WT hDAT most potently, followed by amphetamine-type stimulants [4-fluoroamphetamine (4-FA), amphetamine and MDMA], selective serotonin reuptake inhibitors (SSRI; fluoxetine and citalopram) and arylcyclohexylamines [methoxetamine (MXE) and ketamine].

Compared to DA uptake by WT hDAT, bupropion, methylphenidate, cocaine, and MXE less potently inhibited DA uptake by T356 M hDAT, while citalopram more potently inhibited uptake. The differences in IC_{50} values between T356 M and WT hDAT were considerable (3–45 fold). As such, the presence of this polymorphism could affect treatment efficiency with these substances as well as susceptibly for toxicity and addiction for individuals carrying this polymorphism.

1. Introduction

Over half of the adult American population uses at least one prescription drug and 1 in 7 is considered a polydrug user (≥5 different prescription drugs). Many of these prescription drugs involve psychotropic medication. Of the psychotropic drug users, the majority uses antidepressants (13%), followed by anxiolytics, sedatives, and hypnotics (6%) (Kantor et al., 2015). In addition to a significant proportion of the population being exposed to prescribed psychotropic drugs, exposure to other psychoactive compounds is also of relevance. An estimated 25% of the adults (15–64 years) in the European Union have used illicit drugs at least once during their life-time (EMCDDA, 2018).

Although (prescribed or illicit) psychoactive compounds likely have multiple mechanisms of action, their effects are at least partly attributable to increased concentrations of monoamine neurotransmitters in the brain. This increase in monoamines is caused by the inhibition of neurotransmitter reuptake via inhibition of the dopamine (DA), norepinephrine (NE) or serotonin (5-HT) reuptake transporters (DAT, NET, and SERT). Moreover, several amphetamine-type stimulants are transporter substrates and trigger a transporter-mediated release of monoamines (transporter reversal), resulting in additional monoamine release (Seger, 2010; Torres et al., 2003). Such increases of monoamine

* Corresponding author at: Neurotoxicology Research Group, Division Toxicology, Institute for Risk Assessment Sciences (IRAS), Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80.177, NL-3508 TD, Utrecht, the Netherlands.

E-mail address: r.westerink@uu.nl (R.H.S. Westerink).

[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.tox.2019.04.012
Received 11 February 2019; Received in revised form 29 March 2019; Accepted 18 April 2019
Available online 19 April 2019

concentrations can modulate the activity of postsynaptic neurotransmitter receptors (Torres et al., 2003; Volkow et al., 2006) and trigger both favourable and adverse effects. With the emergence of over 700 new psychoactive substances (NPS)(UNODC, 2018), many substances have entered the drug market that can affect neurotransmitter reuptake transporters (Zwartsen et al., 2017).

Of the three monoamine reuptake transporters, DAT is a primary site of action for many psychoactive substances [for review see (Hondebrink et al., 2018; Zhu and Reith, 2008)]. Disturbed DAT function is implicated in many neurodegenerative and neuropsychiatric disorders like Parkinson's disease, attention deficit hyperactivity disorder (ADHD), depression and addictive disorders (Blum et al., 2013; Dunlop and Nemeroff, 2007; Madras et al., 2005; Nutt et al., 2004). As DAT is coded by a single copy gene (*SLC6A3*), with its expression mostly restricted to the dopaminergic system, it suggests limited opportunity for compensation by other gene products. Therefore, polymorphisms in this gene could have a potentially large impact (Hahn and Blakely, 2002).

Recently, others have described a rare *de novo* hDAT mutation in an autism spectrum disorder patient (Neale et al., 2012). This mutation caused a single nucleotide polymorphism (SNP) in which threonine (Thr) 356 has been substituted by methionine (Met) (T356 M) in the 7th transmembrane domain of hDAT. As this SNP resides in a highly conserved region implicated in ion binding, hDAT containing the T356 M mutation was suspected to functionally differ from wild type (WT) hDAT (Hamilton et al., 2013). Recent research demonstrated that T356 M hDAT indeed has a ˜3-fold reduced affinity for DA and a ˜2.5-fold reduction in maximum uptake velocity compared to WT hDAT (Herborg et al., 2018). Interestingly, IC_{50} values of methylphenidate, cocaine, and amphetamine are also higher in T356 M hDAT (Herborg et al., 2018). The differences in IC_{50} were particularly pronounced for methylphenidate and cocaine, which are considered as specific DAT blockers. The difference in IC_{50} was less striking for amphetamine, which in addition to being a DAT blocker, is also known as a DAT reverser [Herborg et al. (2018); for review on modes of action see Hondebrink et al. (2018)].

To confirm and extend on these observations, we selected 10 psychoactive substances (including therapeutic substances and recreational drugs) from several classes to further investigate the functionality of T356 M hDAT in the presence of psychoactive substances. To this end, we transiently expressed the T356 M polymorphic and WT hDAT in HEK293 cells and compared the inhibitory potencies of the selected psychoactive compounds.

2. Materials and methods

2.1. Substrates and psychoactive substances

[^{3}H]DA (63.6 Ci/mmol) was purchased from PerkinElmer (Groningen, The Netherlands). DA, bupropion hydrochloride [2-(tert-butylamino)-1-(3-chlorophenyl)propan-1-one;hydrochloride], fluoxetine hydrochloride [*N*-methyl-3-phenyl-3-(4-(trifluoromethyl)phenoxy)propan-1-amine;hydrochloride] and citalopram hydrobromide [1-(3-(dimethylamino)propyl)-1-(4-fluorophenyl)3H-2-benzofuran-5-carbonitrile;hydrobromide] were purchased from Sigma-Aldrich (St Louis, MO, USA). Cocaine [methyl(1S,3S,4R,5R)-3-benzoloxy-8-methyl-8-azabicyclo(3.2.1)octane-4-carboxylate], DL-amphetamine (1-phenylpropan-2-amine) and methylphenidate (methyl-2-phenyl-2-piperidin-2-ylacetate) hydrochloride (purity > 98.5%) were obtained from Spruyt Hillen (IJsselstein, the Netherlands). MDMA [3,4-methylenedioxy-*N*-methamphetamine, 1-(1,3-benzodioxol-5-yl)-*N*-methylpropan-2-amine], 4-FA [4-fluoroamphetamine, 1-(4-fluorophenyl)-propan-2-amine] and methoxetamine [MXE; 2-(ethylamino)-2-(3-methoxyphenyl)cyclohexan-1-one] hydrochloride (purity > 97%) were obtained from Lipomed (Weil am Rhein, Germany). (S)-Ketamine [Ketanest-S; (2S)-2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one] was obtained from Eurocept Pharmaceuticals (Ankeveen, the Netherlands). Drug stock solutions (10 mM) were prepared daily in Hank's Balanced Salt Solution (1X) (HBSS) buffer solution (Catalog # 14025; Thermo Fisher, Breda, The Netherlands), and 10 mM HEPES (HBSS-HEPES, pH 7.4), with [^{3}H]DA and DA.

2.2. Cloning of (T356 M) hDAT

The neurotransmitter transporter *SLC6A3* (hDAT; accession number NM_001044.4) was cloned into a pENTR4 vector at the *Bam*HI and *Xho*I sites which introduces a 10 amino acid tag at the N-terminal side of the protein (GenScript, Piscataway, NJ, USA). The T356 M polymorphism was introduced using polymerase chain reaction (PCR) (primer sequence can be found in Supplemental Fig. 2) followed by DpnI digestion. After transformation into DH5α cells clones were picked. After sequencing of the clones, pENTR4-*SLC6A3* WT and T356 M were confirmed. The *SLC6A3* gene was cloned into the destination vector (BacMam) using a Gateway® LR-reaction (Invitrogen™, Carlsbad, CA, USA) as described before (El-Sheikh et al., 2007). The expression clone was transformed into DH10Bac *E.coli*. For both WT and T356 M hDAT, three different bacterial colonies were used to isolate three stocks of recombinant bacmid DNA. Next, Sf9 cells were transfected with these bacmids and supernatant was used to generate the viral stocks. Three different WT and T356 M viruses were created to get an independent n = 3. The three viruses were used each experiment.

2.3. Transient expression in HEK293 cells

Human Embryonic Kidney 293 (HEK293) cells were used to express WT and T356 M hDAT as they are a commonly used overexpression model for (transport) proteins (Thomas and Smart, 2005). These cells are of human origin and have, in general, a low background transport activity (Supplemental Fig. 1; Zwartsen et al., 2017). In addition, they are easily transduced with recombinant baculovirus, making them suitable for the WT and T356 M hDAT transfection.

With some minor deviations, cells were maintained and transduced as described by te Brake et al. (te Brake et al., 2016). Briefly, HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glutamax (Thermo Fisher) with 10% *(v/v)* foetal calf serum (FCS). Cells were seeded on a biocoat poly-D-lysine plate, (VWR International, Amsterdam, The Netherlands) at 120,000 and 20,000 cells/well for a 24-wells and 96-wells plate, respectively. After 24 h, cells were transduced with virus (10% of end volume) and sodium butyrate (2 mM final) was added. Cells transduced with mock virus (enhanced yellow fluorescent protein (EYFP) instead of hDAT cDNA) were used to determine non-specific uptake. Cells were then incubated at 37 °C for 48–72 h.

2.4. WT and T356 M hDAT uptake assay

Transport and inhibition experiments were performed as described by te Brake et al. (2016). Briefly, on the day of the experiment, the culture medium was removed and cells were washed with 37 °C HBSS-HEPES buffer. Uptake was initiated by replacing this solution with 37 °C HBSS-HEPES buffer supplemented with [^{3}H]DA and DA, with or without an inhibitor. Uptake was terminated by washing with ice-cold HBSS-HEPES buffer containing 0.5% (*m/v*) bovine serum albumin (BSA), after which the cells were washed with ice-cold HBSS-HEPES buffer and lysed with NaOH (1 M). Next, samples were transferred to scintillation vials before adding liquid scintillation fluid (PerkinElmer, Waltham, MA, USA). In all experiments, reference samples of the [^{3}H]DA stocks were taken. Radioactivity was measured using the Packard TriCarb™ liquid scintillation analyser (2900 TR; PerkinElmer). In addition, the protein amount was determined using Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories, Veenendaal, The Netherlands) to estimate the number of cells per condition.

To test for the optimal incubation time, mock virus and WT hDAT transduced HEK293 cells in 24-wells plates were incubated with 7.85 nM [^{3}H]DA for different time periods (1, 2, 5 and 10 min). Based on these data, all subsequent experiments were performed using 2 min incubation time (Supplemental Fig. 1). To determine the maximum velocity of uptake (V_{max}) and the Michaelis-Menten constant (K_m) of WT hDAT and the T356 M polymorphism, cells were incubated with a constant concentration of 14.1 nM [^{3}H]DA with or without supplementation of a range of DA (0.03 μM – 300 μM). To determine the uptake of DA by the WT hDAT and the T356 M polymorphism during co-incubation with psychoactive substances, cells were incubated with a constant concentration of 1.0 μM DA and 7.85 nM [^{3}H]DA and a range of inhibitor concentrations. Effects of methylphenidate and bupropion were measured at final concentrations of 0.03–1000 μM, whereas for all other compounds effects were measured at final concentrations of 0.01–1000 μM. Tested concentrations were based on concentrations relevant to human exposure.

2.5. Calculation of kinetics and inhibition assays

For the uptake kinetics and the inhibition assays, transport was expressed as pmol/mg protein/min. Uptake via WT and T356 M hDAT was corrected for the mock transporter uptake on the same plate at corresponding concentrations (2 wells per concentration or vehicle). The number of viruses used (n_{virus}; technical replicates; one or two wells per virus per exposure), the number of experiments performed (N_{plates}; biological replicates) and the total number of wells per type of assay are listed in the legends of the figures.

To determine the V_{max}, and K_m, the Michaelis-Menten equation was fitted to the uptake values using non-linear regression analysis (GraphPad Prism™ version 7.04; GraphPad Software Inc., La Jolla, CA, USA).

To determine the inhibition of DA uptake, technical replicates were normalized to the virus and plate matched control (vehicle-exposed cells). Next, the technical replicates per concentration and vehicle were pooled for each experiment. After that, pooled technical replicates of the three experiments were averaged. Non-linear regression analysis was used to calculate IC_{50} values with GraphPad Prism, and if needed a bottom = 0 constraint was added.

Statistical analyses were performed with one-way ANOVA's followed by a Dunnet's *post-hoc* test to compare the K_m, V_{max} and IC_{50} values between hDAT and T356 M ($p < 0.05$). IC_{50} values are presented with 95% confidence intervals [CI] of $n_{viruses}$ and N_{plates}. Data points and K_m and V_{max} values are shown as the mean ± standard error of the mean (SEM) for $n_{viruses}$ and N_{plates}. Analysed and raw data available upon request.

2.6. Calculation of the estimated human brain concentration

The estimated human brain concentration range was calculated for bupropion, citalopram, and methylphenidate according to Zwartsen et al. (2017). Estimated human brain concentrations of other substances were obtained from literature (Hondebrink et al., 2018; Zwartsen et al., 2018, 2017).

For bupropion, the human blood concentration range between 0.06 – 0.7 μM was established based on volunteer studies (Hoiseth et al., 2015; Jefferson et al., 2005; Johnston et al., 2002; Park et al., 2010; Posner et al., 1984; Viviani et al., 2012). This range was multiplied by the brain partitioning factor (BPF) (2.0–9.8), which was based on blood and brain concentrations reported in animal studies (Butz et al., 1982; DeVane et al., 1986; Suckow et al., 1986) to derive an estimated human brain concentration for bupropion that ranges from 0.1 to 6.7 μM.

The estimated human brain concentration for citalopram was estimated to range from 0.03–7.4 μM. This was based on human blood and serum concentrations of patients using citalopram and people arrested for driving under the influence of drugs [0.03–1.1 μM (Jones et al., 2016; Ostad Haji et al., 2011; Paulzen et al., 2016; Senna et al., 2010)], multiplied by the BPF of 1.0–6.7 [obtained from human lethal case reports (Fu et al., 2000; Luchini et al., 2005; Mari et al., 2012; Nedahl et al., 2018; Rohrig and Hicks, 2015; Wille et al., 2009)].

For methylphenidate, human plasma concentrations ranging between 0.02 and 0.5 μM were found in volunteer studies (Chan et al., 1980; Gualtieri et al., 1982; Modi et al., 2000; Volkow et al., 2003). Based on mice studies, a BPF of 1.0–13 (Balcioglu et al., 2009) was used to estimate the human brain concentration of methylphenidate of 0.02–6.5 μM.

3. Results

3.1. Transport of the WT and T356 M polymorphic hDAT

The T356 M polymorphic hDAT variant was successfully generated and expressed in HEK293 cells. Transport activity of the WT hDAT and T356 M hDAT variant was measured at the predetermined optimal incubation time of 2 min (Supplemental Fig. 1).

To determine the uptake kinetics of WT and T356 M hDAT, cells transduced with the respective transporters were incubated with different concentrations of DA (0.02–300 μM), at a constant concentration of [^{3}H]DA (14 nM). The V_{max} of the WT hDAT was 1381 ± 97 pmol/mg protein/min, which was 2.8 times higher than the V_{max} of 493 ± 21 pmol/mg protein/min determined for T356 M (Fig. 1, $p < 0.001$). K_m values also differed significantly with values for WT hDAT of 8.8 ± 0.5 μM and T356 M hDAT of 26.4 ± 3.1 μM ($p < 0.01$).

3.2. Inhibition of the WT and T356 M polymorphic hDAT by psychoactive substances

To determine the inhibitory potency of psychoactive substances, both the T356 M and the WT hDAT were exposed to a set of 10 substances, consisting of potent DA reuptake inhibitors (methylphenidate, cocaine and bupropion), DAT substrates that reverse DA transport (amphetamine, MDMA and 4-FA), selective serotonin reuptake inhibitors (SSRI) with low affinity for DAT (citalopram and fluoxetine) and arylcyclohexylamines with limited DAT inhibitory properties (ketamine and MXE)(Hondebrink et al., 2018; Korte et al., 2015; Zwartsen et al., 2017) (Fig. 2). In all cases, exposure to these substances concentration-dependently inhibited the uptake of DA. In 4 out of 10 drugs, a difference was observed between the inhibition of uptake via the WT and the T356 M hDAT.

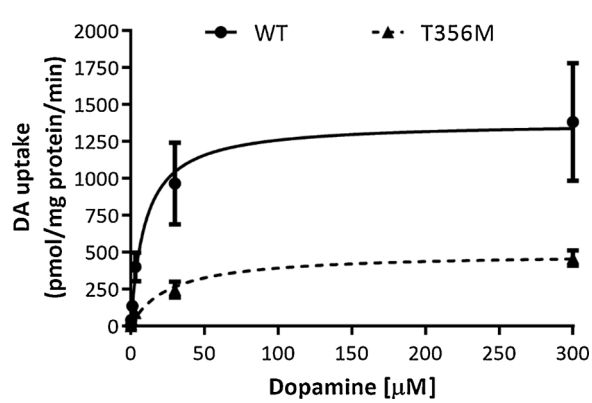

Fig. 1. Dopamine uptake of WT and T356 M polymorphic hDAT. Uptake at different dopamine (DA) concentrations (t = 2 min) was measured as mean pmol/mg protein/min ± SEM for a total of 12 wells (N_{plates} = 3, $n_{viruses}$ = 2).

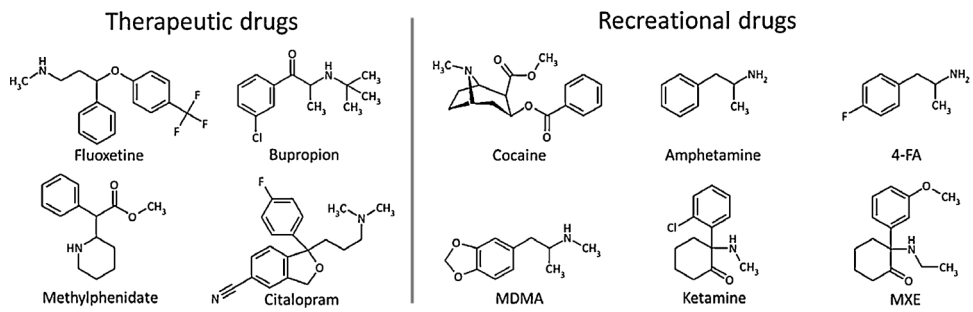

Fig. 2. Chemical structures of the tested therapeutic (fluoxetine, bupropion, methylphenidate, and citalopram) and recreational drugs (cocaine, amphetamine, 4-FA, MDMA, ketamine and MXE).

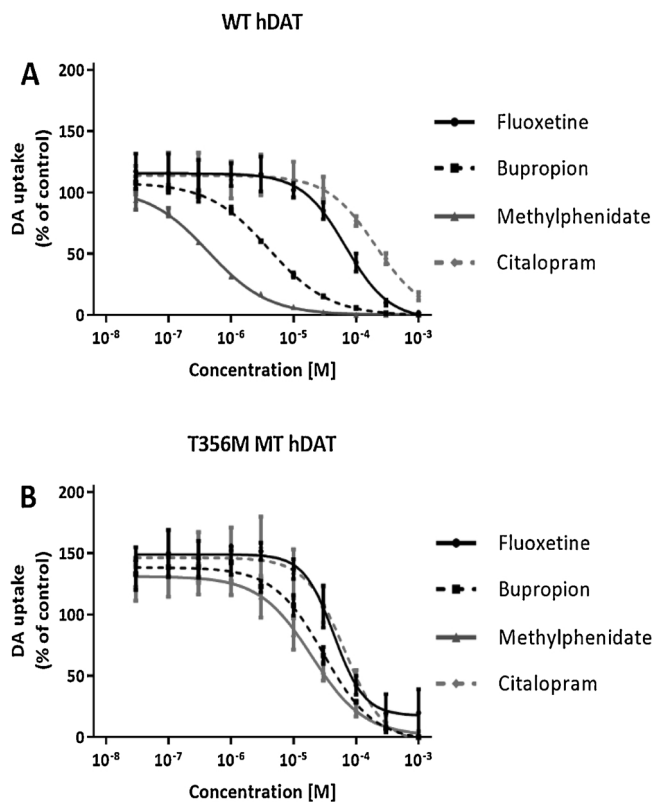

Fig. 3. Concentration-response curves for the inhibition of dopamine uptake via WT (A) and T356 M polymorphic hDAT (B) for different therapeutic drugs. Depicted are fluoxetine, bupropion, methylphenidate, and citalopram. Uptake is depicted as mean ± SEM for a total of 9 wells (N_{plates} = 3, $n_{viruses}$ = 3)], as a percentage of control. IC_{50} values are listed in Table 1.

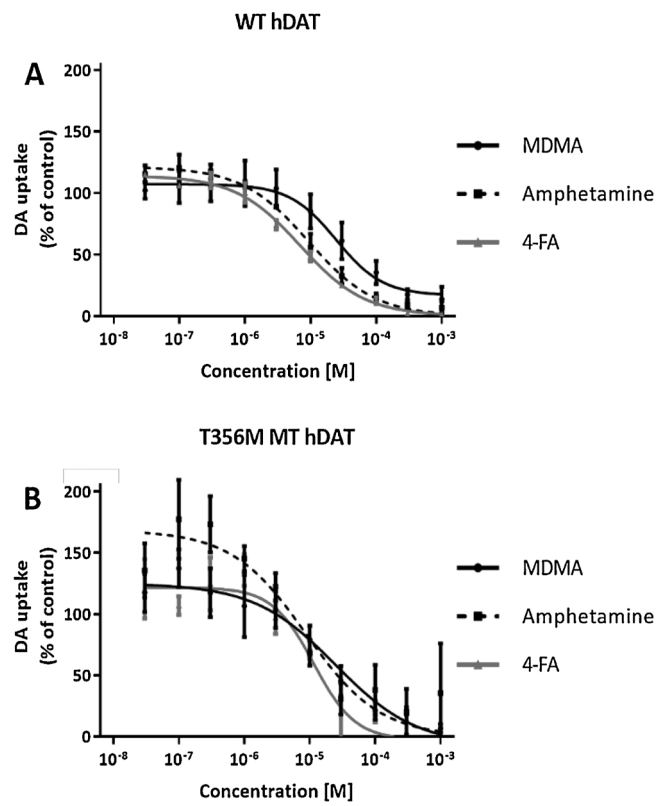

Fig. 4. Concentration-response curves for amphetamine-type stimulants for the inhibition of dopamine uptake via WT (A) and T356 M polymorphic hDAT (B). Depicted are amphetamine-type stimulants MDMA, amphetamine and 4-FA. Uptake is depicted as mean ± SEM for a total of 9 wells (N_{plates} = 3, $n_{viruses}$ = 3), as a percentage of control. IC_{50} values are listed in Table 1.

3.2.1. Therapeutic drugs

Fluoxetine, bupropion, methylphenidate, and citalopram concentration-dependently inhibited the uptake of DA via the WT and the T356 M polymorphic hDAT (Fig. 3). The WT transporter was inhibited with IC_{50} values of 68, 4.0, 0.4 and 203 μM, respectively (Table 1). The inhibition of DA uptake via T356 M did not differ significantly from WT when exposed to fluoxetine [WT vs MT: 45 μM vs 68 μM ($p = 0.26$)]. The IC_{50} values of both bupropion and methylphenidate were significantly increased by the T356 M polymorphism [WT vs MT: 4.0 μM vs 30 μM ($p < 0.001$) and 0.4 μM vs 20 μM ($p < 0.001$), respectively]. This increase in IC_{50} values was 7.5-fold for bupropion and 45-fold for methylphenidate. Notably, citalopram inhibited the T356 M polymorphic hDAT with a significant 3-fold higher potency compared to WT hDAT [WT vs MT: 203 μM vs 67 μM ($p = 0.008$)].

3.2.2. Recreational drugs

As with the therapeutic drugs, all recreational drugs concentration-dependently inhibited DA uptake via WT and T356 M polymorphic hDAT (Figs. 4 and 5). The amphetamine-type stimulants MDMA, amphetamine and 4-FA all inhibited WT transporters with comparable IC_{50} values (Fig. 4). Interestingly, IC_{50} values for inhibition of DA uptake were also comparable between the WT and T356 M hDAT (Table 1; WT vs MT): 34 μM vs 11 μM ($p = 0.08$) (MDMA), 11 μM vs 11 μM ($p = 1.0$) (amphetamine), and 11 μM vs 7.0 μM ($p = 0.2$) (4-FA).

As depicted in Fig. 5, the IC_{50} values of the arylcyclohexylamine ketamine were 10–80 times higher than those of amphetamine-type stimulants and were also comparable between the WT and T356 M hDAT (Table 1; WT vs MT): 383 μM vs 476 μM ($p = 0.3$). The ketamine derivative MXE inhibited T356M hDAT less potently than WT hDAT: a ˜4-fold difference in IC_{50} values was observed [WT vs MT: 108 μM vs 404 μM ($p = 0.0003$)]. Cocaine is a potent DA reuptake inhibitor in WT

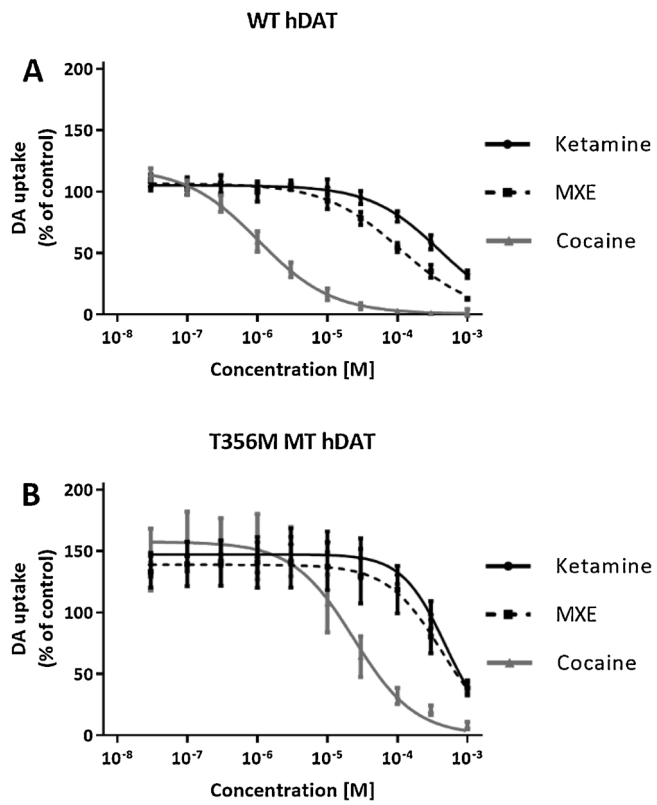

Fig. 5. Concentration-response curves for arylcyclohexylamines and cocaine for the inhibition of dopamine uptake via WT (A) and T356 M polymorphic hDAT (B). Depicted are arylcyclohexylamines ketamine and methoxetamine (MXE) and cocaine. Uptake is depicted as mean ± SEM for a total of 9 wells ($N_{plates} = 3$, $n_{viruses} = 3$), as a percentage of control. IC_{50} values are listed in Table 1.

hDAT (IC_{50} value of 1.1 μM). However, the T356 M polymorphism increased the IC_{50} value of cocaine to inhibit DA uptake by ˜21-fold [Table 1; 23 μM ($p < 0.001$)].

4. Discussion

Over the last decade, research into the pharmacological effects of polymorphisms and mutations in targets of psychoactive substances has gained interest. The current study extends on the earlier observation that T356 M hDAT impairs DA uptake and alters sensitivity to certain psychoactive substances (Herborg et al., 2018).

Our results confirm the functional alterations in T356 M hDAT compared to WT hDAT (Fig. 1), and also indicate differences in the potency of various groups of psychoactive substances to inhibit hDAT. We found a statistically significant decrease in V_{max} and an increase in K_m for T356 M hDAT, in line with both Herborg et al. (2018) and Hamilton et al. (2013). The K_m value determined for WT hDAT is in the range of values reported by others using the same cell line at 37 °C (Moron et al., 2003; Riherd et al., 2008; Storch et al., 1999; Yoon et al., 2009), whilst the V_{max} for WT hDAT observed in this study is ˜2-fold higher than reported by others (Moron et al., 2003; Storch et al., 1999), which may be due to differences in experimental setup, like incubation time (Scholze et al., 2001).

IC_{50} values of WT hDAT DA uptake for amphetamine, cocaine, and methylphenidate determined by Herborg et al. (2018) are ˜10-fold lower compared to our study, possibly due to the use of different transfected cells (COS-7) and measurements at room temperature. However, our IC_{50} values for inhibition of WT hDAT of amphetamine, 4-FA, MDMA, cocaine, MXE, fluoxetine, methylphenidate, and bupropion are comparable with other studies measuring DA uptake at 37 °C in HEK293 cells [for review see Table 3 in Zwartsen et al., 2017; Simmler et al (2013)].

Notably, the differences in inhibition of DA uptake (T356 M vs WT hDAT) by the potent DA reuptake inhibitors methylphenidate and cocaine (Figs. 3 and 5) are similar between our data and Herborg et al

Table 1
IC_{50} values for the inhibition of WT and T356 M polymorphic hDAT by psychoactive drugs compared to the estimated human brain concentration [brain]. IC_{50} values (μM) are presented with 95% confidence intervals [CI]. * and ** indicate that T356 M hDAT IC_{50} values are significantly ($p < 0.01$, $p < 0.001$, respectively) different from WT. IC_{50} values of WT and T356 M hDAT which are > 2 times the estimated human brain concentration (μM) are highlighted in grey. [a] (Zwartsen et al., 2017); [b] (Hondebrink et al., 2018); [c] (Zwartsen et al., 2018).

Group	Drug	IC_{50} WT hDAT	IC_{50} T356M hDAT	[brain]
Therapeutic drugs	Fluoxetine	68 [40-164]	45 [29-74]	4-9[a]
	Bupropion	4.0 [3.3-4.9]	30 [21-48] **	0.1-6.9
	Methylphenidate	0.4 [0.3-0.6]	20 [10-48] **	0.02-6.5
	Citalopram	203 [121-342]	67 [35-193] *	0.03-7.4
Recreational drugs	MDMA	34 [15-191]	11 [3.9-30]	0.2-448[b]
	Amphetamine	11 [7.5-15]	11 [4.3-32]	0.3-336[b]
	4-FA	7.0 [5.3-9.5]	11 [5.6-26]	0.1-12[b]
	Ketamine	383 [299-498]	476 [335-691]	0.1-30[b]
	MXE	108 [78-149]	404 [215-853] **	0.1-6.1[b]
	Cocaine	1.1 [0.7-1.6]	23 [12-42] **	0.02-30[c]

(2018). Additionally, our data show for the first time that bupropion inhibited DA uptake by T356 M hDAT less potently compared to the potent inhibition of WT hDAT (Fig. 3). Interestingly, citalopram inhibited the DA uptake via the T356 M polymorphic hDAT more potently compared to WT hDAT.

These findings are of interest as heterogeneity of the response of individuals to prescribed psychotropic drugs and (illicit) recreational drugs has been a concern from a pharmacological and toxicological point of view for decades (Keers and Aitchison, 2011; Narasimhan and Lohoff, 2012; Rietjens et al., 2012). It is known that interindividual variation is at least partially due to genetic factors. While it is recognized that pharmacokinetics can differ significantly between patients, the role of genetic differences in pharmaco- and toxicodynamics has been hardly investigated, even though differences in expression and/or function of receptors, ion channels or transporters could also alter drug effects.

As the investigated polymorphism was discovered only in one patient during screening of autism spectrum disorder (ASD) patients and their parents, it is not yet possible to estimate the prevalence of this polymorphism in the human population. Future screening of different and larger populations could show a higher prevalence of the T356 M polymorphism. In this case, these findings are of particular relevance as many people are exposed to psychoactive substances (therapeutics and/or drugs of abuse). The effect of the T356 M polymorphism, even though likely not highly prevalent in the human population, also illustrates the need for wider genome screens in the search for other hDAT polymorphisms, as such mutations can also add to the interindividual variation in response to psychoactive substances.

Patients with this polymorphic transporter may be classified as treatment-resistant for bupropion or methylphenidate. Although WT hDAT is inhibited by bupropion and methylphenidate at estimated therapeutic brain concentrations (IC_{50} values for hDAT within estimated brain concentration; Table 1), T356 M hDAT is not (IC_{50} values for hDAT above estimated brain concentration), possibly contributing to the interindividual variation seen with bupropion and methylphenidate treatments. In addition, cocaine users with the T356 M polymorphism likely require higher doses to experience its intended effects, which could increase the risk for toxic effects that are not mediated by DAT inhibition, like hyperthermia, cardiotoxicity or hepatotoxicity (Kontak et al., 2012). On the other hand, users could be less at risk for cocaine dependence, as DA uptake is less potently inhibited.

The relevance of the difference in inhibition of DA uptake of this polymorphism for patients using citalopram is low, as the IC_{50} values on both transporters are well above the estimated brain concentration range (Table 1). Fluoxetine also showed only a weak but equipotent inhibition at both hDATs, in line with the primary target of SSRIs being SERT. Moreover, our data indicate comparable inhibition of T356 M and WT hDAT (Fig. 4) by DA releasing DAT substrates MDMA, amphetamine and 4-FA, at IC_{50} values relevant for human exposure (Table 1). Furthermore, arylcyclohexylamines ketamine and MXE inhibit hDAT at concentrations 100-fold higher than their primary target, the N-methyl-D-aspartate (NMDA) receptor (Hondebrink et al., 2018). Consequently, the minor effect of the T356 M SNP on the IC_{50} of MXE is likely of limited (clinical) relevance.

Attenuation of the inhibition of DA uptake by T356 M hDAT compared to WT hDAT may thus be specific for a select number of potent DA reuptake inhibitors (bupropion, methylphenidate, and cocaine), as this difference is absent for amphetamine-type stimulants, SSRIs and arylcyclohexylamines. Elevated inhibition of DAT uptake was only seen for the SSRI citalopram, but not for fluoxetine. Possible reasons for the difference in inhibition by DA reuptake inhibitors for T356 M hDAT compared to WT hDAT may relate to conformational changes in a binding domain of hDAT (S1 vs S2) and/or the existence of different moieties in the chemical structures [i.e. ketone (= 0) group] and the three-dimensional shape of DA reuptake inhibitors compared to other DAT inhibitors. Future research, including 3D conformational prediction analysis, will have to clarify whether the observed differences are due to general differences in physico-chemical properties or to specific pharmacological properties, e.g. the potency to inhibit or the ability to reverse DAT.

In summary, the polymorphic hDAT is differently affected by several psychoactive substances compared to WT hDAT. Although the prevalence of this mutation is currently unknown, such a mutation could contribute to interindividual differences in, for example, the treatment efficiency with psychoactive substances, but also susceptibly for toxicity and addiction following recreational drug use.

Funding

This research was funded by the Dutch Poisons Information Center (DPIC; University Medical Center Utrecht), the Faculty of Veterinary Medicine (Utrecht University) and the Radboud Institute for Molecular Sciences (Radboud university medical center).

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

We thank the members of the Neurotoxicology Research Group, the department of Pharmacology and Toxicology and Jeannette Wolfswinkel for their helpful discussions.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.tox.2019.04.012.

References

Balcioglu, A., Ren, J.Q., McCarthy, D., Spencer, T.J., Biederman, J., Bhide, P.G., 2009. Plasma and brain concentrations of oral therapeutic doses of methylphenidate and their impact on brain monoamine content in mice. Neuropharmacology 57 (7-8), 687–693.

Blum, K., Oscar-Berman, M., Barh, D., Giordano, J., Gold, M., 2013. Dopamine genetics and function in food and substance abuse. J. Genet. Syndr. Gene Ther. 4 (121), 1000121.

Butz, R.F., Welch, R.M., Findlay, J.W.A., 1982. Relationship between bupropion disposition and dopamine uptake inhibition in rats and mice. J. Pharmacol. Exp. Ther. 221 (3), 676–685.

Chan, Y.M., Soldin, S.J., Swanson, J.M., Deber, C.M., Thiessen, J.J., MacLeod, S., 1980. Gas chromatographic/mass spectrometric analysis of methylphenidate (Ritalin) in serum. Clin. Biochem. 13 (6), 266–272.

DeVane, C.L., Laizure, S.C., Cameron, D.F., 1986. The effect of experimentally-induced renal failure on accumulation of bupropion and its major basic metabolites in plasma and brain of guinea pigs. Psychopharmacology (Berl.) 89 (4), 404–408.

Dunlop, B.W., Nemeroff, C.B., 2007. The role of dopamine in the pathophysiology of depression. Arch. Gen. Psychiatry 64 (3), 327–337.

El-Sheikh, A.A., van den Heuvel, J.J., Koenderink, J.B., Russel, F.G., 2007. Interaction of nonsteroidal anti-inflammatory drugs with multidrug resistance protein (MRP) 2/ABCC2- and MRP4/ABCC4-mediated methotrexate transport. J. Pharmacol. Exp. Ther. 320 (1), 229–235.

EMCDDA, 2018. European Drug Report 2018 - Trends and Developments. . Available online: http://www.emcdda.europa.eu/system/files/publications/8585/20181816_TDAT18001ENN_PDF.pdf [Accessed]2018.

Fu, K., Konrad, R.J., Hardy, R.W., Brissie, R.M., Robinson, C.A., 2000. An unusual multiple drug intoxication case involving citalopram. J. Anal. Toxicol. 24 (7), 648–650.

Gualtieri, C.T., Wargin, W., Kanoy, R., Patrick, K., Shen, C.D., Youngblood, W., Mueller, R.A., Breese, G.R., 1982. Clinical studies of methylphenidate serum levels in children and adults. J. Am. Acad. Child Psychiatry 21 (1), 19–26.

Hahn, M.K., Blakely, R.D., 2002. Monoamine transporter gene structure and polymorphisms in relation to psychiatric and other complex disorders. Pharmacogenomics J. 2 (4), 217–235.

Hamilton, P.J., Campbell, N.G., Sharma, S., Erreger, K., Herborg Hansen, F., Saunders, C., Belovich, A.N., Consortium, N.A.A.S., Sahai, M.A., Cook, E.H., Gether, U., McHaourab, H.S., Matthies, H.J., Sutcliffe, J.S., Galli, A., 2013. De novo mutation in the dopamine transporter gene associates dopamine dysfunction with autism spectrum disorder. Mol. Psychiatry 18 (12), 1315–1323.

Herborg, F., Andreassen, T.F., Berlin, F., Loland, C.J., Gether, U., 2018. Neuropsychiatric disease-associated genetic variants of the dopamine transporter display

heterogeneous molecular phenotypes. J. Biol. Chem. 293 (19), 7250–7262.
Hoiseth, G., Haslemo, T., Uthus, L.H., Molden, E., 2015. Effect of CYP2B6*6 on steady-state serum concentrations of bupropion and hydroxybupropion in psychiatric patients: a study based on therapeutic drug monitoring data. Ther. Drug Monit. 37 (5), 589–593.
Hondebrink, L., Zwartsen, A., Westerink, R.H.S., 2018. Effect fingerprinting of new psychoactive substances (NPS): What can we learn from in vitro data? Pharmacol. Ther. 182, 193–224.
Jefferson, J.W., Pradko, J.F., Muir, K.T., 2005. Bupropion for major depressive disorder: pharmacokinetic and formulation considerations. Clin. Ther. 27 (11), 1685–1695.
Johnston, J.A., Ascher, J., Leadbetter, R., Schmith, V.D., Patel, D.K., Durcan, M., Bentley, B., 2002. Pharmacokinetic optimisation of sustained-release bupropion for smoking cessation. Drugs 62 (2), 11–24.
Jones, A.W., Holmgren, A., Ahlner, J., 2016. Post-mortem concentrations of drugs determined in femoral blood in single-drug fatalities compared with multi-drug poisoning deaths. Forensic Sci. Int. 267, 96–103.
Kantor, E.D., Rehm, C.D., Haas, J.S., Chan, A.T., Giovannucci, E.L., 2015. Trends in prescription drug use among adults in the united states from 1999-2012. JAMA 314 (17), 1818–1830.
Keers, R., Aitchison, K.J., 2011. Pharmacogenetics of antidepressant response. Expert Rev. Neurother. 11 (1), 101–125.
Kontak, A., Vongpatanasin, W., Victor, R.G., 2012. Cocaine Overdose, Primer on the Autonomic Nervous System. pp. 577–581.
Korte, S.M., Prins, J., Krajnc, A.M., Hendriksen, H., Oosting, R.S., Westphal, K.G., Korte-Bouws, G.A.H., Olivier, B., 2015. The many different faces of major depression: it is time for personalized medicine. Eur. J. Pharmacol. 753, 88–104.
Luchini, D., Morabito, G., Centini, F., 2005. Case report of a fatal intoxication by citalopram. Am. J. Forensic Med. Pathol. 26 (4), 352–354.
Madras, B.K., Miller, G.M., Fischman, A.J., 2005. The dopamine transporter and attention-deficit/hyperactivity disorder. Biol. Psychiatry 57 (11), 1397–1409.
Mari, F., Gualco, B., Rensi, R., Bertol, E., 2012. Acute massive pulmonary thromboembolism due to acute intoxication by duloxetine: a case report. Cardiovasc. Toxicol. 12 (3), 258–262.
Modi, N.B., Lindemulder, B., Gupta, S.K., 2000. Single- and multiple-dose pharmacokinetics of an oral once-a-day osmotic controlled-release OROS (methylphenidate HCl) formulation. J. Clin. Pharmacol. 40 (4), 379–388.
Moron, J.A., Zakharova, I., Ferrer, J.V., Merrill, G.A., Hope, B., Lafer, E.M., Lin, Z.C., Wang, J.B., Javitch, J.A., Galli, A., Shippenberg, T.S., 2003. Mitogen-activated protein kinase regulates dopamine transporter surface expression and dopamine transport capacity. J. Neurosci. 23 (24), 8480–8488.
Narasimhan, S., Lohoff, F.W., 2012. Pharmacogenetics of antidepressant drugs: current clinical practice and future directions. Pharmacogenomics 13 (4), 441–464.
Neale, B.M., Kou, Y., Liu, L., Ma'ayan, A., Samocha, K.E., Sabo, A., Lin, C.F., Stevens, C., Wang, L.S., Makarov, V., Polak, P., Yoon, S., Maguire, J., Crawford, E.L., Campbell, N.G., Geller, E.T., Valladares, O., Schafer, C., Liu, H., Zhao, T., Cai, G., Lihm, J., Dannenfelser, R., Jabado, O., Peralta, Z., Nagaswamy, U., Muzny, D., Reid, J.G., Newsham, I., Wu, Y., Lewis, L., Han, Y., Voight, B.F., Lim, E., Rossin, E., Kirby, A., Flannick, J., Fromer, M., Shakir, K., Fennell, T., Garimella, K., Banks, E., Poplin, R., Gabriel, S., DePristo, M., Wimbish, J.R., Boone, B.E., Levy, S.E., Betancur, C., Sunyaev, S., Boerwinkle, E., Buxbaum, J.D., Cook Jr., E.H., Devlin, B., Gibbs, R.A., Roeder, K., Schellenberg, G.D., Sutcliffe, J.S., Daly, M.J., 2012. Patterns and rates of exonic de novo mutations in autism spectrum disorders. Nature 485 (7397), 242–245.
Nedahl, M., Johansen, S.S., Linnet, K., 2018. Reference Brain/Blood concentrations of citalopram, duloxetine, Mirtazapine and sertraline. J. Anal. Toxicol. 42 (3), 149–156.
Nutt, J.G., Carter, J.H., Sexton, G.J., 2004. The dopamine transporter: importance in Parkinson's disease. Ann. Neurol. 55 (6), 766–773.
Ostad Haji, E., Tadic, A., Wagner, S., Dragicevic, A., Muller, M.J., Boland, K., Rao, M.L., Fric, M., Laux, G., Hiemke, C., 2011. Association between citalopram serum levels and clinical improvement of patients with major depression. J. Clin. Psychopharmacol. 31 (3), 281–286.
Park, J., Vousden, M., Brittain, C., McConn, D.J., Iavarone, L., Ascher, J., Sutherland, S.M., Muir, K.T., 2010. Dose-related reduction in bupropion plasma concentrations by ritonavir. J. Clin. Pharmacol. 50 (10), 1180–1187.
Paulzen, M., Lammertz, S.E., Grunder, G., Veselinovic, T., Hiemke, C., Tauber, S.C., 2016. Measuring citalopram in blood and cerebrospinal fluid: revealing a distribution pattern that differs from other antidepressants. Int. Clin. Psychopharmacol. 31 (3), 119–126.
Posner, J., Bye, A., Jeal, S., Peck, A.W., Whiteman, P., 1984. Alcohol and bupropion pharmacokinetics in healthy male volunteers. Eur. J. Clin. Pharmacol. 26 (5), 627–630.
Rietjens, S.J., Hondebrink, L., Westerink, R.H., Meulenbelt, J., 2012. Pharmacokinetics and pharmacodynamics of 3,4-methylenedioxymethamphetamine (MDMA): inter-individual differences due to polymorphisms and drug-drug interactions. Crit. Rev. Toxicol. 42 (10), 854–876.
Riherd, D.N., Galindo, D.G., Krause, L.R., Mayfield, R.D., 2008. Ethanol potentiates dopamine uptake and increases cell surface distribution of dopamine transporters expressed in SK-N-SH and HEK-293 cells. Alcohol 42 (6), 499–508.
Rohrig, T.P., Hicks, C.A., 2015. Brain tissue: a viable postmortem toxicological specimen. J. Anal. Toxicol. 39 (2), 137–139.
Scholze, P., Sitte, H.H., Singer, E.A., 2001. Substantial loss of substrate by diffusion during uptake in HEK-293 cells expressing neurotransmitter transporters. Neurosci. Lett. 309 (3), 173–176.
Seger, D., 2010. Cocaine, metamfetamine, and MDMA abuse: the role and clinical importance of neuroadaptation. Clin. Toxicol. (Phila) 48 (7), 695–708.
Senna, M.C., Augsburger, M., Aebi, B., Briellmann, T.A., Donze, N., Dubugnon, J.L., Iten, P.X., Staub, C., Sturm, W., Sutter, K., 2010. First nationwide study on driving under the influence of drugs in Switzerland. Forensic Sci. Int. 198 (1-3), 11–16.
Simmler, L.D., Wandeler, R., Liechti, M.E., 2013. Bupropion, methylphenidate, and 3,4-methylenedioxypyrovalerone antagonize methamphetamine-induced efflux of dopamine according to their potencies as dopamine uptake inhibitors: implications for the treatment of methamphetamine dependence. BMC Res. Notes 6, 220.
Storch, A., Ludolph, A.C., Schwarz, J., 1999. HEK-293 cells expressing the human dopamine transporter are susceptible to low concentrations of 1-methyl-4-phenylpyridine (MPP+) via impairment of energy metabolism. Neurochem. Int. 35 (5), 393–403.
Suckow, R.F., Smith, T.M., Perumal, A.S., Cooper, T.B., 1986. Pharmacokinetics of bupropion and metabolites in plasma and brain of rats, mice, and guinea pigs. Drug Metab. Dispos. 14 (6), 692–697.
te Brake, L.H.M., van den Heuvel, J.J.M.W., Buaben, A.O., van Crevel, R., Bilos, A., Russel, F.G., Aarnoutse, R.E., Koenderink, J.B., 2016. Moxifloxacin is a potent *In vitro* inhibitor of OCT- and MATE-mediated transport of metformin and ethambutol. Antimicrob. Agents Chemother. 60 (12), 7105.
Thomas, P., Smart, T.G., 2005. HEK293 cell line: a vehicle for the expression of recombinant proteins. J. Pharmacol. Toxicol. Methods 51 (3), 187–200.
Torres, G.E., Gainetdinov, R.R., Caron, M.G., 2003. Plasma membrane monoamine transporters: structure, regulation and function. Nat. Rev. Neurosci. 4 (1), 13–25.
UNODC, 2018. World Drug Report 2018.
Viviani, R., Abler, B., Seeringer, A., Stingl, J.C., 2012. Effect of paroxetine and bupropion on human resting brain perfusion: an arterial spin labeling study. Neuroimage 61 (4), 773–779.
Volkow, N.D., Wang, G.J., Fowler, J.S., Molina, P.E., Logan, J., Gatley, S.J., Gifford, A., Ding, Y.S., Wong, C., Pappas, N.R., Zhu, W., Swanson, J.M., 2003. Cardiovascular effects of methylphenidate in humans are associated with increases of dopamine in brain and of epinephrine in plasma. Psychopharmacology (Berl.) 166 (3), 264–270.
Volkow, N.D., Wang, G.J., Telang, F., Fowler, J.S., Logan, J., Childress, A.R., Jayne, M., Ma, Y., Wong, C., 2006. Cocaine cues and dopamine in dorsal striatum: mechanism of craving in cocaine addiction. J. Neurosci. 26 (24), 6583–6588.
Wille, S.M., De Letter, E.A., Piette, M.H., Van Overschelde, L.K., Van Peteghem, C.H., Lambert, W.E., 2009. Determination of antidepressants in human postmortem blood, brain tissue, and hair using gas chromatography-mass spectrometry. Int. J. Legal Med. 123 (6), 451–458.
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 (12), 1663–1671.
Zhu, J., Reith, M.E., 2008. Role of the dopamine transporter in the action of psychostimulants, nicotine, and other drugs of abuse. CNS Neurol. Disord. Drug Targets 7 (5), 393–409.
Zwartsen, A., Verboven, A.H.A., van Kleef, R., Wijnolts, F.M.J., Westerink, R.H.S., Hondebrink, L., 2017. Measuring inhibition of monoamine reuptake transporters by new psychoactive substances (NPS) in real-time using a high-throughput, fluorescence-based assay. Toxicol. In Vitro 45 (Pt 1), 60–71.
Zwartsen, A., Hondebrink, L., Westerink, R.H., 2018. Neurotoxicity screening of new psychoactive substances (NPS): effects on neuronal activity in rat cortical cultures using microelectrode arrays (MEA). Neurotoxicology 66, 87–97.