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ORIGINAL ARTICLE

Neurochemical binding profiles of novel indole and benzofuran MDMA analogues

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Abstract 3,4-Methylenedioxy-*N*-methylamphetamine (MDMA) has been shown to be effective in the treatment of post-traumatic stress disorder (PTSD) in numerous clinical trials. In the present study, we have characterized the neurochemical binding profiles of three MDMA-benzofuran analogues (1-(benzofuran-5-yl)-propan-2-amine, 5-APB; 1-(benzofuran-6-yl)-*N*-methylpropan-2-amine, 6-MAPB; 1-(benzofuran-5-yl)-*N*-methylpropan-2-amine, 5-MAPB) and one MDMA-indole analogue (1-(1*H*-indol-5-yl)-2-methylamino-propan-1-ol, 5-IT). These compounds were screened as potential second-generation anti-PTSD drugs, against a battery of human and non-human receptors, transporters, and enzymes, and their potencies as 5-HT_2 receptor agonist and monoamine uptake inhibitors determined. All MDMA analogues displayed high binding affinities for 5-$HT_{2a,b,c}$ and $NE_{\alpha 2}$ receptors, as well as significant 5-HT, DA, and NE uptake inhibition. 5-APB revealed significant agonist activity at the 5-$HT_{2a,b,c}$ receptors, while 6-MAPB, 5-MAPB, and 5-IT exhibited significant agonist activity at the 5-HT_{2c} receptor. There was a lack of correlation between the results of functional uptake and the monoamine transporter binding assay. MDMA analogues emerged as potent and selective monoamine oxidase A inhibitors. Based on 6-MAPB favorable pharmacological profile, it was further subjected to IC_{50} determination for monoamine transporters. Overall, all MDMA analogues displayed higher monoamine receptor/transporter binding affinities and agonist activity at the 5-$HT_{2a,c}$ receptors as compared to MDMA.

Keywords 3,4-Methylenedioxy-*N*-methylamphetamine (MDMA) · MDMA analogues · Neurochemical profile · Monoamine receptors · Monoamine transporters

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Introduction

Post-traumatic stress disorder (PTSD) is typically a chronic illness caused by exposure to a traumatic event (Heim and Nemeroff 2009). A large percentage of the population exposed to life-threatening traumatic situations such as terrorist attacks, traffic accidents, violent assaults, and death of a relative develop PTSD, which is often associated with symptoms such as nightmares, apathy, cognitive deficiency, lack of concentration, anxiety, restlessness, insomnia, and suicide attempts (Heim and Nemeroff 2009; Shakespeare-Finch and Lurie-Beck 2014). The therapeutic strategy of treating PTSD in the USA includes psychotherapy and pharmacological intervention, utilizing the selective serotonin (5-HT) reuptake inhibitors sertraline and paroxetine as the only FDA-approved drugs for this application (Marshall et al. 2001; Mithoefer et al. 2011, 2013). However, since a large proportion of the patients (25–50 %) are resistant to the current therapies, more efficient and safer therapeutic alternatives are urgently warranted (Cukor et al. 2009; Mithoefer et al. 2011, 2013). The clinical use of MDMA to enhance psychotherapy was first documented in 1978 (Pentney 2001). In 1977 and

1985, MDMA was classified in the UK and the USA, respectively, as schedule I controlled substance on the grounds of potential abuse and lack of accepted safety (Misuse of Drugs Act 1971; Pentney 2001). Once a drug is classified under schedule I, it is unlikely that any medical value will be discovered for it, as it is extremely difficult to receive an approval for clinical testing and medical research (Nutt 2014). Notwithstanding, a decade ago, the FDA has granted a general consent to support clinical trials utilizing MDMA for the treatment of PTSD (Doblin 2002). More recently, numerous clinical trials clearly demonstrated MDMA efficacy and safety for the treatment of chronic PTSD (Bouso et al. 2008; Mithoefer et al. 2011, 2013; Oehen et al. 2013). There were no drug-related serious adverse events and no evidence of impaired cognitive function as measured by neuropsychological testing. Before the prohibition of MDMA in the USA, it was used off-label for a wide range of psychiatric conditions such as depression, anxiety, and psychosis (Greer and Tolbert 1986; Grinspoon and Bakalar 1986). Moreover, numerous studies indicated MDMA's potential in ameliorating dyskinesia associated with L-DOPA treatment of Parkinson's disease (Lettfuss et al. 2012). The therapeutic potential of MDMA is believed to consist in temporarily reducing anxiety associated with PTSD, thus helping subjects gain access to their emotions and internal conflicts without the overwhelming fear normally associated with these emotions and memories (Bouso et al. 2008; Mithoefer et al. 2011, 2013). MDMA exerts its therapeutic effect through a complex combination of pharmacological effects, which include 5-HT_2 receptor stimulation, monoamine transporter inhibition (5-HT transporter, HTT; dopamine transporter, DAT; norepinephrine transporter, NET), active transport into the presynaptic nerve ending, partial inhibition of brain monoamine oxidase (MAO), and displacement of monoamines from their presynaptic vesicular storage sites, thereby elevating and promoting the release of DA, NE, and 5-HT from presynaptic nerve terminals in the brain (Capela et al. 2009; Leonardi and Azmitia 1994; Rothman et al. 2001; Rothman and Baumann 2003).

The MDMA-benzofuran analogues, 1-(benzofuran-5-yl)-propan-2-amine (5-APB), 1-(benzofuran-6-yl)-*N*-methylpropan-2-amine (6-MAPB), and 1-(benzofuran-5-yl)-*N*-methylpropan-2-amine (5-MAPB), and the MDMA-indole analogue 1-(1*H*-indol-5-yl)-2-methylamino-propan-1-ol (5-IT), are reported to be used as illegal recreational drugs, due to their psychostimulating empathogenic MDMA-like properties (Iversen et al. 2013; King 2013; Nelson et al. 2014; Fig. 1). The synthesis of 5-IT was first reported in 1962 by Albert Hofmann and its pharmacological properties later described by Alexander Shulgin (Hofmann and Troxler 1963; Shulgin 1997). Recently, 5-IT was implicated with several fatal and non-fatal intoxications; however, in the majority of cases, concomitant drug abuse was found in the postmortem examination (Kronstrand et al. 2013; Seetohul and Pounder 2013).

Iversen et al. characterized the neurochemical binding affinities of 14 synthetic MDMA and amphetamine analogues, among which the benzofuran MDMA analogues 5- and 6-APB displayed potent agonistic activity at the 5-HT_{2b} receptor and NET, DAT, and HTT inhibition (Iversen et al. 2013).

Due to the similar chemical structure of the MDMA-benzofuran analogues to MDMA, the aim of the present study was to expand the neurochemical binding profile to the *N*-methyl derivatives of 6-APB and 5-APB, namely 6-MAPB and 5-MAPB, and include 5-APB and 5-IT as well (Fig. 1). MDMA was used as a reference compound. The aforementioned compounds were screened as second-generation MDMA-like drugs with potential indication for PTSD, against a battery of human and non-human receptors, transporters, and enzymes, and their potencies as 5-HT_2 receptor agonist and monoamine uptake inhibitors determined.

Materials and methods

Chemicals

The following three MDMA analogues were supplied as reference materials by Z-CHEM BV (Veemarkt 61,1019 DA Amsterdam, Netherlands): 5-IT, 5-APB, 6-MAPB, and 5-MAPB (Fig. 1).

3,4-Methylenedioxymethamphetamine (MDMA) was supplied as a reference material by CEREP (Le Bois l'Eveque, 86600 Celle L'Evescault, France; http://www.cerep.fr/cerep/users/pages/about/strategy.asp). Certified purities ranged from 92 to 99.9 % (full certificates of analysis for each material are available in the supplemental section). Stock solutions of all the test compounds were prepared in DMSO yielding a concentration of 10^{-2} M. For in vitro pharmacology assays, depending on the assay volume and solvent tolerance, the stock solutions were diluted to [100×], [333×], or [1000×] in 100 % solvent, then either added directly or further diluted to [10×] or [5×] in H_2O or assay buffer before addition to the assay well (final solvent concentration kept constant).

Neurochemical assays

The receptor, transporter, and enzyme binding profiles and functional assays were conducted by CEREP (Le Bois l'Eveque, 86600 Celle L'Evescault, France; http://www.cerep.fr/cerep/users/pages/about/strategy.asp). A total of 5 compounds (including MDMA as a reference compound) were screened against a total of 25 targets (receptors, enzymes, and transporters; Tables S1 and S2); in vitro functional assays were performed against 6 targets as listed in

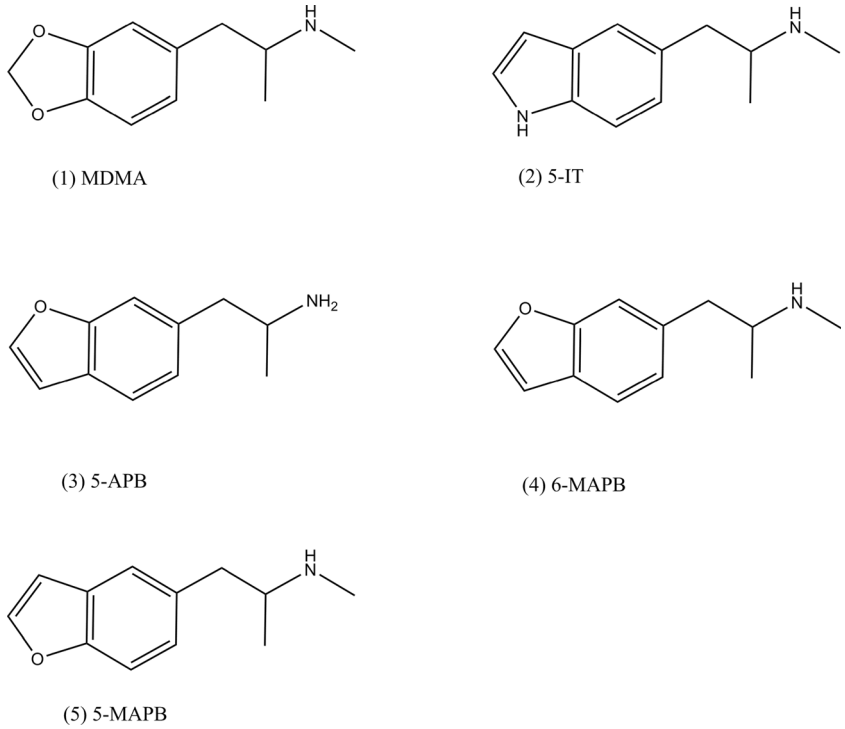

Fig. 1 Chemical structures of MDMA (*1*) and its four novel MDMA analogues: (*2*) 1-(1*H*-indol-5-yl)-2-methylamino-propan-1-ol (*5-IT*), (*3*) 1-(benzofuran-5-yl)-propan-2-amine hydrochloride (5-APB), (*4*) 1-(benzofuran-6-yl)-*N*-methylpropan-2-amine (6-MAPB), and (*5*) 1-(benzofuran-5-yl)-*N*-methylpropan-2-amine (5-MAPB)

Table S3. The compounds were screened in triplicate at a concentration of 10 μM. Assay conditions are summarized in Tables S1–S3 (see for details: www.cerep.fr/cerep/users/pages/ProductsServices/GPCRPlatform.asp).

In each experiment, the respective reference compound was tested concurrently with the test compounds, and the data were compared with historical values determined at CEREP. The experiment was accepted in accordance with CEREP's validation Standard Operating Procedure.

Radioligand binding assay

The radioligand binding assay was conducted according to the CEREP standard protocols (Krejsa et al. 2003; CEREP Binding Assay 2013). In brief, binding experiments were conducted in 96-well microplates in a total volume of 200 μl of appropriate buffers (Krejsa et al. 2003; CEREP Binding Assay 2013). Reaction mix included solution of a tested compound, radioligand, and diluted membranes (15 μg protein per well) or tissue suspensions. Specific assay conditions for each receptor and enzyme are shown in Table S1 and S2, respectively. Cell membranes expressing recombinant human 5-$HT_{2a,b,c}$, DA, and $NE_{\beta 1,2}$ receptors were used. $NE_{\alpha 1,2}$ receptors were obtained from rat cerebral cortex. The radioactivity was measured in MicroBeta TriLux 1450 liquid scintillation counter (PerkinElmer, USA).

Enzyme binding assay

Monoamine oxidase A (MAO A) binding assay was performed according to the protocol published by Weyler and Salach (1985). In brief, MAO A activity was measured spectrophotometrically by monitoring the increase in absorbance at 314 nm on oxidation of kynuramine with formation of 4-hydroxyquinoline (Weyler and Salach 1985). The measurements were carried out, at 30 °C, in 50 mM NaPi buffer, pH 7.2, containing 0.2 % Triton X-100, 1 mM kynuramine, and 40 pmol of the purified enzyme in 1 ml total volume.

MAO B binding assay was performed with slight modifications according to the protocol published by Tsugeno et al. (1995). In brief, the test substances were plated with MAO B and the luciferin derivative substrate was subsequently added to yield a final concentration of 4 μM. The reaction mixture was incubated for 1 h at 37 °C, followed by the addition of the Reporter Luciferin Detection Reagent and the reaction mixture further incubated for 30 min to produce luminescence signal.

The catechol-*O*-methyltransferase (COMT) activity assay was utilized based on previously published work (Müller-Enoch et al. 1976). Porcine COMT was prepared as described earlier (Müller-Enoch et al. 1976). COMT activity was determined by measuring the fluorescence intensity of scopoletin enzymatically produced with esculetin (1 μM) as the substrate and *S*-adenosyl-L-methionine as the co-substrate.

The method of Nagatsu et al. (1964) was used with modifications for the determination of tyrosine hydroxylase activity. Tyrosine hydroxylase was obtained as described, from rat striatum (Nagatsu et al. 1964). In this assay, [3,5-^{3}H]-L-tyrosine was used as substrate (10 μM), and the amount of tritiated water produced during the 3-hydroxylation was measured.

The PKC_α binding assay relied on a modified protocol published by Chen et al. (1993). In brief, PKC_α was incubated at room temperature for 15 min with biotinylated oligopeptide substrate (60 nM) and ATP followed by the addition of a premixed solution of europium cryptate (Eu(K))-labeled antibody and fluorophore-conjugated streptavidin (SA-XL665). A homogeneous time-resolved fluorescence (HTRF) measurement was made after 1 h of incubation.

Cellular receptor/transporter functional assays

The functional characterization of the MDMA analogues at recombinant human 5-HT2A, 5-HT2B, and 5-HT2C receptors was performed with 5-HT_{2a}, 5-HT-2c-transfected HEK-293, and in 5-HT_{2b}-transfected CHO cells according to a previously published work with slight modifications (Porter et al. 1999; Table S3). Activation of the 5-HT_2 receptor resulted in the production of inositol phosphate 1 (IP1), which was subsequently measured using the HTRF assay as described by Canal et al. (2013).

NET and HTT uptake assays were carried out as described previously under minor modifications (Perovic and Müller 1995). In brief, uptake assays of NET and HTT were performed by incubating rat hypothalamus and rat brain synaptosomes with 0.2 μCi/mL ^{3}H-NE and ^{3}H-5-HT at 37 °C for 20 and 15 min, respectively. ^{3}H-NE and ^{3}H-5-HT in the supernatant were quantified by liquid scintillation counting. Specific uptake was determined by subtracting nonspecific uptake from total uptake as previously described (Zhao et al. 2008). DAT uptake assay was conducted as previously described under slight modifications (Janowsky et al. 1986). In brief, uptake assay of DAT was performed by incubating rat striatum synaptosomes with 0.2 μCi/mL ^{3}H-DA at 37 °C for 15 min. ^{3}H-DA in the supernatant was quantified by liquid scintillation counting. Specific uptake was determined by subtracting nonspecific uptake from total uptake as previously described (Janowsky et al. 1986).

Data analysis

Results showing an inhibition or stimulation (for assays run in basal conditions) higher than 50 % are considered to represent significant effects of the test compounds. Fifty percent is the most common cutoff value for further investigation (determination of IC_{50} values from concentration–response curves). Results showing an inhibition or stimulation between 25 and 50 % are indicative of weak to moderate effects, while results showing an inhibition or stimulation lower than 25 % are not considered significant and mostly attributable to variability of the signal around the control level. IC_{50} values for the monoamine uptake inhibition were determined using iterative curve fitting routines (GraphPad/Prism, version 5, San Diego, CA, USA).

Results

Inhibition of ligand-specific binding to 5-$HT_{2a,b,c}$ receptors

All the tested compounds inhibited radioligand (^{125}I-2,5-dimethoxy-4-iodoamphetamine 0.1–0.2 nM) specific binding to human recombinant 5-$HT_{2a,b,c}$ receptors at a concentration of 10 μM (Fig. 2a). MDMA and 5-IT displayed the lowest percent binding inhibition (68 %) at the 5-HT_{2a} and 5-HT_{2b} receptors, respectively, while 5-MAPB was the only compound inhibiting more than 90 % of agonist-specific binding at all 5-HT_2 receptor subtypes (Fig. 2a).

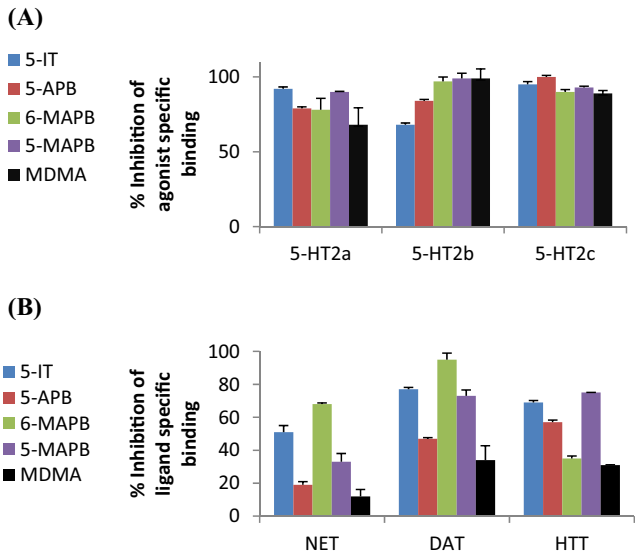

Fig. 2 **a** Percent inhibition of ligand-specific binding (^{125}I-(±)-DOI at 0.2 nM) to 5-$HT_{2a,b,c}$ subtypes at MDMA analogue concentration of 10^{-5} M following incubation for 60 min at room temperature. 5-IT:, 92.3 % (5-HT2a), 67.9 % (5-HT2b), 94.8 % (5-HT2c); 5-APB: 78.7 % (5-HT2a), 84.3 % (5-HT2b), 100.2 % (5-HT2c); 6-MAPB: 78.1 % (5-HT2a), 96.6 % (5-HT2b), 90.6 % (5-HT2c); 5-MAPB: 90.5 % (5-HT2a), 98.5 % (5-HT2b), 92.9 % (5-HT2c); MDMA: 68.1 % (5-HT2a), 99.2 % (5-HT2b), 88.8 % (5-HT2c). **b** Percent inhibition of ligand-specific binding to transporters for norepinephrine (NET; radioligand: ^{3}H-nisoxetine (1 nM)), dopamine (DAT; radioligand: ^{3}H-BTCP 4 nM), and serotonin (HTT; ^{3}H-imipramine 2 nM) at MDMA and its analogues at a concentration of 10^{-5} M. 5-IT :, 51.2 % (NET), 77.3 % (DAT), 69.2 % (HTT); 5-APB: 19.3 % (NET), 47.3 % (DAT), 56.9 % (HTT); 6-MAPB: 68.1 % (NET), 95.2 % (DAT), 34.7 % (HTT); 5-MAPB: 32.5 % (NET), 73.2 % (DAT), 74.9 % (HTT); MDMA: 12.4 % (NET), 34 % (DAT), 31.4 % (HTT)

Inhibition of ligand-specific binding to monoamine transporters

Among the tested compounds, only 5-IT, 5-APB, and 5-MAPB (10 μM) revealed more than 55 % inhibition of radioligand-specific (^{3}H-imipramine 2 nM) binding to human recombinant HTT, with 5-MAPB displaying the highest percent inhibition (75 %) (Fig. 2b). MDMA inhibited less than 50 % of ligand-specific binding at HTT (31 %), indicative of weak to moderate effect. At the human recombinant DAT, more that 50 % inhibition of agonist-specific (^{3}H-BTCP 4 nM) binding was obtained for 5-IT, 6-MAPB, and 5-MAPB, while MDMA displayed the lowest percent inhibition (34 %) (Fig. 2b).

In contrast to HTT and DAT, at the human recombinant NET, only 5-IT and 6-MAPB revealed more than 50 % inhibition of agonist-specific (^{3}H-nisoxetine 1 nM) binding, while MDMA showed the lowest percent inhibition (12 %) (Fig. 2b). Among the tested compounds, MDMA displayed the lowest activity at all transporters tested; 6-MAPB and 5-MAPB showed significant effect (>50 %) at two monoamine transporters, whereas 5-IT was the only compound exhibiting significant activity (>50 %) at all monoamine transporters (Fig. 2b).

Inhibition of radioligand binding to common neurotransmitter receptors

Table 1 summarizes the percent inhibition of radioligand binding to most common receptors associated with amphetamine/MDMA-like compounds. Among the receptors tested, significant percent inhibition of radioligand binding by at least one tested compound was observed for the following receptors: neuronal nicotinic α4β2 and muscarinic (nonspecific) $NE_{\alpha 1,2}$ and $NE_{\beta 2}$ (Table 1). MDMA displayed remarkable percent inhibition (82 %) only at the neuronal nicotinic α4β2 subunit (Table 1). 5-IT was the only compound revealing significant nonspecific percent inhibition at the muscarinic and $NE_{\alpha 1}$ receptors, whereas at the $NE_{\alpha 2}$, all MDMA analogues exhibited significant percent inhibition. A significant percent inhibition of nonspecific radioligand binding at the $NE_{\beta 2}$ receptor was observed for 5-IT and 6-MAPB. No assayed compound had appreciable affinities for any of the following receptors: GABA, NMDA, AMPA, kainate, and $DA_{1,2,3,4}$ (Table 1).

Enzyme inhibition of substrate-specific metabolism

The five compounds were screened against a battery of four central monoamine levels regulating enzymes (monoamine oxidases A and B, catechol-o-methyl transferase, tyrosine hydroxylase) at 10 μM, and their effect on PKC_α as a downstream signaling kinase of 5-HT_2 receptor and HTT was established. The results of the percent inhibition of substrate-specific metabolism are summarized in Table 2. MAO A was the only enzyme targeted by MDMA analogues, revealing 66.2–73.5 % inhibition of substrate-specific (kynuramine 0.15 M) metabolism (Table 2). In contrast, MDMA had only mild inhibitory effect on MAO A. 5-IT was the only compound revealing a remarkable inhibitory activity on PKC_α (84 %) (Table 2).

5-HT_2 and monoamine transporter functional assay

Receptor functional assays were performed with human recombinant 5-$HT_{2a,b,c}$ receptors at drug concentration of 10 μM (Fig. 3a and Table S3). Agonist activity was measured as percent of 5-HT response, and the results are depicted in Fig. 3a. 5-APB was the only MDMA analogue exerting significant partial agonist activity at the 5-HT_{2a} (68 %) and 5-HT_{2b} (80 %), while the remaining compounds showed only moderate (25–50 %) to non-significant (<25 %) activity. On the other hand, all MDMA analogues exerted significant partial agonist activity at 5-HT_{2c} receptor subtype (≥50 %). 5-APB displayed the highest partial agonist activity at all 5-HT_2 receptors studied (68–80 %) (Fig. 3a). MDMA showed only mild (5-HT_{2c}, 33 %; 5-$HT_{2b,}$ 27 %) to non-significant partial agonist activity (5-HT_{2a}, 18 %). Overall, MDMA analogues as well as MDMA tend to show higher agonist activity at the 5-HT_{2c} receptor, except for 5-APB, revealing higher agonist activity at the 5-$HT_{2a,b}$ receptors (Fig. 3a).

The functional assay of monoamine uptake by transporters was performed in synaptosomes isolated from rat brain (Table S3). At the concentration of 10 μM, MDMA and its four analogues were highly active as inhibitors of 5-HT, DA, and NE uptake, demonstrating more than 95 % uptake inhibition of monoamines (Fig. 3b). However, at the drug concentration tested, no potency differences towards the various monoamine transporters could be determined.

5-HT_2 and monoamine transporter functional assay

Due to the relative selectivity towards 5-HT_{2c} receptor and the high potency as monoamine uptake inhibitor in the functional assays (Fig. 3a, b), 6-MAPB was further subjected to IC_{50} determination of monoamine uptake inhibition via four-point concentration–response studies (Fig. 4). The IC_{50} values obtained for DA and NE uptake inhibition were of the same magnitude, namely 0.03 and 0.04 μM, respectively, whereas the IC_{50} value determined for 5-HT uptake inhibition was one magnitude higher (IC_{50} = 0.3 μM). Overall, 6-MAPB displayed a higher inhibitory activity towards NE and DA uptake transport than towards 5-HT transport. On the other hand, 6-MAPB exhibited significant binding affinity towards 5-HT_2 and $NE_{\alpha 2/\beta 2}$ receptors, while no significant binding affinities were observed for the DA receptor subtypes (Fig. 1a; Table 1).

Table 1 Percent inhibition of radioligand binding to neuroreceptors

Receptors	Radioligand	MDMA	5-IT	5-APB	6-MAPB	5-MAPB
N neuronal α4β2	^{3}H-cytisine	81.9 ± 6.0	0 ± 0	6.6 ± 3.8	68.5 ± 1.3	70.6 ± 0.07
Muscarinic	^{3}H-QNB	23.7 ± 5.3	51.5 ± 0.07	10.3 ± 3.2	35.2 ± 4.9	41.2 ± 7.7
NE α1	^{3}H-prazosine	12.5 ± 1.9	55.1 ± 4.4	14.4 ± 0.01	18.9 ± 8.9	39.5 ± 1.9
NE α2	^{3}H-RX821002	39.2 ± 5.2	87.2 ± 6.4	56.2 ± 1.1	80.8 ± 2.1	57.1 ± 1.6
NE β1	^{3}H-CGP12177	12.2 ± 2.5	13.2 ± 12.2	8.2 ± 7.9	26.9 ± 4.4	14.4 ± 8.2
NE β2	^{3}H-CGP12177	14.1 ± 1.7	55.8 ± 0.01	14.1 ± 1.5	51.1 ± 4	20.5 ± 3.8
GABA	^{3}H-GABA	15.8 ± 2.2	11.2 ± 0.1	<1 %	<1 %	11.2 ± 3.3
AMPA	^{3}H-AMPA	15.7 ± 21.3	<1 %	<1 %	<1 %	<1 %
Kainate	^{3}H-kainic acid	<1 %	19.5 ± 7.3	26.8 ± 9.6	<1 %	<1 %
NMDA	^{3}H-CGP39653	12 ± 1.5	<1 %	<1 %	<1 %	<1 %
DA 1	^{3}H-SCH 23390	11.3 ± 0.07	46.1 ± 6.9	<1 %	3.4 ± 17.1	29.2 ± 1.4
DA 2s	^{3}H-methylspiperone	14 ± 3.9	37.6 ± 7.6	8.3 ± 5.3	8.2 ± 6.7	8.7 ± 8.3
DA 3	^{3}H-methylspiperone	40.8 ± 2.5	40.8 ± 2.5	3.2 ± 2.9	14.4 ± 6.6	23.2 ± 7.5
DA 4,4	^{3}H-methylspiperone	34.5 ± 6.7	<1 %	<1 %	22.2 ± 6.8	24.9 ± 12.3

Radioligand displacement assay was conducted by CEREP in duplicates at drug concentration of 10 μM according to CEREP standard, validated assay protocols described in http://www.cerep.fr/cerep/users/pages/ProductsServices/GPCRPlatform.asp. Depicted are the mean values with the corresponding standard deviation

N neuronal α4β2 nicotinic acetylcholine receptor subtype α4β2, *NE* norepinephrine, *GABA* gama-aminobutyric acid, *AMPA* α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, Na^+/K^+ glutamatergic ion channel, *NMDA* *N*-methyl-D-aspartate glutamatergic Ca^{2+} channel, *DA* dopaminergic receptor

Discussion

Numerous studies indicate that the existing pharmacotherapies for PTSD are ineffective for 25–50 % of patients (Cukor et al. 2009; Mithoefer et al. 2011, 2013). The medial costs and patients suffering due to treatment failure are extensive. The US Veterans Health Administration spent in 2010 about 859 million dollars for the treatment of veterans suffering from PTSD (Congressional Budget Office 2012). Consequently, there is an urgent need for the development of new and highly effective, lower cost drugs for the treatment of PTSD. Recent studies clearly indicated MDMA as a highly effective treatment for PTSD patients, without revealing any evidence of harm (Bouso et al. 2008; Mithoefer et al. 2011, 2013; Oehen et al. 2013). Due to the MDMA classification as a schedule I drug, it is currently extremely difficult to receive an approval for clinical studies and medical research. Hence, the aim of the present study was to characterize the neurochemical binding profile of the second-generation MDMA drugs, 5-IT, 5-APB, 6-MAPB, and 5-MAPB, against a battery of receptors, enzymes, and transporters, in order to evaluate their potential to substitute MDMA for the treatment of PTSD.

All of the aforementioned MDMA analogues displayed notably higher binding affinities to 5-$HT_{2a,c}$ receptors as compared to MDMA, while the binding affinity to the 5-HT_{2b} receptor was equal or less than MDMA (Fig. 2b). In the functional assay, the differences in the agonist activities among the tested compounds measured as percent 5-HT response were

Table 2 Percent enzyme inhibition of control values

Enzymes	Substrate	MDMA	5-IT	5-APB	6-MAPB	5-MAPB
MAO A	Kynuramine (0.15 mM)	24.2 ± 2.3	67.5 ± 0.01	73.5 ± 0.9	65.4 ± 4.7	66.2 ± 0.01
MAO B	D-Luciferin derivative (4 μM)	<1 %	3.7 ± 1.1	2.1 ± 0.5	<1 %	<1 %
PKC_α	ATP + biotinyl-βAβAβ AKIQASFRGHMAR KK (60 nM)	36 ± 4.9	83.9 ± 2.3	<1 %	<1 %	<1 %
Tyrosine hydroxylase	^{3}H-tyrosine (10 μM)	<1 %	7.8 ± 0.8	<1 %	9.8 ± 4.2	5.9 ± 0.9
COMT	Esculetin (1 μM)	13.2 ± 4.0	10.7 ± 3.1	<1 %	<1 %	<1 %

Enzyme inhibition assay was conducted by CEREP in duplicates at drug concentration of 10 μM according to CEREP standard, validated assay protocols described in http://www.cerep.fr/cerep/users/pages/ProductsServices/GPCRPlatform.asp. Depicted are the mean values with the corresponding standard deviation

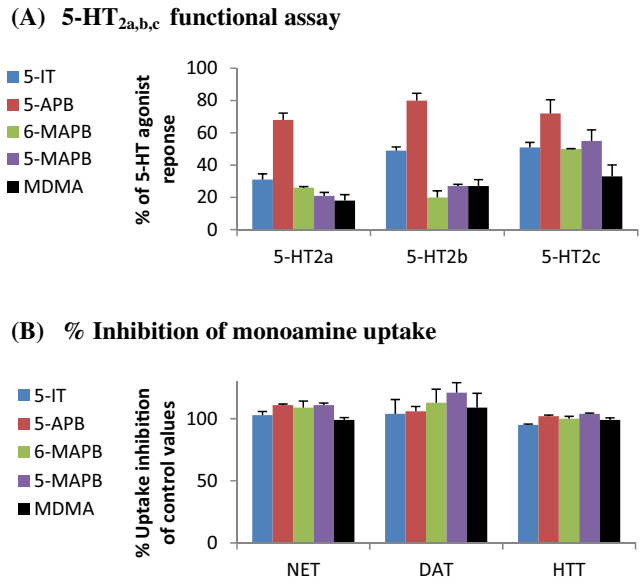

Fig. 3 **a** Functional assay on serotonin receptor subtypes 5-$HT_{2a,b,c}$. MDMA and its analogues were tested at 10^{-5} M and serotonin was used as substrate at 1 μM (5-$HT_{2b,c}$) or 10 μM (5-HT_{2a}). 5-IT percent of control agonist response: 31.4 % (5-HT2a), 48.5 % (5-HT2b), 51 % (5-HT2c); 5-APB: 67.5 % (5-HT2a), 79.5 % (5-HT2b), 71.9 % (5-HT2c); 6-MAPB: 26.3 % (5-HT2a), 19.8 % (5-HT2b), 47.5 % (5-HT2c); 5-MAPB: 21.7 % (5-HT2a), 27.0 % (5-HT2b), 55.1 % (5-HT2c); MDMA: 17.9 % (5-HT2a), 27.4 % (5-HT2b), 33.6 % (5-HT2c). **b** Percent inhibition of monoamine uptake of transporters for norepinephrine (NET; substrate: ^{3}H-NE), dopamine (DAT; substrate: ^{3}H-DA), and serotonin (HTT; substrate: ^{3}H-5-HT) by MDMA and its analogues at a concentration of 10^{-5} M. 5-IT percent inhibition: 102.7 % (NET), 104.2 % (DAT), 94.9 % (HTT); 5-APB: 111.5 % (NET), 106.0 % (DAT), 102.0 % (HTT); 6-MAPB: 108.6 % (NET), 113.6 % (DAT), 99.9 % (HTT); 5-MAPB: 110.5 % (NET), 121.6 % (DAT), 103.5 % (HTT); MDMA: 98.8 % (NET), 109.0 % (DAT), 99.2 % (HTT)

notably higher than in the receptor binding assay (Figs. 2a and 3a). All of the MDMA analogues tested herein acted as more potent agonists at the 5-$HT_{2a,c}$ receptors than MDMA, while at the 5-HT_{2b} receptor, only 6-MAPB revealed significant

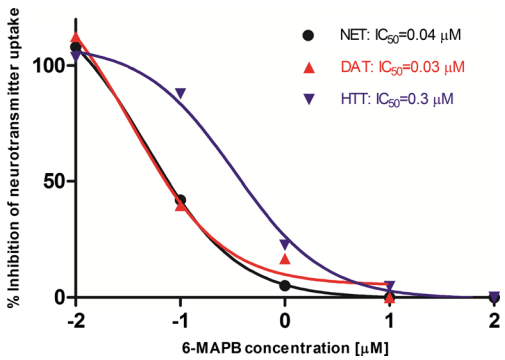

Fig. 4 IC_{50} determination of 6-MAPB on norepinephrine (NET), dopamine (DAT), and serotonin transporter (HTT) activity at a 6-MAPB concentration range of 10^{-8}–10^{-4} M. The dose–response assay was conducted with the following substrates: ^{3}H-NE (0.2 μCi/mL), ^{3}H-DAT (0.2 μCi/mL), and ^{3}H-HTT (0.2 μCi/mL) incubated at 37 °C for 15 (NET) or 20 min (DAT and HTT)

lower agonist activity than MDMA. This finding is potentially important, since activation of the 5-HT_{2b} receptor was shown to be associated with increased prevalence of valvular heart disease as well as partially mediating the effects of hallucinogenic amphetamine analogues (Baumann and Rothman 2009; Thomas 2010), while the activation of the 5-$HT_{2a,c}$ receptors regulates in part dopamine levels in the striatum, limbic system, and prefrontal cortex, as well as oxytocin and prolactin release, thereby exerting mood- and anxiety-modulating activities (Capela et al. 2009; Dumont et al. 2009). One could argue that 6-MAPB displays a more favorable in vitro pharmacological profile than MDMA, in terms of safety and efficacy based on the in vitro results.

Moreover, MDMA and its aforementioned analogues inhibited the monoamine uptake of all three transporters (DAT, NET, and HTT) to a similar extend, and no selectivity to a specific transporter was observed at a concentration of 10 μM (Fig. 3b). This finding might explain the psychostimulant effects of these drugs commonly reported by recreational drug users and in clinical studies with MDMA as well as the sympathomimetic effects of these drugs on the cardiovascular system (King 2013; Nelson et al. 2014).

Conversely, in the monoamine transporter binding assay, only the MDMA-indole-analogue, 5-IT, exhibited significant inhibition of ligand-specific binding to all three monoamine transporters, while MDMA showed only a weak to moderate percent inhibition of ligand-specific binding at HTT and DTA and non-significant effect at the NET (Fig. 3b). Among the MDMA-benzofuran analogues, 6-MAPB was the only compound inhibiting ligand-specific binding at the NET, whereas at the DAT and HHT, a significant percent binding inhibition was observed for 5- and 6-MAPB as well as 5-APB and 5-MAPB, respectively. The poor correlation observed between the functional assay and the percent inhibition of ligand-specific binding assay might be associated with the fact that transporter inhibitors and radioligands used for displacement assays bind to different sites on the transporter, and that in contrast to the binding assay, the functional assay was not conducted under steady-state conditions (Iversen et al. 2013).

Based on its favorable pharmacological profile, 6-MAPB was further selected for IC_{50} determination of monoamine uptake inhibition (Fig. 3). 6-MAPB was found to be 10 and 100 times more potent HTT and DAT inhibitor, respectively, than MDMA under similar assay conditions as reported by Bogen et al. and Escubedo et al. (Fig. 4; Bogen et al. 2003; Escubedo et al. 2011). In rat synaptosomes, it was found that the rank potency order of MDMA to inhibit monoamine transporters was HTT > NET > DAT (Capela et al. 2009; Rothman et al. 2001), whereas in the present study, 6-MAPB was found to be 10 times more potent DAT and NET inhibitor than HTT inhibitor (Fig. 4). On the other hand, due to a lack of correlation between the potency of monoamine uptake inhibition and the stimulation of monoamine release via the same

transporters, additional assays are required in order to determine separately the capabilities of 6-MAPB to release DA, NE, and 5-HT via their corresponding transporters (Escubedo et al. 2011; Rothman and Baumann 2003; Verrico et al. 2007).

In the enzyme inhibition assay (Tables 2 and S2) at 10 μM, all MDMA analogues significantly inhibited MAO A, while no inhibitory effect was observed for the other major monoamine levels regulating enzymes, MAO B, COMT, and tyrosine hydroxylase. MDMA revealed at 10 μM weak inhibitory activity of MAO A, while no inhibitory effect was observed on the other hereby tested enzymes (Table 2). These results are in accordance with the preferential inhibition of MAO A by MDMA (IC_{50} = 44 μM) reported by Leonardi and Azmitia (1994). The selective in vitro inhibition of MAO A by the MDMA analogues might have a clinical contribution to the sympathomimetic and psychostimulant effects observed with the recreational use of the aforementioned analogues (Wood et al. 2015). The selective MAO A inhibition along with the monoamine uptake inhibition might also have utility in treating depression (Hamon and Blier 2013).

The results obtained in the enzyme activity assay for 5-IT are in agreement with the results published by Herraiz and Brandt, who reported a selective inhibition of MAO A by 5-IT with an IC_{50} value of 1.6 μM (Herraiz and Brandt 2013). 5-IT was also the only MDMA analogue revealing highly potent inhibition of PKC_{α}, a central intracellular protein kinase known to be involved in diverse cellular signaling pathways (Konopatskaya and Poole 2010). Recently, de Quervain et al. reported a linkage between a genetic variability of the gene encoding PKCα and susceptibility to PTSD (de Quervain et al. 2012). Further studies are needed in order to determine whether PKC_{α} inhibition by selective inhibitors in PTSD patients might be of a potential clinical target.

Screening the drugs against a wide battery of human and non-human receptors and other CNS targets revealed a variety of other potentially significant interactions. A common target shared by all MDMA analogues was the high affinity binding to the $NE_{\alpha 2}$ receptor (Table 1). MDMA as well as 5- and 6-MAPB showed a remarkable percent of radioligand displacement at the nicotinic neuronal α4β2 receptor. α4β2 neuronal nicotinic receptors are ligand-gated ion channels, proven to play a major role in neuropathic and inflammatory pain modulation as well as in anxiety relief and reward behavior via regulation of multiple neurotransmitters (McGranahan et al. 2011; Pandya and Yakel 2013). The substantial displacement of radioligand binding at the nicotinic neuronal α4β2 receptor by MDMA at 10 μM is in alignment with the results published in several studies describing complex interaction with the same receptor (Garcia-Ratés et al. 2007, 2010).

In conclusion, all MDMA analogues displayed higher monoamine receptor binding affinities and agonist activity at the $5\text{-}HT_{2a,c}$ and $NE_{\alpha 2}$ receptors as compared to MDMA, as well as selectively inhibited MAO A activity. 6-MAPB was the only analogue displaying non-significant $5\text{-}HT_{2b}$ agonist activity and therefore was further subjected to IC_{50} determination for monoamine transporters. MDMA and its benzofuran and indole analogues at a concentration of 10 μM emerged as highly potent inhibitors of DAT, NET, and HTT. The therapeutic value of all of the above observed in vitro results remains to be elucidated in clinical trials. However, current regulations in the USA and UK make it extremely difficult for researchers to study the potential therapeutic benefits of MDMA-like compounds, resulting in a substantial hindrance in the development of new beneficial psychopharmacological substances.

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