

Monoamine receptor interaction profiles of 4-thio-substituted phenethylamines (2C-T drugs)

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ABSTRACT

Background: 4-Thio-substituted phenethylamines (2C-T drugs) are potent psychedelics with poorly defined pharmacological properties. Because of their psychedelic effects, 2C-T drugs are sometimes sold as new psychoactive substances (NPSs). The aim of the present study was to characterize the monoamine receptor and transporter interaction profiles of a series of 2C-T drugs.

Methods: We determined the binding affinities of 2C-T drugs at monoamine receptors and transporters in human cells that were transfected with the respective receptors or transporters. We also investigated the functional activation of serotonergic 5-hydroxytryptamine 2A (5-HT_{2A}) and 5-HT_{2B} receptors, activation of human trace amine-associated receptor 1 (TAAR₁), and inhibition of monoamine uptake transporters.

Results: 2C-T drugs had high affinity for 5-HT_{2A} and 5-HT_{2C} receptors (1–54 nM and 40–350 nM, respectively). With activation potencies of 1–53 nM and 44–370 nM, the drugs were potent 5-HT_{2A} receptor and 5-HT_{2B} receptor, respectively, partial agonists. An exception to this were the benzylthiophenethylamines, which did not potently activate the 5-HT_{2B} receptor (EC₅₀ > 3000 nM). Furthermore, the compounds bound to serotonergic 5-HT_{1A} and adrenergic receptors. The compounds had high affinity for the rat TAAR₁ (5–68 nM) and interacted with the mouse but not human TAAR₁. The 2C-T drugs did not potently interact with monoamine transporters (K_i > 4000 nM).

Conclusion: The receptor binding profile of 2C-T drugs predicts psychedelic effects that are mediated by potent 5-HT₂ receptor interactions.

Abbreviations: 4-bromo-2,5-dimethoxyphenethylamine, 2C-B; 2,5-dimethoxy-4-methylthiophenethylamine, 2C-T-1; 2,5-dimethoxy-4-(β-methylthio)phenethylamine, 2C-T-3; 2,5-dimethoxy-4-isopropylthiophenethylamine, 2C-T-4; 2,5-dimethoxy-4-propylthiophenethylamine, 2C-T-7; 2,5-dimethoxy-4-allylthiophenethylamine, 2C-T-16; 2,5-dimethoxy-4-*n*-butylthiophenethylamine, 2C-T-19; 2,5-dimethoxy-4-(2,2-difluoroethylthio)phenethylamine, 2C-T-21.5; 2,5-dimethoxy-4-(2,2,2-trifluoroethylthio)phenethylamine, 2C-T-22; 2,5-dimethoxy-4-isobutylthiophenethylamine, 2C-T-25; 2,5-dimethoxy-4-benzylthiophenethylamine, 2C-T-27; 2,5-dimethoxy-4-(3-fluoropropylthio)phenethylamine, 2C-T-28; 2,5-dimethoxy-4-(4-fluorobutylthio)phenethylamine, 2C-T-30; 2,5-dimethoxy-4-(4-trifluoromethylbenzylthio)phenethylamine, 2C-T-31; 2,5-dimethoxy-4-(3-methoxybenzylthio)phenethylamine, 2C-T-33; 5-hydroxytryptamine (serotonin), 5-HT; dopamine, DA; dopamine transporter, DAT; fluorescence imaging plate reader, FLIPR; high-performance liquid chromatography, HPLC; lysergic acid diethylamide, LSD; norepinephrine, NE; norepinephrine transporter, NET; new psychoactive substance, NPS; serotonin transporter, SERT; trace amine-associated receptor 1, TAAR₁.

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1. Introduction

Substituted phenethylamines are a class of drugs that includes several potent psychedelics that exert their effects through interactions with the serotonergic 5-hydroxytryptamine 2 (5-HT₂) receptor site (Glennon et al., 1982, 1984; Nelson et al., 1999; Nichols, 2004; Titeler et al., 1988). Many psychedelic phenethylamines were first synthesized by Alexander Shulgin during the 1970s and 1980s and were described in the book *PiHKAL: A Chemical Love Story* (Shulgin and Shulgin, 1995). 2C drugs are a subfamily of substituted phenethylamines, consisting of 2,5-dimethoxy-4-substituted phenethylamines. The term 2C refers to the two carbon atoms between the benzene ring and amino group (Shulgin and Shulgin, 1995). Originally proposed as psychotropic agents for psychotherapy (Shulgin and Shulgin, 1995; Shulgin and Carter, 1975), 2C drugs are now popular among recreational drug users because of their psychedelic and entactogenic properties (de Boer and Bosman, 2004;

Gonzalez et al., 2015). Today, the Internet appears to be the main source for both acquiring information on and purchasing 2C drugs and other NPSs (Brandt et al., 2014; Orsolini et al., 2017; Schifano et al., 2005). Although classic 2C drugs are considered physiologically safe, several incidences, including sympathomimetic toxicity, psychosis, and death, have been documented (Bosak et al., 2013; Curtis et al., 2003; Huang and Bai, 2011; Miyajima et al., 2008; Stoller et al., 2017). Additionally, several 2C fatalities have been reported in the media (Dean et al., 2013). Moreover, newly emerged highly potent phenethylamine hallucinogens, including *N*-(2-methoxybenzyl)-2,5-dimethoxy-4-substituted ("NBOMe") phenethylamines, were found to be unexpectedly toxic and recently associated with several fatalities (Nichols, 2016; Nikolaou et al., 2015; Poklis et al., 2014; Rose et al., 2013; Suzuki et al., 2015). We previously reported the receptor and transporter interaction profiles of 2C drugs compared with their NBOMe analogs (Rickli et al., 2015). The sulfur-containing 2C drugs (2C-T-2, 2C-T-4, and 2C-T-7) that were included in the study proved to be potent agonists at 5-HT₂ receptors (Rickli et al., 2015). Several other compounds of the 2C-T series have been described (Shulgin and Shulgin, 1995; Trachsel, 2003), but little information is available regarding their interactions with monoamine receptors and transporters. On Internet drug discussion websites such as bluelight.org, the most commonly discussed 2C-T drugs are 2C-T-2, 2C-T-4, 2C-T-7, and 2C-T-21. Other compounds of the series are only sporadically mentioned and their use does currently not seem to be widespread. However, NPSs constantly emerge and it is possible that several other 2C-T compounds will appear on the drug market in the future.

In the present study, we determined and compared the monoamine receptor and transporter affinities of 14 compounds of the 2C-T series (Fig. 1). The numbering of the compounds of the 2C-T series describes the sequence of construction and has no structural relationship (Shulgin and Shulgin, 1995). 2C-T-3 was first named 2C-T-20; however, because its amphetamine analog 2,5-dimethoxy-4-(beta-methylthio)amphetamine was originally named Aleph-3, 2C-T-20 was later renamed 2C-T-3 to maintain consistency between the 2C-T and Aleph series (Shulgin and Shulgin, 1995). The unusual number of 2C-T-21.5 is based on the fact that with its difluoroethylthio substitution, 2C-T-21.5 lies between the mono-fluorinated 2C-T-21 and tri-fluorinated 2C-T-22 (Shulgin and Shulgin, 1995).

2. Material and methods

2.1. Drugs

The 2C-T drugs were synthesized as hydrochlorides as described previously (Shulgin and Shulgin, 1995; Trachsel, 2003) and provided by ReseaChem GmbH. High-performance liquid chromatography (HPLC) purity was >98.5%. 4-Bromo-2,5-dimethoxyphenethylamine (2C-B) hydrochloride, *D*-methamphetamine hydrochloride, and lysergic acid diethylamide (LSD) were purchased from Lipomed (Arlesheim, Switzerland), with high-performance liquid chromatography (HPLC) purity > 98.5%.

2.2. 5-HT_{1A} and 5-HT_{2A} receptor radioligand binding assays

For membrane preparations, HEK 293 cells that were transiently transfected with the 5-HT_{1A} or 5-HT_{2A} receptor were released from the culture flasks using trypsin/ethylenediaminetetraacetic acid (EDTA), harvested, washed twice with ice-cold phosphate-buffered saline (PBS; without Ca²⁺ and Mg²⁺), pelleted at 1000 rotations per minute (rpm) for 5 min at 4 °C, frozen, and stored at –80 °C. Frozen pellets were suspended in 20 ml HEPES-NaOH (20 mM, pH 7.4) that contained 10 mM EDTA and homogenized with a Polytron (PT

6000, Kinematica, Luzern, Switzerland) at 14,000 rpm for 20 s. The homogenates were centrifuged at 48,000 × *g* for 30 min at 4 °C. Subsequently, the supernatants were removed and discarded, and the pellets were resuspended in 20 ml HEPES-NaOH (20 mM, pH 7.4) that contained 0.1 mM EDTA using the Polytron (20 s at 14,000 rpm). This procedure was repeated, and the final pellets were resuspended in HEPES-NaOH that contained 0.1 mM EDTA and homogenized using the Polytron. Typically, aliquots of 2 ml membrane portions were stored at –80 °C. With each new membrane batch, the dissociation constant (*K_d*) was determined by a saturation curve.

For the competitive binding assays, [³H]-8-OH-DPAT and [³H]-ketanserin were used as 5-HT_{1A} and 5-HT_{2A} receptor radioligands, respectively, at concentrations equal or close to the *K_d* values. Specific binding of the radioligands to the target receptors was defined as the difference between total binding (binding buffer alone) and nonspecific binding that was determined in the presence of 10 μM pindolol (for the 5-HT_{1A} receptor radioligand) or 10 μM spiperone (for the 5-HT_{2A} receptor radioligand). The compounds were tested at a broad range of concentrations (30 pM–30 μM) in duplicate. The test compounds were diluted in binding assay buffer at pH 7.4 (50 mM Tris/HCl, 10 mM MgCl₂, and 1 mM EGTA), and dilution curves were constructed in assay microplates (Greiner, 96-well, U-bottom, PS). Radioligand (50 μl) and the membrane suspension (100 μl) were added to the assay plates to a final volume of 200 μl in each well and incubated and shaken for 30 min at room temperature. Incubations were terminated by rapid filtration through Unifilter-96 plates (Packard Instrument Company, PerkinElmer, Schwerzenbach, Switzerland) and GF/C glass filters (PerkinElmer) that were presoaked for a minimum of 1 h in 0.3% polyethylenimine and washed three times with ice-cold washing buffer (50 mM Tris/HCl, pH 7.4). After the addition of Microscint 40 (45 μl/well, PerkinElmer), the Unifilter-96 plates were sealed. After 1 h, radioactivity was counted using a TopCount Microplate Scintillation Counter (Packard Instrument Company). IC₅₀ values were determined by calculating nonlinear regression curves for a one-site model using at least three independent 10-point concentration-response curves, run in duplicate, for each compound. *K_i* (affinity) values, which correspond to the dissociation constants, were determined using the Cheng-Prusoff equation: $K_i = IC_{50} / (1 + \text{radioligand concentration} / K_d)$. *K_i* values are presented as means ± SD (in μM).

2.3. 5-HT_{2C} receptor radioligand binding assay

For membrane preparations, HEK 293 cells that were transiently transfected with the 5-HT_{2C} receptor were released from the culture flasks using trypsin/EDTA, harvested, washed twice with ice-cold PBS (without Ca²⁺ and Mg²⁺), pelleted at 1000 rpm for 5 min at 4 °C, frozen, and stored at –80 °C. Frozen pellets were suspended in 20 ml HEPES/NaOH (20 mM, pH 7.4) that contained 10 mM EDTA and homogenized with a Polytron (PT 6000, Kinematica) at 14,000 rpm for 20 s. The homogenates were centrifuged at 48,000 × *g* for 30 min at 4 °C. Subsequently, the supernatants were removed and discarded, and the pellets were resuspended in 20 ml HEPES-NaOH (20 mM, pH 7.4) that contained 0.1 mM EDTA using the Polytron (20 s at 14,000 rpm). This procedure was repeated, and the final pellets were resuspended in HEPES/NaOH that contained 0.1 mM EDTA and homogenized using the Polytron. Typically, 2 ml aliquots of membrane portions were stored at –80 °C. With each new membrane batch, the dissociation constant (*K_d*) was determined by a saturation curve.

For the competitive binding assay, [³H]-mesulergine was used as the 5-HT_{2C} receptor radioligand at a concentration equal or close to the *K_d* value. Specific binding of the radioligand to the target

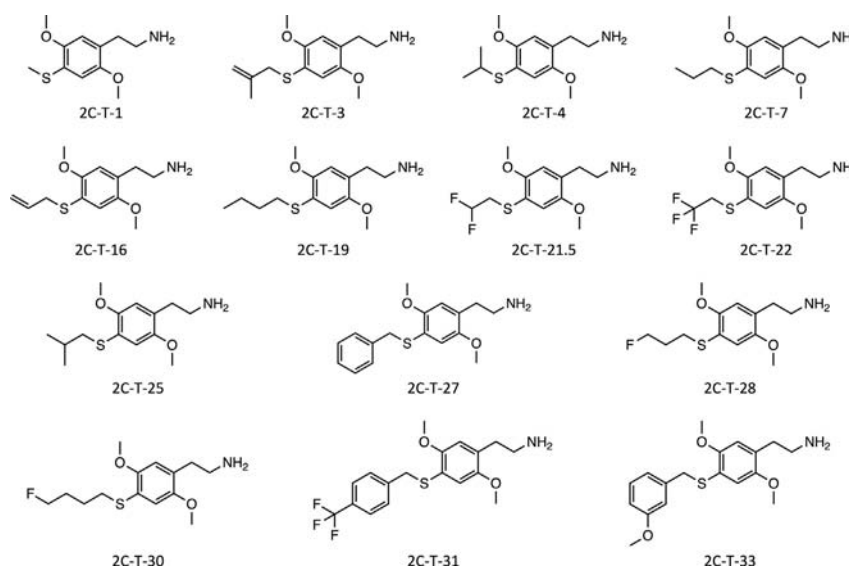


Fig. 1. Structures of 4-thio-substituted phenethylamines (2C-T drugs).

receptor was defined as the difference between total binding (binding buffer alone) and nonspecific binding that was determined in the presence of 10 μ M mianserin. The compounds were tested at a broad range of concentrations (30 pM–30 μ M) in duplicate. The test compounds were diluted in binding assay buffer at pH 7.4 (50 mM Tris/HCl, 10 mM MgCl₂, 1 mM EGTA, and 10 μ M pargyline), and dilution curves were constructed in 96-well white polystyrene assay plates (Sigma-Aldrich, Buchs, Switzerland). Membrane stocks were thawed and resuspended to a concentration of approximately 0.04 mg protein/ml binding assay buffer using a Polytron tissue homogenizer. The membrane homogenate (40 μ g/ml) was then lightly mixed for 5–30 min with YSi-poly-L-lysine (PerkinElmer) at 0.5 mg beads/well. The membrane/bead mixture (50 μ l) was added to each well of the assay plate that contained the radioligand (50 μ l) and the test compounds (final volume in each well, 200 μ l) to start the assay. The assay plates were sealed, incubated for 2 h at room temperature with agitation, and then counted in the PVT SPA counting mode of a TopCount Microplate Scintillation Counter (Packard Instrument Company). IC₅₀ values were determined by calculating nonlinear regression curves for a one-site model using at least three independent 10-point concentration-response curves, run in duplicate, for each compound. K_i (affinity) values, which correspond to the dissociation constants, were determined using the Cheng-Prusoff equation: $K_i = IC_{50} / (1 + \text{radioligand concentration} / K_d)$. K_i values are presented as means $\pm$ SD (in μ M).

2.4. Rat and mouse TAAR₁ receptor radioligand binding assays

HEK 293 cells that stably expressed rat or mouse TAAR₁ were used as described previously (Revel et al., 2011). All of the cell lines were maintained at 37 °C and 5% CO₂ in high-glucose Dulbecco's modified Eagle's medium (DMEM) that contained 10% fetal calf serum (heat-inactivated for 30 min at 56 °C), 1% penicillin/streptomycin, and 375 μ g/ml Geneticin (Gibco, Zug, Switzerland). For membrane preparation, the cells were released from the culture flasks using trypsin/EDTA, harvested, washed twice with ice-cold PBS (without Ca²⁺ and Mg²⁺), pelleted at 1000 $\times$ g for 5 min at 4 °C, frozen, and stored at –80 °C. Frozen pellets were suspended in buffer A (20 ml HEPES-NaOH [20 mM, pH 7.4] that contained 10 mM EDTA) and homogenized with a Polytron (PT 6000;

Kinematica) at 14,000 rpm for 20 s. The homogenate was centrifuged for 30 min at 48,000 $\times$ g at 4 °C. The supernatant was removed and discarded, and the pellet was resuspended in buffer A using the Polytron (20 s at 14,000 rpm). The centrifugation and removal of the supernatant was repeated, and the final pellet was resuspended in buffer A and homogenized using the Polytron. Typically, 2-ml aliquots of membrane portions were stored at –80 °C. With each new membrane batch, the dissociation constant (K_d) was determined by a saturation curve.

For the competitive binding assays, the TAAR₁ agonist [³H]-RO5166017 was used as a TAAR₁ radioligand at a concentration equal or close to the K_d values, which were usually around 0.7 nM (mouse TAAR₁) and 2.3 nM (rat TAAR₁). Nonspecific binding was defined as the amount of radioligand that bound in the presence of 10 μ M RO5166017. Compounds were tested at a broad range of concentrations (30 pM–30 μ M) in duplicate. Compounds (20 μ l/well) were transferred to a 96-deep-well plate (TreffLab, Deger-sheim, Switzerland), and 180 μ l of binding buffer (20 mM HEPES-NaOH, 10 mM MgCl₂, and 2 mM CaCl₂, pH 7.4), 300 μ l of radioligand, and 500 μ l of membranes (resuspended at 60 μ g protein/ml) were added. The plates were incubated at 4 °C for 90 min. Incubations were terminated by rapid filtration through Unifilter-96 plates (Packard Instrument Company) and GF/C glass filters (PerkinElmer) that were presoaked for 1 h in 0.3% polyethylenimine and washed three times with 1 ml of cold binding buffer. After the addition of Microscint 40 (45 μ l/well, PerkinElmer), the Unifilter-96 plate was sealed. After 1 h, radioactivity was counted using a TopCount Microplate Scintillation Counter (Packard Instrument Company). IC₅₀ values were determined by calculating nonlinear regression curves for a one-site model using at least three independent 10-point concentration-response curves, run in duplicate, for each compound. K_i (affinity) values, which correspond to the dissociation constants, were determined using the Cheng-Prusoff equation. K_i values are presented as means $\pm$ SD (in μ M).

2.5. Adrenergic α_{1A} and α_{2A} receptor radioligand binding assays

CHO cells were stably transfected with the adrenergic α_{1A} receptor and maintained at 37 °C and 5% CO₂ in HAM's F12 medium (Invitrogen) that contained 10% fetal calf serum (heat inactivated for 30 min at 56 °C), 150 μ g/ml Geneticin (Gibco, Zug, Switzerland),

and 1% penicillin/streptomycin. CHL cells that stably expressed the adrenergic α_{2A} receptor were maintained at 37 °C and 5% CO₂ in high-glucose DMEM that contained 5% fetal calf serum (heat inactivated for 30 min at 56 °C) and 250 µg/ml Geneticin (Gibco, Zug, Switzerland).

For membrane preparations, the cells were released from the culture flasks using trypsin/EDTA, harvested, washed twice with ice-cold PBS (without Ca²⁺ and Mg²⁺), pelleted at 1000 rpm for 5 min at 4 °C, frozen, and stored at –80 °C. Frozen pellets were suspended in 20 ml HEPES-NaOH (20 mM, pH 7.4) that contained 10 mM EDTA and homogenized with a Polytron (PT 6000, Kinematica) at 14,000 rpm for 20 s. The homogenates were centrifuged at 48,000 × g for 30 min at 4 °C. Subsequently, the supernatants were removed and discarded, and the pellets were resuspended in 20 ml of HEPES-NaOH (20 mM, pH 7.4) that contained 0.1 mM EDTA using the Polytron (20 s at 14,000 rpm). This procedure was repeated, and the final pellets were resuspended in HEPES-NaOH that contained 0.1 mM EDTA and homogenized using the Polytron. Typically, 2 ml aliquots of membrane portions were stored at –80 °C. With each new membrane batch, the dissociation constant (K_d) was determined by a saturation curve.

For the competitive binding assays, [³H]-prazosin and [³H]-rauwolscine were used as adrenergic α_{1A} and adrenergic α_{2A} receptor radioligands, respectively, at concentrations equal or close to the K_d values. Specific binding of the radioligands to the target receptors was defined as the difference between total binding (binding buffer alone) and nonspecific binding that was determined in the presence of 10 µM chlorpromazine (for the α_{1A} receptor radioligand) and 10 µM phentolamine (for the α_{2A} receptor radioligand). The compounds were tested at a broad range of concentrations (30 pM–30 µM) in duplicate. The test compounds were diluted in binding assay buffer (50 mM Tris/HCl, pH 7.4), and dilution curves were constructed in assay microplates (Greiner, 96 well, U-bottom, PS). Radioligand (50 µl) and the membrane suspension (100 µl) were added to the assay plates and incubated and shaken for 1 h at room temperature. Incubations were terminated by rapid filtration through Unifilter-96 plates (Packard Instrument Company) and GF/C glass filters (PerkinElmer) that were presoaked for 1 h in 0.3% polyethylenimine and washed three times with 1 ml cold binding assay buffer. After the addition of Microscint 40 (45 µl/well, PerkinElmer), the Unifilter-96 plates were sealed. After 1 h, radioactivity was counted using a TopCount Microplate Scintillation Counter (Packard Instrument Company). IC₅₀ values were determined by calculating nonlinear regression curves for a one-site model using at least three independent 10-point concentration-response curves, run in duplicate, for each compound. K_i (affinity) values, which correspond to the dissociation constants, were determined using the Cheng-Prusoff equation: $K_i = IC_{50} / (1 + \text{radioligand concentration} / K_d)$. K_i values are presented as means ± SD (in µM).

2.6. D₂ receptor radioligand binding assay

For membrane preparations, HEK 293 cells that were transiently transfected with the dopamine D₂ receptor were released from the culture flasks using trypsin/EDTA, harvested, washed twice with ice-cold PBS (without Ca²⁺ and Mg²⁺), pelleted at 1000 rpm for 5 min at 4 °C, frozen, and stored at –80 °C. Frozen pellets were suspended in 20 ml of HEPES-NaOH (20 mM, pH 7.4) that contained 10 mM EDTA and homogenized with a Polytron (PT 6000, Kinematica) at 14,000 rpm for 20 s. The homogenates were centrifuged at 48,000 × g for 30 min at 4 °C. Subsequently, the supernatants were removed and discarded, and the pellets were resuspended in 20 ml of HEPES-NaOH (20 mM, pH 7.4) that contained 0.1 mM EDTA using the Polytron (20 s at 14,000 rpm). This procedure was

repeated, and the final pellets were resuspended in HEPES-NaOH that contained 0.1 mM EDTA and homogenized using the Polytron. Typically, 2 ml aliquots of membrane portions were stored at –80 °C. With each new membrane batch, the dissociation constant (K_d) was determined by a saturation curve.

For the competitive binding assays, [³H]-spiperone was used as the dopamine D₂ receptor radioligand at a concentration equal or close to the K_d value. Specific binding of the radioligand to the target receptor was defined as the difference between total binding (binding buffer alone) and nonspecific binding that was determined in the presence of 10 µM spiperone. The compounds were tested at a broad range of concentrations (30 pM–30 µM) in duplicate. The test compounds were diluted in binding assay buffer at pH 7.4 (50 mM Tris/HCl, 5 mM MgCl₂, 1 mM EDTA, 5 mM KCl, 1.5 mM CaCl₂, and 120 mM NaCl), and dilution curves were constructed in assay microplates (Greiner, 96 well, U-bottom, PS). Radioligand (50 µl) and the membrane suspension (100 µl) were added to the assay plates (final volume in each well, 200 µl) and incubated and shaken for 1 h at room temperature. Incubations were terminated by rapid filtration through Unifilter-96 plates (Packard Instrument Company) and GF/C glass filters (PerkinElmer) that were presoaked for a minimum of 1 h in 0.3% polyethylenimine and washed three times with ice-cold washing buffer (50 mM Tris/HCl, pH 7.4). After the addition of Microscint 40 (45 µl/well, PerkinElmer), the Unifilter-96 plates were sealed. After 1 h, radioactivity was counted using a TopCount Microplate Scintillation Counter (Packard Instrument Company). IC₅₀ values were determined by calculating nonlinear regression curves for a one-site model using at least three independent 10-point concentration-response curves, run in duplicate, for each compound. K_i (affinity) values, which correspond to the dissociation constants, were determined using the Cheng-Prusoff equation: $K_i = IC_{50} / (1 + \text{radioligand concentration} / K_d)$. K_i values are presented as means ± SD (in µM).

2.7. Monoamine transporter radioligand binding assays

HEK 293 cells that stably expressed the human norepinephrine transporter (hNET), human serotonin transporter (hSERT), or human dopamine transporter (hDAT) were cultured, and cells were collected and washed three times with ice-cold PBS, pelleted at 1000 rpm for 5 min at 4 °C, frozen, and stored at –80 °C. Frozen pellets were then resuspended in 400 ml of HEPES-NaOH (20 mM, pH 7.4) that contained EDTA (10 mM) at 4 °C. After homogenization with a Polytron (PT 6000, Kinematics) at 10,000 rpm for 15 s, the homogenates were centrifuged at 48,000 × g for 30 min at 4 °C. Aliquots of the membrane stocks were frozen at –80 °C.

For the competitive binding assays, *N*-methyl-[³H]-nisoxetine, [³H]-citalopram, and [³H]-WIN35,428 were used as hNET, hSERT, and hDAT radioligands, respectively, at concentrations equal or close to the K_d values. Specific binding of the radioligand to the target transporters was defined as the difference between total binding (binding buffer alone) and nonspecific binding that was determined in the presence of 10 µM indatraline. The compounds were tested at a broad range of concentrations (30 pM–30 µM) in duplicate. The test compounds were diluted in binding assay buffer at pH 7.4 (126 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.76 mM KH₂PO₄), and dilution curves were constructed in 96-well Opti-Plates (PerkinElmer). Membrane stocks were thawed and resuspended to a concentration of approximately 60 µg protein/ml binding assay buffer using a Polytron tissue homogenizer. The membrane homogenates were then lightly mixed for 5–30 min with polyvinyl toluene (PVT) wheatgerm agglutinin-coated scintillation proximity assay beads (WGA-SPA, Amersham Biosciences) at 11.5 mg beads/well. The membrane/bead mixture (50 µl) was

added to each well of the assay plate that contained the radioligand (50 μ l) and the test compounds (final volume in each well, 200 μ l) to start the assay. The assay plates were sealed, incubated for 2 h at room temperature with agitation, and counted in the PVT SPA counting mode of a TopCount Microplate Scintillation Counter (Packard Instrument Company). IC₅₀ values were determined by calculating nonlinear regression curves for a one-site model using at least three independent 10-point concentration-response curves, run in duplicate, for each compound. K_i (affinity) values, which correspond to the dissociation constants, were determined using the Cheng-Prusoff equation: $K_i = IC_{50} / (1 + \text{radioligand concentration} / K_d)$. K_i values are presented as means $\pm$ SD (in μ M).

2.8. Functional activity at the serotonin 5-HT_{2A} receptor

Mouse embryonic fibroblasts (NIH-3T3 cells) that expressed the human 5-HT_{2A} receptor were incubated in HEPES-Hank's Balanced Salt Solution (HBSS) buffer (Gibco, Zug, Switzerland; 70,000 cells/100 μ l) for 1 h at 37 °C in 96-well poly-D-lysine-coated plates. To each well, 100 μ l of dye solution (fluorescence imaging plate reader [FLIPR] calcium 5 assay kit; Molecular Devices, Sunnyvale, CA, USA) was added, and the plates were incubated for 1 h at 37 °C. The plates were placed in a FLIPR, and 25 μ l of the test drugs that were diluted in HEPES-HBSS buffer that contained 250 mM probenidol was added online. The increase in fluorescence was then measured, and EC₅₀ values were derived from the concentration-response curves using nonlinear regression. The maximal receptor activity (efficacy) is expressed relative to 5-HT activity, which was set to 100%.

2.9. Functional activity at the serotonin 5-HT_{2B} receptor

HEK 293 cells that expressed the human 5-HT_{2B} receptor were incubated in growth medium (high-glucose DMEM; Invitrogen, Zug, Switzerland), 10 ml/L penicillin/streptomycin (Gibco, Zug, Switzerland), 10% fetal calf serum (non-dialyzed, heat-inactivated), and 250 mg/L Geneticin at a density of 50,000 cells/well at 37 °C in poly-D-lysine-coated 96-well plates overnight. The growth medium was then removed by snap inversion, and 100 μ l of the calcium indicator Fluo-4 solution (Molecular Probes, Eugene, OR, USA) was added to each well. The plates were incubated for 45 min at 31 °C before the Fluo-4 solution was removed by snap inversion, and 100 μ l of Fluo-4 solution was added a second time for 45 min at 31 °C. The cells were washed with HBSS and 20 mM HEPES (assay buffer) immediately before testing using an EMBLA cell washer, and 100 μ l assay buffer was added. The plates were placed in a FLIPR, and 25 μ l of the test substances that were diluted in assay buffer was added online. The increase in fluorescence was then measured, and EC₅₀ values were derived from the concentration-response curves using nonlinear regression. The maximal receptor activity (efficacy) is expressed relative to 5-HT activity, which was set to 100%.

2.10. Functional activity at the human TAAR₁ receptor

Recombinant HEK 293 cells that expressed human TAAR₁ were grown at 37 °C and 5% CO₂/95% air in 250 ml Falcon culture flasks in 30 ml of culture medium. The cell culture medium contained high-glucose DMEM, 10% fetal calf serum (heat inactivated for 30 min at 56 °C), 500 μ g/ml Geneticin (Gibco), and 500 μ g/ml hygromycin B. Cells were harvested when 80–90% confluence was reached. The culture medium was removed from the culture flasks, and the cells were washed once with 5 ml of PBS. After removing the wash solution, 5 ml of trypsin/EDTA solution was added for 5 min at 37 °C. Afterward, 45 ml of culture medium was added to the 5 ml

detached cell solution, and 50 ml was transferred to a Falcon tube. The tube was centrifuged at 900 rpm for 3 min at room temperature, and the supernatant was removed. The cell pellet was resuspended in fresh culture medium and brought to 5×10^5 cells per ml. The cells were then plated into 96-well plates (BIOCOAT 6640, Becton Dickinson, Allschwil, Switzerland) with a multipipette (100 μ l/well, 80,000 cells/well) and incubated for 20 h at 37 °C. For the cAMP assay, the cell culture medium was removed, and 50 μ l of PBS (without Ca²⁺ and Mg²⁺) was added. Afterward, PBS was removed by snap inversion followed by gently tapping of the plate on a tissue. Krebs-Ringer bicarbonate buffer (90 μ l; Sigma-Aldrich) that contained 1 mM IBMX was added, and the plates were incubated for 60 min at 37 °C and 5% CO₂/95% air. All of the compounds were tested at a broad range of concentrations (300 pM–30 μ M) in duplicate, and a standard curve (0.13 nM–10 μ M cAMP) was constructed on each plate. A reference plate that included RO5256390, β -phenylethylamine, and p-tyramine also accompanied each experiment. Typically, 30 μ l of a compound solution, 30 μ l of β -phenylethylamine (as maximal response), or a basal control in PBS that contained 1 mM IBMX was then added, and the cells were incubated for 40 min at 37 °C. Afterward, the cells were lysed with 50 μ l of 3 $\times$ detection mix solution that contained Ru-cAMP Alexa700 anti-cAMP antibody and lysis buffer for 120 min at room temperature under strong shaking using black lids. Fluorescence was measured using a NanoScan (IOM reader; 456 nm excitation wavelength; 630 and 700 nm emission wavelengths). The FRET signal was calculated as the following: FRET (700 nm) – P $\times$ FRET (630 nm), where P = Ru (700 nm) / Ru (630 nm).

2.11. Monoamine uptake transporter inhibition

The monoamine transporter inhibition potential of the 2C-T drugs was assessed for a single high concentration of 10 μ M to exclude activity. Monoamine uptake was determined in HEK 293 cells that stably expressed the hNET, hDAT, and hSERT (Tatsumi et al., 1997) as previously described in detail (Hysek et al., 2012). Briefly, cells were cultured to 70–90% confluence in DMEM (10% fetal calf serum and 250 μ g/ml Geneticin, both from Gibco), detached, and resuspended in Krebs-Ringer bicarbonate buffer (Sigma-Aldrich) at a density of 3×10^6 cells/ml. For [³H]-DA uptake experiments, the uptake buffer was supplemented with 0.2 mg/ml ascorbic acid. The cell suspension (100 μ l) was incubated with 25 μ l of the test drugs, vehicle control, and transporter-specific inhibitors (10 μ M nisoxetine for NET, 10 μ M mazindol for DAT, and 10 μ M fluoxetine for SERT) dissolved in buffer for 10 min in a round-bottom 96-well plate at room temperature at 450 rpm on a rotary shaker. Monoamine uptake transport was then initiated by adding 50 μ l of [³H]-NE (13.1 Ci/mmol; PerkinElmer), [³H]-DA (30.0 Ci/mmol, PerkinElmer), or [³H]-5-HT (80.0 Ci/mmol; Anawa, Zürich, Switzerland) dissolved in buffer at a final concentration of 5 nM for an additional 10 min. The cell suspension (100 μ l) was then transferred to 500 μ l microcentrifuge tubes that contained 50 μ l of 3 M KOH and 200 μ l silicon oil (1:1 mixture of silicon oil types AR 20 and AR 200; Sigma-Aldrich). To separate the cells from the uptake buffer, they were centrifuged through silicone oil for 3 min at $16,550 \times g$, and the tubes were frozen in liquid nitrogen immediately afterward. The cell pellet was then cut into 6 ml scintillation vials (PerkinElmer) that contained 0.5 ml lysis buffer (0.05 M Tris-HCl, 50 mM NaCl, 5 mM EDTA, and 1% NP-40 in water), and the samples were shaken for 1 h before 5 ml of scintillation fluid (Ultimagold, PerkinElmer) was added. Monoamine uptake was then quantified by liquid scintillation counting on a Packard 1900 TR Tri-Carb Liquid Scintillation Counter (Packard Instrument Company). Nonspecific uptake that was determined in the presence of selective inhibitors was subtracted from the total counts, and

monoamine uptake was compared with the vehicle control.

2.12. Statistical analysis

IC₅₀ values of radioligand binding were determined by calculating nonlinear regression curves for a one-site model using at least three independent 10-point concentration-response curves for each substance. K_i (affinity) values, which correspond to the dissociation constants, were calculated using the Cheng-Prusoff equation. Nonlinear regression concentration-response curves were used to determine EC₅₀ values for 5-HT_{2A} and 5-HT_{2B} receptor activation. Efficacy (maximal activity) is expressed relative to the activity of 5-HT, which was used as a control set to 100%. Monoamine uptake of three independent experiments was compared with vehicle controls using analysis of variance followed by Dunnett's multiple-comparison test.

3. Results

3.1. Interactions with serotonin receptors

The binding affinities and activation potency of 2C-T drugs to serotonin receptors are listed in Table 1. All of the drugs bound to the 5-HT_{1A} receptor in the range of 660–2370 nM and bound to the 5-HT_{2A} receptor in the range of 1–54 nM, with an activation potency of 1–53 nM and activation efficacy of 3–75%. All of the drugs except 2C-T-27 and 2C-T-33 activated the 5-HT_{2B} receptor, with an activation potency of 44–3310 nM and activation efficacy of 28–75%. All of the drugs bound to the 5-HT_{2C} receptor with affinities of 40–350 nM. 2C-B and LSD were included in Table 1 as reference psychedelics for comparison.

3.2. Binding to monoamine receptors and transporters

Binding affinities of 2C-T drugs to monoamine transporters and receptors are listed in Table 2. None of the drugs bound to the tested binding region of the NET at the investigated concentration range. Only 2C-T-27, 2C-T-31, and 2C-T-33 bound to the DAT, with affinities of 4.8–7.7 μ M. Only 2C-T-3 bound to the SERT, with a K_i of 7.2 μ M. No affinity for adrenergic α_{1A} or dopamine D₂ receptors was detected *in vitro*, with the exception of 2C-T-28, 2C-T-30, 2C-T-31, and 2C-T-33, which bound to the α_{1A} receptor with a K_i of 2.3–3.6 μ M. However, the drugs bound to the α_{2A} receptor in the range of 97–804 nM. Furthermore, the drugs bound to rat and mouse TAAR₁, with affinities of 5–68 nM and 55–2340 nM, respectively, but did not activate the human TAAR₁ at the investigated concentration range. As reference, the TAAR₁ interactions of the partial agonist (Simmler et al., 2016) D-methamphetamine are listed in Table 2.

3.3. Monoamine uptake transporter inhibition

No significant NE, DA, or 5-HT uptake inhibition was observed for any of the 2C-T drugs at 10 μ M (data not shown).

4. Discussion

The compounds had 17- to 830-fold higher affinity for the 5-HT_{2A} vs. 5-HT_{1A} receptor and 4- to 44-fold higher affinity for the 5-HT_{2A} vs. 5-HT_{2C} receptor. Similarly, selectivity for the 5-HT₂ receptor site has been shown for 2C drugs, NBOMe derivatives, and phenylisopropylamine hallucinogens (substituted amphetamines) but not for other psychedelics of the tryptamine class or LSD (Fantegrossi et al., 2005; Halberstadt and Geyer, 2011; Pierce and Peroutka, 1989; Rickli et al., 2015, 2016; Titeler et al., 1988). In

accordance with those findings, in our study 2C-B had selectivity ratios in the range of the 2C-T drugs and LSD was less selective with 5-HT_{2A} vs. 5-HT_{1A} and 5-HT_{2A} vs. 5-HT_{2C} ratios of 0.28 and 2.6, respectively.

The three benzylthiophenethylamines (2C-T-27, 2C-T-31, and 2C-T-33) most potently bound to the 5-HT_{2A} receptor, with affinities of 1.6–3.8 nM. However, they had the lowest receptor activation potential for the 5-HT_{2A} receptor (26–53 nM) and negligible or no activation potential for the 5-HT_{2B} receptor. These three benzylthiophenethylamines also most potently bound to the 5-HT_{2A} receptor in a previous test series of 11 2C-T drugs using [³H]-LSD as the radioligand (Trachsel et al., 2013). High affinity and antagonistic properties could be expected because of the bulky and lipophilic 4-substituent (Hansen et al., 2014; Nichols et al., 1977; Seggel et al., 1990). The remaining compounds all activated the 5-HT_{2A} and 5-HT_{2B} receptors in the range of 1–15 nM and 44–370 nM, respectively, with an activation efficacy of 36–75% and 28–75%, respectively. Drugs of the 2C-T series can therefore be classified as partial agonists as previously shown for other 2C drugs (Moya et al., 2007; Rickli et al., 2015) and similar psychedelic effects may be expected. Several fluorine-containing compounds were investigated. The 5-HT_{2A} and 5-HT_{2C} receptor affinity of 2C-T-7 was higher (3.2- and 2.5-fold, respectively) compared with its monofluorinated analog 2C-T-28. Compared with its monofluorinated analog 2C-T-30, 5-HT₂ binding affinity also increased for 2C-T-19 (1.4- and 1.6-fold increase in affinity for 5-HT_{2A} and 5-HT_{2C} receptors, respectively). A decrease in affinity by fluorine has previously been described for 2C-T-30 in radioligand binding assays using [³H]-LSD-labeled cloned 5-HT_{2A} receptors (Trachsel, 2012). However, although 2C-T-7 had a higher activation potential for 5-HT_{2A} and 5-HT_{2B} receptors compared with 2C-T-28, the activation potential of 2C-T-19 for both receptors was lower compared with 2C-T-30. The difluoroethyl derivative 2C-T-21.5 and trifluoroethyl derivative 2C-T-22 had comparable affinities for 5-HT_{2A} and 5-HT_{2C} receptors. However, 2C-T-21.5 had a higher activation potential for the 5-HT_{2A} receptor, whereas 2C-T-22 had a higher activation potential for the 5-HT_{2B} receptor. In a previous study (Rickli et al., 2015), 2C-T-2, the non-fluorinated analog of 2C-T-21.5 and 2C-T-22, had slightly higher affinity for 5-HT_{2A} and 5-HT_{2C} receptors (9 and 67 μ M, respectively) compared with 2C-T-21.5 and 2C-T-22. In the present study, however, the 5-HT_{2A} and 5-HT_{2B} receptor activation potency of 2C-T-21.5 and 2C-T-22, respectively, was higher than the previously determined receptor activation potency for 2C-T-2 (Rickli et al., 2015). Therefore, although terminal fluorinating appears to decrease 5-HT receptor affinity, it may increase activation potential in some cases.

In the radioligand binding assay, none of the substances bound to the investigated NET binding site, and none of the compounds significantly inhibited NE uptake at 10 μ M. Only 2C-T-27, 2C-T-31, and 2C-T-33 bound to the DAT, with affinities of 4.8–7.7 μ M. Only 2C-T-3 bound to the SERT, with an affinity of 7.2 μ M. However, no DA or 5-HT uptake inhibition was observed for any of the compounds at 10 μ M. Transporter binding and inhibition may occur at higher concentrations, which has been shown previously for 2C-T-2, 2C-T-4, and 2C-T-7 (Rickli et al., 2015). However, given the potent interactions with serotonergic receptors, the monoamine transporter interactions of 2C-T drugs are likely not clinically relevant and were not investigated at higher concentrations in the present study. No interactions with dopaminergic D₂ or adrenergic α_{1A} receptors were observed in the radioligand binding assays, with the exception of low-affinity binding of 2C-T-28, 2C-T-30, 2C-T-31, and 2C-T-33 to the α_{1A} receptor. However, all of the drugs bound to the α_{2A} receptor, with an affinity of 97–804 nM. The selectivity for α_{2A} over α_{1A} receptors has previously been reported for 2C drugs but not NBOMe compounds, which bound to both receptors with

Table 1

Serotonin receptor binding affinities and activation potencies.

	5-HT _{1A}	5-HT _{2A}			5-HT _{2B}		5-HT _{2C}	Selectivity (binding ratios)	
	Receptor binding	Receptor binding	Activation potency	Activation efficacy	Activation potency	Activation efficacy	Receptor binding	5-HT _{2A} /5-HT _{1A}	5-HT _{2A} /5-HT _{2C}
	K _i ± SD [nM]	K _i ± SD [nM]	EC ₅₀ ± SD [nM]	max ± SD [%]	EC ₅₀ ± SD [nM]	max ± SD [%]	K _i ± SD [nM]		
2C-T-1	1035 ± 125	49 ± 21	2.0 ± 0.1	75 ± 3	57 ± 38	58 ± 11	347 ± 129	21	7.1
2C-T-3	812 ± 161	11 ± 5	7.7 ± 1.6	44 ± 6	44 ± 8	28 ± 7	40 ± 6	74	3.6
2C-T-4	916 ± 86	54 ± 21	5.5 ± 0.7	56 ± 5	63 ± 11	75 ± 10	295 ± 28	17	5.5
2C-T-7	878 ± 97	5.3 ± 0.6	1.2 ± 0.4	49 ± 12	52 ± 10	46 ± 12	54 ± 25	166	10
2C-T-16	660 ± 74	9.2 ± 3.6	1.3 ± 0.6	57 ± 9	47 ± 32	36 ± 1	67 ± 15	72	7.3
2C-T-19	1019 ± 129	6.9 ± 3.3	12 ± 2	55 ± 6	369 ± 188	40 ± 3	101 ± 25	148	15
2C-T-21.5	1321 ± 193	14 ± 3	4.6 ± 1.4	66 ± 7	182 ± 12	40 ± 4	159 ± 77	94	11
2C-T-22	1915 ± 53	16 ± 1	15 ± 5	36 ± 2	110 ± 55	35 ± 15	151 ± 45	120	9.4
2C-T-25	1036 ± 134	21 ± 7	12 ± 2	49 ± 7	108 ± 35	32 ± 11	80 ± 32	49	3.8
2C-T-27	1166 ± 147	1.6 ± 0.5	26 ± 2	27 ± 4	>10,000		52 ± 12	729	33
2C-T-28	1904 ± 42	17 ± 6	5.7 ± 0.3	45 ± 7	81 ± 23	34 ± 16	135 ± 38	112	7.9
2C-T-30	2368 ± 22	9.5 ± 2.0	5.7 ± 2.4	40 ± 1	51 ± 34	61 ± 10	158 ± 45	249	17
2C-T-31	1063 ± 51	3.8 ± 1.1	53 ± 12	2.8 ± 0.7	3309 ± 1084	44 ± 13	157 ± 8	280	41
2C-T-33	1411 ± 38	1.7 ± 1.3	26 ± 8	40 ± 1	>10,000		75 ± 6	830	44
2C-B	311 ± 46	6.9 ± 1.8	2.1 ± 0.8	92 ± 8	75 ± 14	52 ± 26	43 ± 4	45	6.2
LSD	1.5 ± 0.4	5.3 ± 3.4	44 ± 14	73 ± 2	>10,000		14 ± 3	0.28	2.6

K_i and EC₅₀ values are given as nM (mean ± SD); activation efficacy (E_{max}) is given as percentage of maximum ± SD.**Table 2**

Monoamine receptor and transporter binding affinities.

	human TAAR ₁	rat TAAR ₁	mouse TAAR ₁	α _{1A}	α _{2A}	D ₂	NET	DAT	SERT
	EC ₅₀ [nM]	K _i [nM]	K _i [nM]	K _i [nM]	K _i [nM]	K _i [nM]	K _i [nM]	K _i [nM]	K _i [nM]
2C-T-1	>30,000	52 ± 8	1877 ± 661	>6510	334 ± 43	>4400	>9710	>8710	>8580
2C-T-3	>30,000	8.0 ± 1.3	465 ± 236	>6510	97 ± 12	>4400	>9710	>8710	7221 ± 470
2C-T-4	>30,000	19 ± 5	2337 ± 911	>6510	217 ± 36	>4400	>9710	>8710	>8580
2C-T-7	>30,000	10 ± 0	311 ± 61	>6510	335 ± 49	>4400	>9710	>8710	>8580
2C-T-16	>30,000	17 ± 7	453 ± 133	>6510	229 ± 17	>4400	>9710	>8710	>8580
2C-T-19	>30,000	4.8 ± 1.5	96 ± 33	>6510	458 ± 45	>4400	>9710	>8710	>8580
2C-T-21.5	>30,000	68 ± 11	1674 ± 185	>6510	383 ± 26	>4400	>9720	>8710	>7510
2C-T-22	>30,000	38 ± 15	974 ± 17	>6510	592 ± 39	>4400	>9720	>8710	>7510
2C-T-25	>30,000	11 ± 1	359 ± 95	>6510	279 ± 6	>6270	>9720	>8710	>7510
2C-T-27	>30,000	10 ± 2	596 ± 197	>6510	351 ± 43	>6270	>9720	4760 ± 569	>7510
2C-T-28	>30,000	62 ± 19	426 ± 116	2730 ± 653	331 ± 31	>6270	>9720	>8710	>7510
2C-T-30	>30,000	29 ± 3	182 ± 52	2297 ± 134	408 ± 42	>6270	>9720	>8710	>7510
2C-T-31	>30,000	5.2 ± 0.6	55 ± 9	2534 ± 88	804 ± 126	>6270	>9720	5474 ± 54	>7510
2C-T-33	>30,000	38 ± 0	761 ± 117	3628 ± 308	343 ± 72	>6270	>9720	7706 ± 249	>7510
<i>d</i> -Methamphetamine	3707 ± 587	257 ± 15	889 ± 49						

K_i and EC₅₀ values are given as nM (mean ± SD).

submicromolar affinity (Rickli et al., 2015). All of the drugs potently bound to the rat TAAR₁ in the range of 5–68 nM and less potently to the mouse TAAR₁ in the range of 55–2340 nM. However, the compounds were inactive at the human TAAR₁ in the functional assays (EC₅₀ > 30 μM). Consistent with our results, a rank order affinity for rat > mouse > human TAAR₁ has previously been described for substituted phenethylamines with bulky residues (Lewin et al., 2008; Simmler et al., 2016; Waincott et al., 2007).

The psychoactive dose of phenethylamines cannot be explained solely by data from *in vitro* assays. For example, Shulgin proposed a dose of 60–100 mg and duration of 3–5 h for 2C-T-1; for 2C-T-4, he proposed a human dose of 8–20 mg and duration of 12–18 h (Shulgin and Shulgin, 1995). However, receptor binding affinities in the radioligand assays for these two compounds were comparable, and the activation potential for the 5-HT_{2A} receptor was even higher for 2C-T-1 than for 2C-T-4. Therefore, other factors, such as lipophilicity, functional selectivity, and monoamine oxidase (MAO) and cytochrome P450 (CYP) metabolism, may influence the dose and effect of the compounds. Shulgin mentioned an unusual subjective variability for 2C-T drugs in the book *PIHKAL* (Shulgin and

Shulgin, 1995). MAO-A, MAO-B, and to a lesser extent CYP2D6 were identified as the main enzymes that are involved in the deamination of 2C-T-2 and 2C-T-7 (Theobald and Maurer, 2007). A study with methoxylated and alkylthio amphetamine derivatives, however, did not find a correlation between the MAO inhibitory potential of the drugs and hallucinogenic potency reported in humans (Scorza et al., 1997). Other human doses were reported for most of the 2C-T drugs, and dose estimates started at 8 mg for the most potent compounds (Shulgin and Shulgin, 1995; Trachsel et al., 2013). A higher human dose (80–130 mg) was described for 2C-T-27 (Trachsel, 2012), consistent with the lower 5-HT_{2A} receptor activation potency that was found in the present study.

5. Conclusion

We characterized sparsely studied potent psychoactive drugs, providing insights into the relationship between the structure and 5-HT₂ receptor binding and activation of psychedelic phenethylamines. 2C-T drugs potently bound to 5-HT_{2A} and 5-HT_{2C} receptors and had affinity for 5-HT_{1A} and α_{2A} receptors. Furthermore, 2C-T

drugs were potent 5-HT_{2A} and 5-HT_{2B} partial agonists with the exception of benzylthiophenethylamines, which had no or negligible activation potential for the 5-HT_{2B} receptor.

Author contributions

D.L., D.T., and M.E.L. designed the research. D.L. and M.C.H. performed the research. D.L., M.C.H., and M.E.L. analyzed the data. D.L., M.C.H., and M.E.L. wrote the paper.

Conflict of interest

D.T. is an employee of ReseaChem GmbH. M.C.H. is an employee of F. Hoffmann-La Roche. The other authors do not have any conflicts of interest to declare for this work.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.neuropharm.2017.07.012>.

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