In vitro pharmacological characterization of novel psychoactive substances and *in vivo* analysis of psilocin for clinical applications

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Prof. Dr Marcel Mayor

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The Dean of Faculty

To my wonderful family and friends.

Thank you for all the love and support.

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“Nic dwa razy się nie zdarza
i nie zdarzy. Z tej przyczyny
zrodziliśmy się bez wprawy
i pomrzemy bez rutyny.

Choćbyśmy uczniami byli
najtępszymi w szkole świata,
nie będziemy repetować
żadnej zimy ani lata… ”

“Nic dwa razy”, Wisława Szymborska
Nobel Prize Laureate in Literature, 1996

I. Project Overview

This PhD thesis is divided into two major project themes. In Part A, the *in vitro* pharmacological characterization of novel psychoactive substances and new psychedelic derivatives are described and are accompanied by two publications. In Part B, the *in vivo* bioanalysis of psilocin for clinical applications is explored and is accompanied by one methodological publication. Data included in these three publications were completed in the Clinical Pharmacology and Toxicology/Psychopharmacology Research laboratory and at the Neuroscience Research (pRED) Roche Innovation Centre based in Basel, Switzerland. The projects were conducted between January 2018 to August 2021 and all herein described publications were published in peer-reviewed research journals.

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II. Abbreviations

2,4,5-trimethoxyamphetamine (**TMA-2**)

2,4,5-trimethoxyphenethylamine (**2C-O-1**)

2,5-dimethoxy-4-(2-fluoroethoxy) amphetamine (**MFEM**)

2,5-dimethoxy-4-(2-fluoroethoxy) phenethylamine (**2C-O-21**)

2,5-dimethoxy-4-(2,2,2-trifluoroethoxy) amphetamine (**MTFEM**)

2,5-dimethoxy-4-(2,2,2-trifluoroethoxy) phenethylamine (**2C-O-22**)

2,5-dimethoxy-4-ethoxyamphetamine (**MEM**)

2,5-dimethoxy-4-ethoxyphenethylamine (**2C-O-2**)

2,5-dimethoxy-4-ethylamphetamine (**DOET**)

2,5-dimethoxy-4-isopropoxyamphetamine (**MIPM**)

2,5-dimethoxy-4-isopropoxyphenethylamine (**2C-O-4**)

2,5-dimethoxy-4-methallyloxyamphetamine (**MMALM**)

2,5-dimethoxy-4-methallyloxyphenethylamine (**2C-O-3**)

2,5-dimethoxy-4-methylamphetamine (**DOM**)

2,5-dimethoxy-4-methylphenethylamine (**2C-D**)

2,5-dimethoxy-4-propoxyampehtamine (**MPM**)

3,4-dihydroxyamphetamine (**HHA**)

3,4-dihydroxymethamphetamine (**HHMA**)

3,4-dihydroxymethcathinone (**HHMC**)

3,4-dihydroxypyrovalerone (**HHPV**)

3,4-methylenedioxyamphetamine (**MDA**)

3,4-methylenedioxycathinone (**MDC**)

3,4-methylenedioxymethamphetamine (**MDMA**)

3,4-methylenedioxymethcathinone (**methylon**e)

3,4-methylenedioxypyrovalerone (**MDPV**)

3,4,5-trimethoxyamphetamine (**TMA**)

3,4,5-trimethoxyphenethylamine (**mescaline**)

4-(2,2-difluoroethoxy)-2,5-dimethoxyamphetamine (**MDFEM**)

4-(2,2-difluoroethoxy)-2,5-dimethoxyphenethylamine (**2C-O-21.5**)

4-alkoxy-2,5-dimethoxyamphetamine (**3C-O**)

4-alkoxy-2,5-dimethoxyphenethylamine (**2C-O**)

4-allyloxy-2,5-dimethoxyamphetamine (**MALM**)

4-allyloxy-2,5-dimethoxyphenethylamine (**2C-O-16**)
4-benzyloxy-2,5-dimethoxyphenethylamine (**2C-O-27**)
4-bromo-2,5-dimethoxyamphetamine (**DOB**)
4-bromo-2,5-dimethoxyphenethylamine (**2C-B**)
4-butoxy-2,5-dimethoxyamphetamine (**MBM**)
4-hydroxy-3-methoxyamphetamine (**HMA**)
4-hydroxy-3-methoxymethamphetamine (**HMMA**)
4-hydroxy-3-methoxymethcathinone (**HMMC**)
4-hydroxy-3-methoxypyrovalerone (**HMPV**)
4-hydroxy-*N,N*-dimethyltryptamine (**psilocin**)
4-hydroxyindole-3-acetic acid (**4-HIAA**)
4-hydroxytryptophol (**4-HTP**)
4-phosphoryloxy-N,N-dimethyltryptamine (**psilocybin**)
5-hydroxtryptamine (**Serotonin, 5-HT**)
8-hydroxy-2-(di-n-propylamino)tetralin (**8-OH-DPAT**)
Adenylyl cyclase (**AC**)
Atmospheric pressure chemical ionization (**APCI**)
Attention deficit/hyperactivity disorder (**ADHD**)
Calibrator (**CAL**)
Cyclic adenosine monophosphate (**cAMP**)
Cytochrome P450 (**CYP**)
Diacyl glycerol (**DAG**)
Dopamine (**DA**)
Dopamine uptake transporter (**DAT**)
Drug-drug interaction (**DDI**)
Electrospray ionization (**ESI**)
European Medicines Agency (**EMA**)
European Monitoring Centre for Drugs and Drug Addiction (**EMCCDA**)
Food and Drug Administration (**FDA**)
G-protein coupled inwardly-rectifying potassium channels (**GIRKs**)
G-protein coupled receptor (**GPCR**)
Gas chromatography (**GC)**
Good Laboratory Practice (**GLP**)
High pressure liquid chromatography (**HPLC**)

High pressure liquid chromatography and tandem mass spectrometry (**HPLC-MS/MS**)
Inositol triphosphate (**IP_3**)
Internal standard (**IS**)
Liquid chromatography (**LC**)
Liquid-liquid extraction (**LLE**)
Lower limit of quantification (**LLOQ**)
Lysergic acid diethylamide (**LSD**)
Mass spectrometry (**MS**)
Mass-to-charge (**m/z**)
Maximal serum concentration (**C_{max}**)
Methylphenidate (**Ritalin**)
Multidisciplinary Association for Psychedelic Studies (**MAPS**)
Multiple reactions monitoring (**MRM**)
N-methyl-D-aspartate receptor (**NMDA-R**)
N,N-dimethyltryptamine (**DMT**)
Norepinephrine (**NE**)
Norepinephrine uptake transporter (**NET**)
Novel molecular entity (**NME**)
Novel psychoactive substances (**NPS**)
Phosphatidylinositol 4,5-bisphosphate (**PIP_2**)
Phospholipase C (**PLC**)
Post-traumatic stress disorder (**PTSD**)
Protein kinase A (**PKA**)
Protein kinase C (**PKC**)
Protein precipitation (**PPT**)
Quality control (**QC**)
Selective serotonin reuptake inhibitor (**SSRI**)
Serotonin uptake transporter (**SERT**)
Solid-phase extraction (**SPE**)
Solute carrier protein (**SLC**)
Standard operating procedure (**SOP**)
Time-to-reach maximal serum concentration (**T_{max}**)
UDP-glucuronosyltransferases (**UGT**)

Ultraviolet (**UV**)

United Nations Office on Drug and Crime (**UNODC**)

γ-aminobutyric acid (**GABA**)

III. Project Rationale

Novel psychoactive substances (NPS) are psychotropic compounds that appear on the recreational drug market as safe alternatives to their chemically homologous counterparts, usually classical drugs of abuse such as 3,4-methylenedioxymethamphetamine (MDMA), but also narcotics or medical drugs, which have been repurposed for recreational use (Liechti 2015, Tracy, Wood et al. 2017, Luethi and Liechti 2020). Often referred to as "legal highs", "research chemicals" or "designer drugs", these compounds are not scheduled, imitate the central effects of classical drugs of abuse, and are associated with similar or more severe adverse drug reactions (Liechti 2015, Tracy, Wood et al. 2017, Luethi and Liechti 2020). Moreover, these compounds are easy to access as they can be purchased via internet forums and the dark web, and are often misbranded by sellers and sold at low costs. Overall, NPS's uncharacterized pharmacological and toxicological profiles are a significant threat to public health and drug legislature agencies, who are unable to keep up with the rate at which these compounds are synthesized and detected on the market (Liechti 2015, Tracy, Wood et al. 2017, Luethi and Liechti 2020).

For a decade now, the number of NPS has been on the rise. However, recently, current trends indicate that the number of new substances entering the recreational drug market is slowly stabilizing when compared to previous years (European Monitoring Centre for Drugs and Drug Addiction 2020, United Nations Office of Drug and Crime 2021). Up to the end of 2019, over 790 NPS were actively monitored in Europe, with 53 new compounds appearing in 2019 (European Monitoring Centre for Drugs and Drug Addiction 2020, United Nations Office of Drug and Crime 2021). Nevertheless, new compounds still appear on the drug market, which require pharmacological (mechanism of action, potency, associated effects, abuse liability) and toxicological characterization (short- and long-term adverse effects, clinical toxicities). As a result, both *in vitro* and *in vivo* investigations as well as case study reports, are essential to understand how they act in humans. This knowledge and the development of bioanalytical drug screening methods to detect NPS in users helps clinicians to adequately treat consumers in cases of intoxication. Finally, it enables the monitoring bodies (United Nations Office on Drug and Crime [UNODC] or European Monitoring Centre for Drugs and Drug Addiction [EMCCDA]) to adequately detect, track, schedule these compounds, and to provide reliable, scientific-based legislation

to control them. Thereby protecting the public from their potential harms and toxicities (King and Sedefov 2007).

Concurrently, the field of psychedelic research has been reignited, particularly in its application of classical psychedelics and related compounds (e.g., psilocybin, lysergic acid diethylamide [LSD] or the entactogenic-psychedelic MDMA) in drug-assisted psychotherapy for various affective disorders including depression, anxiety, and post-traumatic stress disorder (PTSD) (Reiff, Richman et al. 2020). The Schedule I status of these compounds (defined as little to no medical value and carrying a huge abuse liability), overall lack of funding from governmental agencies and a tainted research past during the 1950-60s, have all have been major contributors to the difficulty of investigating these substances. Despite all these unfavourable circumstances, the development of psychedelic organizations [e.g., Multidisciplinary Association for Psychedelic Studies (MAPS)], funding from private industry and start-up companies, and the high-quality studies conducted with psychedelics nowadays, has strengthened their potential application as therapeutic agents. For example, MDMA-assisted therapy in PTSD is currently in the works and is scheduled for FDA licensing as early as 2023 (Mitchell, Bogenschutz et al. 2021). More psychedelics are investigated for their therapeutic purposes e.g., mescaline and *N,N*-dimethyltryptamine (DMT). The ultimate goal of researchers in the field is to submit drug applications to the FDA, in order to offer these substances as licensed medicines. To achieve this, clinical trials need to be conducted with these compounds in humans. The pharmacokinetics of these compounds and how they interact with other drugs (drug-drug interaction studies, DDI) are just a few important aspects that need to be studied further. Both parameters require validated bioanalytical assays that can allow scientists to examine these necessary parameters (e.g., quantification of these compounds in plasma or urine).

One part of my PhD thesis project focused on the *in vitro* pharmacological characterization of novel psychoactive substances, mainly the metabolites of three ring-substituted stimulants, MDMA, methylone, and MDPV; as well as a series of structurally related 4-alkyloxy-subsituted 2,5-dimethoxyamphetamines and their phenethylamine counterparts, which are related to the potent psychedelic 2,4,5-trimethoxyamphetamine (TMA-2). Since the pharmacology of the latter compounds was largely undefined, my research project aimed to shed light into their structure-activity relationship and their potential psychedelic potency, by examining their

receptor interaction profiles at the various monoamine receptors and transporters. In parallel, the former compounds (i.e., metabolites of ring-substituted stimulants) were examined for their potential activity at the human monoamine uptake transporters in order to assess their role in pharmacological properties of their parent compounds, which have been previously determined via *in vivo* and *in vitro* studies in rats. The second part of my PhD thesis project focused on the development and subsequent full validation of liquid chromatography and tandem mass spectrometry (LC-MS/MS) bioanalytical method for the bioanalysis of psilocybin's main metabolites, psilocin, 4-hydroxyindole-3-acetic acid (4-HIAA), and psilocin glucuronide in human plasma. The bioanalytical method was applied to assess the pharmacokinetics of psilocybin in two clinical studies conducted in our department. The first study examined the interaction of the SSRI, escitalopram with 25 mg psilocybin in healthy subjects. The goal of the investigation was to better understand the effect of escitalopram pre-treatment on the psilocybin-induced subjective effects and to assess whether the SSRI had any influence on the pharmacokinetics of psilocin. In the second study, the difference in subjective effects of LSD and psilocybin (15 mg and 30 mg) in healthy subjects were examined and compared. The validated psilocin bioanalytical method was used to assess the dose-response relationship of psilocin in both studies at all three administered doses.

IV. Project Summary

In **Chapter 2.1**, the metabolites of three popular amphetamine analogues containing a 3,4-methylenedioxy ring (MDMA, methylone, and MDPV) were examined at the human monoamine uptake transporters and various human monoamine receptors, using transfected cell lines. The aim of the study was to determine the pharmacological interactions of the different metabolites compared to their parent compounds with the human monoaminergic system.

The *N*-demethylated metabolites of MDA and MDC exhibited similar inhibition profiles at the transporters as their parents with a potent inhibition at the NET (IC_{50} = 0.38 – 2.3 μM), and selectivity for the SERT (DAT/SERT ratio < 1). The overall inhibition profile of MDC exhibited a slight decrease in potency when compared to methylone, whereas for MDA it remained unchanged when compared to MDMA. Similar to MDMA, MDA also exhibit relevant binding affinity to the 5-HT_{2A} receptor (K_i = 3.2 μM). The *O*-demethylenated metabolites of the three amphetamine analogues (e.g., HHMA and HHA) displayed a reduction in SERT inhibition potency, while maintaining their NET and DAT inhibition potency. The *O*-methylated metabolites (e.g., HMMA and HMA) however exhibited a significant attenuation of inhibition potency at the NET (IC_{50} = 1.7 – 30 μM). Taking all the findings together, several metabolites exhibited relevant interactions at the human monoamine transporters and receptors. The *N*-demethylated metabolites of methylone and MDMA were the most pharmacologically relevant metabolites as they are not inactivated in humans by conjugation with glucuronic acid. In conclusion, these metabolites may potentially contribute to the activity associated with their parent compounds in users.

In **Chapter 2.2**, numerous 4-alkyloxy-substituted 2,5-dimethoxyamphetamine and phenethylamine derivatives of TMA-2 were examined for their receptor binding and activation properties at the human monoamine receptors and transporters. The aim of the investigation was to determine the pharmacological profile of these derivatives specifically at the serotonergic 5-HT_{2A} receptor to reveal their structure-activity relationship as well as to predict their potential psychedelic activity.

All derivatives exhibited moderate to high affinity to the 5-HT_{2A} receptor (K_i = 8–1700 nM), with a selectivity for the 5-HT_{1A} vs. 5-HT_{2C} receptors. Extension of the 4-alkoxy group enhanced the 5-HT_{2A} and 5-HT_{2c} receptor binding affinities, with mixed

effects on 5-HT_{2A} receptor activation potency (amount of drug need to elicit response) and efficacy (maximal drug effect). Presence of fluoro substituent at the 4-alkoxy group attenuated the 5-HT_{2A} and 5-HT_{2c} receptor binding affinities, however additional fluorination had the opposite effect at the receptors. The 4-allyl and 4-methallyl derivatives (e.g., 2C-O-16, MALM, 2C-O-3, and MMALM) exhibited the most promising profiles at the 5-HT_{2A} receptor (highest affinities, activation potencies, and efficacies) and were predicted to produce psychedelic-like effects in humans.

In **Chapter 4.1**, a bioanalytical method to quantify psilocybin's main metabolites, psilocin, 4-HIAA, and psilocin glucuronide in human plasma was developed and validated according to regulatory guidelines. The purpose of the project was to develop a method that was user-friendly, sensitive, and overall reliable. Moreover, the method was developed to use only small amounts of sample and to minimize the run time. The developed method was used in order to quantify samples from two ongoing clinical studies investigating the pharmacokinetics of psilocybin in healthy volunteers.

Psilocin and 4-HIAA were detected by multiple reaction monitoring in positive and negative ionisation modes, respectively. The method showed a linear relationship between concentration and signal intensity in the range observed in clinical study samples. The method was accurate and precise with an inter-assay accuracy (100 – 109%) and precision (≤8.9%) measured in three separate validation runs. A simple methanol protein precipitation extraction method was employed to process the plasma samples, with almost complete analyte recovery (≥94.7%). The matrix effect was consistent across several plasma batches and importantly, did not interfere with the analysis of the analytes. Both analytes showed little degradation (≤10%) after three thaw-freeze cycles, room temperature for 8 h and 1 month storage at -20 ^{o}C. Moreover, the conjugation of the analytes was examined using *Escherichia coli* β-glucuronidase. The clinical application of the method was examined by analysing samples from three healthy volunteers treated with an oral dose of 25 mg psilocybin. The maximal plasma concentration (C_{max}) of psilocin and 4-HIAA was on average 19.2 ng/ml and 137 ng/ml, respectively. Time to achieve maximal plasma concentration (T_{max}) was observed between 120 – 140 min post treatment. Psilocin underwent glucuronidation reaching maximal plasma concentrations of 78.3 ng/ml after 220 min, whereas 4-HIAA was not conjugated.

In conclusion, my PhD thesis furthered the pharmacological characterization of several popular NPS metabolites and potentially psychedelic novel derivatives of

TMA-2 at the monoamine uptake transporters and receptors. The *in vitro* pharmacological knowledge that was gained can be used to understand the pharmacological and potential toxicological properties that may be associated with some of these compounds when ingested by recreational users. Moreover, the monitoring bodies can better control compounds which are associated with a higher risk of abuse (e.g., more dopaminergic). Furthermore, my PhD thesis also furthered the quantification of psilocybin's metabolites in plasma by the development and validation of an optimized bioanalytical method. The method was used to investigate the dose-response relationship of psilocybin and was also used to investigate drug-drug interactions of escitalopram and psilocybin in healthy subjects. As a result, the method has furthered the knowledge of psilocybin's pharmacokinetics *in vivo*.

Part A: *In vitro* pharmacological characterization of novel psychoactive substances

Chapter 1: Pharmacology of drugs of abuse

1.1 Monoamines and the monoaminergic system

Dopamine (DA), norepinephrine (NE), and serotonin (5-HT) are three monoamines that are responsible for the neurotransmission between monoaminergic neurons in the central and peripheral nervous systems (Elhwuegi 2004). These neurotransmitters are synthesized in the nerve terminals from precursor amino acids, tyrosine (DA and NE) or tryptophan (5-HT) (Iversen 1967, Fuller 1980) (**Figure 1**). As signaling molecules, they interact with their respective pre- and/or post-synaptic targets thereby modulating a diverse set of physiological functions including movement, attention, motivation, reward processes, temperature and vasculature regulation, perception, and many other important processes (Ressler and Nemeroff 1999, Vallone, Picetti et al. 2000, Tzschentke 2001, Berger, Gray et al. 2009). Unsurprisingly, the main role of the monoaminergic system is to keep all of these different processes in state of homeostasis (Berger, Gray et al. 2009, Kurian, Gissen et al. 2011). However, when the system is impaired, a lack of homeostasis in the above mentioned processes is often linked to the development of neuropsychiatric disorders such as depression, anxiety, attention deficit/hyperactivity disorder (ADHD) or schizophrenia (Heninger and Charney 1988, Carlsson, Waters et al. 2001, Morilak and Frazer 2004, Clark, Chamberlain et al. 2009).

Nowadays, these disorders and many neurological conditions are treated with medications that target different components of the monoaminergic life cycle including the postsynaptic receptors, presynaptic uptake transporters or vesicular transporters (**Figure 1**) (Greenhill, Kollins et al. 2006, Di Giovanni, Svob Strac et al. 2016, Cipriani, Furukawa et al. 2018). One such example would be the selective serotonin reuptake inhibitors (SSRIs) which target the serotonin uptake transporter and are used in the treatment of depression or methylphenidate (Ritalin), a stimulant, which is used in the treatment of ADHD, and targets all three monoamine uptake transporters, but to different degrees (Greenhill, Kollins et al. 2006, Cipriani, Furukawa et al. 2018). Moreover, a huge variety of designer drugs like 3,4-methylenedioxymethamphetamine (MDMA) also interact with the monoamine uptake transporters. The main role of these transporters is to terminate the monoaminergic neurotransmission and therefore maintain presynaptic homeostasis (Torres, Gainetdinov et al. 2003, Luethi and Liechti 2020).

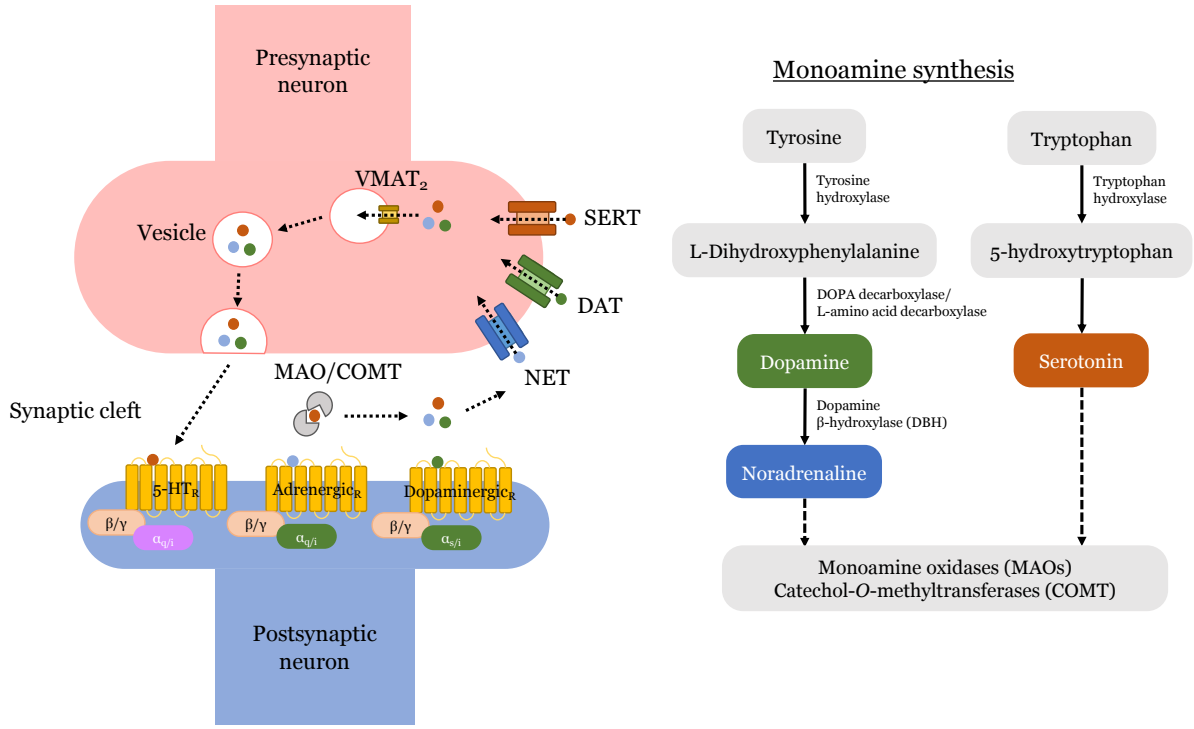

Figure 1: Monoamine synthesis and transport in neurons. Left: Monoamine neurotransmitters (NE/DA/5-HT) are packaged into vesicles via the vesicular monoamine transporter 2 ($VMAT_2$). When the presynaptic neuron is activated by a depolarizing action potential, the vesicles are released (thereby releasing the monoamines inside) into the synaptic cleft via a calcium-dependent exocytosis. The released monoamines bind to their respective receptors to potentiate their effects. Monoamines are then either recycled back into the presynaptic neuron via their respective uptake transporters (NET/DAT/SERT) or are metabolized by enzymes found in the synapse (e.g., MAOs or COMT). **Right:** Tyrosine is hydroxylated to form L-dihydroxyphenylalanine (L-DOPA) via tyrosine hydroxylase (rate limiting step). L-DOPA is then decarboxylated to dopamine via DOPA decarboxylase or L-amino acid decarboxylase, and can be further hydroxylated to noradrenaline by dopamine β-hydroxylase. Serotonin is produced from the amino acid tryptophan which undergoes an oxidation via tryptophan hydroxylase to produced 5-hydroxytryptophan. This intermediate can then be decarboxylated to produce serotonin via L-amino acid decarboxylase. Figure concept inspired and adapted from Elhwuegi (2004). **Legend**: dopamine, DAT (green); norepinephrine, NET (blue); serotonin, SERT (orange).

1.2 Drugs of abuse: Stimulants, entactogens, and psychedelics

Stimulants, entactogens, and psychedelics are three subgroups of designer drugs that interact with the monoaminergic system, albeit in different ways, to produce a psychoactive or "mind-altering" effect. Other subclasses of designer drugs such as cannabinoids, dissociatives, or sedatives interact with different molecular targets like the cannabinoid, *N*-methyl-D-aspartate (NMDA), γ-aminobutyric acid (GABA) or the opioid receptors (Pathan and Williams 2012, Wallach, Kang et al. 2016, Alves, Goncalves et al. 2020). The aforementioned subclasses of designer drugs will not be discussed further in this thesis, as they are outside the scope of the doctoral project. The detailed review of the pharmacology and toxicology of these designer drugs have

been summarized by Pourmand, Mazer-Amirshahi et al. (2018) and Luethi and Liechti (2020).

1.3 Stimulants and entactogens

Stimulants and entactogens are structurally diverse and psychoactive substances which mainly but not exclusively interact with the monoamine uptake transporters to enhance monoaminergic neurotransmission (Liechti 2015, Tracy, Wood et al. 2017, Luethi and Liechti 2020). They can also be further subdivided based on their chemical structure into e.g., amphetamines, cathinones, pyrovalerones and more (**Figure 2**). Other monoaminergic receptors like the serotoninergic, dopaminergic, adrenergic, and trace-amine associated receptor 1 (TAAR1) are also targeted by these compounds but to varying degrees (Di Cara, Maggio et al. 2011, Simmler, Rickli et al. 2014, Rickli, Hoener et al. 2015).

In general, at the monoamine uptake transporters, stimulants and entactogens act as either reuptake inhibitors, which block the uptake of monoamines or as transporter-substrates, which cause non-exocytotic monoamine release (Fleckenstein, Gibb et al. 2000, Rothman and Baumann 2003, Sitte and Freissmuth 2015). Moreover, metabolites of these compounds may also interact with the transporters and can therefore potentiate the parent's psychoactive or toxic effects, which will be addressed further in **Chapter 2.1,** as this was one of the key focuses of my doctoral project (de la Torre, Farre et al. 2004, Baumann, Ayestas et al. 2012, Schindler, Thorndike et al. 2014). Prototypical examples of compounds which fall under these two groups include cocaine, a potent inhibitor of the NET and DAT, or MDMA, an entactogen and potent SERT releaser (Simmler, Rickli et al. 2014).

Favorable effects associated with these substances include an overall increase in energy, cognition, sense of well-being, as well as euphoria, increase in emotional warmth and empathy, which are more common to entactogens (Morgan, Noronha et al. 2013, Hysek, Schmid et al. 2014, Hysek, Simmler et al. 2014). Moreover, acute psychedelic-like effects are also observed with entactogens like MDMA (Nichols 1986, Liechti, Saur et al. 2000). Acute toxicity associated with these substances includes agitation, hypertension, and tachycardia, which are often associated with more sympathomimetic compounds. Hyperthermia, hyponatremia, agitation, and sweating are typically symptoms associated with serotonin syndrome and often are connected to the toxicity of more serotonergic compounds (Parrott 2002, Liechti 2015, Logan,

Mohr et al. 2017). In the worst of cases and often at high doses, renal toxicity, hepatotoxicity, cardiotoxicity, and neurotoxicity can occur (Carvalho, Pontes et al. 2010, Rudin, Liechti et al. 2021). Overall, this subclass of designer drugs is often associated with acute toxicity, a high abuse liability (especially for very dopaminergic compounds), and potential development of dependence, as they are often short acting and used on a more regular basis by users (Luethi and Liechti 2020).

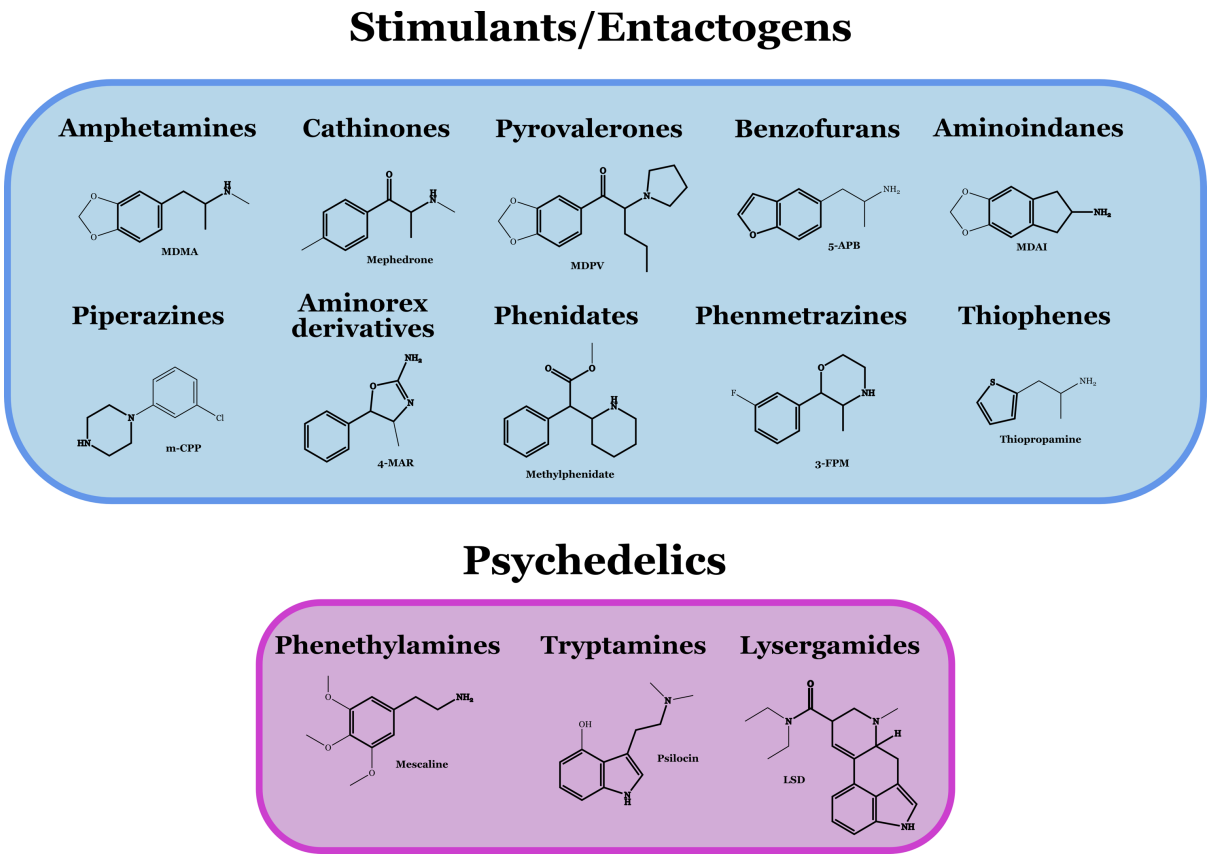

Figure 2: Subclassification of stimulants/entactogens and psychedelics by their chemical structure. Each structural subgroup includes an example compound with the core structure of the subgroup in bold e.g., the amphetamine subgroup shows the structure of MDMA with the amphetamine structure highlighted in bold. Figure concept inspired and adapted from Luethi and Liechti (2020).

1.4 Psychedelics

Psychedelics are a diverse group of compounds (**Figure 2**) which mainly target serotonergic transmission by their agonism at the 5-HT_{2A} receptor (Vollenweider, Vollenweider-Scherpenhuyzen et al. 1998, Nichols 2004, Geyer and Vollenweider 2008, Nichols 2016, Kraehenmann, Pokorny et al. 2017, Preller, Burt et al. 2018, Madsen, Fisher et al. 2019). Structurally similar to serotonin, these compounds are often found naturally-occurring e.g., psilocybin which is found in mushrooms of the *Psilocybe* genus, or can be synthetically derived e.g., lysergic acid diethylamide (LSD),

a semisynthetic compound originally derived from ergot fungus's ergotamine (Schwartz and Smith 1988, Aghajanian and Marek 1999, Nichols 2016). Furthermore, these serotonergic psychedelics produce "mind-altering" effects by modulating perception, mood, and cognition (Nichols 2004, Nichols 2016).

Similar to the stimulants and entactogens, psychedelics do not exclusively interact with the 5-HT_{2A} receptors, albeit this interaction is the most significant as it is responsible for the associated psychedelic effects (Kraehenmann, Pokorny et al. 2017, Preller, Burt et al. 2018, Madsen, Fisher et al. 2019). Serotonergic (5-HT_{1A}, 5-HT_{2B}, and 5-HT_{2c}), dopaminergic, and adrenergic receptors are also targeted by various psychedelics, as well as monoamine uptake transporters by some phenethylamine and tryptamine-based compounds (Fantegrossi, Reissig et al. 2008, Halberstadt and Geyer 2011, Rickli, Luethi et al. 2015, Rickli, Moning et al. 2016, Eshleman, Wolfrum et al. 2018, Luethi, Trachsel et al. 2018).

Classical psychedelics such as LSD, psilocybin, 3,4,5-trimethoxyphenethylamine (mescaline) or *N,N*-dimethyltryptamine (DMT), have been historically used in the religious setting and for recreational purposes (Nichols 2004, Carod-Artal 2015, Nichols 2016). In the last 50 years, a vast array of structural analogues of these prototypical psychedelics (e.g., phenethylamine derivatives) have been derived in order to study the serotonin system and also to investigate the psychedelic potential of these compounds (Glennon, Liebowitz et al. 1980, Glennon, McKenney et al. 1986, Shulgin and Shulgin 1991, Glennon, Dukat et al. 1994, Nichols, Frescas et al. 1994, Chambers, Kurrasch-Orbaugh et al. 2001, Chambers, Kurrasch-Orbaugh et al. 2002, Trachsel 2002, Trachsel, Lehmann et al. 2013, Nichols, Sassano et al. 2015, Rickli, Luethi et al. 2015, Rickli, Moning et al. 2016, Luethi, Trachsel et al. 2018). The phenethylamine structural analogues have been altered at different key structural positions e.g., at the phenyl ring with small or large lipophilic substituents (extension of the alkyl chain or cumulative fluorination) or introduction of α-methyl group (amphetamine counterparts) with the intention of better understanding the structure-activity relationship (SAR) of these compounds. A new subfamily of the phenethylamines based on 2,4,5-trimethoxyamphetamine (TMA-2) and their phenethylamine counterparts (2C-O derivatives) have been investigated as the second key focus of my doctoral project and will be discussed in **Chapter 2.2**.

For several years now, a huge research effort has been made to study psychedelics. This endeavor has not been easy as psychedelics have had a poor

reputation in the past and are still scheduled under the US Controlled Substance Act, which prohibits their use and research (Nichols 2004, Nichols 2016, Liechti 2017). Currently, several classical psychedelics including LSD, are being investigated in healthy volunteers for dose-finding studies or in small cohorts of patients e.g., in anxiety associated with life-threatening diseases (Gasser, Holstein et al. 2014, Carhart-Harris, Bolstridge et al. 2016, Belouin and Henningfield 2018, Carhart-Harris, Bolstridge et al. 2018, Holze, Vizeli et al. 2020, Holze, Vizeli et al. 2021). Additionally, psilocybin clinical research gained great momentum in recent years, with the intention of repurposing the drug for the treatment of neuropsychiatric disorders like anxiety or depression (Grob, Danforth et al. 2011, Griffiths, Johnson et al. 2016, Ross, Bossis et al. 2016). The application of psilocybin in clinical research will be discussed in **Part B**.

In healthy volunteers', psychedelics typically induce both psychological and autonomic effects. Favorable psychological effects observed include loss of subjective self (ego dissolution), mystical experiences, positive mood, alteration in sense of time or thinking, increase in sense of oneness and trust, as well as visual and sensory synesthesia (Schmid, Enzler et al. 2015, Liechti 2017, Liechti, Dolder et al. 2017, Holze, Vizeli et al. 2020, Holze, Vizeli et al. 2021). Weak autonomic effects associated with psychedelic ingestion mainly present as increases in body temperature, heart rate, blood pressure, as well as increases in pupil size (Schmid, Enzler et al. 2015, Liechti 2017, Liechti, Dolder et al. 2017, Holze, Vizeli et al. 2020, Holze, Vizeli et al. 2021). Acute adverse effects associated with psychedelics mainly include exhaustion, dry mouth, nausea, headaches, dizziness and increases in anxiety (Schmid, Enzler et al. 2015, Dolder, Schmid et al. 2016). Moreover, acute panic or psychosis, agitation, aggression, and more severe adverse effects like serotonin syndrome, exacerbation of underlying psychiatric conditions, delusions or acute renal toxicities or drug-related fatalities have been reported, but are mostly linked to new designer psychedelics (Luethi and Liechti 2020, Rudin, Liechti et al. 2021). In general, however, the infrequent ingestion of classical psychedelics in the supervised and supportive setting is associated with a good safety profile and low drug dependency as these compounds are longer-lasting and very potent when compared to most stimulants/entactogens (Nichols 2004, Nichols 2016, Liechti 2017).

1.5 Monoamine uptake transporters: Role in the psychoactive effects of stimulants and entactogens

The norepinephrine, dopamine and serotonin uptake transporters (NET, DAT, and SERT, respectively) are plasma membrane bound proteins (peri-synaptically) which are essential for the termination of monoaminergic neurotransmission by taking up released monoamines from the synapse (Torres, Gainetdinov et al. 2003, Sitte and Freissmuth 2015). In the central nervous system, the NET is highly localized in the locus coeruleus and related brainstem nuclei, which are involved in processes like the stress response, attention and memory acquisition, and additionally, cardiac and respiratory processes e.g. heart rate regulation (Hoffman, Hansson et al. 1998). The DAT is extensively expressed in the cell bodies of the substantia nigra and the ventral tegmental area, both of which are heavily involved in reward pathways and movement (Hoffman, Hansson et al. 1998). The SERT is mostly expressed in the median and dorsal raphe nuclei, which innervate the hippocampus, forebrain, and amygdala and are linked to memory, emotions, thermoregulation, and pain perception (Hoffman, Hansson et al. 1998).

As solute carrier proteins (NET; SLC6A2, DAT; SLC6A3 and SERT; SLC6A4) the monoamine uptake transporters function as symporters transporting their respective monoamine alongside the transport of sodium (Na^+) and chloride (Cl^-) ions (Torres, Gainetdinov et al. 2003, Kristensen, Andersen et al. 2011). Driven by the Na^+ and potassium (K^+) ion gradients created by the sodium/potassium adenosine triphosphate pump (Na^+/K^+ ATPase), the DAT mediates the transport of DA alongside two Na^+ ions and single Cl^- ion, while the SERT and NET mediate transport of 5-HT and NE, respectively with the co-transport of one Na^+ ion and one Cl^- ion (Gu, Wall et al. 1994) (**Figure 3**). Overall, this classical substrate transporter mechanism indicates that the binding of the neurotransmitter and the co-transported ions (outward facing conformation) induces a conformational change of the transporter (shifts to inward facing conformation), thereby transporting the neurotransmitter and co-transported ions back into the neurons from the synapse (Fischer and Cho 1979, Gu, Wall et al. 1994, Rudnick 1998).

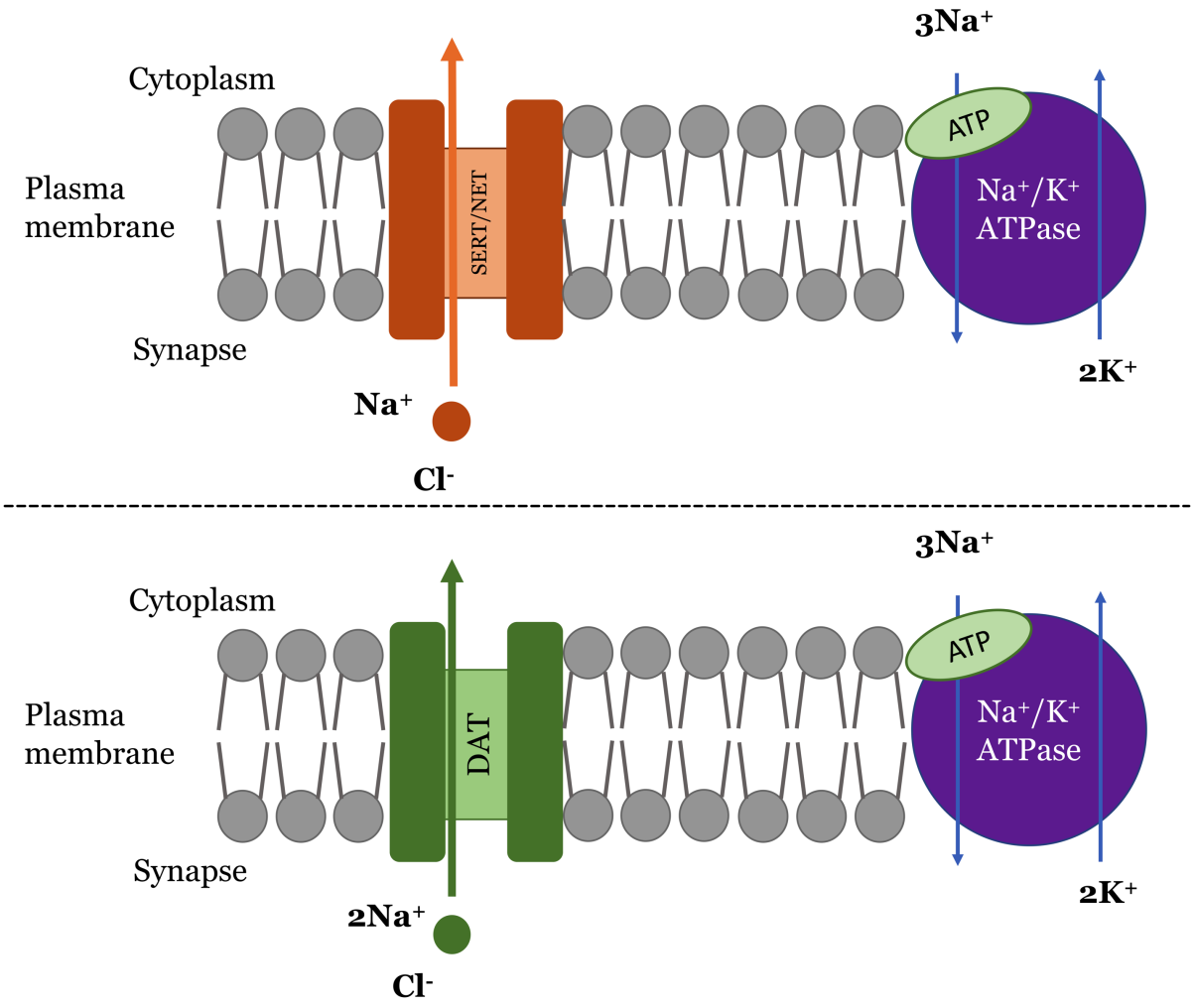

Figure 3: Simple schematic monoamine transport through the monoamine uptake transporters. Monoamines (dopamine; green circle, serotonin; orange circle) are co-transported with sodium and chloride ions from the synapse into the cytoplasm of the presynaptic neuron. The sodium/potassium ATPase creates the ion gradient across the membrane which under normal conditions drives the monoamines into the cytoplasm. Figure concept inspired and adapted from German, Baladi et al. (2015).

The transformation of the transporter back into the outward facing conformation requires the binding and transport of K^+ ions, as proposed for SERT (Nelson and Rudnick 1979, Rudnick 1998). Furthermore, the transporter cycle can be reversed, thereby releasing instead of taking up monoamines. Another proposed mechanism for the transporters is that they can function as channel-like proteins, whereby the outward and inward gates can open and allow the flow of the taken up neurotransmitters and co-transported ions into the neurons, creating transported-associated currents, which are observed for the transporters and cannot be accounted for by the classical transporter mechanism (Sonders, Zhu et al. 1997, Sitte, Huck et al. 1998).

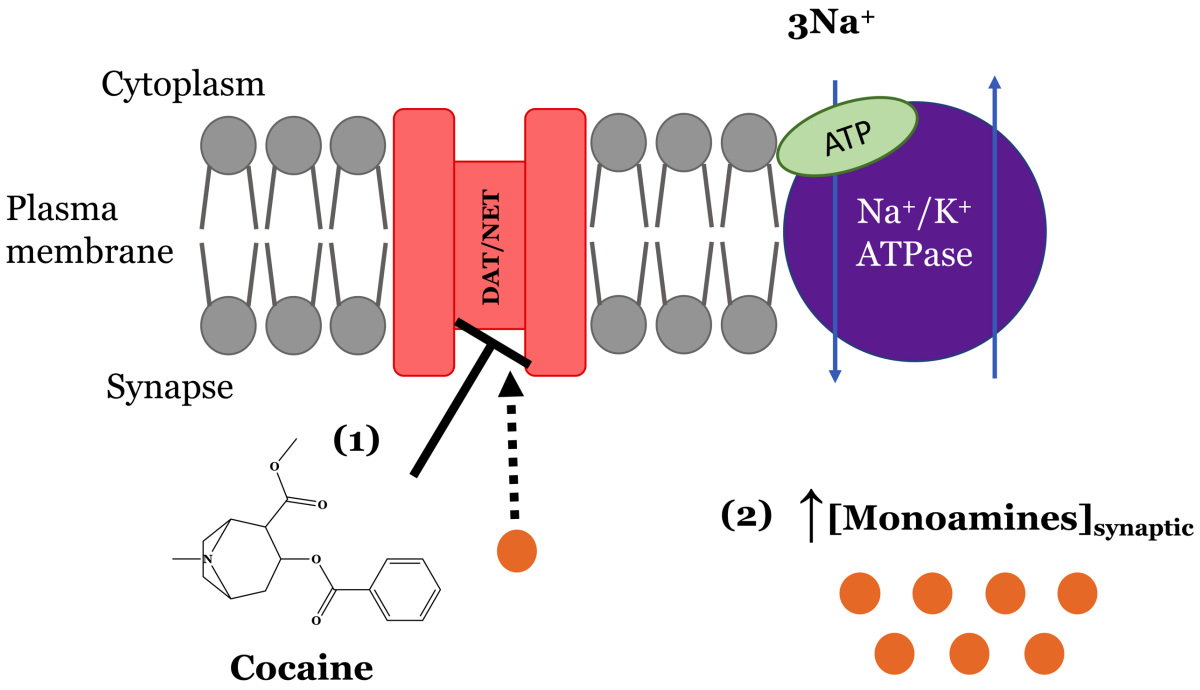

Figure 4: Simple mechanism of a reuptake inhibitor at the monoamine uptake transporter. (1) Cocaine binds to the monoamine uptake transporter (MAT) and blocks the transporter from transporting released monoamines (orange circles). Cocaine does not itself get transported by MAT into the cytoplasm. (2) This causes an accumulation of monoamines in the synapse thereby augmenting synaptic transmission. Figure concept inspired and adapted from Torres (2003).

Often drugs of abuse like the stimulants or entactogens as well as many therapeutic drugs, interact with the monoamine uptake transporters in order to induce their pharmacological effects. They act at these proteins in two distinct ways, either as reuptake inhibitors (**Figure 4**) or as substrate-type releasers (**Figure 5**) (Fleckenstein, Gibb et al. 2000, Rothman and Baumann 2003, Sitte and Freissmuth 2015). Cocaine, 3,4-methylenedioxypyrovalerone (MDPV), fluoxetine, paroxetine, mazindol, and nisoxetine are all reuptake inhibitors, which bind to the monoamine uptake transporters, but are not transported into the cytoplasm of the nerve terminals (Rothman and Baumann 2003). They augment extracellular monoamine concentrations by essentially preventing the transporter from recycling back released monoamines from the synapse (Fleckenstein, Gibb et al. 2000, Rothman and Baumann 2003). On the other hand, amphetamine and MDMA are substrate-type releasers, which also bind and are transported by monoamine uptake transporters into the nerve terminals (Fleckenstein, Gibb et al. 2000, Rothman and Baumann 2003, Sitte and Freissmuth 2015). Once inside, MDMA or other substrate-type releasers disrupt the packing of monoamines into the synaptic vesicles by accumulating inside the vesicles and depleting the synaptic vesicles stores (Fleckenstein, Gibb et al. 2000, Rothman and Baumann 2003, Sitte and Freissmuth 2015). Additionally, they reverse

the monoamine uptake transporters (non-exocytotic release), inducing monoamine efflux, which all together elevates the extracellular monoamine concentration (Fleckenstein, Gibb et al. 2000, Rothman and Baumann 2003, Sitte and Freissmuth 2015).

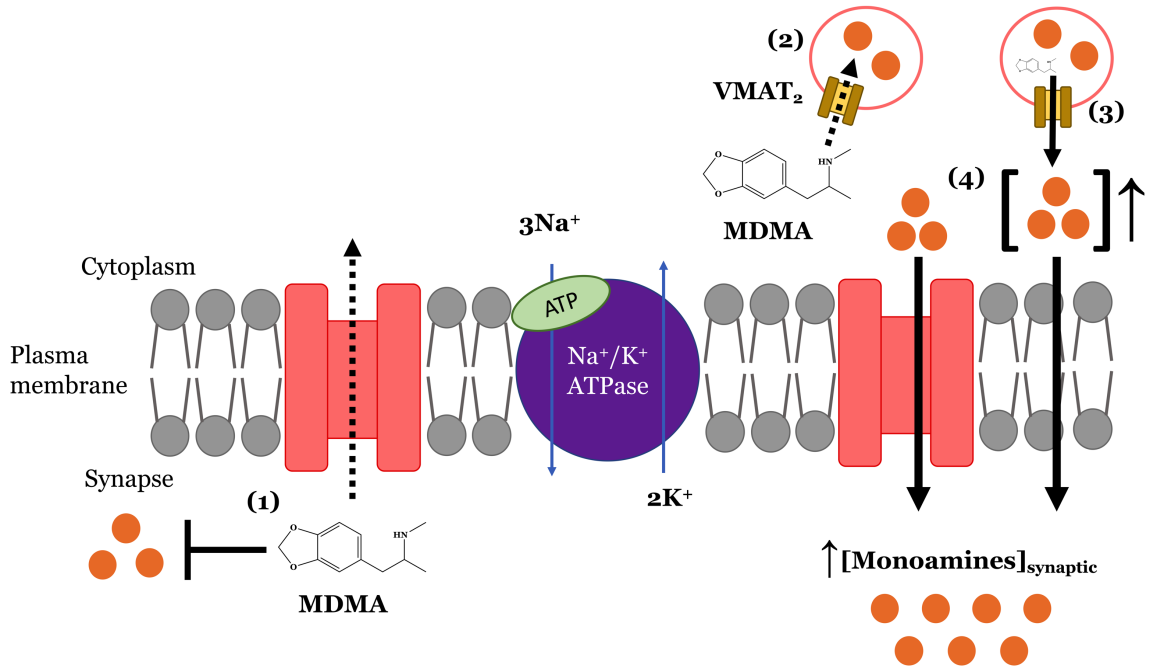

Figure 5: Simple step-by-step mechanism of substrate-type releaser at the monoamine uptake transporters. (1) MDMA binds the monoamine uptake transporter (MAT) and prevents the transport of released monoamines (orange balls) from synapse. MDMA is then transported along the concentration gradient into the cytoplasm of the presynaptic neurone (2) MDMA then is transported into the synaptic vesicles full of monoamines via the vesicular monoamine transporter 2 ($VMAT_2$). (3) MDMA reverses transport of the $VMAT_2$ and depletes the monoamine vesicular stores. (4) MDMA also reverses the flow of the MAT and the monoamines are released via the transporters and also by passive diffusion into the synapse, thereby prolonging synaptic transmission. Figure concept inspired and adapted from Torres (2003).

The pharmacological profiles of various stimulants and entactogens are largely dependent on their specific interactions at the monoamine uptake transporters. The produced pharmacological effects, potential clinical potency, and assessment of their abuse liability can often be predicted *in vitro* and *in vivo* by assessing the monoamine inhibition profiles of each compound at the three monoamine transporters (Wee, Anderson et al. 2005, Simmler, Buser et al. 2013, Luethi and Liechti 2018). *In vitro*, the DAT vs. SERT inhibition ratio can be determined to assess and compare the inhibition potency of a substance at the dopamine and serotonin transporter. Stronger selectivity for the DAT (DAT vs. SERT ratio > 1) and therefore increased DA extracellular levels are linked to substances which produce greater psychostimulant effects, similar to cocaine and are associated with high abuse liability (Rothman, Baumann et al. 2001, Suyama, Sakloth et al. 2016, Suyama, Banks et al. 2019).

Moreover, strong DAT inhibition is often associated with potent NET inhibition, which enhances noradrenergic activity leading to sympathomimetic effects (Rothman, Baumann et al. 2001, Simmler, Buser et al. 2013). On the other hand, a stronger selectivity for the SERT (DAT vs. SERT ratio < 1) and therefore increased 5-HT extracellular levels are associated with more entactogenic-like acute effects, similar to MDMA and lower associated abuse potential (Rothman, Baumann et al. 2001, Wee, Anderson et al. 2005).

1.6 Serotonergic receptors: The role of the 5-HT_{2A} receptor in the mechanism of psychedelics

The serotonergic (5-HT) receptors can be divided into seven distinct subgroups (5-HT_{1-7} receptors) and function mostly as G-protein coupled receptors (GPCRs) whereas, the 5-HT_3 receptors are ligand-gated ion channels (Nichols and Nichols 2008). The 5-HT receptors mediate various normal physiological processes including sleep, mood, cognition, memory, cardiovascular function, and sensory perception (Liu, van den Pol et al. 2002, Nichols and Nichols 2008). Within this section, I will discuss mainly four 5-HT subtypes (5-HT_{2A}, 5-HT_{2C}, 5-HT_{2B}, and 5-HT_{1A}, receptors; **Figure 6**) which are involved in the mechanism of action of classical psychedelics and novel synthetic phenethylamines and related amphetamines.

The 5-HT_{2A} receptors are located mostly post-synaptically on dendritic spines of pyramidal cells, are expressed in the neocortex, frontal and temporal cortices, as well as the basal ganglia and thalamus (**Figure 6**) (McKenna and Saavedra 1987, Pazos, Probst et al. 1987, Wong, Lever et al. 1987, Cornea-Hebert, Riad et al. 1999). LSD, psilocybin, psychedelic phenethylamines, and amphetamines mediate their psychedelic effects via agonistic binding and activation of the 5-HT_{2A} receptor. Therefore, 5-HT_{2A} receptor is a prime target site for psychedelic action, which can be blocked by 5-HT_{2A} receptor antagonist ketanserin that attenuates psychedelic subjective effects in humans (Vollenweider, Vollenweider-Scherpenhuyzen et al. 1998, Aghajanian and Marek 1999, Nichols 2004, Nichols 2016, Preller, Burt et al. 2018, Holze, Vizeli et al. 2021). *In vitro*, LSD binds to the 5-HT_{2A} receptor in sub nanomolar range while psilocin and various psychedelic phenethylamines and amphetamines bind with low to moderate nanomolar concentrations (Rickli, Luethi et al. 2015, Rickli, Moning et al. 2016, Luethi, Trachsel et al. 2018).

The 5-HT_{2C} receptor shares a high degree of sequence homology at the ligand binding site with the 5-HT_{2A} receptor (**Figure 6**) (Boess and Martin 1994, Nichols and Nichols 2008). Therefore, 5-HT_2 receptor agonists or antagonists which are said to exhibit selectivity for each type of receptor, in reality bind both receptor subtypes and thus show low selectivity between these two isoforms (Chambers, Kurrasch-Orbaugh et al. 2001, Chambers, Kurrasch-Orbaugh et al. 2002). Additionally, the 5-HT_{2C} receptor may play a role in the overall psychological profile associated with psychedelics (Nichols 2004, Nichols 2016). In general, the 5-HT_{2C} receptor is located in several brain structures like the cortex, thalamus, amygdala, basal ganglia, hippocampus and found in great abundance at the choroid plexus (Pasqualetti, Ori et al. 1999, Clemett, Punhani et al. 2000). The mesolimbic pathway (reward pathway), where dopaminergic signaling connects the ventral tegmental area of the midbrain to the striatum in the basal ganglia of the forebrain is regulated by 5-HT_{2C} receptors, which attenuate dopaminergic transmission (Gobert, Rivet et al. 2000, Bubar and Cunningham 2007). As a result, 5-HT_{2C} receptors agonists can be potentially used in the treatment of psychostimulant abuse. Moreover, the receptor is involved in the mechanism of action of some antidepressants such as SSRIs and is also considered as a potential anti-anxiety target, as it is found highly expressed in the amygdala and positively associated with anxiety states (Di Matteo, Cacchio et al. 2002, Kuznetsova, Amstislavskaya et al. 2006, Cremers, Rea et al. 2007, Hackler, Turner et al. 2007).

The 5-HT_{2B} receptor is abundantly expressed in the gastrointestinal machinery. Mainly the kidneys, liver and gut, but is also found in the heart and lungs (**Figure 6**) (Bonhaus, Bach et al. 1995, Choi, Ward et al. 1997, Nebigil, Choi et al. 2000, Nebigil, Etienne et al. 2001). The receptor plays a significant role in the physiological development of the heart and brain during developmental years and is also very important in the development of cardiac valve related heart disease (Choi, Ward et al. 1997, Fitzgerald, Burn et al. 2000, Nebigil, Choi et al. 2000, Rothman, Baumann et al. 2000, Nebigil, Etienne et al. 2001). Agonists at the 5-HT_{2B} receptor induce the expansion of myofibroblasts and are linked to the development of valvopathies, which are commonly associated with appetite suppressant, fenfluramine and are also induced by some amphetamine-based psychoactive stimulants (off-target adverse effect) (Fitzgerald, Burn et al. 2000, Rothman, Baumann et al. 2000).

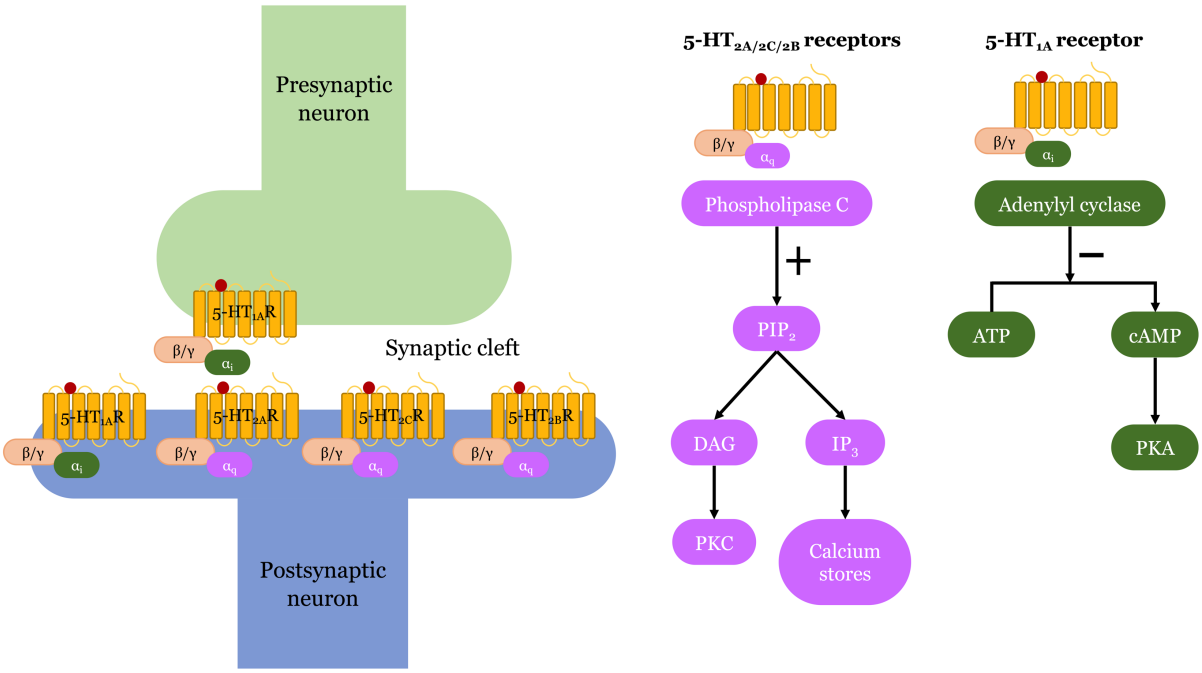

Figure 6: Localization and signalling mechanisms of 5-HT receptors. Predominately, the 5-HT_{2A}, 5-HT_{2C}, and 5-HT_{2B} receptors are found postsynaptically and coupled to the G_q-protein which activates membrane-bound phospholipase C (PLC) to cleave phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacyl glycerol (DAG) and inositol triphosphate (IP_3). Increased levels of DAG and PIP_2 lead to the activation protein kinase C (PKC) and release intracellular calcium stores, respectively (Rosenbaum, Rasmussen et al. 2009). Moreover, activation of the G-protein also leads to the activation of G-protein independent processes e.g., deactivation of G-proteins, receptor internalization or desensitization via the activation β-arrestins pathways (Böhm, Grady et al. 1997, Ferguson 2001). The 5-HT_{1A} receptors are found both pre- and post-synaptically and couple to the G_i-protein (Nichols and Nichols 2008). Activation of the G_i-protein leads to the inhibition of adenylyl cyclase (AC), which normally stimulates the production of cyclic adenosine monophosphate (cAMP), and activate protein kinase A (PKA), to initiate further downstream effects. Figure concept inspired and adapted from Millan, Marin et al. (2008).

The 5-HT_{1A} receptor is localized in the brain on presynaptic (auto-receptors) and postsynaptic neurons (**Figure 6**) (Newman-Tancredi 2011). 5-HT_{1A} autoreceptors are abundantly expressed in the brainstem, on dorsal and median raphe nuclei, while postsynaptic 5-HT_{1A} receptors are expressed in limbic system like the hippocampus, septum and the entorhinal cortex (Pazos and Palacios 1985, Aghajanian 1995). The brainstem 5-HT_{1A} auto-receptors are found on neurons which extend their projections to various parts of the forebrain. As a result, activation of these receptors, initiates $G_{i/o}$-protein coupled mechanisms like the activation of inward rectifying potassium channels (GIRKs) which causes neuronal hyperpolarization, decreasing neuronal firing and further hindering the biosynthesis of serotonin and its release via these innervations (Aghajanian 1995). Postsynaptic 5-HT_{1A} receptors are located on neurons which also expressed 5-HT_{2A} receptors and it is believed that the two receptor subtypes have opposing effects, as the induced functional effects of activated 5-HT_{2A} receptors can be attenuated by 5-HT_{1A} receptors agonists like 8-OH-DPAT (Araneda and

Andrade 1991, Willins and Meltzer 1997). The 5-HT_{1A} receptors may play role in anxiety, as full or partial agonists of the receptor e.g., buspirone elevates anxiety behaviors with a much safer off-target profile compared to benzodiazepines (Sprouse and Aghajanian 1987, Heisler, Chu et al. 1998). The receptor may also be a potential target in depression or schizophrenia (Lucki 1991, Bantick, Deakin et al. 2001). Moreover, the 5-HT_{1A} auto-receptors play a significant role in the mechanism of action of SSRIs like fluoxetine, where they initially oppose the increase of 5-HT causes by the inhibition of the uptake transporter (therapeutic lag) but over time become desensitized, allowing for normal neuronal firing and the onset of the therapeutic effects associated with antidepressants (Briley and Moret 1993, Celada, Puig et al. 2004). Importantly, the receptor is also implicated in mechanism of action of psychostimulants (e.g., amphetamine) and their potential addiction properties, whereby the activation of 5-HT_{1A} auto-receptors and the postsynaptic 5-HT_{1A} receptors can indirectly and directly, respectively, decrease serotonergic modulation and enhanced addictive behaviors associated with these drugs of abuse (Muller, Carey et al. 2007).

1.7 Involvement of α_1 and α_2 adrenergic receptors

The adrenergic receptors (α_1–α_2 and β_1–β_3) are GPCRs which are activated by norepinephrine and epinephrine and mediate various physiological effects including cardiac and respiratory functions and the fight-or-flight response (Graham, Perez et al. 1996, Schmidt and Weinshenker 2014, Motiejunaite, Amar et al. 2021). The α1-adrenoceptors which couple to the $G_{q/11}$-protein are found primarily on the smooth muscle cells of blood vessels, heart, and urinary tract, where the receptor's activation induces smooth muscle contraction leading to blood vessels vasoconstriction, increase in blood pressure (hypertension) and increase in heart rate (Graham, Perez et al. 1996, Motiejunaite, Amar et al. 2021). The receptor is also targeted by antihypertensives like prazosin (α_1-adrenoceptor antagonist) and is involved in the physiological effects induced by stimulant drugs including vasoconstriction of the blood vessels, and increase in body temperature (hyperthermia) (Hysek, Schmid et al. 2012, Schmidt and Weinshenker 2014).

On the other hand, the α_2-adrenoceptors which couple to the $G_{i/o}$-protein are found mostly centrally on presynaptic (auto-receptors) neurons where they produce membrane hyperpolarization and cause a decrease in neuronal firing via negative

feedback loop (Graham, Perez et al. 1996, Schmidt and Weinshenker 2014, Motiejunaite, Amar et al. 2021). Moreover, post-synaptically α_2-adrenoceptors can be also found in the heart tissues where they cause vasoconstriction (Graham, Perez et al. 1996, Motiejunaite, Amar et al. 2021). The receptor is targeted by antihypertensive drugs like clonidine (α_2-adrenoceptor agonist) and is implicated in the mechanism of action of stimulant drugs as it modulates norepinephrine release and also is involved in the potential toxicity associated with sympathomimetic effects of these substances (Hysek, Brugger et al. 2012, Schmidt and Weinshenker 2014, Giovannitti, Thoms et al. 2015).

Moreover, both receptors are implicated in stimulant-induced behaviors related to addiction, where they seem to induced opposing effects e.g α_{2A}-adrenoreceptor antagonism increases acute locomotion while α_{1A}-adrenoreceptor antagonism decreases these responses (Schmidt and Weinshenker 2014).

1.8 Involvement of D_1 and D_2 dopaminergic receptors

The dopamine receptors are GPCRs which are divided into two distinct types; the D_1-type includes the D_1 and D_5 receptor subtypes, and the D_2-type consists of D_2, D_3, and D_4 receptor subtypes (Tiberi, Jarvie et al. 1991, Vallone, Picetti et al. 2000, Beaulieu and Gainetdinov 2011). The D_1 and D_5 receptors coupled to the G_s-protein mediated their effects by activating AC, which increases the production of cAMP and further activates PKA (Beaulieu and Gainetdinov 2011). Both receptors are found post-synaptically, with the D1 receptor expressed at high levels in the nucleus accumbens, substantia nigra, striatum, frontal cortex, and amygdala (Beaulieu and Gainetdinov 2011). The D_2, D_3, and D_5 receptors couple to the $G_{i/o}$-protein and inhibit the downstream effects of AC (Beaulieu and Gainetdinov 2011). The D_2 and D_3 receptors are found both pre-and postsynaptically and the D_2 receptor is expressed prominently in the striatum, nucleus accumbens, substantia nigra, ventral tegmental area, and olfactory tubercle (Vallone, Picetti et al. 2000, Beaulieu and Gainetdinov 2011).

In general, dopamine is a critical neurotransmitter which plays a major role in many essential CNS processes like reward, sleep, movement, and attention (Iversen and Iversen 2007). Moreover, dysfunction of the dopaminergic inputs in these various processes is linked to several neurological disorders including depression, bipolar disorder, schizophrenia, Parkinson's disease and Huntington's disease (Carlsson, Waters et al. 2001, Cepeda, Murphy et al. 2014, Marino, de Souza et al. 2020). Classical

anti-psychotics like chlorpromazine and haloperidol (antagonists) target the D_2 receptors (Seeman 2010). Moreover, dopamine and the dopaminergic receptors are also implicated in the action of psychoactive drugs like cocaine or amphetamine and their associated high abuse and reinforcing effects (Wu, Reith et al. 2001, Pierce and Kumaresan 2006). Enhancement of the dopamine levels in the brain has been observed in nucleus accumbens, a key brain area linked to the reinforcing properties of several addictive drugs. Chronic drug use of these substances in individuals vulnerable to addiction may lead to neuroadaptations in the dopaminergic system that can result in potential compulsive abuse of these substances (Di Chiara and Imperato 1988, Wu, Reith et al. 2001, Volkow, Fowler et al. 2003, Di Chiara, Bassareo et al. 2004, Volkow, Fowler et al. 2009).

1.9 Involvement of trace-amine associated receptor 1

The trace-amine associated receptor 1 (TAAR1) is key member of the TAAR family which is a GPCR that binds endogenous trace amines like tryptamine, or β-phenethylamine to elevates cAMP levels and mediated further downstream effects (Borowsky, Adham et al. 2001, Bunzow, Sonders et al. 2001). The receptor is highly expressed in the limbic regions of the brain and monoaminergic dense sites like the amygdala, ventral tegmental area, and substantia nigra (Borowsky, Adham et al. 2001, Bunzow, Sonders et al. 2001). The TAAR1 is a modulator of several CNS processes like mood, attention, and addiction (Lindemann, Ebeling et al. 2005, Rutigliano, Accorroni et al. 2017). Several psychoactive drugs of abuse including amphetamines and phenethylamines, which are structurally similar to the endogenous ligands of TAAR1, bind and interact with the receptor as either full or partial agonists (Bunzow, Sonders et al. 2001, Simmler, Buchy et al. 2016). TAAR1 is implicated as a potential drug target for some neuropsychiatric disorders and seems to play an important role in regulation of psychostimulant effects likely by auto-inhibition (Wolinsky, Swanson et al. 2007, Lindemann, Meyer et al. 2008, Di Cara, Maggio et al. 2011, Revel, Moreau et al. 2013, Jing and Li 2015). In particular, TAAR1 modulates neuronal firing influencing psychoactive effects and abuse potential associated with many of these substances (Di Cara, Maggio et al. 2011). Activation of the TAAR1 using partial agonists like RO5203648 has been shown to attenuate stimulant-induced drug seeking behaviours like self-administration, drug reinstatement, and hyperlocomotion

in rodents exposed to classical stimulants like cocaine or methamphetamine (Revel, Moreau et al. 2012, Jing and Li 2015, Pei, Mortas et al. 2015).

Chapter 2

2.1 Publication 1

Metabolites of the ring-substituted stimulants MDMA, methylone and MDPV differentially affect human monoaminergic systems.

Dino Luethi and **Karolina E. Kolaczynska**, Melanie Water, Masaki Suzuki, Kenner C. Rice, Bruce E. Blough, Marius C. Hoener, Michael H. Baumann, and Matthias E. Liechti

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Metabolites of the ring-substituted stimulants MDMA, methylone and MDPV differentially affect human monoaminergic systems

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Abstract

Background: Amphetamine analogs with a 3,4-methylenedioxy ring-substitution are among the most popular illicit drugs of abuse, exerting stimulant and entactogenic effects. Enzymatic *N*-demethylation or opening of the 3,4-methylenedioxy ring via *O*-demethylenation gives rise to metabolites that may be pharmacologically active. Indeed, previous studies in rats show that specific metabolites of 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxymethcathinone (methylone) and 3,4-methylenedioxypyrovalerone (MDPV) can interact with monoaminergic systems.

Aim: Interactions of metabolites of MDMA, methylone and MDPV with human monoaminergic systems were assessed.

Methods: The ability of parent drugs and their metabolites to inhibit uptake of tritiated norepinephrine, dopamine and serotonin (5-HT) was assessed in human embryonic kidney 293 cells transfected with human monoamine transporters. Binding affinities and functional activity at monoamine transporters and various receptor subtypes were also determined.

Results: MDMA and methylone displayed greater potency to inhibit norepinephrine uptake as compared to their effects on dopamine and 5-HT uptake. *N*-demethylation of MDMA failed to alter uptake inhibition profiles, whereas *N*-demethylation of methylone decreased overall transporter inhibition potencies. *O*-demethylenation of MDMA, methylone and MDPV resulted in catechol metabolites that maintained norepinephrine and dopamine uptake inhibition potencies, but markedly reduced activity at 5-HT uptake. *O*-methylation of the catechol metabolites significantly decreased norepinephrine uptake inhibition, resulting in metabolites lacking significant stimulant properties.

Conclusions: Several metabolites of MDMA, methylone and MDPV interact with human transporters and receptors at pharmacologically relevant concentrations. In particular, *N*-demethylated metabolites of MDMA and methylone circulate in unconjugated form and could contribute to the in vivo activity of the parent compounds in human users.

Keywords
MDMA, methylone, MDPV, metabolite, transporter

Introduction

3,4-Methylenedioxymethamphetamine (MDMA, "ecstasy") is a popular drug of abuse which exerts stimulant and entactogenic effects by evoking release and inhibiting uptake of presynaptic norepinephrine, 5-HT and dopamine (Del Bello et al., 2015; Rickli et al., 2015a). The enzymatic biotransformation of MDMA includes two main pathways: (1) *N*-demethylation to form 3,4-methylenedioxyamphetamine (MDA) and (2) *O*-demethylenation to form catechol metabolites 3,4-dihydroxymethamphetamine (HHMA) and 3,4-dihydroxyamphetamine (HHA) (de la Torre et al., 2004; Kreth et al., 2000; Meyer et al., 2008; Segura et al., 2005). The latter metabolites may contribute to MDMA-induced adverse clinical effects, since the cytotoxicity of highly reactive catechols is well established (Antolino-Lobo et al., 2011; Carmo et al., 2006). Additionally, the 4-hydroxy-3-methoxy metabolites of MDMA exhibit increased potency to stimulate vasopressin secretion, which could increase the risk of fatal hyponatremia (Fallon et al., 2002; Forsling et al., 2002).

Previous investigations in rats show that subcutaneous injection of MDMA or its *N*-demethylated metabolite MDA (1–10 mg/kg) increases blood pressure, heart rate and locomotor

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activity (Schindler et al., 2014). By contrast, injection of the *O*-demethylenated catechol metabolites HHMA and HHA (1–10 mg/kg) induces potent sympathomimetic effects on the cardiovascular system but fails to affect locomotor activity, suggesting these more polar compounds do not readily cross the blood–brain barrier and target norepinephrine transporters or receptors in the periphery (Mueller et al., 2009; Schindler et al., 2014). Escobedo and colleagues demonstrated that HHMA can be detected in mouse brain after intraperitoneal administration of high doses of HHMA (30 mg/kg) but not MDMA (30 mg/kg) (Escobedo et al., 2005), which indicates that catechol metabolites can cross the blood–brain barrier under certain circumstances.

The synthetic cathinones 3,4-methylenedioxymethcathinone (methylone) and 3,4-methylenedioxypyrovalerone (MDPV) share the methylenedioxy ring-substitution with MDMA but their pharmacological effects are different. Methylone is a transporter substrate like MDMA (Baumann et al., 2012; Simmler et al., 2013), but it inhibits dopamine uptake more potently than 5-HT uptake in transfected cells (Eshleman et al., 2013; Simmler et al., 2013). MDPV is a potent blocker of the norepinephrine transporter (NET) and dopamine transporter (DAT) devoid of substrate activity, similar to the mechanism of action for methylphenidate (Baumann et al., 2013; Eshleman et al., 2013; Luethi et al., 2018a; Simmler et al., 2013) but with increased potency and toxicity. Importantly, the metabolism pattern of methylone and MDPV is similar to MDMA, giving rise to potentially active metabolites (Baumann et al., 2012; de la Torre et al., 2004; Schindler et al., 2014). In the present study, we wished to assess the potential clinical relevance of in vivo and in vitro studies in rats (Anizan et al., 2016; Elmore et al., 2017; Schindler et al., 2014) by examining the interactions of metabolites of MDMA, methylone and MDPV with human monoamine transporters and receptors, and rat and mouse trace amine-associated receptor 1 (TAAR1).

Methods

Drugs/test substances

Mazindol, MDA HCl, MDMA HCl, MDPV HCl, methylone HCl and fluoxetine HCl were purchased from Lipomed (Arlesheim, Switzerland). Nisoxetine HCl was obtained from Sigma-Aldrich (Buchs, Switzerland). 3,4-Dihydroxyamphetamine (HHA) HCl, 3,4-dihydroxymethamphetamine (HHMA) fumarate, 4-hydroxy-3-methyoxyamphetamine (HMA) fumarate and 4-hydroxy-3-methoxymethamphetamine (HMMA) HCl were synthesized and analyzed for purity at the Research Triangle Institute (RTI, Durham, NC, USA) (Schindler et al., 2014). 3,4-Dihydroxymethcathinone (HHMC) HBr, 4-hydroxy-3-methyoxymethcathinone (HMMC) HCl and 3,4-methylenedioxycathinone (MDC) HCl were synthesized as described in Ellefsen et al. (2015); 3,4-dihydroxypyrovalerone (HHPV) HBr and 4-hydroxy-3-methyoxypyrovalerone (HMPV) HCl were synthesized as described in Anizan et al. (2014). All drugs were used as racemic mixtures. Radiolabeled [^{3}H] norepinephrine (13.1 Ci/mmol) and [^{3}H] dopamine (30.0 Ci/mmol) were attained from Perkin-Elmer (Schwerzenbach, Switzerland). Radiolabeled [^{3}H] 5-HT (80 Ci/mmol) was obtained from Anawa (Zurich, Switzerland).

Monoamine uptake transporter inhibition

Human embryonic kidney (HEK) 293 cells (Invitrogen, Zug, Switzerland) stably overexpressing the human NET, DAT or 5-HT transporter (SERT) were used to investigate the inhibition of monoamine uptake as previously described (Luethi et al., 2018b). Briefly, cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Life Technologies, Zug, Switzerland) containing 10% fetal bovine serum (Gibco), and upon 70–90% confluency, the cells were detached and resuspended (3×10^6 cells/mL) in Krebs-Ringer Bicarbonate Buffer (KRB; Sigma-Aldrich, Buchs, Switzerland). For [^{3}H] dopamine uptake experiments, the uptake buffer was additionally supplied with 0.2 mg/mL of L-ascorbic acid (Sigma-Aldrich). To 100 µL of cell suspension, 25 µL of uptake buffer containing test compounds (at concentrations of 1 nM to 900 µM), transporter-specific inhibitors (10 µM nisoxetine for NET, 10 µM mazindol for DAT and 10 µM fluoxetine for SERT) or vehicle control were added in a round bottom 96-well plate. After 10 min shaking on a rotary shaker at 450 rotations per min at room temperature, 50 µL of radiolabeled [^{3}H] norepinephrine, [^{3}H] dopamine or [^{3}H] 5-HT dissolved in uptake buffer was added for an additional 10 min to initiate uptake transport. Thereafter, 100 µL of the suspension was transferred into microcentrifuge tubes containing 50 µL of 3 M KOH and 200 µL of a 1:1 mixture of silicone oil type AR 200 and type AR 20 (Sigma-Aldrich). The tubes were centrifuged for 3 min (13,200 rotations per min) and frozen in liquid nitrogen. The cell pellet was then cut into 6-mL scintillation vials (Perkin-Elmer) containing lysis buffer (1% NP-40, 5 mM EDTA, 50 mM NaCl, 0.05 M TRIS HCl). The vials were shaken for 1 h, filled with 4.5 mL of scintillation fluid (Ultimagold, Perkin-Elmer), and, subsequently, the uptake was quantified using a scintillation counter (Packard Tri-Carb Liquid Scintillation Counter 1900 TR). Uptake in the presence of the selective inhibitors was subtracted to determine specific uptake. A summary of cell culture and assay conditions for the monoamine reuptake inhibition assays is given in Supplemental Table S1.

Receptor and transporter binding

Receptor and transporter binding affinities were determined as previously described in detail for each receptor and transporter (Luethi et al., 2018c). Briefly, cell membrane preparations were derived from various cell lines (Supplemental Table S2) and overexpressed the respective monoamine receptors or transporters (human genes, with the exception of rat and mouse genes for TAAR1). The membrane preparations were incubated with radiolabeled selective ligands at concentrations equal to K_d, and ligand displacement by the compounds was measured. The difference between total binding (binding buffer alone) and nonspecific binding (in the presence of specific competitors) was determined to be specific binding. The following radioligands and competitors, respectively, were used: 2.9 nM *N*-methyl-[^{3}H] nisoxetine and 10 µM indatraline (NET), 3.3 nM [^{3}H]WIN35,428 and 10 µM indatraline (DAT), 1.5 nM [^{3}H]citalopram and 10 µM indatraline (SERT), 0.90 nM [^{3}H]8-hydroxy-2-(di-n-propylamine)tetralin and 10 µM pindolol (5-HT_{1A} receptor), 0.40 nM [^{3}H]ketanserin and 10 µM spiperone (5-HT_{2A} receptor), 1.4 nM [^{3}H]mesulgerine and 10 µM mianserin (5-HT_{2C} receptor), 0.11 nM [^{3}H]prazosin and 10 µM chlorpromazine (α_1 adrenergic

receptor), 2 nM [^{3}H]rauwolscine and 10 μM phentolamine (α_2 adrenergic receptor), 1.2 nM [^{3}H]spiperone and 10 μM spiperone (dopamine D_2 receptors), and 3.5 nM (rat TAAR1) or 2.4 nM (mouse TAAR1) [^{3}H]RO5166017 and 10 μM RO5166017 (rat and mouse TAAR1). IC_{50} values were assessed by calculating non-linear regression curves for a one-site model using three independent 10-point concentration–response curves. K_i values were determined by the Cheng–Prusoff equation. A summary of cell culture and assay conditions for the radioligand binding assays is given in Supplemental Table S2.

Activity at the 5-HT$_{2B}$ receptor

Human embryonic kidney (HEK) 293 cells expressing the human 5-HT_{2B} receptor were incubated in growth medium at a density of 50,000 cells per well at 37°C in poly-D-lysine-coated 96-well plates overnight. Thereafter, the growth medium was removed by snap inversion, and 200 μl of no wash dye (FLIPR calcium 6 assay kit Cat # R8191; Molecular Devices, Sunnyvale, CA, USA) was added to each well. The plates were incubated for 2 h at 37°C. Thereafter, the plates were placed into a FLIPR, and 50 μL of the test drugs diluted in assay buffer were added to each well online. The increase in fluorescence was measured for 51 s, and EC_{50} values were derived from the concentration–response curves using non-linear regression. A summary of cell culture and assay conditions for the 5-HT_{2B} receptor activation assay is given in Supplemental Table S3.

Functional activity at the human TAAR1

Activity at the human TAAR1 was assessed as previously described in detail (Luethi et al., 2018c). Recombinant HEK 293 cells that expressed the human TAAR1 were harvested and pelleted by centrifugation at 900 rotations per min for 3 min at room temperature. Thereafter, the supernatant was removed and the cell pellet was resuspended in fresh culture medium. The cells were plated into 96-well plates (100 μL, containing 80,000 cells per well) and incubated for 20 h at 37°C. The cell culture medium was removed and 50 μl phosphate buffered saline (PBS) (without Ca^{2+} and Mg^{2+}) was added. The PBS was removed by snap inversion, 90 μL of KRB containing 1 mM IBMX was added and the plates were incubated for 60 min at 37°C. All the compounds were tested at a broad concentration range (300 pM–30 μM) in duplicate, and a standard curve (0.13 nM–10 μM cAMP) was included on each plate. Additionally, a reference plate containing RO5256390, β-phenylethylamine and p-tyramine was included in each experiment. Compound solution, β-phenylethylamine (as maximal response), or basal control were added at a volume of 30 μL, and the cells were incubated for 40 min at 37°C. Finally, the cells were lysed with 50 μL of detection mix solution containing Ru-cAMP Alexa700 anti-cAMP antibody and lysis buffer for 120 min at room temperature under heavy shaking; fluorescence was then measured. A summary of cell culture and assay conditions for the human TAAR1 activation assay is given in Supplemental Table S3.

Data analysis

Prism software (version 7.0a, GraphPad, San Diego, CA, USA) was used for calculations. Monoamine uptake data were fitted by non-linear regression to variable-slope sigmoidal dose–response curves, and IC_{50} values and 95% confidence intervals were derived from three to five individual 11-point inhibition curves. The DAT/SERT ratio is expressed as 1/DAT IC_{50}:1/SERT IC_{50}. For radioligand binding assays, logistic regression was used to calculate IC_{50} values derived from three 10-point curves. No observed affinity within a concentration range indicates that there was no binding at the highest tested concentration as well as no binding at nine different lower concentrations. Unpaired two-tailed Student's *t*-test was used to compare individual K_i values and P values < 0.05 were considered to be statistically significant. EC_{50} values for 5-HT_{2B} receptor activation were determined using non-linear regression concentration–response curves. The maximal activity at the receptors was calculated relative to 5-HT activity, which was defined as 100%. The activity at the human TAAR1 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) – $P \times$ Å~ FRET (630 nM), where P = Ru (700 nM)/Ru (630 nM).

Results

Monoamine transporter inhibition

The uptake inhibition curves for MDMA, methylone, MDPV and their respective metabolites are shown in Figures 1–3, with corresponding IC_{50} values and DAT/SERT inhibition ratios listed in Table 1. Numbers in parentheses indicate the number of individual 11-point curves (NET/DAT/SERT): MDMA (3/3/3), MDA (3/3/3), HHMA (4/–/5), HHA (3/–/3), HMMA (3/3/4), HMA (3/4/3), methylone (3/3/3), MDC (4/3/3), HHMC (3/–/3), HMMC (3/4/4), MDPV (3/3/3), HHPV (3/4/3) and HMPV (3/3/4). No sigmoidal uptake curves could be plotted for dopamine uptake inhibition in the cases of HHA, HHMA and HHMC but the curve intercepts between 0 and 100% uptake were included in Figures 1–3 for comparison; the full non-sigmoidal curves of three of individual 11-point curves are shown in Supplemental Figure S1.

MDMA potently inhibited norepinephrine uptake (IC_{50} = 0.38 μM) and had a distinct preference to inhibit 5-HT (IC_{50} = 2.5 μM) vs. dopamine (IC_{50} = 21 μM) uptake. The *N*-demethylation of MDMA did not alter potency to inhibit norepinephrine and barely influenced its selectivity for 5-HT (IC_{50} = 4.3 μM) vs. dopamine (IC_{50} = 17 μM) uptake inhibition. The catechol metabolites of MDMA and MDA that are formed by *O*-demethylenation maintained potent norepinephrine uptake inhibition with IC_{50} values of 0.35 and 0.18 μM for HHMA and HHA, respectively. However, both catechol metabolites displayed a substantial decrease in inhibition of 5-HT uptake (IC_{50} of 63–65 μM) compared to the parent compounds. As no IC_{50} values could be calculated for HHMA and HHA for dopamine uptake inhibition, the intercepts of the dopamine uptake curves and 50% uptake inhibition values were determined as estimates of their transporter inhibition potencies. For these metabolites, 50% dopamine uptake inhibition was reached at 9.6–9.8 μM.

O-methylation of the catechol metabolites decreased the potency to inhibit norepinephrine uptake, which resulted in IC_{50} values of 10 and 22 μM for HMMA and HMA, respectively. Dopamine uptake inhibition for HMMA (IC_{50} = 5.6 μM) and HMA (IC_{50} = 0.13 μM) was significantly increased compared to MDMA and MDA (confidence intervals of the IC_{50} values do not overlap). However, HMA only partially inhibited dopamine

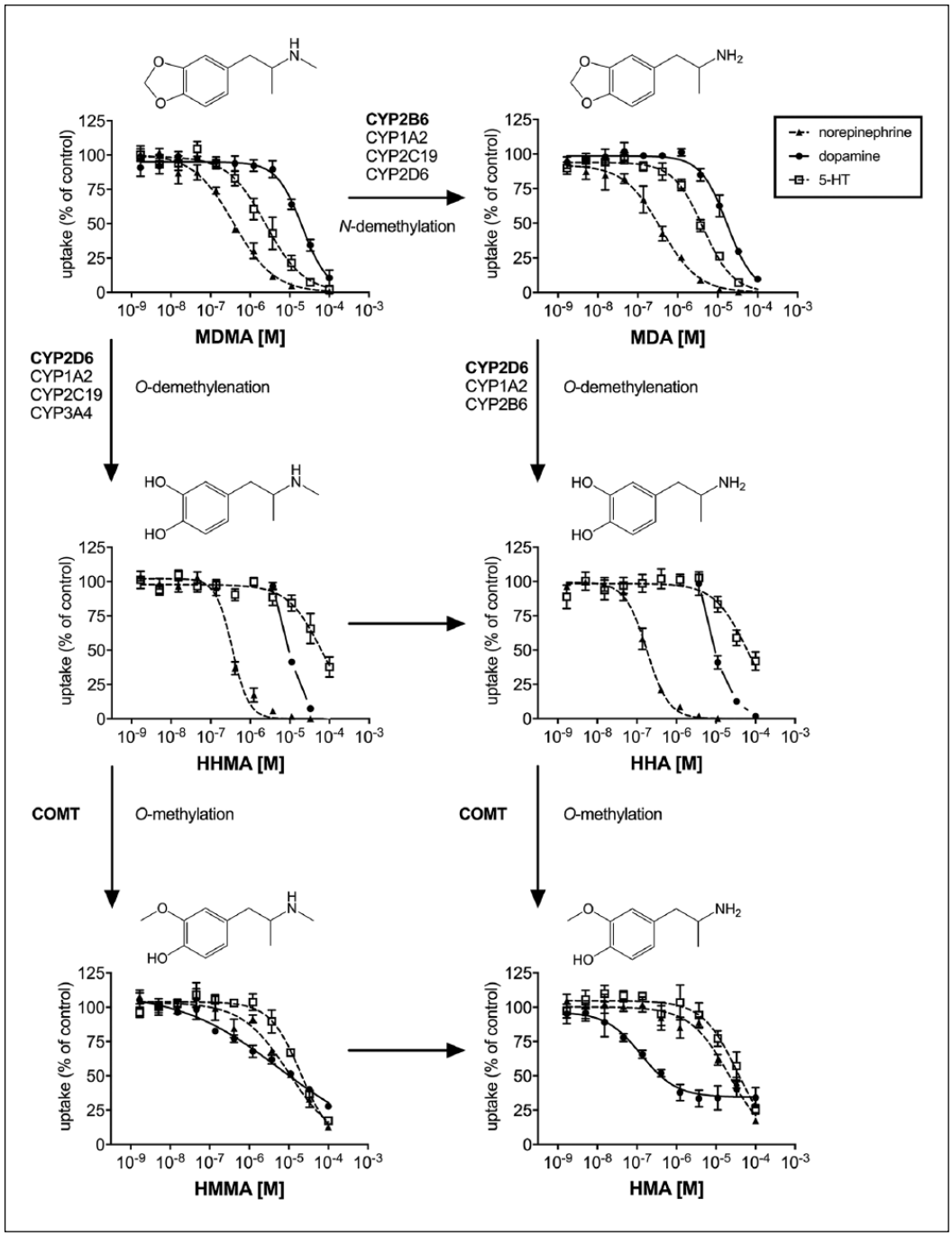

Figure 1. Metabolism of MDMA and corresponding monoamine uptake inhibition curves. Major (bold) and minor enzymes involved in MDMA metabolism as described in Kreth et al. (2000) and Meyer et al. (2008). Monoamine uptake curves were fitted by non-linear regression and the data are presented as the mean ± SEM. For HHMA and HHA no IC_{50} value was calculated for DAT, due to the lack of sigmoidal shape of the uptake curves. For these compounds, the DAT uptake curve intercept between 0 and 100% is shown in the figure for comparison, and full curves are shown in Supplemental Figure S1.

uptake, with a maximum of 66% transporter inhibition (34% dopamine uptake).

Similar to MDMA, the β-keto analog methylone potently inhibited norepinephrine uptake (IC_{50} = 0.58 μM). Unlike MDMA, methylone had higher preference to inhibit dopamine (IC_{50} = 6.6 μM) vs. 5-HT (IC_{50} = 18 μM) uptake. MDC, the metabolite formed by *N*-demethylation of methylone, was more selective for 5-HT vs. dopamine inhibition but showed lower

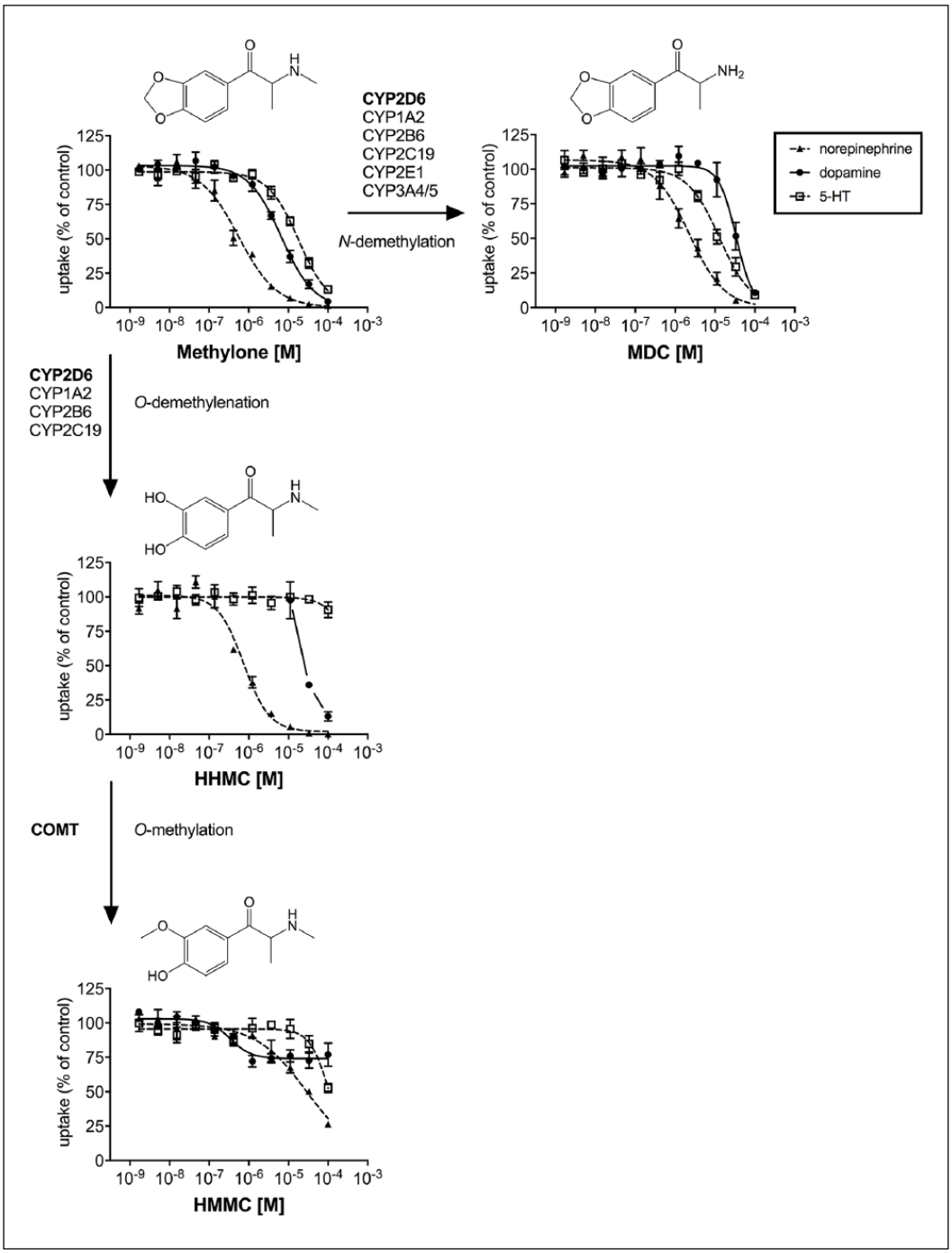

Figure 2. Metabolism of methylone and corresponding monoamine uptake inhibition curves. Major (bold) and minor enzymes involved in methylone metabolism as described in Pedersen et al. (2013). Monoamine uptake curves were fitted by non-linear regression and the data are presented as the mean ± S.E.M. No DAT IC_{50} value was calculated for HHMC, due to the lack of sigmoidal shape of the uptake curve. The DAT uptake curve intercept between 0 and 100% for HHMC is shown in the figure for comparison, and the full curve is shown in Supplemental Figure S1.

overall potency for all transporters. As observed for MDMA metabolites, the *O*-demethylenated catechol metabolite HHMC had substantially decreased 5-HT uptake inhibition potency (IC_{50} > 100 μM) without significant change in norepinephrine uptake inhibition (IC_{50} = 0.78 μM). HMMC, which is formed by *O*-methylation of HHMC, had decreased the potency to inhibit norepinephrine uptake (IC_{50} = 30 μM) while altering DAT function at lower concentrations (IC_{50} = 0.34 μM) compared to the

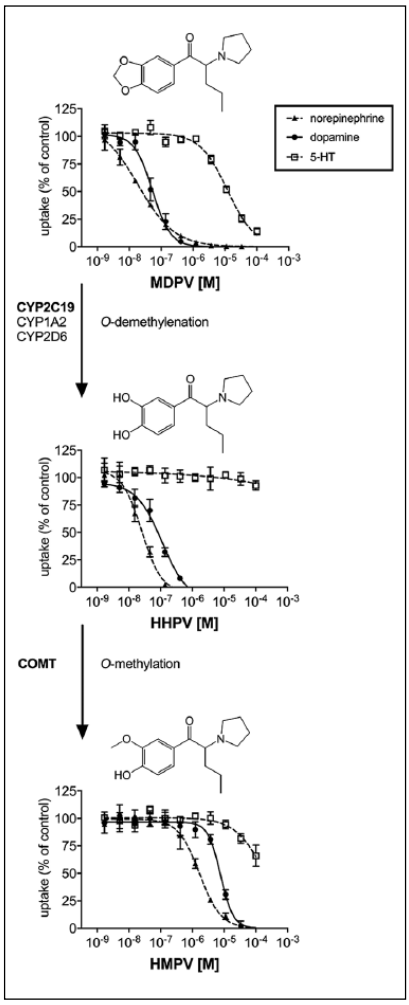

Figure 3. Metabolism of MDPV and corresponding monoamine uptake inhibition curves. Major (bold) and minor enzymes involved in methylone metabolism as described in Meyer et al. (2010). Monoamine uptake curves were fitted by non-linear regression, and the data are presented as the mean ± SEM.

parent compound. Like HMA, HMMC only partially (26%) inhibited dopamine uptake transport (74% dopamine uptake).

MDPV inhibited NET and DAT with high potency (IC_{50} = 0.018 and 0.053 μM, respectively) but had much lower potency at SERT (IC_{50} = 12 μM). The catechol metabolite HHPV potently inhibited NET and DAT as well (IC_{50} = 0.024 and 0.092 μM, respectively) but was devoid of SERT inhibition activity (IC_{50} > 100 μM). *O*-methylation of HHPV resulted in substantially decreased NET and DAT inhibition potency (IC_{50} = 1.7 and 7.7 μM, respectively) for the corresponding hydroxy-methoxy metabolite HMPV.

Monoamine receptor and transporter binding affinities

The binding affinities and activation potencies of MDMA, methylone, MDPV and their metabolites at monoamine transporters and receptors are shown in Table 2 (*n*=3). MDMA did not bind to any monoamine transporters in the investigated concentration range. However, the catechol metabolites HHMA and HHA displayed sub-micromolar affinity at the NET and low micromolar affinity at the DAT. MDMA and MDA bound to adrenergic α_1 and α_2 receptors (K_i of 2.6–8.8 μM) whereas the catechol metabolites HHMA and HHA bound to α_2 receptors only (K_i of 1.3 and 2.0 μM, respectively). Neither MDMA nor any of its metabolites bound to dopaminergic D_2 receptors. MDMA and MDA showed affinity at serotonergic 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors in the range of 3–11 μM. The remaining metabolites of MDMA were devoid of any binding to serotonergic receptors apart from HHA, which bound to the 5-HT_{2A} receptor (K_i = 9.2 μM). MDMA and its metabolites showed high affinity to both rat and mouse TAAR1 in the range of 0.1–3.1 μM, except for HHMA, which did not bind to the mouse TAAR1 in the investigated concentration range (K_i > 4.4 μM).

Methylone bound to the DAT (K_i = 2.3 μM) but not to the NET or SERT in the investigated concentration range. The *N*-demethylated metabolite MDC showed no affinity to any monoamine transporter. The dihydroxy and hydroxy-methoxy metabolites HHMC and HMMC both bound to the NET with K_i of 4 μM, and HHMC additionally bound to the DAT (K_i = 7.1 μM). Methylone and its metabolites did not bind to any adrenergic, dopaminergic or serotonergic receptors in the investigated concentration range. Binding to the rat TAAR1 was observed for MDC and HHMC in the range of 2–5 μM, and binding to the mouse TAAR1 was observed for MDC only (K_i = 3.5 μM).

MDPV displayed high affinity at the NET and DAT (K_i = 0.10 and 0.01 μM, respectively) with less potent affinity at the SERT (K_i = 2.2 μM). The dihydroxy metabolite HHPV maintained potent NET inhibition potency (K_i = 0.11 μM, respectively) but displayed a slight but significantly lower DAT inhibition (0.02 μM). The hydroxy-methoxy metabolite HMPV bound to the NET and DAT significantly less potently (K_i = 6.2 and 2.0 μM, respectively) compared to MDPV and HHPV. HHPV and HMPV did not bind to the SERT in the investigated concentration range (K_i >7.4 μM). MDPV and its metabolites did not bind to adrenergic or dopaminergic receptors in the investigated concentration range. Moderate affinity at serotonergic receptors was observed for MDPV at the 5-HT_{1A} and 5-HT_{2C} receptor (K_i = 12 and 2 μM, respectively) and for HHPV at the 5-HT_{1A} receptor only (K_i = 4.3 μM). MDPV and its metabolite did not bind to rat or mouse TAAR1 in the investigated concentration range.

Activity at the serotonergic 5-HT_{2B} receptor and human TAAR1

No binding affinity at the 5-HT_{2B} receptor was examined; however, to assess for possible 5-HT_{2B} interactions, the activation potency for this receptor subtype was determined (*n*=3). Of all the parent compounds and metabolites, MDA was the only compound to partially activate the 5-HT_{2B} receptor with EC_{50} of 0.20 ± 0.01 μM and activation efficacy of 51 ± 7%. The remaining drugs did not activate the 5-HT_{2B} receptor at concentrations up to 10 μM. Activation of the human TAAR1 was assessed for compounds with considerable affinity (K_i < 2 μM) for either rat or mouse TAAR1. HMA was the only compound to partially activate human TAAR1 with EC_{50} of 10.4 ± 0.2 μM and activation efficacy of 51 ± 12%.

Table 1. Monoamine uptake inhibition.

	NET	DAT	SERT	DAT/SERT ratio
	IC_{50} [μM]	IC_{50} [μM]	IC_{50} [μM]	
MDMA	0.38 (0.28–0.52)	21 (17–26)	2.5 (1.7–3.6)	0.12 (0.07–0.21)
HHMA	0.35 (0.30–0.41)	9.8[a]	65 (46–94)	
HMMA	10 (7–15)	5.6 (2.8–11.4)	20 (16–25)	3.6 (1.4–8.9)
MDA	0.38 (0.27–0.54)	17 (14–21)	4.3 (3.5–5.3)	0.25 (0.17–0.38)
HHA	0.18 (0.15–0.21)	9.6[a]	63 (43–93)	
HMA	22 (16–30)	0.13 (0.06–0.26)[b]	35 (25–50)	270 (96–830)
Methylone	0.56 (0.41–0.76)	6.6 (5.5–7.9)	18 (15–21)	2.7 (1.9–3.8)
HHMC	0.78 (0.58–1.05)	25[a]	>100	
HMMC	30 (20–43)	0.34 (0.16–0.71)[b]	>100	
MDC	2.3 (1.6–3.2)	34 (27–42)	13 (10–16)	0.38 (0.24–0.59)
MDPV	0.018 (0.010–0.033)	0.053 (0.040–0.070)	12 (10–14)	226 (143–350)
HHPV	0.024 (0.018–0.031)	0.092 (0.067–0.126)	>100	
HMPV	1.7 (1.2–2.2)	7.7 (6.2–9.6)	>100	

Values are means and 95% confidence intervals. DAT/SERT ratio = 1/DAT IC_{50}:1/SERT IC_{50}.
[a]No IC_{50} value could be calculated as the curve did not display a sigmoidal shape; given concentrations represent 50% uptake inhibition.
[b]Partial uptake inhibition.

Table 2. Monoamine transporter and receptor binding.

	NET	DAT	SERT	D_2	α_{1A}	α_{2A}	5-HT_{1A}	5-HT_{2A}	5-HT_{2C}	$TAAR1_{rat}$	$TAAR1_{mouse}$
	K_i [μM]	K_i [μM]	K_i [μM]	K_i [μM]	K_i [μM]	K_i [μM]	K_i [μM]	K_i [μM]	K_i [μM]	K_i [μM]	K_i [μM]
MDMA	>8.7	>8.5	>7.5	>13	6.9±1.2	4.6±0.1	11±2	4.6±1.1	4.4±0.8	0.25±0.01	3.1±0.7
HHMA	0.94±0.14	1.9±0.0	>7.4	>13	>9.5	1.3±0.1	>18	>12	>5.1	3.0±0.3	>4.4
HMMA	>8.8	>8.5	>7.4	>13	>9.5	>4.7	>18	>12	>5.1	0.25±0.04	0.45±0.05
MDA	>8.8	>8.5	>7.4	>13	8.8±0.6	2.6±0.1	9.0±0.8	3.2±0.8	4.8±0.4	0.22±0.03	0.18±0.03
HHA	0.79±0.30	2.9±0.3	>7.4	>13	>9.5	2.0±0.4	>18	9.2±1.7	>5.1	1.9±0.4	1.4±0.2
HMA	>8.8	>8.5	>7.4	>13	>9.5	>4.7	>18	>12	>5.1	0.15±0.02	0.05±0.02
Methylone	>8.7	2.3±0.1	>7.5	>13	>9.5	>5.0	>17	>12	>14	>5.0	>4.7
HHMC	4.0±1.0	7.1±0.3	>7.4	>13	>9.5	>4.7	>18	>12	>5.1	2.2±0.1	>4.4
HMMC	4.2±1.0	>8.5	>7.4	>13	>9.5	>4.7	>18	>12	>5.1	>5.2	>4.4
MDC	>8.8	>8.5	>7.4	>13	>9.5	>4.7	>17	>12	>5.1	4.8±0.5	3.5±0.3
MDPV	0.10±0.01	0.011±0.001	2.2±0.1	>13	>9.5	>5.0	12±2	>12	2.1±0.2	>5.0	>4.7
HHPV	0.11±0.01	0.023±0.003	>7.4	>13	>9.5	>4.7	4.3±1.6	>12	>5.1	>5.2	>4.4
HMPV	6.2±0.7	2.0±0.2	>7.4	>13	>9.5	>4.7	>17	>12	>5.1	>5.2	>4.4

K_i values are given as mean ± SD.

Discussion

Pharmacological effects of MDMA metabolites

The metabolism of MDMA in humans consists of two pathways: (1) *N*-demethylation to form MDA and (2) *O*-demethylenation to form HHMA and HHA (de la Torre et al., 2004, 2012; Kreth et al., 2000; Meyer et al., 2008; Schmid et al., 2016). MDA formation is primarily mediated by cytochrome P450 (CYP) 2B6 with contributions from CYP1A2, CYP2C19 and CYP2D6 (Kreth et al., 2000; Meyer et al., 2008; Schmid et al., 2016; Vizeli et al., 2017). CYP2D6 is the primary enzyme involved in *O*-demethylenation of MDMA and MDA, with contributions from CYP1A2, CYP2C19 and CYP3A4 (Kreth et al., 2000; Meyer et al., 2008; Schmid et al., 2016; Vizeli et al., 2017). The demethylenated catechol metabolites HHMA and HHA are subsequently *O*-methylated by catechol-*O*-methyltransferase (COMT) to yield HMMA and HMA (de la Torre et al., 2004, 2012). A study in healthy human subjects revealed T_{max} values of 2.2–2.6 h for MDMA, 4.6–5.9 h for MDA and 2.7–5.0 h for HMMA after oral administration of different MDMA doses (Schmid et al., 2016). C_{max} levels of MDA accounted for 4–8% of the C_{max} of MDMA. The C_{max} of HMMA strongly depended on CYP2D6 activity and ranged from 9 to 48% of the C_{max} of MDMA; however, the C_{max} of unconjugated HMMA was only about 1–3% of the C_{max} of MDMA, suggesting a high proportion of conjugated HMMA (Schmid et al., 2016). It is noteworthy that the biotransformation of MDMA is generally similar between rats and humans, with the notable exception that rats metabolize

the parent compound at a much faster rate (Baumann et al., 2009; Concheiro et al., 2014).

In the current study, both MDMA and MDA displayed their most potent effects at norepinephrine uptake inhibition, along with an entactogenic pharmacological profile, which is expressed as more potent effects at 5-HT vs. dopamine uptake inhibition (Liechti, 2015; Simmler et al., 2013). MDA showed activity at the serotonergic 5-HT_{2B} receptor. The affinity of MDA and MDMA at the 5-HT_{2A} receptor did not significantly differ, but MDA has previously been shown to activate the 5-HT_{2A} receptor about 10-fold more potently compared to MDMA (Rickli et al., 2015b). However, a recent study showed that the hallucinogenic potency of psychedelic drugs correlates with their 5-HT_{2A} and 5-HT_{2C} receptor binding affinities but not with their 5-HT_{2A} receptor functional activities. This intriguing observation may be explained by higher sensitivity of the ligand-binding assay or inherent limitations of the calcium mobilization assay to provide a valid index of in vivo receptor subtype activity (Luethi and Liechti, 2018). Taken together, MDA might exert mild psychedelic effects that are slightly more pronounced than MDMA, but an increase in such effects may not be noticeable over the time course after MDMA use, as only a small fraction of the parent compound ($<$10%) is metabolized to MDA.

Our findings show that *O*-demethylenation resulted in more pronounced dopaminergic activity of the metabolites, which is not surprising given the similarities in chemical structures for the hydroxy metabolites and dopamine. The catechol metabolites HHMA and HHA maintained the NET inhibition potency of their precursors MDMA and MDA, respectively. Furthermore, the corresponding catechol metabolites displayed substantial binding affinity to both NET and DAT. In contrast to the overall reduced potency at monoamine transporters, the hydroxy-methoxy metabolites showed high affinity to rodent TAAR1. However, HMA was the only compound that partially activated human TAAR1 in this study, with only moderate potency (EC_{50} of 10 μM). Further research is needed to decipher the effect of MDMA metabolites on TAAR1.

The catechol and hydroxy-methoxy metabolites of MDMA are almost exclusively found in conjugated form as sulfates (HHMA and partially HMMA) or glucuronides (HMMA) in plasma and urine (de la Torre et al., 2004; Schmid et al., 2016; Segura et al., 2001; Steuer et al., 2015). Thus, despite the rather modest amount of MDA generated from biotransformation of MDMA, it is the most relevant contributor to the pharmacological effects among all MDMA metabolites. Transporter inhibition data and animal studies (Schindler et al., 2014) suggest that the fraction of unconjugated catechol metabolites could contribute to cardiovascular effects of MDMA, while a substantial contribution of the hydroxy-methoxy metabolites seems unlikely. The anomalous increase of dopamine uptake to more than 200% as observed for HHMA and HHA has been reported before for unlabeled dopamine and other DAT substrates (Henry et al., 2018), but the underlying mechanisms responsible for this phenomenon are not yet understood and more research is needed to provide better insight. It is worth mentioning that Escubedo et al. (2011) reported that HHMA induces wash-resistant inhibition of dopamine uptake in rat brain tissue suggesting that dihydroxy metabolites might induce long-term changes in DAT structure and function related to cytotoxic effects. Furthermore, various mechanistic studies suggest that MDMA metabolites are in fact responsible for the neurotoxic effects related to MDMA use (Moratalla et al., 2017). Besides metabolism in the liver, CYP enzymes located near drug targets in the brain may affect local metabolism of centrally acting drugs (Miksys and Tyndale, 2002). Therefore, metabolite concentrations in the brain may differ from the measured plasma levels, and potential effects of the metabolites cannot solely be derived from transporter and receptor interaction studies.

Pharmacological effects of methylone metabolites

In vitro studies with human liver microsomes demonstrate that methylone is *N*-demethylated to form MDC and *O*-demethylenated to form HHMC, analogous to the metabolism of MDMA. These biotransformations mainly involve the actions of CYP2D6 with minor contributions from other CYP enzymes (Pedersen et al., 2013). HHMC is further metabolized by COMT to yield HMMC and to a lesser extent to 3-hydroxy-4-methoxymethcathinone (Pedersen et al., 2013). Following a 5 mg/kg injection of methylone in rats, about 3% of total methylone was excreted in urine in unchanged form within 48 h post-dosing, whereas the amount of MDC and HMMC accounted for about 2% and 26%, respectively (Kamata et al., 2006). Additionally, 5% of the HMMC isomer 3-hydroxy-4-methoxy-methcathinone was formed. However, $>$80% of the hydroxy-methoxy metabolites of methylone were excreted as conjugates (Kamata et al., 2006). In the same study, a single human urinary sample of a patient admitted to an emergency department after ingestion of an unknown amount of methylone powder was analyzed. In accordance with the rat data, the analysis revealed that HMMC was the most abundant metabolite with MDC and 3-hydroxy-4-methoxy-methcathinone being minor metabolites (Kamata et al., 2006). HMMC was the most abundant metabolite also in rat plasma following subcutaneous injections of methylone, reaching 12–22% of the parent compound; HHMC and MDC were detected in amounts of 10–13% of injected methylone (Elmore et al., 2017). C_{max} was reached after 15 min for methylone, after 30–45 min for MDC, after 60–70 min for HHMC and after 90–120 min for HMMC (Elmore et al., 2017). In another study, maximum brain and serum concentrations of methylone were reached 30 min after subcutaneous injection with approximately five times higher brain vs. serum concentrations (Stefkova et al., 2017). Serum levels of MDC peaked 30 min later than those of methylone and reached about 20% of the methylone levels (Stefkova et al., 2017). In that study, HMMC was found to be the second most abundant metabolite in serum after MDC. However, the precise amount of HMMC could not be quantified. Lopez-Arnau and colleagues reported T_{max} values of 0.5 and 1 h for 15 mg/kg and 30 mg/kg oral doses, respectively (Lopez-Arnau et al., 2013). The similar T_{max} values observed after subcutaneous injection and oral administration suggests fast absorption of the drug. Different observations have been made regarding the linearity of methylone pharmacokinetics, with one study suggesting linear pharmacokinetics after oral administration (Lopez-Arnau et al., 2013) and another suggesting non-linear pharmacokinetics after subcutaneous administration (Elmore et al., 2017).

In the current study, methylone was a potent inhibitor of norepinephrine uptake and to a lesser extent an inhibitor of dopamine and 5-HT uptake. The *N*-demethylated metabolite MDC

exerted similar 5-HT uptake inhibition potency to methylone but much weaker potency at norepinephrine and dopamine uptake inhibition, suggesting weaker psychotropic effects compared to the parent compound (Luethi and Liechti, 2018), which is in accordance with animal studies (Elmore et al., 2017). The partial dopamine inhibition by HHMC at sub-micromolar concentrations may not be sufficient to produce discernable dopaminergic effects over the course of time after methylone intake. In fact, neither HHMC nor HMMC increase basal dialysate dopamine concentrations when administered intravenously to rats (Elmore et al., 2017). Like methylone (Baumann et al., 2012), MDC and HHMC are known transporter substrates (Elmore et al., 2017). Such substrate-type activity has been previously observed for phase I metabolites of the transporter substrate 4-methylmethcathinone (mephedrone) (Mayer et al., 2016). The binding of methylone to DAT and of HHMC to both DAT and NET suggests that these compounds may adopt both substrate-type and inhibitory binding modes, as has been described before for certain MDMA analogs (Sandtner et al., 2016). As observed for the catechol metabolites of MDMA and other hDAT substrates (Henry et al., 2018), HHMC caused a more than 200% increase in dopamine uptake at some concentrations.

Pharmacological effects of MDPV metabolites

The main metabolic pathway of MDPV biotransformation is *O*-demethylenation by CYP2D6 to form HHPV, followed by *O*-methylation to yield HMPV (Meyer et al., 2010; Strano-Rossi et al., 2010). Meyer and colleagues found HMPV to be the most abundant metabolite in human and rat urine (Meyer et al., 2010). Following subcutaneous injections of 0.5–2 mg/kg MDPV to rats, T_{max} was reached after 13–19 min (Anizan et al., 2016). In that study, HMPV was the most abundant metabolite, with maximal concentrations reaching 53–61% of the parent compound and T_{max} of 189–206 min; HHPV was detected at maximal concentrations of 12–19% of MDPV and T_{max} of 206–257 min (Anizan et al., 2016). MDPV and its metabolites displayed linear pharmacokinetics and both metabolites are mainly present as conjugates (Anizan et al., 2014; Meyer et al., 2010; Strano-Rossi et al., 2010). Anizan and colleagues did not observe a correlation between concentrations of the hydroxylated MDPV metabolites and horizontal locomotor activity or stereotypy in rats; the authors therefore hypothesized that the metabolites either do not cross the blood–brain barrier or potentially exert an inhibitory effect on locomotor activity (Anizan et al., 2016).

The results of the current study confirm that MDPV and HHPV are both highly potent NET and DAT inhibitors (Baumann et al., 2017; Meltzer et al., 2006). In contrast to the entactogen MDMA, the high selectivity for dopaminergic vs. serotonergic activity indicates stronger reinforcing properties and a higher addictive liability for MDPV (Liechti, 2015). The radioligand binding data indicate that both metabolites of MDPV are devoid of any transporter substrate activity, as observed for the parent compound (Baumann et al., 2013; Rickli et al., 2015a). However, previous studies in rats indicate that HHPV may not significantly contribute to stimulant effects due to low blood–brain barrier permeability and high proportion of formed conjugates (Schindler et al., 2016). Consistent with this hypothesis, microdialysis studies in conscious rats show that intravenous administration of HHPV fails to alter locomotor activity or substantially increase extracellular dopamine concentrations in the nucleus accumbens (Baumann et al., 2017). Unlike the catechol metabolites of MDMA and methylone, HHPV displayed a sigmoidal uptake curve and did not cause an increase in dopamine uptake more than 100%, which may be explained by the lack of substrate activity for this metabolite.

Conclusion

Metabolites of the 3,4-methylenedioxy ring-substituted stimulants MDMA, methylone and MDPV interact with monoamine transporters. The *N*-demethylation of MDMA only slightly changes the monoamine uptake inhibition profile but potentially increases the 5-HT_{2A} receptor-mediated psychedelic properties of the formed metabolite MDA. By contrast, *N*-demethylation of methylone substantially decreases the norepinephrine and dopamine uptake inhibition potencies of the corresponding metabolite MDC. The *O*-demethylenated catechol metabolites of all substances maintained the norepinephrine uptake inhibition potency of the parent compounds but showed marked reductions in 5-HT uptake inhibition potency. *O*-methylation of the catechol metabolites significantly decreased the inhibition potency across all monoamine transporters. The hydroxy-methoxy metabolites of MDMA and methylone displayed rather uncommon dopamine uptake inhibition curves, expressed by an unusual flat slope for HMMA and only partial uptake inhibition for HMA and HMMC. The DAT inhibition curve of the hydroxy-methoxy metabolite of MDPV displayed a common sigmoidal shape, which suggests that the irregularities in shape for DAT uptake curves are caused by the substrate properties of the respective metabolites. The catechol metabolites of MDMA, methylone and MDPV could potentially contribute to cardiovascular effects in humans, due to potent norepinephrine uptake inhibition. However, pharmacokinetic data from human and rat studies show a high proportion of conjugates for the catechol and hydroxy-methoxy metabolites, suggesting only minor contribution of these metabolites to in vivo pharmacological effects. On the other hand, *N*-demethylated metabolites MDA and MDC are found in unconjugated form at pharmacologically relevant amounts. The effects of MDA are expected to contribute more to the pharmacological effects compared to MDC, as the monoamine inhibition potency of the former is similar to the parent compound. Placebo-controlled clinical studies are needed to gain better insight into the pharmacokinetics of methylone and MDPV in humans and therefore a clearer interpretation of the results of the current study.

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Authors' contributions

D.L., M.H.B. and M.E.L. designed the research. M.S., K.C.R. and B.E.B. synthesized substances. D.L., K.E.K., M.W. and M.C.H. conducted the research. D.L., K.E.K., M.C.H. and M.E.L. analyzed data. D.L., K.E.K., M.H.B. and M.E.L. wrote the manuscript.

Declaration of conflicting interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: 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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Supplemental material

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2.2. Publication 2

Receptor interaction profiles of 4-alkoxy-substituted 2,5-dimethoxyphenthylamines and related amphetamines.

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Receptor Interaction Profiles of 4-Alkoxy-Substituted 2,5-Dimethoxyphenethylamines and Related Amphetamines

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Background: 2,4,5-Trimethoxyamphetamine (TMA-2) is a potent psychedelic compound. Structurally related 4-alkyloxy-substituted 2,5-dimethoxyamphetamines and phenethylamine congeners (2C-O derivatives) have been described but their pharmacology is mostly undefined. Therefore, we examined receptor binding and activation profiles of these derivatives at monoamine receptors and transporters.

Methods: Receptor binding affinities were determined at the serotonergic 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} receptors, trace amine-associated receptor 1 (TAAR1), adrenergic α_1 and α_2 receptors, dopaminergic D_2 receptor, and at monoamine transporters, using target-transfected cells. Additionally, activation of 5-HT_{2A} and 5-HT_{2B} receptors and TAAR1 was determined. Furthermore, we assessed monoamine transporter inhibition.

Results: Both the phenethylamine and amphetamine derivatives (K_i = 8–1700 nM and 61–4400 nM, respectively) bound with moderate to high affinities to the 5-HT_{2A} receptor with preference over the 5-HT_{1A} and 5-HT_{2C} receptors (5-HT_{2A}/5-HT_{1A} = 1.4–333 and 5-HT_{2A}/5-HT_{2C} = 2.1–14, respectively). Extending the 4-alkoxy-group generally increased binding affinities at 5-HT_{2A} and 5-HT_{2C} receptors but showed mixed effects in terms of activation potency and efficacy at these receptors. Introduction of a terminal fluorine atom into the 4-ethoxy substituent by trend decreased, and with progressive fluorination increased affinities at the 5-HT_{2A} and 5-HT_{2C} receptors. Little or no effect was observed at the 5-HT_{1A} receptor for any of the substances tested ($K_i \geq 2700$ nM). Phenethylamines bound more strongly to the TAAR1 (K_i = 21–3300 nM) compared with their amphetamine analogs (K_i = 630–3100 nM).

Conclusion: As seen with earlier series investigated, the 4-alkyloxy-substituted 2,5-dimethoxyamphetamines and phenethylamines share some trends with the many other phenethylamine pharmacophore containing compounds, such as when increasing the size of the 4-substituent and increasing the lipophilicity, the affinities at the 5-$HT_{2A/C}$ subtype also increase, and only weak 5-$HT_{2A/C}$ subtype selectivities were achieved. At

least from the binding data available (i.e., high affinity binding at the 5-HT_{2A} receptor) one may predict mainly psychedelic-like effects in humans, at least for some of the compound investigated herein.

Keywords: 2,4,5-trimethoxyamphetamine, phenethylamine, 2C-O, 3C-O, receptor, transporter, psychedelic

INTRODUCTION

The serotonin (5-hydroxytryptamine, 5-HT) 5-HT_2 receptor family is involved in monitoring the balance of several central nervous system processes including sleep, appetite or sexual activity as well as maintaining the regulation of the cardiovascular system. Unsurprisingly, a lack of homeostasis in the 5-HT_2 receptor mediated processes controlling neurotransmission is thought to play a large role in the occurrence of several mental disorders like anxiety, depression or schizophrenia.

The 5-HT_2 receptor family can be subdivided into the 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} isoforms, all of which are activated by their natural but non-selective, neurotransmitter agonist, 5-HT (**1**) (**Figure 1**; structures **1**-**5**). The lack of selectivity observed for the endogenous neurotransmitter limited its use as a pharmacological tool to characterize the role of each receptor subtype. Investigations using selective ligands associated to selective functional activity are one approach to overcome this issue. Simple aryl-substituted phenethylamines are one example of ligands which lack specific receptor selectivity but show high affinity binding at the 5-HT receptor family (Glennon et al., 1992; Monte et al., 1996; Chambers et al., 2001; Chambers et al., 2002; Whiteside et al., 2002).

The thoroughly investigated 2,5-dimethoxy-4-bromoamphetamine (DOB; **2**, Y = Br, R = Me) (**Figure 1**) binds to the 5-HT_{2A} and 5-HT_{2C} (and 5-HT_{2B}) receptors with high affinity but shows relatively low selectivity between the receptor subtypes (Glennon et al., 1992; Monte et al., 1996; Chambers et al., 2001; Chambers et al., 2002; Whiteside et al., 2002). This lack of selectivity is thought to be due to the a high degree of sequence homology between the two receptor subtypes in the ligand binding site located in the transmembrane region (Boess and Martin, 1994).

Up-to-date, hundreds of phenethylamines, mostly synthetic derivatives, are known and have either been investigated for 5-HT_2 receptor affinities and/or for their psychoactive properties (Aldous et al., 1974; Barfknecht and Nichols, 1975; Nichols et al., 1977; Glennon et al., 1980; Domelsmith et al., 1981; Glennon and Young, 1982; Shulgin and Shulgin, 1991; Glennon et al., 1994; Jacob and Shulgin, 1994; Trachsel, 2003; Trachsel et al., 2013; Rickli et al., 2015; Luethi et al., 2018; Luethi et al., 2019). Many of them have been established as potent psychedelics in man, with mescaline (**3**), the psychedelic ingredient of cacti such as *peyote*, being the first psychedelic phenethylamine synthetically available to man since the year 1919 (Späth, 1919). For this reason, they have been extensively investigated for the past hundred years in animal models and in humans.

The most potent compounds carry the prototypical structure of a phenethylamine with a 2,4,5-subtitution pattern (structure **2**). The 2- and 5- positions are occupied by MeO (methoxy-) groups while the 4-position bares a lipophilic substituent like a halogen, alkyl, alkylsulfanyl or other. Since the introduction of an α-Me (methyl) group (R = Me) onto **2** has a minor influence on affinity binding to the 5-$HT_{2A/C}$ receptors, racemic α-methyl congeners (amphetamines) display about the same affinity at these receptors as their phenethylamine counterparts (Johnson et al., 1990; Glennon et al., 1992; Dowd et al., 2000; Parrish et al., 2005). On the other hand, significant effects of this modification on the dosage and duration of action *in vivo* have been observed in humans (Shulgin and Shulgin, 1991). These are thought to be partially caused by an increased metabolic stability (Glennon et al., 1983; Glennon et al., 1992) and increased hydrophobicity (Nichols et al., 1991). Furthermore, intrinsic activity at the receptor also appear to play a significant role (Nichols et al., 1994), as the α-Me group-containing amphetamines show higher intrinsic activity compared to their phenethylamine counterparts (Parrish et al., 2005).

Overall, a general trend among compounds with the structure **2** which contain small lipophilic substituents (Y = halogen, Me, CF_3 etc.) on the pivotal 4-position exhibit agonist properties. Conversely, compounds which contain large lipophilic 4-substituents like a longer alkyl chain (such *n*-butyl, 3-phenylpropyl etc.) exhibit antagonist activity (Dowd et al., 2000). Regardless of these observed trends, the transition between these structures is yet to be thoroughly defined.

In continuation of expanding the data available, we investigated a series of hitherto relatively unknown 4-substituted 2,5-dimethoxyphenethylamines and some of their corresponding α-Me congeners the 4-alkoxy derivatives 2C-O (structures **6**-**12**)

Abbreviations: 2,4,5-trimethoxyamphetamine, TMA-2; 2,4,5-trimethoxyphenethylamine, 2C-O-1; 2,5-dimethoxy-4-(2-fluoroethoxy) amphetamine, MFEM; 2,5-dimethoxy-4-(2-fluoroethoxy)phenethylamine, 2C-O-21; 2,5-dimethoxy-4-(2,2,2-trifluoroethoxy)amphetamine, MTFEM; 2,5-dimethoxy-4-(2,2,2-trifluoroethoxy)phenethylamine, 2C-O-22; 2,5-dimethoxy-4-ethoxyamphetamine, MEM; 2,5-dimethoxy-4-ethylamphetamine, DOET; 2,5-dimethoxy-4-isopropoxyamphetamine, MIPM; 2,5-dimethoxy-4-isopropoxyphenethylamine, 2C-O-4; 2,5-dimethoxy-4-methallyloxyamphetamine, MMALM; 2,5-dimethoxy-4-methallyloxyphenethylamine, 2C-O-3; 2,5-dimethoxy-4-methylamphetamine, DOM; 2,5-dimethoxy-4-methylphenethylamine, 2C-D; 2,5-dimethoxy-4-propoxyampehtamine, MPM; 2,5-dimethoxy-4-ethoxyphenethylamine, 2C-O-2; 3,4,5-trimethoxyamphetamine, TMA; 3,4,5-trimethoxyphenethylamine, mescaline; 4-(2,2-difluoroethoxy)-2,5-dimethoxyamphetamine, MDFEM; 4-(2,2-difluoroethoxy)-2,5-dimethoxyphenethylamine, 2C-O-21.5; 4-alkoxy-2,5-dimethoxyamphetamine, 3C-O derivatives; 4-alkoxy-2,5-dimethoxyphenethylamines, 2C-O derivatives; 4-allyloxy-2,5-dimethoxyamphetamine, MALM; 4-allyloxy-2,5-dimethoxyphenethylamine, 2C-O-16; 4-benzyloxy-2,5-dimethoxyphenethylamine, 2C-O-27; 4-bromo-2,5-dimethoxyamphetamine, DOB; 4-bromo-2,5-dimethoxyphenethylamine, 2C-B; 4-butoxy-2,5-dimethoxyamphetamine, MBM; 5-hydroxytryptamine (serotonin), 5-HT; dopamine transporter, DAT; dopamine, DA; norepinephrine transporter, NET; norepinephrine, NE; serotonin transporter, SERT; trace amine-associated receptor 1, TAAR1.

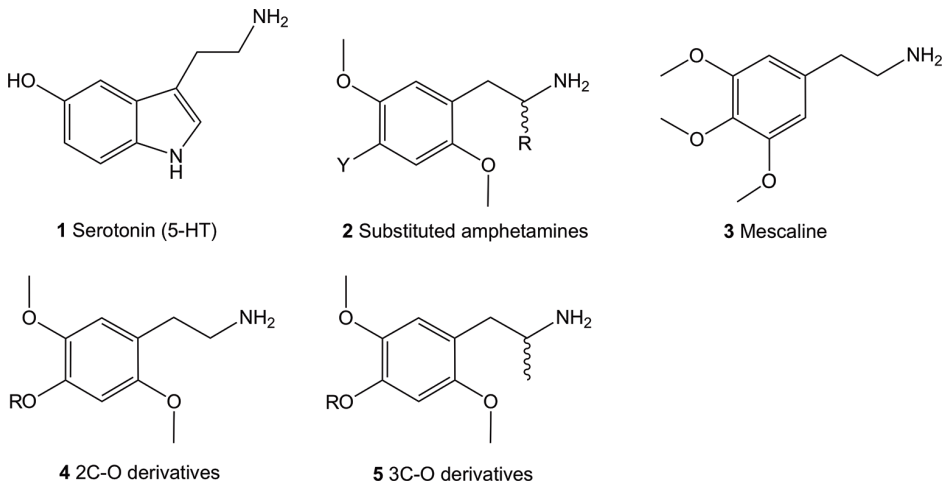

FIGURE 1 | Chemical structures of 5-hydroxytryptamine (5-HT; **1**), 2,5-dimethoxy-4-substituted phenethylamines (**2**), 3,4,5-trimethoxyphenethylamine (mescaline; **3**) and the general structure of the 4-alkoxy-2,5-dimethoxyphenethylamine (**4**) and 4-alkoxy-2,5-dimethoxyamphetamine (**5**) compounds. All amphetamines contain the phenethylamine core.

and 3C-O (structures **13-19**) compounds (**Figure 2**). Within these compounds, 2,4,5-trimethoxyamphetamine (TMA-2; **19**, **Figures 2** and **3**) can be considered as the archetypical representative. It is a synthetic compound that induces psychedelic effects in users likely *via* agonistic binding to the serotonergic 5-HT_{2A} receptor, similar to other classic psychedelics (Shulgin and Shulgin, 1991; Vollenweider et al., 1998; Preller et al., 2017). Back in 1966, varying the 3,4,5-trimethoxy substitution pattern to all possible position isomers revealed the importance of a 2,4,5-trimethoxy arrangement in aryl-substituted amphetamines and phenethylamines (Shulgin, 1966). Unlike classical amphetamines, **19** is characterized as a psychedelic with minor stimulant properties and is roughly 6 times more potent in humans than its structural and psychedelic isomer 3,4,5-trimethoxyamphetamine (TMA, structure not shown) (Shulgin and Shulgin, 1991; EMCDDA, 2004).

Since the 1960s, only a handful 4-alkoxy analogs of **19** have been synthesized and described (Shulgin and Shulgin, 1991; Trachsel, 2012). In accordance to the nomenclature introduced by the chemist Alexander Shulgin, they can generally be named as 2C-O derivatives (4-alkoxy-2,5-dimethoxyphenethylamines) and as 3C-O derivatives (4-alkoxy-2,5-dimethoxyamphetamines) (Shulgin and Shulgin, 1991) (structures **4** and **5**, **Figure 1**). The subsequent number is random and has been assigned in the order of planning the chemical synthesis of the compounds. However, as for the 3C-O derivatives naming is following a somewhat different approach: the initials for the three substituents in the 2,4,5-positions are used to build the name. For example, the name MMALM (compound **13**, **Figure 2**) derives from *m*ethoxy *m*eth*a*llyl *m*ethoxy (Shulgin and Shulgin, 1991). Up-to-date, the effects of only few of the 2C-O and 3C-O derivatives have been described chemically and (psycho)pharmacologically (Shulgin and Shulgin, 1991; Trachsel et al., 2013). Although the 2C-O derivatives initially examined by Shulgin were shown to be fairly inactive in humans (2C-O-1; **21** and 2C-O-4; **22**, **Figure 3**) some

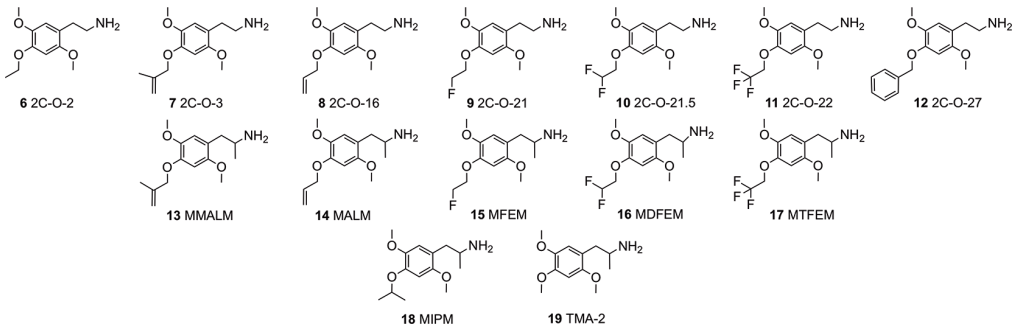

FIGURE 2 | Chemical structures of 4-alkyloxy-2,5-dimethoxyphenethylamines (2C-O derivatives) and 4-alkyloxy-2,5-dimethoxyamphetamines (3C-O) examined in the investigation.

derivatives such as **19** and 2,5-dimethoxy-4-ethoxyamphetamie (MEM) (**24**) displayed psychedelic activity (**Figure 3**) (Shulgin and Shulgin, 1991). However, upon further increasing chain length to a 4-propyloxy (MPM; **26**) or 4-butyloxy (MBM; structure not shown) substituent, again no psychoactive effects could be observed on comparable doses as used for **19** and **24**. The rather mixed results of low human potency and inactivity was one of the reasons Shulgin did not further evaluate the structure-activity relationship (SAR) of the 2C-O and 3C-O derivatives. Up-to-date, it remains unclear whether the early observations are due to pharmacokinetic properties such as a difference in metabolism or pharmacodynamic properties like differences in 5-HT receptor target interaction potency. Thus, in order to get more insight into SAR, a series of new 2C-O and 3C-O analogs (**6-19, Figure 2**) with potential to induce psychedelic properties (Trachsel, 2012) was prepared and pharmacologically investigated herein.

In the current study, we investigated the receptor binding and activation properties of several 2C-O derivatives and some of their 3C-O counterparts (**Figure 2**) at serotonergic, dopaminergic, and adrenergic receptors and trace-amine associated receptor 1 (TAAR1). The modifications found on these derivatives at the 4-position included introduction of one or more fluorines (mono-/di-/tri-fluorination at the terminal H_3C-group of the 4-ethoxy substituent) or extending of the 4-alkoxy-group by homologation, alkenylation and/or branching. Furthermore, we investigated the potential of these derivatives to bind and inhibit norepinephrine, dopamine, and 5-HT transporters (NET, DAT, and SERT, respectively) in human transporter-transfected human embryonic kidney (HEK) 293 cells.

MATERIALS AND METHODS

Drugs

All of the 2,5-dimethoxy-4-substituted phenethylamines (2C-O-2, 2C-O-16, 2C-O-3, 2C-O-27, 2C-O-21, 2C-O-21.5 and 2C-O-22) and the 2,5-dimethoxy-4-substituted amphetamines (TMA-2, 2,5-dimethoxy-4-isopropoxyamphetamine (MIPM), 4-allyloxy-2,5-dimethoxyamphetamine (MALM), 2,5-dimethoxy-4-methallyloxyamphetamine (MMALM), 2,5-dimethoxy-4-(2-fluoroethoxy)amphetamine (MFEM), 4-(2,2-difluoroethoxy)-2,5-dimethoxyamphetamine (MDFEM), and 2,5-dimethoxy-4-(2,2,2-trifluoroethoxy)amphetamine (MTFEM). all racemates) were synthetized in the hydrochloride form by ReseaChem (Burgdorf, Switzerland) as previously described (Shulgin and Shulgin, 1991; Trachsel, 2003; Trachsel et al., 2013). Purity for all described substances was >98%. [^{3}H]5-HT (80.0 Ci/mmol) was acquired from Anawa (Zurich, Switzerland). [^{3}H]dopamine (30.0 Ci/mmol) and [^{3}H]norepinephrine (13.1 Ci/mmol) were purchased from Perkin-Elmer (Schwerzenbach, Switzerland).

Radioligand Receptor and Transporter Binding Assays

The radioligand receptor and transporter binding assays were performed according to methods previously described in detail

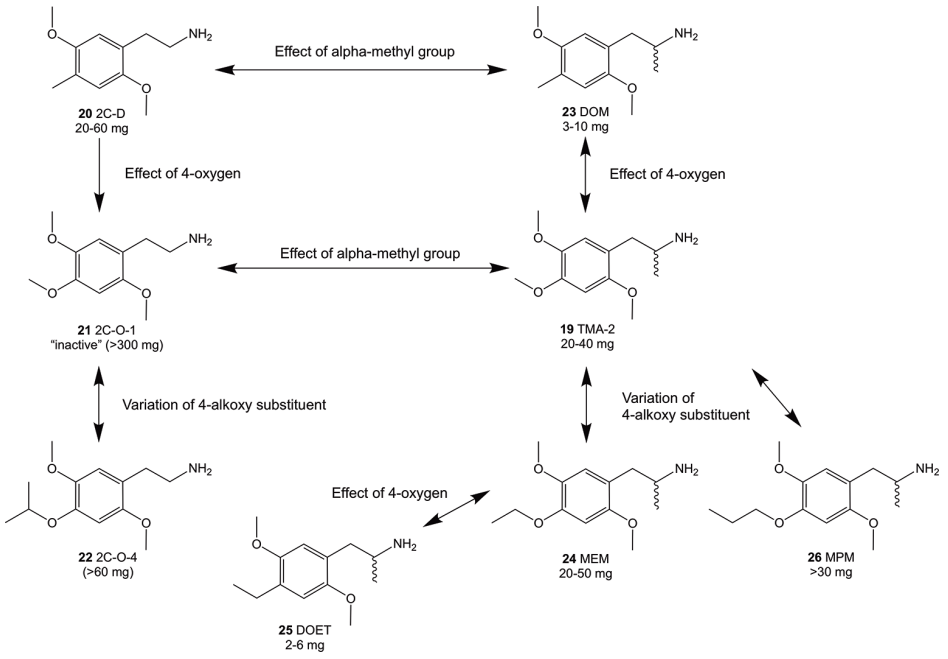

FIGURE 3 | Summary of Shulgin's early findings on structural modifications and their relation to psychoactive oral doses in humans using phenethylamines and amphetamines related to the 2C-O and 3C-O compounds. Doses were taken from PiHKAL (Shulgin and Shulgin, 1991). In general, an alpha-methylation (compounds **23** and **19**) of the 4-substituted 2,5-dimethoxy compounds increased the psychoactive potency while addition of 4-oxygen decreased potency (compounds **21** and **19**).

(Luethi et al., 2018). In summary, cell membrane preparations overexpressing respective receptors (human genes, except for rat and mouse genes for TAAR1) or transporters (human genes) were briefly incubated with radiolabeled selective ligands at a concentration equal to the dissociation constant (K_d). The cell membrane preparations were obtained from various cell lines including a Chinese hamster ovary cell line (α_{1A} adrenergic receptor), Chinese hamster lung cell line (α_{2A} adrenergic receptor) and HEK 293 cell line (5-HT_{1A}, 5-HT_{2A}, 5-$HT_{2C,}$ TAAR1, D_2, NET, DAT, SERT). Ligand displacement by the substances was measured. Specific binding of the radioligand to the target site was defined by measuring the difference between total binding and nonspecific binding (calculated in the presence of the respective receptor competitor in excess).

The following radioligands and the respective competitors were used: 0.90 nM [^{3}H]8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT) and 10 μM pindolol (5-HT_{1A} receptor), 0.40 nM [^{3}H]ketanserin and 10 μM spiperone (5-HT_{2A} receptor), 1.4 nM [^{3}H]mesulgerine, and 10 μM mianserin (5-HT_{2C} receptor), 3.5 nM or 2.4 nM (rat and mouse TAAR1, respectively) [^{3}H] RO5166017 and 10 μM RO5166017 (rat and mouse TAAR1), 0.11 nM [^{3}H]prazosin and 10 μM chlorpromazine (α_1 adrenergic receptor), 2 nM [^{3}H]rauwolscine and 10 μM phentolamine (α_2 adrenergic receptor), 1.2 nM [^{3}H]-spiperone and 10 μM spiperone (dopaminergic D_2 receptor), 2.9 nM N-methyl-[^{3}H]-nisoxetine and 10 μM indatraline (NET), 1.5 nM [^{3}H]citalopram, and 10 μM indatraline (SERT), 3.3 nM [^{3}H]WIN35,428 and 10 μM indatraline (DAT).

Activity At the Serotonin 5-HT_{2A} Receptor

Activity at the 5-HT_{2A} receptor was examined as previously described (Luethi et al., 2018). In summary, mouse embryonic fibroblasts (NIH-3T3 cells) expressing the human 5-HT_{2A} receptor were seeded at a density of 70,000 cells per 100 μl in poly-D-lysine-coated 96-well plates. The cells were then incubated in HEPES-Hank's Balanced Salt Solution (HBSS) buffer (Gibco) for 1 h at 37°C. Next, the plates were incubated in 100 μl (per well) dye solution for 1 h at 37°C (fluorescence imaging plate reader [FLIPR] calcium 5 assay kit; Molecular Devices, Sunnyvale, CA, USA). Once inside the FLIPR, the plates were exposed to 25 μl of test drugs which were diluted in HEPES-HBSS buffer composed of 250 mM probenecid while online. Using nonlinear regression, the rise in fluorescence was measured and EC_{50} values were calculated from the concentration-response curves. The efficacy was calculated relative to 5-HT activity which was defined as 100%. This assay was mainly used to determine whether the compounds were active, while 5-HT_{2A} receptor binding is considered to be more relevant to predict hallucinogenic potency in humans (Luethi and Liechti, 2018).

Activity At the Serotonin 5-HT_{2B} Receptor

Activity at the 5-HT_{2B} receptor was examined as previously described (Luethi et al., 2018). In summary, HEK 293 cells expressing the human 5-HT_{2B} receptor were seeded at a density of 50,000 cells per well in 96-well poly-D-lysine-coated plates overnight at 37°C. The cells were then incubated in growth medium consisting of high glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Zug, Switzerland), 10% fetal calf serum (non-dialyzed, heat-inactivated), 250 mg/l Geneticin and 10 ml/l PenStrep (Gibco), overnight at 37°C. After removal of the growth medium *via* snap inversion, calcium indicator Fluo-4-solution (100 μl) was injected into each well (Molecular Probes, Eugene, OR, USA) and incubated for 45 min at 31°C. Afterwards, the Fluo-4 solution was removed (snap inversion) and a further 100 μl of the Fluo-4 solution was added and incubated (45 min, 31°C). Thereafter, using the EMBLA cell washer, the cells were washed just before testing with HBSS and 20 mM HEPES and exposed to 100 μl of assay buffer. The well plate was positioned in the FLIPR and while online, 25 μl of the test compounds diluted in assay buffer were added. Concentration-response curves were calculated using nonlinear regression, and EC_{50} values were obtained. The maximal activity at the receptors was calculated relative to 5-HT activity which was defined as 100%. Since setting up a stable and reliable binding assay for the 5-HT_{2B} receptor has been proven to be difficult in the past, we did not try to include binding data for this receptor in our investigation. However, because the 5-HT_{2B} receptor activity is important for determining the potential cardiotoxicity of a derivative, we have included this data to estimate whether any substances have the potential to induced endocardial fibrosis.

Activity At the Human TAAR1

Activity at the human TAAR1 was examined as previously described in full detail (Luethi et al., 2018). In summary, human TAAR1 expressing recombinant HEK 293 cells were grown in 250 ml falcon culture flasks containing 30 ml of high glucose DMEM (10% heat inactivated fetal calf serum, 500 μg/ml Geneticin [Gibco, Zug, Switzerland] and 500 μg/ml hygromycin B) at 37°C and 5% CO_2/95% air. At 80-90% confluency, the cells were collected. The medium was aspirated, cells were washed with phosphate-buffered saline (PBS) and then trypsinized for 5 min at 37°C with 5 ml of trypsin/EDTA solution. Next, 45 ml of medium was added and the mixture was transferred into a falcon tube. After centrifugation (900 rpm, 3 min, RT), the supernatant was aspirated and the remaining cell pellet was resuspended in fresh medium to 5×10^5 cells/ml. Using a multipipette, 100 μl of cells were transferred (80,000 cells/well) into a 96-well plate (BIOCOAT 6640, Becton Dickinson, Allschwil, Switzerland) and incubated for 20 h at 37°C. For the cAMP assay, the medium was aspirated replaced with 50 μL PBS without Ca^{2+} and Mg^{2+} ions. The PBS was then extracted using snap inversion and the plate was softly tapped against tissue; 90 μL of Krebs-Ringer Bicarbonate buffer (Sigma-Aldrich) containing 1 mM IBMX was added and incubated for 60 min at 37°C and 5% CO_2/95% air. A concentration range between 300 pM and 30 μM of test compounds was examined in duplicate. A standard curve with a range of cAMP concentrations (0.13 nM to 10 μM) was created per 96-well plate. Each experiment was accompanied with a reference plate that included three compounds; RO5256390, β-phenylethylamine, and p-tyramine. The cells were exposed to either 30 μl of compound solution, 30 μl of β-phenylethylamine (as maximal response), or a basal control in PBS (containing 1

mM IBMX) for 40 min at 37°C. Next, under forceful shaking using black lids, the cells were exposed to 50 µl of 3x detection mix solution (composed of Ru-cAMP Alexa700 anti-cAMP antibody and lysis buffer) for 120 min at room temperature in order to lyse the cells. Using the NanoScan reader (Innovative Optische Messtechnik, Berlin, Germany; 456 nm excitation wavelength; 630 and 700 nm emission wavelengths), the fluorescence was examined and the fluorescence resonance energy transfer (FRET) signal was determined using the following equation; FRET (700 nm) – P × FRET (630 nm), where P = Ru (700 nm)/ Ru (630 nm). Receptor binding affinity at the human TAAR1 was not determined since unfortunately, there are no suitable radioligands available for this receptor.

Functional activity of the derivatives was only examined at the human TAAR1 and not at the mouse and rat TAAR1, because functional assays are not set up.

Monoamine Uptake Transporter Inhibition

The monoamine uptake transporter inhibition for 2,5-dimethoxy-4-alkyloxy phenethylamines and amphetamines was examined in HEK 293 cells stably transfected with the human 5-HT, norepinephrine and dopamine transporters (hSERT, hNET, hDAT) as previously described (Luethi et al., 2018). Only one single high concentration was tested to exclude activity at the transporters at pharmacologically relevant concentrations. Briefly, the cells were cultured in DMEM (Gibco, Life Technologies, Zug, Switzerland) containing 10% fetal bovine serum (Gibco) and 250 µg/ml Geneticin (Gibco). At 70-90% confluency, the cells were detached and resuspended in Krebs-Ringer Bicarbonate Buffer (Sigma-Aldrich, Buchs, Switzerland) at a density of 3×10^6 cells per ml of buffer. For [^{3}H]dopamine uptake experiments, the buffer additionally contained 0.2 mg/ml of ascorbic acid. 100 µL of cell suspension was added to each well into a round bottom 96-well plate. The cells were then incubated with 25 µL buffer containing the test drug (10 µM), vehicle control (0.1% dimethyl sulfoxide) or transporter-specific inhibitors (10 µM fluoxetine (SERT), 10 µM mazindol (DAT) or 10 µM nisoxetine (NET)) for 10 min by shaking on a rotary shaker (450 rpm) at room temperature. Uptake transport was initiated by adding [^{3}H]5-HT, [^{3}H]dopamine, or [^{3}H]norepinephrine at a final concentration of 5 nM to the mixture. After 10 min, 100 µL of the cell mixture was transferred to 500 µL microcentrifuge tubes containing 50 µL of 3 M KOH and 200 µL silicon oil (1:1 mixture of silicon oil types AR 20 and 200; Sigma-Aldrich). The tubes were centrifuged for 3 min at 16,550 g, to allow the transport of the cells through the silicon oil layer into the KOH layer. The tubes were frozen in liquid nitrogen and the cell pellet was cut into 6 ml scintillation vials (Perkin-Elmer) containing 0.5 ml lysis buffer (1% NP-40, 50 mM NaCl, 0.05 M TRIS-HCl, 5 mM EDTA, and deionized water). The samples were shaken for 1 h before 3 ml of scintillation fluid (Ultimagold, Perkin Elmer, Schwerzenbach, Switzerland) was added. Monoamine uptake was then quantified by liquid scintillation counting on a Packard Tri-Carb Liquid Scintillation Counter 1900 TR. Uptake in the presence of the selective inhibitors was determined to be nonspecific and subtracted from the total counts.

Statistical Analysis

Calculations were performed using Prism 7.0a software (GraphPad, San Diego, CA, USA). IC_{50} 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. The K_i values correspond to the dissociative constant for the inhibitor and were calculated using the Cheng-Prusoff equation. Nonlinear regression concentration-response curves were used to determine EC_{50} values for 5-HT_{2A} and 5-HT_{2B} receptor activation. Maximal activation activity (efficacy) is expressed relative to the activity of 5-HT, which was used as a control and set to 100%. Monoamine uptake of three to four independent experiments was compared to control using 1-way analysis of variance followed by a Dunett's multiple-comparison test. Monoamine uptake of 3,4-methylenedioxymethamphetamine (MDMA) was included as comparison. Receptor affinity binding (K_i) <50 nM was defined as high affinity binding, <500 nM moderate affinity binding while K_i >1000 nM was defined as low affinity binding. Activation efficacy (max %) < 85% was defined as partial agonism while max % > 85% was defined as full agonism.

RESULTS

Binding and Activation At 5-HT Receptors

5-HT receptor binding affinities and activation potencies are listed in **Table 1**. The well-explored phenethylamine 2C-B (Kurrasch-Orbaugh et al., 2003; Rickli et al., 2015; Papaseit et al., 2018) was included for comparison. All of the 2C-O derivatives (**Figure 2**, structures **6–12**) exhibited only very weak binding properties to the 5-HT_{1A} receptor (K_i = 2.7–5.5 µM) and the 2,5-dimethoxy-4-alkyloxy substituted amphetamines (**Figure 2**, structures **13–19**) did not bind to the receptor (K_i > 5600 nM).

The 2C-O derivatives bound to the 5-HT_{2A} receptor with submicromolar affinity (K_i = 8–1000 nM), with the exception of 2C-O-21 (**9**) (K_i = 1700 nM). Additionally, the 2C-O derivatives were partial agonists with EC_{50} values in the ranges of 16–2600 nM and activation efficacies of 30–84%. Some of the 3C-O derivatives, the 4-alkoxy-2,5-dimethoxy substituted amphetamines bound to the 5-HT_{2A} receptor with submicromolar affinity (K_i = 61–980 nM), with the exception of MFEM (**15**), MIPM (**18**), and TMA-2 (**19**) (K_i = 1300–4400 nM). The 3C-O compounds activated the receptor with EC_{50} values in the ranges of 2–990 nM. Compounds MALM (**14**) and MMALM (**13**) proved to be full agonists with activation efficacies of 89% and 95%, respectively. The remaining compounds activated the receptor as partial agonists with activation efficacies in the range of 47–84%.

All compounds activated the 5-HT_{2B} receptor at submicromolar concentrations. Compounds 2C-O-2 (**6**), MALM (**14**), and MMALM (**13**) were full agonists with activation efficacies of 85–101%. The remaining compounds were partial agonists (activation efficacies in the range of 20–78%) with the exception of MTFEM (**17**) which had only negligible activation efficacy (5%).

Compounds 2C-O-3 (**7**), 2C-O-16 (**8**), 2C-O-27 (**12**), MALM (**14**), and MMALM (**13**) potently bound to the 5-HT_{2C} receptor

TABLE 1 | Serotonin receptor binding affinities and activation potencies of 4-alkoxy-substituted 2,5-dimethoxyphenethylamines and amphetamines.

		h5-HT_{1A}	h5-HT_{2A}			h5-HT_{2B}		h5-HT_{2C}	Selectivity (binding ratios)	
		Receptor binding K_i ± SD [nM] [^{3}H]8-OH-DPAT	Receptor binding K_i ± SD [nM] [^{3}H]ketanserin	Activation potency EC_{50} ± SD [nM]	Activation efficacy max ± SD [%]	Activation potency EC_{50} ± SD [nM]	Activation efficacy max ± SD [%]	Receptor binding K_i ± SD [nM] [^{3}H] mesulgerine	5-HT_{2A}/5-HT_{1A}	5-HT_{2A}/5-HT_{2C}
4-alkoxy-substituted 2,5-dimethoxyphenethylamines										
6	2C-O-2	3600 ± 400	670 ± 90	16 ± 3	50 ± 10	49 ± 44	85 ± 17	2300 ± 1200	5.4	3.4
7	2C-O-3	2700 ± 200	40 ± 8	0.5 ± 0.3	70 ± 4	68 ± 21	50 ± 22	150 ± 30	68	3.8
8	2C-O-16	4500 ± 500	140 ± 40	4.9 ± 2.5	84 ± 4	120 ± 70	42 ± 13	470 ± 40	32	3.4
9	2C-O-21	5500 ± 0	1700 ± 600	53 ± 15	44 ± 17	900 ± 450	39 ± 26	3600 ± 1900	3.2	2.1
10	2C-O-21.5	2900 ± 200	1000 ± 100	2600 ± 1600	30 ± 8	480 ± 260	28 ± 23	4200 ± 1500	2.9	4.2
11	2C-O-22	3300 ± 200	440 ± 60	460 ± 140	35 ± 8	250 ± 10	28 ± 20	1900 ± 400	7.5	4.3
12	2C-O-27	2700 ± 100	8.1 ± 1	76 ± 80	36 ± 16	480 ± 190	39 ± 16	110 ± 50	333	14
4-alkoxy-substituted 2,5-dimethoxyamphetamines										
13	MMALM	NA	61 ± 0	1.5 ± 0.1	95 ± 4	29 ± 13	90 ± 13	290 ± 110	>92	4.8
14	MALM	>5600	150 ± 0	2.9 ± 0.5	89 ± 10	9.5 ± 3.3	101 ± 6	900 ± 220	>37	6
15	MFEM	>5600	1900 ± 900	330 ± 180	56 ± 36	270 ± 210	41 ± 22	11000 ± 3000	>2.9	5.8
16	MDFEM	>5600	980 ± 620	140 ± 80	48 ± 23	130 ± 80	21 ± 8	6700 ± 2400	>5.7	6.8
17	MTFEM	>5600	460 ± 240	19 ± 1	80 ± 6	200 ± 30	4.8 ± 3.5	2400 ± 800	>12	5.2
18	MIPM	>5600	4400 ± 2100	990 ± 330	47 ± 13	180 ± 120	20 ±14	9030 ± 2390	>1.4	2.1
19	TMA-2	>17000	1300 ± 700	190 ± 90	84 ± 10	270 ± 0	78 ± 5	5300 ± 1600	>13	4.1
Reference substance										
	2C-B[a]	311±46	6.9 ± 1.8	2.1 ± 0.8	92 ± 8	75 ± 14	52 ± 26	43 ± 4	45	6.2

K_i and EC_{50} values are given as nM (mean ± SD); activation efficacy (E_{max}) is given as percentage of maximum ± SD. NA, not assessed.
[a]Data previously published in (Luethi et al., 2018).

(K_i = 15–900 nM). The remaining compounds bound with affinities in the range of 1900–11000 nM.

Interactions With Non-Serotonergic Monoamine Receptors and Transporters

The non-serotonergic monoamine receptors and transporters binding affinities are listed in **Table 2** with MDMA as reference for comparison. The 2C-O derivatives did not activate the human TAAR1 except for 2C-O-2 (**6**) and 2C-O-22 (**11**), which activated the receptor in the micromolar range (EC_{50} = 3600–9600 nM). The 4-alkyloxy-2,5-dimethoxy substituted amphetamines did not activate the human TAAR1.

All 2C-O derivatives showed moderate to relatively high affinities to the rat TAAR1 (K_i = 21–410 nM). In contrast, the 3C-O derivatives generally only weakly bound to the rat TAAR1 (K_i = 1100–3100 nM), with exception for MMALM (**13**) (K_i = 630 nM), which showed a modest affinity. The 2C-O derivatives also weakly bound to the mouse TAAR1 (K_i = 650–4000 nM) with 2C-O-27 (**12**) being the most potent compound. Compounds 2C-O-21 (**9**) and 2C-O-21.5 (**10**) did not bind to mouse TAAR1 in the examined concentration range (K_i > 4800 nM). The amphetamine derivatives did not bind to the mouse TAAR1 in the examined concentration range (K_i > 4400 nM), except for MMALM (**13**), MALM (**14**) and MTFEM (**17**) which showed weak binding properties (K_i = 1700–4500 nM).

None of the phenethylamines or amphetamines bound to the adrenergic α_{1A} (K_i > 6500 nM) or dopaminergic D_2 receptors (K_i > 4400 nM) in the examined concentration range. Compounds 2C-O-3 (**7**), 2C-O-16 (**8**), and 2C-O-27 (**12**) bound to the α_{2A} receptor in the submicromolar range (K_i = 180–620 nM); the other 2C-O derivatives bound only weakly to the α_{2A} receptor (K_i = 1800–3600 nM). With exception for MMALM (**13**) (K_i = 2400 nM), none of the 3C-O derivatives bound to the α_{2A} receptor in the examined concentration range (K_i > 4700 nM). Among the tested 5-HT, dopamine and norepinephrine transporter interactions, **12** proved to be the only compound able to bind to the DAT (K_i = 6.1 μM), none of the remaining 2C-O and 3C-O compounds bound to the monoamine transporters at investigated concentrations.

Monoamine Transporter Inhibition

None of the investigated compounds significantly inhibited monoamine uptake transporters (IC_{50} > 10 μM) (**Supplementary Figure 1**).

DISCUSSION

Serotonin 5-HT_{2A} *vs.* 5-HT_{2C} Receptor Binding and Subtype Selectivity

Despite the use of a limited set of compounds, some trends were observed. We found that 2,5-dimethoxy-4-alkyloxy substituted phenethylamine derivatives (**Figure 2**, structures **6-12**) showed binding preference at the 5-HT_{2A} over the 5-HT_{2C} receptor and bound to the 5-HT_{1A} receptor. The corresponding 3C-O derivatives (**Figure 2**, structures **13-19**) displayed a comparable 5-HT_{2A} *vs.* 5-HT_{2C} binding preference but did not bind to the 5-HT_{1A} receptor (section 4.2). The observed moderate 5-HT_{2A} *vs.*

TABLE 2 | Monoamine receptor and transporter binding affinities of 4-alkoxy-subsituted 2,5-dimethoxyphenethylamines and amphetamines.

		human TAAR1	rat TAAR1	mouse TAAR1	α_{1A}	α_{2A}	D_2	hNET	hDAT	hSERT
		EC_{50} ± SD [nM]	K_i ± SD [nM] [^{3}H] RO5166017	K_i ± SD [nM] [^{3}H] RO5166017	K_i ± SD [nM] [^{3}H] prazosin	K_i ± SD [nM] [^{3}H] rauwolscine	K_i ± SD [nM] [^{3}H] spiperone	K_i ± SD [nM] [^{3}H] N-methyl-[^{3}H] nisoxetine	K_i ± SD [nM] [^{3}H] WIN35,428	K_i ± SD [nM] [^{3}H] citalopram
4-alkoxy-substituted 2,5-dimethoxyphenethylamines										
6	2C-O-2	9600 ± 6340	340 ± 70	4000 ± 1100	>6500	2600 ± 100	>4800	>9700	>8700	>8600
7	2C-O-3	> 30000	130 ± 0	1100 ± 400	>6500	180 ± 10	>4800	>9700	>8700	>8600
8	2C-O-16	> 30000	260 ± 70	2500 ± 1000	>6500	620 ± 20	>4800	>9700	>8700	>8600
9	2C-O-21	> 30000	410 ± 40	> 4900	>6500	3600 ± 500	>4800	>9700	>8700	>8600
10	2C-O-21.5	> 30000	250 ± 20	> 4800	>6500	1900 ± 100	>4800	>9700	>8700	>8600
11	2C-O-22	3600 ± 2400	240 ± 60	2500 ± 400	>6500	1800 ± 100	>4800	>9700	>8700	>8600
12	2C-O-27	> 30000	21 ± 3	650 ± 190	>6500	570 ± 30	>4800	>9700	6100 ± 400	>8600
4-alkoxy-substituted 2,5-dimethoxyamphetamines										
13	MMALM	> 30000	630 ± 150	1700 ± 900	>6500	2400 ± 200	>4800	>9700	>8700	>8600
14	MALM	> 30000	1100 ± 200	2000 ± 400	>6500	>4800	>4400	>9700	>8700	>8600
15	MFEM	> 30000	1640 ± 300	> 4900	>6500	>4800	>4800	>9700	>8700	>8600
16	MDFEM	> 30000	1200 ± 200	> 4800	>6500	>4800	>4800	>9700	>8700	>8600
17	MTFEM	> 30000	1300 ± 100	4500 ± 500	>6500	>4800	>4800	>9700	>8700	>8600
18	MIPM	> 30000	2900 ± 700	> 4800	>6500	>4800	>4800	>9700	>8700	>8600
19	TMA-2	NA	3100 ± 100	> 4400	>6500	>4700	>13000	>8700	>8500	>7500
Reference substance										
	MDMA[a]	NA	370 ± 120	2400 ± 1100	>6000	15000 ± 200	25200 ± 2000	30500 ± 8000	6500 ± 2500	13300 ± 600

K_i and EC_{50} values are given as nM (mean ± SD); activation efficacy (E_{max}) is given as percentage of maximum ± SD. NA, not assessed.
[a]Data previously published in (Simmler et al., 2013).

5-HT_{2C} subtype selectivity is in accordance with the many other phenethylamine and amphetamine ligands described by others (Glennon et al., 1980; Glennon et al., 1986; Glennon et al., 1994; Nichols et al., 1994; Nelson et al., 1999; Nichols et al., 2015; Rickli et al., 2015; Nichols, 2016; Rickli et al., 2016; Luethi et al., 2018).

The extension of the 4-alkoxy group was found to increase the binding affinity at the 5-HT_{2A} and 5-HT_{2C} receptors for both phenethylamine and amphetamine derivatives, a trend also in accordance with previous studies on extending a lipophilic 4-substituent in 2,5-dimethoxyphenethylamines (Dowd et al., 2000; Luethi et al., 2018). For 2C-O-16 (**8**; 4-allyloxy) and 2C-O-3 (**7**; 4-methallyloxy) it remains unclear whether the increased affinities at the 5-HT_{2A} and 5-HT_{2C} receptors originate from increasing substituent size solely or whether oxidation to their alkenyl pharmacopore contributes to this as well. Generally, an increased lipophilicity also increases affinities to these receptors and thus both effects may be contributory. Introduction of one (2C-O-21; **9**) or two fluorines (2C-O-21.5; **10**) onto the terminal carbon atom of the 4-ethoxy group lead to decreasing affinities at both 5-HT_{2A} and 5-HT_{2C} receptors compared to 2C-O-2 (**6**). In comparison, 2C-O-22 (**11**) bearing a 4-trifluoroethoxy substituent showed slightly increased affinity over the non-fluorinated compound **6** at the 5-HT_{2A} and 5-HT_{2C} receptors. However, when considering the fluorinated compounds **9**-**11** solely, progressive fluorination leads to increased affinities.

A similar trend was observed for the 4-alkoxy substituted 2,5-dimethoxyamphetamines. Increasing the 4-alkoxy substituent lead to increased affinities, with MIPM (**18**) being an exception: although its 4-isopropyloxy substituent may be considered to be more lipophilic than an ethoxy group, it may have some unfavorable steric bulkiness in that binding area. Even though a benzyloxy substituent is even bulkier (and leads to the highest affinities among these and other compounds at this receptor) (Luethi et al., 2018), the isopropyl group is branched directly at its carbon bound to the oxygen. This potentially may force the 5-MeO group more prominently to an out-of-plane orientation leading to decreased 5-HT_{2A} and 5-HT_{2C} receptor interactions (Monte et al., 1996). Yet, within the 4-alkylthio substituted 2,5-dimethoxyphenethylamines (2C-T and ALEPH derivatives; structures not shown), a 4-isopropylthio substituent proved to be highly efficient in causing psychedelic effects in man, while its affinities where distinctly lower than e.g. those of the corresponding 4-methallylthio or 4-propylthio derivatives, which are both active in man at similar doses (Shulgin and Shulgin, 1991; Luethi et al., 2018). Clearly, other factors, such as lipophilicity, receptor activation, functional selectivity, and monoamine oxidase (MAO) and cytochrome P450 (CYP) metabolism, may influence the dose and effects in man. Also, branching the alkyl chain geminally to the attached oxygen may be more detrimental than when attached to a sulphur atom in respect to 5-$HT_{2A/C}$ affinities, considering the former to have a significantly lower steric bulkiness. Being branched "closer" to the aromatic nucleus due to the smaller oxygen atom, the negligibly active or even psychedelically inactive 2C-O-4 (**22**) (Shulgin and Shulgin, 1991) may also be affected by these steric effects.

Increasing fluorination of the terminal carbon in the 4-ethoxy substituent of the 3C-O derivatives investigated lead to increased affinities at the 5-HT_{2A} and 5-HT_{2C} receptor subtypes (the fluorine-free counterpart MEM; **24** (Shulgin and Shulgin, 1991; Halberstadt et al., 2019) was not available for this study). Additionally, extension of the 4-alkoxy group and increasing the

number of fluoro substituents in 3C-O derivatives increased the binding selectivity for 5-HT_{2A} over 5-HT_{1A} receptors.

It is well known that psychedelic phenethylamines and amphetamines bind to both 5-HT_{2C} and 5-HT_{2A} receptors (Kurrasch-Orbaugh et al., 2003; Nichols, 2004; Moya et al., 2007; Nichols, 2016). Although the interaction with the 5-HT_{2C} receptor is thought to be involved to some extent in overall profile of psychological effects induced by psychedelics, the 5-HT_{2A} receptor is considered as the main primary target mediating the action of psychedelics in humans (Vollenweider et al., 1998; Nichols, 2004; Nichols, 2016; Kraehenmann et al., 2017; Preller et al., 2017).

The moderate selectivity for the 5-HT_{2A} receptor over 5-HT_{1A} and 5-HT_{2C} receptors observed for the 4-alkyloxy substituted derivatives (structures **6**–**8, 12**–**14, 18**–**19**) is in agreement with previously reported selectivity ratios for various substituted phenethylamines (Barfknecht and Nichols, 1975; Glennon et al., 1983; Pierce and Peroutka, 1989; Glennon et al., 1994; Nichols et al., 1994; Nelson et al., 1999; Rickli et al., 2015; Luethi et al., 2018). However, this selectivity for the 5-HT_{2A} receptor is not seen for tryptamine psychedelics or LSD, which are non-selective at these serotonergic receptors (Halberstadt and Geyer, 2011; Rickli et al., 2015; Luethi et al., 2018). Furthermore, a previous study shows that the 5-$HT_{2A/2C}$ receptor binding of various psychedelics *in vitro* can be used to predict the clinical potency in humans (Luethi and Liechti, 2018).

Binding to the 5-HT_{1A} Receptor

The 2C-O derivatives bound with low affinities to the 5-HT_{1A} receptor (K_i = 2700–5500 nM), similar to 2,4,5-trisubsituted *N*-2-methyoxybenzyl (NBOMe) derivatives (K_i = 1800–7100 nM) (Rickli et al., 2015) and slightly weaker than 2C-T derivatives (K_i = 660–2368 nM) (Luethi et al., 2018). Some 2C derivatives lacking 4-oxo or 4-thio substitution, such as the 4-bromo derivative 2C-B, and some psychoactive tryptamines displayed more noteworthy binding at the receptor in earlier studies (Rickli et al., 2015; Rickli et al., 2016) but did not reach the low nanomolar 5-HT_{1A} affinity of LSD (Luethi et al., 2018).

Structural modifications of the amphetamine derivatives **13**-**19** did not result in 5-HT_{1A} receptor binding in the examined concentration range for any of the compounds. Although the aryl-unsubstituted derivatives amphetamine and phenethylamine share little to no pharmacological properties with the psychedelic phenethylamines, it has been shown that amphetamine (*K*i = 6700 nM) (Simmler et al., 2013) has an affinity of more than one order of magnitude lower than phenethylamine at the 5-HT_{1A} receptor. This suggests an unfavorable role of the α-methyl group towards binding abilities at this receptor. This diminishment of binding affinities upon α-methyl introduction into phenethylamines is in accordance with several other aryl-substituted phenethylamines investigated (Rickli et al., 2019).

Binding and Activation of the 5-HT_{2A} Receptor

Carbon chain length extension and/or oxidation of the 4-alkyloxy substituent increased the binding affinity at the 5-HT_{2A} receptor 5-fold (2C-O-16; **8**) and 17-fold (2C-O-3; **7**), compared to 2C-O-2 (**6**), the derivative bearing the shortest carbon chain. Similarly, the binding affinity was increased 2-fold for the difluorinated 2C-O-21.5 (**10**) and 4-fold for the trifluorinated 2C-O-22 (**11**) when compared to the monofluorinated derivative 2C-O-21 (**9**). However, **9** displayed the most potent 5-HT_{2A} receptor activation and the highest activation efficacy among all fluorinated 2C-Os. Surprisingly, activation potency of 2C-O-21.5 (**10**) was in the high nanomolar range, 50-fold and 6-fold higher than for 2C-O-21 (**9**) and 2C-O-22 (**11**), respectively. Likely, difluorination on **10** is less favorable for higher activation potency than monofluorination (2C-O-16; **8**) or trifluorination (2C-O-22; **11**).

Similar trends were observed for 3C-O derivatives. The extension and/or oxidation of the 4-alkyloxy substituent increased the binding affinity up to 9-fold for MALM (**14**) and 21-fold for MMALM (**13**) when compared to TMA-2 (**19**). Furthermore, the activation potency at the 5-HT_{2A} receptor was 66-fold and 127-fold increased for compounds **14** and MFEM (**15**), respectively, when compared to **19**. The binding affinity was slightly increased (2-fold and 4-fold) for the difluorinated MDFEM (**16**) and trifluorinated MTFEM (**17**), respectively, when compared to the monofluorinated **15**. Increasing number of fluoride substituents also increased the activation potency, resulting in a 2-fold increase for **16** and a 17-fold increase for **17** when compared to **15**.

Where available, direct comparison of the 2C-Os to their amphetamine counterparts revealed slightly higher 5-HT_{2A} receptor binding, higher activation, and lower efficacy for 4-alkoxy-substituted phenethylamine compounds (2C-O-16 *vs.* MALM and 2C-O-3 *vs.* MMALM). Similar observations were made for the fluorinated derivatives with the exception of receptor activation, which differed for 2C-O and 3C-O derivatives. Overall, the 4-alkoxy-substituted 2,5-dimethoxyphenethylamines activated the 5-HT_{2A} receptor as partial agonists (A_E = 30 – 84%), meanwhile the 4-alkoxy-substituted 2,5-dimethoxyamphetamine counterparts showed slightly higher activation efficacy (A_E), with some amphetamine counterparts activating the 5-HT_{2A} receptor as full agonists (A_E > 85% –95%; compounds **13** and **14**). These results suggest that the α-methyl group plays a minor role in 5-HT_{2A} interactions for the tested compounds. This finding is in line with previous reports that the racemic α-Me introduction causes largely unchanged effects on the binding affinity and functional potency at the 5-HT_{2A} receptor affinity but does augment the intrinsic activity [Nichols et al., 1994; Parrish et al., 2005; Trachsel et al., 2013)].

Compounds 2C-O-1 (**21**) and 2C-O-4 (**22**), two members of the 2C-O family, were not psychoactive in humans, at least at the doses tested so far (Shulgin and Shulgin, 1991). It has been suggested that this may be due to a rapid metabolism or low binding affinity to the 5-HT_{2A} receptor (Clark et al., 1965; Nelson et al., 1999; Trachsel, 2012). The 5-HT_{2A} activation mediates psychedelic effects (Glennon et al., 1992; Chambers et al., 2002; Kraehenmann et al., 2017) and receptor binding affinity has been shown to be a good predictor of the dose needed (clinical potency) to induce a psychedelic effect (Luethi and Liechti, 2018).

The amphetamine derivatives and 2C-Os studied herein bound with moderate to high affinity to the 5-HT_{2A} receptor and are partial or full agonists at the 5-HT_{2A} receptor, rendering them potentially psychedelic. In previous studies, 2C-T and NBOMe derivatives

were shown to bind to the 5-HT_{2A} receptor in the low nanomolar range, and therefore more potently than most 4-alkyloxy substituted derivatives of the current study. However, with a binding affinity of 6.3 μM (Rickli et al., 2015), mescaline (**3**) exemplifies that even low binding affinities may result in strong psychedelic effects, when a sufficiently high dose (> 200 mg) is ingested (Shulgin and Shulgin, 1991). Therefore, by sharing many typical structural features with known phenethylamine-type psychedelics (Shulgin and Shulgin, 1991; Trachsel et al., 2013), all compounds investigated in this study may potentially elicit strong psychedelic effects.

In the present study, 2C-O-27 (**12**) showed the highest affinity at the 5-HT_{2A} receptor (K_i = 8.1 nM). This is consistent with previous studies that suggest that bulky 4-substituents, such as 4-benzylthio, of phenethylamines result in high affinity 5-HT_{2A} binding and antagonistic behavior (Dowd et al., 2000; Luethi et al., 2018; Luethi et al., 2019). Measured by head-twitch response (HTR), Halberstadt et al. reported equipotent behavioral potency for TMA-2 (**19**) and two 4-homologated analogs (MEM; **24** and MPM; **26**) (Halberstadt et al., 2019). Furthermore, the authors demonstrated that the determined potency for the investigated psychedelics *in vivo* using HTR correlated highly (r = 0.98) with previously reported human potency data. Among HTR, drug discrimination (DD) is an extremely powerful tool and has been used for decades in order to compare psychedelic compounds in rats. Thus, the 4-alkyloxy substituted derivatives of the current study which are predicted to be potentially psychedelic in humans (based on their 5-HT_{2A} receptor interactions determined herein) should be further investigated using HTR and/or DD to characterize their potential psychedelic effects in human.

Overall, within the series of compounds investigated herein, the highest activation potencies at the 5-HT_{2A} receptor subtype were observed for 2C-O-3 (**7**) and MMALM (**13**) (EC_{50} = 0.5 nM and EC_{50} = 1.5 nM, respectively). Compared to the reference psychedelic 2C-B, both **7** and **13** activate the receptor in the same range, with **7** showing 4-fold higher activation potency than 2C-B.

Activation of the 5-HT_{2B} Receptors

At the 5-HT_{2B} receptor, for the 2C-O derivatives, the effect of increasing carbon chain length and/or bulkiness showed mixed effects on activation potency. The activation efficacy however was between 2-fold to 10-fold lower for the derivatives with increasing carbon chain (2C-O-16; **8**, 2C-O-3; **7** and 2C-O-27; **12**) when compared to 2C-O-2 (**6**). For the derivatives with increasing number of fluorine substituents, the activation potency was increased 2-fold (2C-O-21.5; **10**) and 4-fold (2C-O-22; **11**) compared to the monofluorinated derivative 2C-O-21 (**9**).

For the amphetamine-based derivatives, the effect of increasing carbon chain length/bulkiness, showed increasing activation potency at the 5-HT_{2B} receptor, that was 2-fold (MIPM; **18**), 28-fold (MALM; **14**), and 9-fold (MMALM; **13**) higher when compared to TMA-2 (**19**). However, whereas the activation efficacy which was substantially decreased for **18** in comparison to **19**, both **14** and **13**, were full agonists (for **18**, see discussions in Section 4.1). The derivatives with varying fluorinations showed similar activation potency at the 5-HT_{2B} receptor. The activation efficacy was however reduced by −20% and −55% for the difluorinated and trifluorinated, MDFEM (**16**) and MTFEM (**17**), respectively, when compared to the monofluorinated MFEM (**22**).

Taken together, these findings indicate that regarding 5-HT_{2B} activation, the extension of the carbon chain has the strongest effect on the amphetamine-based derivatives, whereas fluorination has the strongest effect on the phenethylamine-based derivatives. Previously, it has been reported that 5-HT_{2B} receptor activation may play a role in the mechanism of action of some substituted amphetamine type stimulants and mediate adverse effects like endocardial fibrosis (Fitzgerald et al., 2000; Rothman et al., 2000; Doly et al., 2008). For this reason, we examined the 5-HT_{2B} receptor activity for the 2C-O and 3C-O derivatives to estimate their potential to cause such adverse effects. We observed full agonist activation efficacy for 2C-O-2 (**6**) and amphetamine-based derivatives (MALM; **14** and MMALM; **13**) which could potentially lead to similar drug induced adverse effects (Rickli et al., 2015) for regular users.

Binding to the 5-HT_{2C} Receptors

At the 5-HT_{2C} receptor, the effect of structural modifications resulted in similar differences in 5-HT_{2C} receptor interactions as observed at the 5-HT_{2A} receptor. The 2C-O derivatives with increasing carbon chain length showed an increase in binding affinity by 5-fold (2C-O-16; **8**), 153-fold (2C-O-3; **7**), and 20-fold (2C-O-27; **12**) when compared to 2C-O-2 (**6**). All fluorinated 2C-O derivatives (compounds **9-11**) displayed comparable affinity at the 5-HT_{2C} receptor.

For 3C-O derivatives, the extension of the carbon chain length enhanced the receptor binding affinity of MALM (**14**) and MMALM (**13**) 6-fold and 18-fold, respectively, when compared to the shortest carbon chain containing TMA-2 (**19**) derivative; extension of the carbon chain by addition of a propyl group (**18**) reduced the receptor binding affinity 2-fold when compared to the TMA-2 (**19**). In the case of the varying number of fluorine substituents, the binding affinity was increased 2-fold and ~5-fold for MDFEM (**16**) and MTFEM (**17**), respectively, when compared to monofluorinated MFEM (**15**).

Overall, highest affinity (K_i < 1000 nM) at the receptor was observed for 2C-O derivatives with extended carbon chain modifications (2C-O-16; **8**, 2C-O-3; **7** and 2C-O-27; **12**) and for their amphetamine-based counterparts (MALM; **14** and MMALM; **13**). Previous findings show high affinity binding for the 5-HT_{2C} receptor in the range of 4.6–640 nM for NBOMe and 2C-T derivatives (Rickli et al., 2015; Luethi et al., 2018).

Non-Serotonergic Monoamine Receptor and Transporter Binding Interactions

In regards to binding at other monoamine receptors, only 2C-O-2 (**7**) and 2C-O-22 (**11**) activated the human TAAR1. However, activity at the human TAAR1 is known to be lower for many psychoactive substances compared to rodent TAAR1 (Simmler et al., 2016). At the rat TAAR1, the binding affinity was increased ~2-fold (2C-O-16; **8**), 3-fold (2C-O-3; **7**), and 16-fold (2C-O-27; **12**) for derivatives with increasingly longer carbon chains when compared to 2C-O-2 (**6**). The same was observed at the mouse TAAR1 with 2-fold (**8**), 4-fold (**7**), and 6-fold (**12**) increase in binding affinity. The binding affinity was slightly increased

(2-fold) for the difluorinated (2C-O-21.5; **10**) and trifluorinated (2C-O-22; **11**) 2C-O derivatives when compared to the monofluorinated derivative, 2C-O-21 (**9**). At the mouse TAAR1, only the trifluorinated derivative **11** bound (K_{i} = 2500 nM) while the monofluorinated and difluorinated derivatives did not.

None of the investigated amphetamine derivatives interacted with the human TAAR1 but all compounds bound to the rat TAAR1. MALM (**14**) and MMALM (**13**) bound the receptor with 3- and 5-fold higher affinity, respectively, than observed for TMA-2 (**19**) and MIPM (**18**). The addition of mono, di- or trifluorine substituents for MFEM (**15**), MDFEM (**16**), and MTFEM (**17**), respectively, had little effect on the extent of affinity at the rat TAAR1. The interactions of the amphetamine-based derivatives at the rat TAAR1 were mostly similar to those observed for their 2C-O counterparts. However, the increasing length of the carbon chain for 2C-O derivatives increased the extent of affinity at the receptor, which was not observed for the amphetamine-based derivatives. The extension of the carbon chain length for **14** and **13** allowed these derivatives to bind to the mouse TAAR1. Compounds **19** and **18** did not bind to the receptor in the examined concentration range. Similarly, only the tri-fluorinated derivative **17** bound to the receptor while the mono- and difluorinated derivatives **15** and **16** did not. A similar observation was made for fluorinated 2C-O derivatives, for which only the trifluorinated 2C-O-22 (**11**) bound to mouse TAAR1 in the investigated concentration range.

The rank order of affinity observed in our study for all the 2C-O and amphetamine-based derivatives at TAAR1 (rat > mouse > human TAAR1) was consistent with previous studies that investigated substituted phenethylamines with various bulky modifications (Lewin et al., 2008; Luethi et al., 2018; Luethi et al., 2019).

The 2C-O derivatives interacted with adrenergic α_{2A} receptors but there was no relevant binding to adrenergic α_{1A} and dopamine D_2 receptors or any of the monoamine transporters. Binding selectivity for substituted phenethylamines for the α_{2A} over α_{1A} receptor is in support of previously published studies of 2C-T but not NBOMe derivatives, which have been shown to bind to both adrenoceptor subtypes (Rickli et al., 2015; Luethi et al., 2018). Additionally, the lack of binding to the monoamine uptake transporters observed for both phenethylamine-based and amphetamine-based derivatives is in line with previous studies of 2C derivatives which did not display significant affinity at monoamine transporters (Rickli et al., 2015; Luethi et al., 2018). An exception to this is 2C-O-27 (**12**), which bound to the DAT at 6.1 μM. Moderate affinity at monoamine transporters has recently been demonstrated for a series of 4-aryl substituted 2,5-dimethoxy phenethylamines (2C-BI derivatives) (Luethi et al., 2019). Compound **12** carries a phenyl ring in its 4-substituent as well and this feature therefore seems to increase transporter binding and potentially inhibition of 2C derivatives.

Conclusion

In summary, we investigated the monoamine receptor and transporter binding and activation properties of several 4-alkyloxy-2,5-dimethoxy substituted phenethylamine and amphetamine derivatives *in vitro*. The compounds mainly interacted with serotonergic receptors and bound with the highest affinity to the 5-HT_{2A} receptor. This suggests that some of these amphetamine-based and phenethylamine-based derivatives could be potent psychedelics in humans.

The most active compounds with highest affinities, activation potencies, and activation efficacies were the 4-allyl and 4-methallyl derivatives 2C-O-16 (**8**) and MALM (**14**) as well as 2C-O-3 (**7**) and MMALM (**13**), respectively. Alterations of the 4-alkoxy group or introduction of fluorine substituents resulted in altered binding affinity at 5-HT_{2A} and 5-HT_{2C} receptors. Their low subtype selectivity is in line with the many other phenethylamine pharmacophore ligands tested so far. Nonetheless, subtle changes in chemical structure went in hand with changes in receptor profiles – and most probably in pharmacodynamics/pharmacokinetics – and would therefore likely lead to different types of psychedelic activities.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

KK, DL, DT, and ML designed the research. KK and MH performed the research. KK and M.L analyzed the data. KK, DT, and ML wrote the manuscript with significant input from all other authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.01423/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Monoamine transporter inhibition. Compounds were tested at a single high concentration (10 μm). Data are mean and SEM of three experiments. None of the compounds with the exception of MDMA relevantly reduced monoamine uptake (all EC_{50} values > 10 μM). As expected, the positive control MDMA markedly reduced NE and 5-HT uptake and moderately reduced DA uptake into the cells at 10 μM indicating activity at all transporters. The moderate inhibition of the DAT is due to the known low potency of MDMA at this transporter in this assay (EC_{50} = 17 μM).

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The reviewer SB declared a past co-authorship with some of the authors DL, ML to the handling editor.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Part B: Bioanalysis of psilocin using HPLC-MS/MS

Chapter 3: Bioanalysis of drugs of abuse

3.1 Bioanalysis in drug development

The discovery of novel molecular entities (NMEs) and their subsequent development into pharmaceutical drugs is a strenuous process (>10 years), costing on average 1 billion dollars (DiMasi, Feldman et al. 2010, DiMasi, Grabowski et al. 2016). The objective of drug development process is to uncover chemical compounds, evaluate their properties, and then identify a single compound which can be marketed as an effective drug with minimal adverse effects and a relatively good safety profile (Pandey, Pandey et al. 2010). In drug discovery preclinical and clinical development, the accurate and reliable quantification of the NME is an essential component in generating bioanalytical data which is used to successfully progress the development of the compound (Pandey, Pandey et al. 2010, Mohs and Greig 2017).

Bioanalysis is a process of developing a bioanalytical method to identify and accurately quantify exogenous/endogenous compounds and their related metabolites in different biological matrices e.g., plasma, serum, urine or other tissues (James, Breda et al. 2004, Moein, El Beqqali et al. 2017). In the development process and its clinical application, bioanalysis plays an important role in several processes including the characteristics of a drug's absorption, distribution, metabolism, and elimination, also known as a drug's pharmacokinetics. Additionally, bioanalysis also plays a key role in the identification of a drug's potentially toxic and unstable metabolites, and later in therapeutic drug monitoring of the compound (Humphrey 1996, Kiorpes 2014). Importantly, preliminary bioanalytical data enables better decision making at each stage of the drug development, increasing the probability that a compound can be approved therapeutically (Pandey, Pandey et al. 2010, Cook, Brown et al. 2014). Bioanalysis is also implicated in various other fields like forensic science, sports doping, environmental sciences, and drugs of abuse. In particular, it plays a key role in the study of novel drugs of abuse both forensically and also in order to investigate their pharmacological and toxicological properties (Wohlfarth and Weinmann 2010, Rebe Raz and Haasnoot 2011, Pozo, De Brabanter et al. 2013, Wagmann and Maurer 2018, Protti, Mandrioli et al. 2019).

Bioanalytical methods or assays need to be validated so that they can be used e.g., to conduct a pharmacokinetic analysis of samples from a clinical trial or to be used for diagnostic or forensic purposes. The validation of a bioanalytical method or assay is

carried out based on a standardized criteria (discussed further in **Chapter 3.3.5**) set by the FDA/EMA guidelines in accordance with Good Laboratory Practice (GLP) standards. The development and validation of a bioanalytical method in accordance with industry standards was the third and final focus of my doctoral project. In this project, I established a method for quantification of psilocybin's metabolites, psilocin, 4-hydroxyindole-3-acetic acid (4-HIAA), and psilocin glucuronide in plasma (**Chapter 4.1**).

3.2 High-pressure liquid chromatography and tandem mass spectrometry

Currently, high-pressure liquid chromatography and tandem mass spectrometry (HPLC-MS/MS) (**Figure 7**) is the most commonly used technique in the bioanalysis of drugs. Mainly because the technique has overcome several shortcomings of analytical systems preceding its creation. Long established techniques like colorimetry were adequate initially but lacked in specificity as it was often difficult to detect parent analytes separately from their metabolites (Hill 2009, Hamidi 2018). This was largely improved by the development of chromatographic techniques like paper or thin-layer chromatography, which enabled separation of the analyte of interest from their corresponding metabolites (Hill 2009, Attimarad, Ahmed et al. 2011). Conversely, these techniques were mostly not able to quantify very low amounts of analytes (ng/ml or pg/ml range) in biological samples among endogenous matrix components (Hill 2009). The development of gas chromatography (GC) in conjunction with progressively more sensitive detectors like mass spectrometers (MS) overcame the sensitivity issue and enabled a fast and selective analysis using only small amounts of sample. However, gas chromatography was limited to thermostable and volatile compounds, thus often requiring work-intense sample preparation including analyte derivatization (Hill 2009, Sanchez-Guijo, Hartmann et al. 2013). The emergence of liquid chromatography (LC) coupled to ultraviolet (UV), and later to MS changed the analytical field. It allowed the measurement of small and large molecular weight low volatility compounds. These low volatility compounds did not require work-intensive sample preparation, and could be rapidly prepared, and provided high level of selectivity and sensitivity (Hill 2009, Pitt 2009).

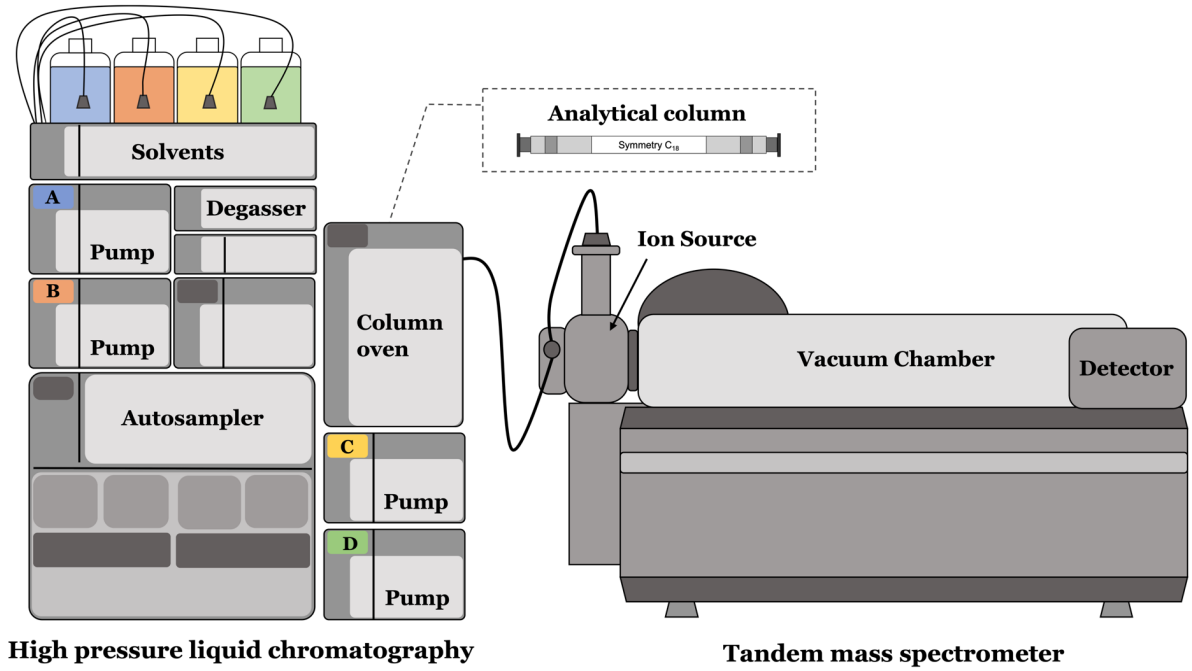

Figure 7: Liquid chromatography and tandem mass spectrometer. High pressure liquid chromatography (HPLC) consists of several components including the (1) solvents reservoir (four bottles on the top) which consist of different mobile phases and additives that are mixed to and carry the sample to the column, (2) degasser, which removes the bubbles from the liquid mobile phases, (3) pumps (pump A to D) which pump the liquid sample in the mobile phase at a specific flow rate and at a specific mobile phase composition, (4) autosampler, which injects the liquid sample into the flow of the mobile phase, (5) column oven, which heats up the analytical column inside to a set temperature and finally the (6) the analytical column (e.g., Symmetry C18), which provides the stationary phase that separates the sample components. HPLC is connected to the (right) tandem mass spectrometer (MS/MS). (8) In the ion source, the liquid sample is pumped through a capillary under voltage which converts the sample into a gaseous mist and ionizes it by ESI or APCI. The ionized analytes are then transferred to the mass analyzers in the (9) vacuum chamber which can be set to screen for specific mass-to-charge (m/z) ratio values in Q1. These are then collided with inert gas in the collision chamber (Q2) and the specific fragmented product ions can be isolated in Q3. The specific precursor and product ion m/z values are detected in the (10) detector (McLafferty 1981, Pitt 2009). Figure concept inspired and adapted from Shimadzu (2021) and Sciex (2010).

High performance liquid chromatography (HPLC) is a technique which separates analyte(s) of interest according to their physiochemical properties (e.g., size, polarity, lipophilicity) (**Figure 7**). A small volume of the sample containing the analytes is injected into the stream of the flowing solvent mixture (serves as the mobile phase) and is pumped into the system at a specific flow rate and at high pressure by the pumps. The pumps then push the sample in the mobile phase through the analytical column (stationary phase). The transport over the column depends on the interactions of the column (and its packing material) with the analytes and also the solubility of the analytes in the mobile phase composition (Hamilton and Sewell 1982, Brown and Hartwick 1988, Lindsay and Barnes 1992). In reverse-phase chromatography, the sample is transported in a polar mobile phase (e.g., water or

methanol) under high pressure (100 – 500 bar) through an analytical column e.g., a non-polar/hydrophobic stationary phase C18 column (Brown and Hartwick 1988, Lindsay and Barnes 1992). The mobile phase composition can be maintained (isocratic elution) or can be changed during the entirety of the analyte separation (gradient elution) (Schellinger and Carr 2006). Based on the mobile phase composition the analytes are solved or interact/bind more or less to the analytical column. Rapid transport over the stationary phase indicates weak interactions, while slower transport indicates stronger interactions (Lindsay and Barnes 1992, Schellinger and Carr 2006). Various types of analytical columns are available with different physiochemical properties e.g., 8-carbon chain (C8), C18, or biphenyl ring columns. These chemically bonded groups interact with the analytes of interest via different Van der Waal and hydrophobic interactions (Lindsay and Barnes 1992). Depending on the chemical attributes of the analyte, other chromatographic separations are required such as normal phase chromatography, ion exchange, and size exclusion chromatography (Pitt 2009). A chromatographic separation for different analytes may employ different configurations e.g., different mobile phase composition, flow rate, and stationary phase with compatible packaging material. For example, normal phase chromatography employs a non-polar mobile phase like *n*-hexane and a polar stationary phase (the opposite of reverse phase HPLC) (McLafferty 1981, Brown and Hartwick 1988, Lindsay and Barnes 1992).

After the analyte(s) of interest are separated by the HPLC they enter the tandem mass spectrometer (MS/MS) (**Figure 7**). First, the analytes which are solved in the mobile phase enter the interface, where they are most commonly ionized by electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). The liquid sample is pumped through a voltage-dependent capillary, which converts the liquid sample into a gaseous mist (nebulization) and ionizes the analyte molecules (Whitehouse, Dreyer et al. 1985, Pitt 2009, Urban 2016). The ion source can be operated in positive and negative ionization modes, which in turn either add or remove protons from the analyte molecules (Pitt 2009). The ionized analytes are then transferred to the mass analyzer inside the MS/MS which is under high vacuum. The mass analyzer can be set to screen specific mass-to-charge (m/z) ratio values corresponding to the analytes of interest (Pitt 2009, Unger, Li et al. 2013, Urban 2016). A commonly used mass analyzer like the quadrupole analyzer is made up of four metal rods that sit parallel to each other and are exposed to different voltage that only allow

the continuous flow of specific m/z ratio values through, while all remaining m/z ratio values are deflected (Pitt 2009). In tandem mass spectrometry, two independent mass analyzers (Q1 and Q3) are separated by collision cell (Q2). In the Q1, a specific m/z ratio can be selected and then can collide with the inert gas e.g., nitrogen in the collision chamber. In the Q3, the specific and fragmented product ions can then be isolated (McLafferty 1981, Pitt 2009). The product ions produced from the collision of a specific parent ion are very specific to each compound and are therefore a "fingerprint" fragmentation pattern of the compound. So, there is little risk that another analyte would have the exact same fragmentation pattern (McLafferty 1981, Pitt 2009). As a result, screening for a specific precursor and then product ion m/z values (known as multiple reactions monitoring, MRM), using tandem mass spectrometry enables the detection of analytes of interest with a high degree of specificity within a sample containing several analytes (McLafferty 1981, Pitt 2009).

3.3 Bioanalytical method development and validation

To develop and validate bioanalytical method in line with regulatory (FDA or EMA) guidelines is often a rigours process. The overall goal of a bioanalytical method is to detect the analyte(s) of interest in the intended matrices (e.g., plasma, whole blood, urine), and to also quantify them reliably.

3.3.1 Method development

The development of an acceptable bioanalytical method requires a preparation of the biological sample for analysis and an appropriate system to detect/measure our analyte(s) of interest. In brief, the extracted biological sample containing the analyte(s) needs to undergo a cleaning procedure to remove any endogenous components (e.g., proteins or lipids) which can often interfere with the detection and quantification of target analyte(s) (Schuhmacher, Zimmer et al. 2003, Zhou, Yang et al. 2017, Li, Jian et al. 2019). Different analytical systems can be applied to detect the target analyte(s) such as liquid or gas chromatography (LC/GS), most often coupled to ultraviolet detection (UV) or mass spectrometry (MS) (Moein, El Beqqali et al. 2017, Li, Jian et al. 2019). The most commonly used technique however consists of high performance liquid chromatography (HPLC) coupled to tandem mass spectrometry

(MS/MS), which offers sufficient analyte sensitivity and accuracy (Pandey, Pandey et al. 2010, Moein, El Beqqali et al. 2017, Li, Jian et al. 2019).

3.3.2 Sample preparation

The first consideration to assess thoroughly is the preparation of the sample before analysis, which should be consistent, sufficient, and as simple as possible to allow for high sample throughput. The sample extraction is key to achieve the necessary sensitivity and inconsistent extraction recovery might affect accurate and precise quantification of the target analytes(s). Three of the most commonly used techniques to clean the sample from the high amount of matrix components include protein precipitation (PPT), liquid-liquid extraction (LLE), and solid phase extraction (SPE) (Ashri and Abdel-Rehim 2011, Moein, El Beqqali et al. 2017, Li, Jian et al. 2019). Complex matrices like plasma, urine or whole blood often contain salts, proteins and other organic components which if left unextracted can interfere with the detection of the target analytes e.g., lead to suppression of the target analyte signal or can even clog the analytical column. Sample preparation is therefore an important step in producing a concentrated sample of the target analyte(s), to improve sensitivity, and reduce the risk of ion suppression or enhancement, which can be problematic if there are differences between subjects (Ashri and Abdel-Rehim 2011, Moein, El Beqqali et al. 2017, Li, Jian et al. 2019).

PPT is a simple and fast extraction method, often applied to plasma or blood samples to extract hydrophilic or hydrophobic analytes (Ashri and Abdel-Rehim 2011, Moein, El Beqqali et al. 2017, Li, Jian et al. 2019). The technique involves exposure of the sample to a miscible organic solvent such as methanol or acetonitrile, which precipitates the proteins found in the sample, releasing all bound analyte(s) of interest. A subsequent centrifugation step, separates the precipitated proteins and leaves behind a supernatant with the target analyte(s). The ratio of organic solvent to sample volume is essential in this technique in order to achieve maximal protein removal; 1 to 4 of organic solvent: 1.0 of sample (v/v) is sufficient to achieve a protein displacement of close to 98% (Ashri and Abdel-Rehim 2011). The extraction recovery of this technique is high for some analytes when compared to LLE or SPE, which means that close to 100% of the analyte(s) of interest can be detected in a sample post extraction (Ashri and Abdel-Rehim 2011, Moein, El Beqqali et al. 2017, Li, Jian et al. 2019).

LLE is a more work-intensive extraction method, often applied to water-based and biological samples (Ashri and Abdel-Rehim 2011, Moein, El Beqqali et al. 2017, Li, Jian et al. 2019). The technique consists of the addition of an immiscible organic solvent such as chloroform or hexane, which then separates the analyte(s) of interest from the matrix components in one liquid phase and transfer it to the organic solvent phase (Ashri and Abdel-Rehim 2011, Moein, El Beqqali et al. 2017, Li, Jian et al. 2019). A subsequent centrifugation step is needed. Often the organic phase must then be evaporated and the sample has to be reconstituted in mobile phase before it can be injected to the analytical instrumentation.

SPE is a commonly used extraction technique that is also work-intensive and rather expensive. The technique employs cartridge full of C8 or C18 sorbent (25 –50 mg) which separates the analytes from the matrix, in a similar fashion to the analytical separation occurring in the HPLC with the analytical column (Ashri and Abdel-Rehim 2011, Moein, El Beqqali et al. 2017, Li, Jian et al. 2019). The cartridge however can be used only once, thus making this extraction method financially burdensome. Often the separated analyte(s) can be directly injected into the analytical machinery or can undergo evaporation and reconstitution steps. The technique is used often for trace analysis for very small quantities of samples, with the purpose of producing concentrated and clean sample extracts for quantification (Ashri and Abdel-Rehim 2011, Moein, El Beqqali et al. 2017, Li, Jian et al. 2019).

3.3.3 Analyte physiochemical properties and HPLC considerations

During the method development process, assessment of the analytes physiochemical properties (e.g., hydrophilicity/lipophilicity; how well an analyte dissolves in water vs. fat) is essential to select an adequate type of column, mobile phase, mobile phase additives, and extraction method (Li, Jian et al. 2019). A column (e.g., C18 packed column provides hydrophobic surface for interacting with nonpolar analytes) is selected so that it interacts well with the analyte(s) of interest. Mobile phases (e.g., water, methanol) and mobile phase additives (e.g., formic acid, with consideration for pH) are screened to alter the interactions of the analyte(s) in terms of their separation on the analytical column and also their ionization in the ion source (Li, Jian et al. 2019). The extraction method used to purify the samples is also considered to achieve the necessary sensitivity needed for the assay. **Figure 8,** shows

a summary of the method development process that was applied for developing the bioanalytical method of psilocybin's metabolites (**Chapter 4.1**).

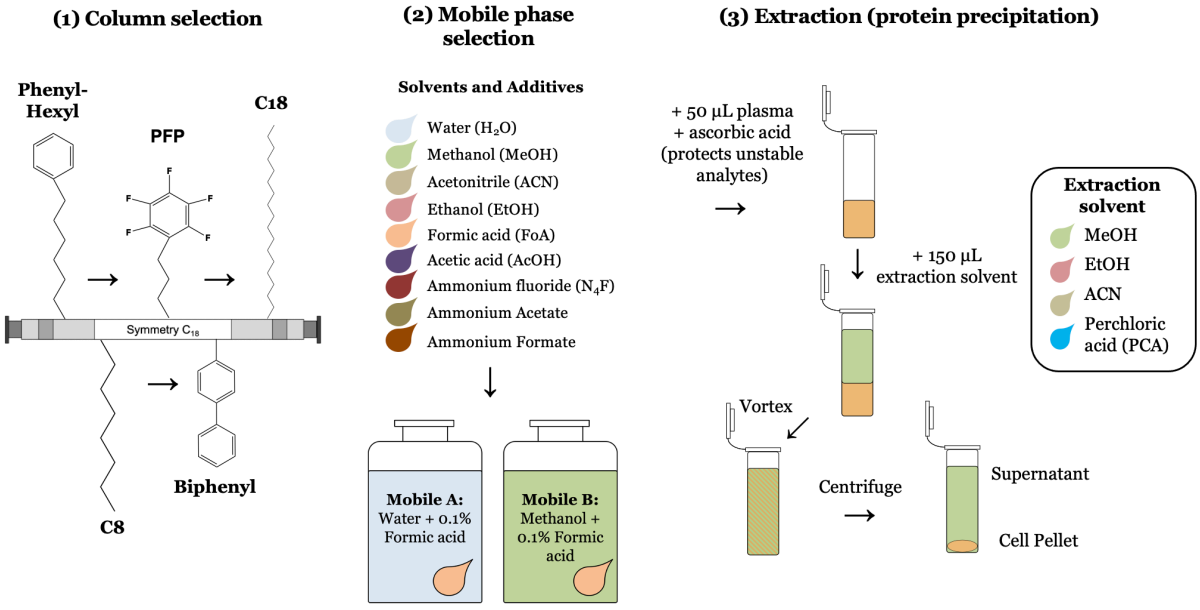

Figure 8: Summary of method development process employed for the bioanalysis of psilocybin's metabolites. (1) Column selection was an important step in the development of the method. Several columns were screened including phenyl-hexyl, pentafluorophenyl (PFP), biphenyl, and C18 phases. These columns retained psilocin and 4-HIAA to different degrees e.g., the PFP column produced a good analyte peak for psilocin but produced unsymmetrical double peaks for 4-HIAA. The optimal retention of both analytes (e.g., peak symmetry, thickness, and overall shape) were achieved with the C_{18} column, therefore this column was chosen for further development and method validation. (2) Different mobile phase were (e.g., water, methanol, acetonitrile) concurrently assessed during column screening. Several additives (e.g., formic acid, acetic acid) were tested to the various mobile phase in order to enhance analyte intensities, improved the linearity range, as well as aid in the interactions of the analytes with the column packing material. Water and methanol supplemented with ammonium fluoride (N_4F) was employed, however due to corrosive nature of the N_4F to the column longevity, we ultimately, supplemented both mobile phases with formic acid. (3) Protein precipitation extraction was examined using various solvents including methanol and perchloric acid. Initially, perchloric acid extraction was promising (analyte peak shape was excellent), however the produced supernatant had to be mixed with buffer to reduce the acidic pH of the supernatant to ensure that the column was not damaged. Therefore, a methanolic protein precipitation was preferred, as it was simple and time-effective, as well as produce enough sharp and symmetrical peak shapes for both analytes.

3.3.4 Analyte(s) and MS/MS setting considerations

Next, the mass spectrometry settings needed to be further improved. First, it is important to select a sufficient sensitive system that can detect and measure our analyte(s) of interest. One has to know the expected concentration range (e.g., ng/ml or pg/ml) or the observed range of analyte(s) in clinical samples (e.g., animals or subjects' post-treatment). This is important to determine the correct range that the analyte should be quantified within the bioanalytical method (Sargent 2013, Moein, El Beqqali et al. 2017). Signal intensity and analyte selectivity are optimized by selecting

the appropriate mode of detection for each analyte (e.g., positive or negative ionization). Screening for the most abundant analyte fragments and selecting them according to the signal-to-noise ratio and their interferences with co-eluting matrix components also enhances better method sensitivity and selectivity (Sargent 2013). Moreover, choosing a suitable mass transition for each analyte (e.g., product ion) which exhibits the best signal-to-noise ratio and adjusting the source temperature also further improves signal intensity and analyte selectivity. Selection of structurally similar and stably labelled internal standards (IS) for the target analyte(s) is key for accurate and precise analyte(s) quantification as they behave in a similar way to their respective analytes e.g., exhibit similar ion suppression and chromatographic properties (Moein, El Beqqali et al. 2017). The gradient program (composition of the mobile phases, flow rate etc.) can be adjusted to obtain adequate chromatographic peaks. Good peak shape consists of symmetrical peaks with a narrow peak width (Sargent 2013, Wahab, Armstrong et al. 2017). Unsymmetrical peaks, peak-fronting/tailing, or thick peak width are all characteristics of bad peak shape. The gradient program parameters also affect the separation of the analytes on the column. In general, several parameters in the bioanalytical method need to be optimized during the method development process. Each parameter influences the analyte(s) to varying degrees, thus complicating optimization of a method which detects multiple analytes at the same time. Once all these parameters have been optimized, the method can then undergo the method validation process.

3.3.5 Method validation

The validation of a bioanalytical method assesses whether the development method is sufficiently reliable to measure the target analyte(s) in the intended biological matrix. Several key parameters need to be evaluated during the validation process, which have been described by the FDA/EMA (European Medicines Agency 2011, Food and Drug Administration 2018).

Different types of method validations exist including a full validation, partial validation, and a cross validation (European Medicines Agency 2011, Food and Drug Administration 2018). In short, a full validation is conducted to assess the quality of a novel developed bioanalytical method, while a partial and cross validation are often applied to the assessment of different parameters or modifications of already validated bioanalytical methods. Since I focused on the assessment of a full validation, I will

discuss the key parameters (linearity, accuracy and precision, selectivity and sensitivity, extraction recovery, matrix effect, and stability) in the next section. Summary of these parameters and their corresponding acceptance criteria is presented in **Table 1**.

Linearity: As a first step, the relationship between the analyte(s) concentration and the signal intensity needs to be assessed. A linear relationship between the two components is preferred over quadratic relationship. A concentration range of the analyte(s) is selected that matches the observed concentration range in e.g., clinical samples. The standard curve is created by preparing calibrator (CAL) and quality control (QC) samples in the matrix of interest (e.g., plasma). This implemented matrix also needs to match the matrix that the analyte(s) are found in which later need to be measured by the method. Once this is achieved, this standard curve is then fitted to a linear regression. This enables the proportional back calculation of the analyte concentration in an unknown sample from the standard curve. Often a correlation coefficient is used to assess the linearity of a standard curve (European Medicines Agency 2011, Food and Drug Administration 2018).

Accuracy and precision: Next, the accuracy and precision of a method is assessed using quality control samples (QCs) which are set at the lowest concentration in the range (known as the lower limit of quantification, LLOQ), and at a low (QC low), medium (QC med), and high concentrations (QC high). The accuracy of the method is examined by calculating the accuracy bias of the nominal analyte concentration to the observed analyte concentration. The precision of the method is determined by the coefficient of variation (CV%) per QC level. The methods accuracy and precision are assessed within a single assay (intra-assay) and between independent assays (inter-assay). The accuracy of bioanalytical method demonstrates how close a measured value of a sample is to its true value and the closeness of multiple measurements of a sample to each other (precision). An inaccurate and imprecise bioanalytical method therefore would produce unreliable results (European Medicines Agency 2011, Food and Drug Administration 2018).

Table 1: Summary of method validation parameters. The key elements for each parameter are summarized with their corresponding acceptance criteria. Figure concept inspired and adapted from Food and Drug Administration (2018).

Parameters	Key elements	Acceptance criteria
Linearity	▪ Blank sample (with IS) ▪ At least 6 CALs that cover quantification range ▪ Double blank sample (without IS) ▪ All samples in intended matrix for analysis	▪ Relationship between analyte concentration and signal intensity should fit simplest regression e.g., linear ▪ ± 15% deviation of observed analyte concentration (CALs) to nominal concentration (± 20 % LLOQ) per run ▪ At least 75% of all CALs need to be in for valid and accepted run
Accuracy and Precision	▪ At least three independent runs on three separate days ▪ Four QC levels (LLOQ, QC low to high) ▪ At least 5 replicates per QC level ▪ Include calibration curves between QC replicates	▪ Accuracy: ± 15% deviation of observed analyte concentration (QCs) to nominal concentration (± 20 % LLOQ) per run ▪ Precision: coefficient of variation (CV) ± 15% for all QCs except LLOQ (CV ± 20 %) ▪ CALs meet acceptance criteria ▪ At least 67% of all QCs need to be in for valid run (with at least 50% of QCs per level)
Selectivity/ Sensitivity	▪ Blank and double blank samples in matrix for analysis ▪ Blank samples spiked to LLOQ level ▪ At least 6 individual sources	▪ No interference in blank or double blank samples (no signal in retention time of analytes or IS) ▪ Analyte response in spiked samples should be at least 5 times the analyte response in double blank sample ▪ Accuracy: ± 20% deviation of observed analyte concentration to nominal concentration (at least 5 replicates in at least 3 runs) ▪ Precision: CV ± 20% for samples (at least 5 replicates in at least 3 runs)
Extraction recovery	▪ At low, mid, and high QC levels ▪ Extract samples compared to extracted blank samples spiked to low, mid, and high QC levels (post extraction)	▪ Recovery should be consistent and reproducible at all levels
Matrix effect	▪ Samples prepared in matrix and compared to samples prepared without matrix (pure water or IS only) ▪ Use multiple sources of matrix	▪ ME should be consistent and reproducible at all levels
Stability	▪ In autosampler at 10° C ▪ Bench top 8 h at room temperature ▪ Freeze-thaw cycles ▪ Stocks stability ▪ Long term stability ▪ At least 3 replicates at low and high QC levels	▪ Accuracy: ± 15% deviation of observed analyte concentration to nominal concentration at each level

Internal standard (IS), calibrator (CAL), quality control (QC), matrix effect (ME), coefficient of variation (CV), low limit of quantification (LLOQ)

Selectivity and sensitivity: Thereafter, the method's selectivity and sensitivity are determined to ensure that the method is selective for the detection of the analyte(s) of interest in order to enable reliable and accurate quantification at low

concentrations in a complex matrix. As a result, the matrix components need to be examined to see if they do not interfere with the analysis of the target analyte(s). The method selectivity is examined by the processing of blank samples from different sources to determine whether matrix components interference occurs. On the other hand, the method sensitivity is assessed by spiking blank samples at the LLOQ concentration and comparing the signal intensity to the background signal (European Medicines Agency 2011, Food and Drug Administration 2018). A signal-to-noise ratio of at least 1 to 5 or 10 should be achieved. The lower limit of detection is considered at a ratio of 1 to 3.

Extraction recovery: Next, the method's extraction recovery was assessed to see how efficacious the employed extraction procedure is at cleaning the target analyte from other components in the sample and how much of the analyte can be recovered from the procedure (European Medicines Agency 2011, Food and Drug Administration 2018). Employing a highly efficacious extraction procedure could enables analyte recovery closer to 100%, which means that very little of the analyte is lost during the sample preparation process. Extraction recovery is examined by spiking unextracted blank samples and extracted blank samples with equal analyte amounts. The spiked extracts correspond to 100% recovery. Overall, the recovery observed should be consistent in the different batches of plasma and at all examined QC levels.

Matrix effects: The matrix components can potentially influence the ionization of the analyte(s) of interest e.g., suppress or enhance the ionization. Thus, it is important to evaluate that these effects are similar and consistent between different sources of matrix. Matrix effects are examined by comparing the analyte amount observed in samples with matrix (e.g., in plasma) and without the matrix (e.g., in pure solvent), which are spiked with the same amount of analyte. The matrix effect observed should be consistent at different QC levels (European Medicines Agency 2011, Food and Drug Administration 2018). A signal less than 85% would indicate ion suppression of the matrix, while a signal beyond 115% would indicate ion enhancement.

Stability: The stability of analytes in the samples needs to be assessed under different conditions (**Table 1**) that are encountered during sample analysis. For example, it is important to examine if the samples remain stable on the bench for 8 h at room temperature or inside the autosampler for 24 h at 10° C. Moreover, the effect of several thawing and re-freezing cycles of the samples needs to be evaluated.

Additionally, the stability of the stock solutions used to make calibrator and quality control samples needs to be examined. The analyte(s) stability under the different storage conditions is assessed by comparing the accuracy of the analytes in stored samples to freshly prepared samples on the day of analysis (European Medicines Agency 2011, Food and Drug Administration 2018).

Method application: The method's applicability is often the final test of the developed bioanalytical method. The purpose of this validation step is to assess if the method is able to successful quantify the target analyte(s) in the matrix of interest (Food and Drug Administration 2018). Preliminary pharmacokinetic parameters e.g., time to reach and maximal plasma concentration (T_{max} and C_{max}) can be determined and compared to previously published data. The method can be assessed for its capability in quantifying samples from ongoing clinical studies in order to see if the method is sufficiently sensitive and selective. For example, one may find unknown metabolites which interfere with the analysis e.g., in-source fragmentation which can produce fragments with the same mass to target analyte(s). As a result, the method would have to be adjusted in order to avoid these unwanted fragments.

3.4 Bioanalysis of psilocybin and drug-assisted psychotherapy

The use of psilocybin-containing mushrooms has been around for centuries, mainly in Central and South America, where it has been used for religious and spiritual purposes (Carod-Artal 2015). In the 1950-60s, psilocybin was investigated and studied clinically as a potential adjuvant in psychotherapy (Reiff, Richman et al. 2020). During these times, most studies were conducted on a small scale and were not as rigorous as nowadays, thus limited progress in psilocybin's research was made. Furthermore, psilocybin use was also becoming more prominent on the recreational drug market. The emergence of the Controlled Substances Act of 1970, later the commencement of the "War on Drugs" by President Nixon, and scheduling of all classical psychedelic substances as lacking any medical application, harshly stopped all clinical research with psychedelics (Reiff, Richman et al. 2020). Fortunately, for the last two decades, psilocybin's potential application in drug-assisted psychotherapy has been reinitiated. Lots of progress has been made in repurposing psilocybin from a recreational drug into a psychotherapeutic tool for psychiatrists. This is largely due to the support of several key scientific agencies around the world (e.g., Multidisciplinary Association for Psychedelic Studies, MAPS), which have not only worked together with the FDA and

EMA to ensure the scientific quality of these novel clinical studies, but have also provided essential financial support for clinical studies (Reiff, Richman et al. 2020).

Currently, psilocybin (as well as other psychedelics) is studied clinically in several countries including Switzerland, United Kingdom, and the United States (Reiff, Richman et al. 2020). As of October 2021, 67 studies are listed and actively investigating psilocybin's potential in psychotherapy (ClinicalTrials.gov 2021). Most of these clinical studies involve healthy volunteers or patients suffering from a range of psychological disorders. Research within these studies divides into investigating psilocybin's pharmacological properties or evaluating its therapeutic potential against the various psychological disorders (Reiff, Richman et al. 2020). So far, the positive effect of psilocybin has been examined for its therapeutic potential in patients suffering from depression, anxiety, alcohol use disorder, obsessive compulsive disorder, and cluster headaches (Moreno, Wiegand et al. 2006, Sewell, Halpern et al. 2006, Grob, Danforth et al. 2011, Carhart-Harris, Bolstridge et al. 2016, Griffiths, Johnson et al. 2016, Ross, Bossis et al. 2016, Carhart-Harris, Roseman et al. 2017, Johnson, Garcia-Romeu et al. 2017, Bogenschutz, Podrebarac et al. 2018, Carhart-Harris, Bolstridge et al. 2018). For example, psilocybin's application in treatment-resistance depression, has led to commencement of large phase 2B clinical trial at various test sites and a special FDA approval of using psilocybin in treatment-resistance depression (COMPASS 2018, Hartman 2018).

The comprehensive investigation of psilocybin for treatment of different psychological disease states, facilitates that psilocybin could one day become a licensed FDA medicine. In order to achieve this enormous task (considering the dire past of psychedelics with the public and also the associated politics) psilocybin's clinical pharmacology has to be thoroughly researched. Therefore, reliable bioanalytical methods are required. Thus far, it is well-established, that oral psilocybin ingestion leads to a rapid dephosphorylation to the active metabolite psilocin, which produces the psychedelic effects associated with the substance (**Figure 9**) (Nichols 2004, Rickli, Moning et al. 2016). Peak plasma levels of psilocin (10 – 40 ng/ml range) are reached after 1.5 – 2 h post administration. Psilocin is largely conjugated via the UDP-glucuronosyltransferases (UGT) in the liver and small intestine, to psilocin-*O*-glucuronide (Brown and Hartwick 1988, Hasler, Bourquin et al. 1997, Grieshaber, Moore et al. 2001, Hasler, Bourquin et al. 2002, Manevski, Kurkela et al. 2010). Moreover, psilocin can also be deaminated and oxidized to metabolites 4-

hydroxytryptophol (4-HTP) and 4-hydroxyindole-3-acetic acid (4-HIAA) via an intermediate metabolite (Hasler, Bourquin et al. 1997, Lindenblatt, Kramer et al. 1998, Passie, Seifert et al. 2002). Overall, subjective effects of psilocybin last for 6 h and peak at approximately 2 h post administration, with an elimination half-life of 2 – 3 h (Hasler, Bourquin et al. 1997, Passie, Seifert et al. 2002, Griffiths, Johnson et al. 2016).

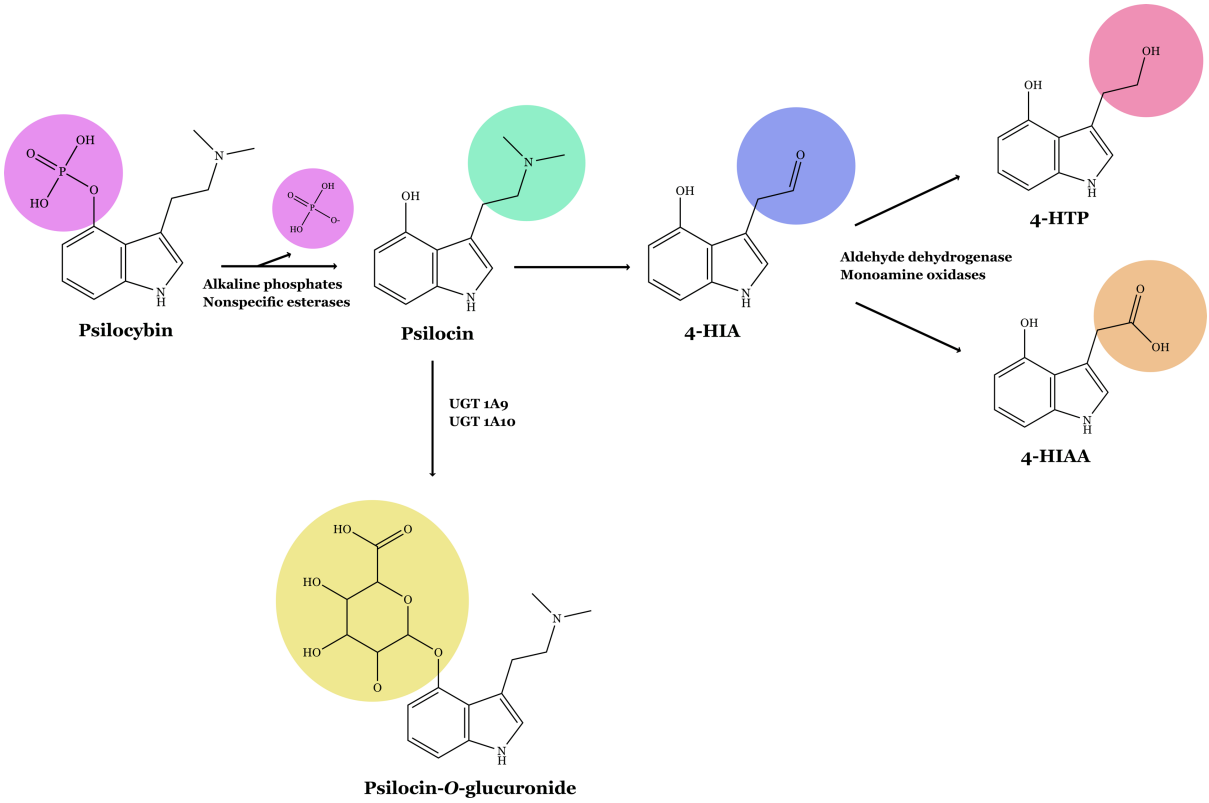

Figure 9: Psilocybin metabolism in humans. Oral psilocybin is rapidly dephosphorylated to psilocin by alkaline phosphatases and nonspecific esters. Psilocin can then be glucuronidated to psilocin-*O*-glucuronide by the UDP-glucuronosyltransferases (UGT) 1A9 in the liver and UGT 1A10 in the small intestine. Psilocin-*O*-glucuronide is considered to be a major metabolite of psilocin. Psilocin can also be deaminated and oxidized likely by liver aldehyde dehydrogenase and monoamine oxidases to 4-hydroxytryptophol (4-HTP) and 4-hydrxoyindole-3-acetic acid, via an unstable intermediate 4-hydroxyindol-3-yl-acetaldehyde (4-HIA) (Hasler, Bourquin et al. 1997, Passie, Seifert et al. 2002, Lindemann, Ebeling et al. 2005). Figure concept inspired and adapted from Hasler, Bourquin et al. (1997) and (Dinis-Oliveira 2017).

Although the knowledge of psilocybin's pharmacological properties has been investigated for the past two decades, there are still several parameters that require in-depth examination. Investigation of the psilocybin's pharmacokinetics in large-scale cohorts using validated bioanalytical methods is still needed, as in the past, most studies were conducted in small and specialized subject populations. Additionally, the relationship between dose and response, psilocybin's interactions with other drugs (e.g., antidepressant medication), *in vivo* metabolism studies, and the relationship between pharmacokinetic and pharmacodynamics, remain to be studied. Importantly,

to uncover the aforementioned clinical pharmacology parameters, rigorous and properly validated bioanalytical methods are of great importance.

Quantification of psilocybin's relevant metabolites including psilocin, psilocin-*O*-glucuronide, 4-HIAA, and 4-HTP in different human matrices (e.g., plasma, urine, hair) has been established since the mid 1990s (Hasler, Bourquin et al. 1997, Lindenblatt, Kramer et al. 1998, Sticht and Kaferstein 2000, Grieshaber, Moore et al. 2001, Hasler, Bourquin et al. 2002, Kamata, Nishikawa et al. 2003, Kamata, Nishikawa et al. 2006, del Mar Ramirez Fernandez, Laloup et al. 2007, Bjornstad, Hulten et al. 2009, Martin, Schurenkamp et al. 2012, Brown, Nicholas et al. 2017). Initially, most methods employed either liquid or gas chromatography coupled to electrochemical detectors or single mass spectrometers, and consisted of work-intensive sample processing procedures (liquid or solid-phase extractions), and long run times (15 – 20 min) per sample (Hasler, Bourquin et al. 1997, Lindenblatt, Kramer et al. 1998, Sticht and Kaferstein 2000, Grieshaber, Moore et al. 2001, Hasler, Bourquin et al. 2002). In time, these methods were superseded by methods employing liquid chromatography coupled to tandem mass spectrometry, which not only possess greater sensitivity but also included progressively shorter sample processing procedures and run time (~ 10 min) (Kamata, Nishikawa et al. 2003, Kamata, Nishikawa et al. 2006, del Mar Ramirez Fernandez, Laloup et al. 2007, Bjornstad, Hulten et al. 2009, Martin, Schurenkamp et al. 2012, Brown, Nicholas et al. 2017). Although some progress has been made in developing sensitive bioanalytical methods, there is still a pressing need to establish and validate methods that are more practical for the analysis of large quantities of samples. As a result, developing and validating a state of the art bioanalytical method to quantify psilocin, psilocin glucuronide, and 4-HIAA in human plasma for upcoming clinical studies was the third and final focus of my doctoral project and is presented in **Chapter 4.**

Chapter 4

4.1 Publication 3

Development and validation of LC-MS/MS method for the bioanalysis of psilocybin's main metabolites, psilocin and 4-hydroxyindole-3-acetic acid, in human plasma.

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Development and validation of an LC-MS/MS method for the bioanalysis of psilocybin's main metabolites, psilocin and 4-hydroxyindole-3-acetic acid, in human plasma

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ABSTRACT

Psilocin is the active metabolite of psilocybin, a serotonergic psychedelic substance. It is used recreationally and investigated in substance-assisted psychotherapy. The pharmacokinetic properties of psilocin are only partially characterized. Therefore, we developed and validated a rapid LC-MS/MS method to quantify psilocin and its metabolite 4-hydroxyindole-3-acetic acid (4-HIAA) in human plasma.

Plasma samples were processed by protein precipitation using methanol. The injected sample was mixed with water in front of a C_{18} analytical column to increase retention of the analytes. Psilocin and 4-HIAA were detected by multiple reaction monitoring (MRM) in positive and negative electrospray ionisation mode, respectively.

An inter-assay accuracy of 100–109% and precision of ≤8.7% was recorded over three validation runs. The recovery was near to complete (≥94.7%) and importantly, consistent over different concentration levels and plasma batches (CV%: ≤4.1%). The plasma matrix caused negligible ion suppression and endogenous interferences could be separated from the analytes. Psilocin and 4-HIAA plasma samples could be thawed and re-frozen for three cycles, kept at room temperature for 8 h or 1 month at −20 °C without showing degradation (≤10%). The linear range ($R \geq 0.998$) of the method covered plasma concentrations observed in humans following a common therapeutic oral dose of 25 mg psilocybin and was therefore able to assess the pharmacokinetics of psilocin and 4-HIAA. The LC-MS/MS method was convenient and reliable for measuring psilocin and 4-HIAA in plasma and will facilitate the clinical development of psilocybin.

1. Introduction

Psilocybin is a popular recreational substance found in several species of psychedelic mushrooms (*Psilocybe*) which cause "mind-altering" effects in humans [1,2]. Isolated in 1958 by Albert Hofmann, psilocybin's psychoactive effects are predominately mediated via 5-HT_{2A} receptors [3,4]. Recently, psilocybin has been repurposed and investigated for the treatment of cluster headache, obsessive compulsive disorder, anxiety and depression, and in alcohol use disorder [5–12].

Psilocybin is an indole alkaloid and structurally resembles the neurotransmitter serotonin [13,14] (Fig. 1). Once ingested, the prodrug psilocybin is rapidly metabolised by intestinal alkaline phosphates and nonspecific esterases to psilocin, which is responsible for psilocybin's psychoactive effects [2,4]. Subjective effects of psilocybin peak 2 h after oral administration and last for 6 h [9,13]. Consistently, psilocin reaches peak concentrations of 10–40 ng/ml in plasma 1.5–2 h after oral administration and is eliminated with a half-life of 2–3 h [14,15]. Importantly, psilocin undergoes glucuronidation by UDP-glucuronosyltransferases (UGT) 1A9 in the liver and UGT1A10 in the small intestine to psilocin-*O*-glucuronide, the major metabolite of psilocin considering that 80% is excreted from body in this form [16–18]. Alternatively, psilocin is deaminated and oxidized by liver aldehyde dehydrogenase and monoamine oxidase to 4-hydroxytryptophol (4-HTP) and 4-hydroxyindole-3-acetic acid (4-HIAA) [13,14,19].

With a rapidly growing interest of applying psilocybin as a potential therapeutic agent for various psychiatric disorders, it is essential to expand our knowledge of its clinical pharmacology. For instance, valid pharmacokinetic (PK) data in larger populations is needed and dose

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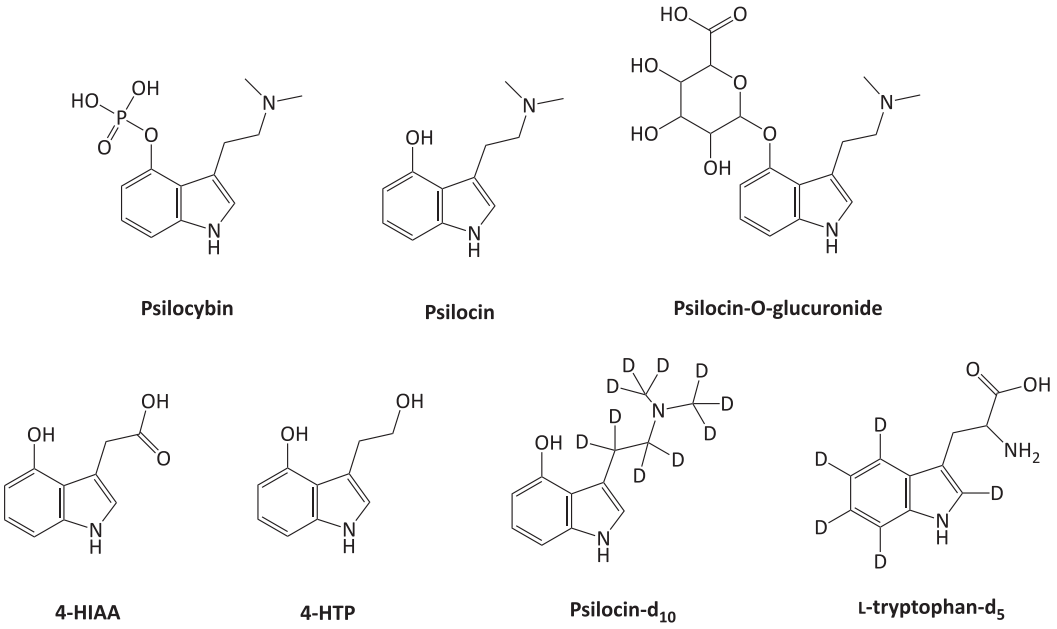

Fig. 1. Chemical structures of psilocybin, its metabolites, and internal standards. Oral psilocybin is rapidly hydrolysed to psilocin, which further undergoes a glucuronidation to psilocin-*O*-glucuronide, or is deamined and oxidized to 4-hydroxytryptophol (4-HTP) and 4-hydroxyindole-3-acetic acid (4-HIAA). Psilocin-d_{10} and L-tryptophan-d_5 were used as internal standards.

finding studies investigating the PK–pharmacodynamic (PD) relationship and drug-drug interaction studies are pending and require suitable bioanalytical methods.

Up-to-date, most methods have focused on quantification psilocybin's metabolites for the purpose of drug screening or preliminary pharmacokinetic studies involving limited sample size (Table 1). Quantification of psilocin in plasma or urine samples were achieved by processing large amounts of sample including work-intensive extraction procedures and selective analysis was warranted using lengthy chromatographic gradient programs. Over the past years, gas chromatography (GC) and high-pressure liquid chromatography (HPLC) methods were developed detecting psilocin by single or tandem mass spectrometry. Even though less sample volume was required, the methods made use of time-consuming liquid–liquid or solid-phase extractions and the run time was rarely below 10 min.

Herein, we present a thorough development and full validation of an LC-MS/MS method for the analysis of psilocin and 4-HIAA in human plasma. In contrast to other methods, we reduced the amount of sample used per analysis, simplified the sample extraction and shortened the run time. Furthermore, we demonstrate the clinical application of the method by assessing the PK of psilocin and 4-HIAA in three healthy participants in a clinical study.

2. Experimental section

2.1. Chemicals, reagents, and reference compounds

Psilocin was purchased from Lipomed (Arlesheim, Switzerland), psilocin-d_{10} (isotopic purity: 99.9%) and L-ascorbic acid (AA) from Sigma-Aldrich (St. Louis, USA) and L-tryptophan-d_5 (isotopic purity: 98.0%) from Toronto Research Chemicals (Toronto, Canada). Deuterium atoms on psilocin-d_{10} were located on the amine side chain, while for L-tryptophan-d_5, five deuterium atoms were on the indole moiety. 4-hydroxyindole-3-acetic acid (4-HIAA) and 4-hydroxytryptophole (4-HTP) were obtained from ReseaChem (Burgdorf, Switzerland). LC-MS grade water and methanol were purchased from Merck (Darmstadt, Germany). Formic acid and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich. Drug free human blood was obtained from the local blood donation center (Basel, Switzerland). Blood was collected in Lithium heparin coated S-Monovette® tubes (Sarstedt, Nümbrecht, Germany). Plasma for calibration and quality control (QC) samples was produced by centrifugation for 10 min at 1811 × *g* (Eppendorf Centrifuge 5810 R).

2.2. LC-MS/MS instrumentation and settings

The analytes were separated using a modular ultra-high performance liquid chromatography (UHPLC) system (Shimadzu, Kyoto, Japan) consisting of four pumps (A, B, C and D). The UHPLC system was connected to a 4000 QTRAP tandem mass spectrometer (AB Sciex, Ontario, Canada).

Psilocin and 4-HIAA were retained on a Symmetry C_{18} analytical column (3.5 µm, 100 Å, 4.6 × 75 mm, Waters, Massachusetts, USA), which was heated to 45 °C in the column oven. Water was used as mobile phase A and methanol as mobile phase B. Both mobile phases contained 0.1% formic acid. The injected sample (10 µl) was mixed before the analytical column in a T-union with mobile phase A delivered by pump C. The initial flow rate of pump C was 1.3 ml/min, which was turned off after 0.5 min of each run. Concurrently, pump A and B loaded the sample onto the analytical column using 10% mobile phase B and a flow rate of 0.3 ml/min. The flow rate was kept at 0.3 ml/min for the first 0.5 min of each run and afterwards set to 0.5 ml/min until the end of the run (0.5–4.5 min). In order to elute the analytes, mobile B concentration was linearly increased to 95% between 0.5 min and 3 min. Afterwards, the column was washed for 1 min at 95% mobile B and finally reconditioned for 0.5 min at 10% mobile B. Between each sample injection the autosampler port was washed with a washing solution composed of water-methanol–acetonitrile-isopropanol (1:1:1:1, v/v). The gradient program resulted in a retention time of 2.17 min for psilocin and psilocin-d_{10}, 2.81 min for L-tryptophan-d_5, and 3.36 min for 4-HIAA. Therefore, the UHPLC flow was connected with the tandem mass spectrometer only between 1.5 min and 3.8 min of the run and otherwise directed into the solvent waste.

In the first 2.5 min of the analytical run, electrospray ionisation in

Table 1
Overview of previously published bioanalytical methods that quantify psilocin, 4-HIAA or psilocin glucuronide in human plasma and urine.

Publication	Analytical method	Analytes quantified	Matrix examined	Sample processing	Sample volume	Quantification range	Run time	Reference
Hasler et al. (1997)	LC-ECD	Psilocin and 4-HIAA	Plasma	Microdialysis used to deproteinize the samples Freeze-drying used to concentrate the samples	3 ml	0.8–50 ng/ml (psilocin) 5–500 ng/ml (4-HIAA)	20 min	[14]
Lindenblatt et al. (1998)	LC-ECD	Psilocin	Plasma	(1) Liquid-liquid extraction and samples evaporated using nitrogen (2) On-line solid-phase extraction	(1) 2 ml (2) 0.4 ml	(1) 200–5800 pg (2) 250–5250 pg	(1) ~15 min (2) 30 min	[19]
Sticht et al. (2000)	GS-MS	Psilocin	Serum Urine	Solid-phase extraction Samples evaporated using nitrogen and derivatization	0.5 ml	10–100 ng/ml	~5 min	[27]
Grieshaber et al. (2001)	GC–MS	Psilocin	Urine	Solid-phase extraction Samples evaporated using nitrogen and derivatization	5 ml	10–200 ng/ml	~11 min	[16]
Hasler et al. (2002)	LC-ECD	Psilocin and psilocin glucuronide	Urine	Freeze-drying to concentrate the samples, methanol extraction and membrane filtration to deproteinize the samples	6 ml	10–1000 ng/ml	20 min	[18]
Kamata et al. (2003)	LC/MS and -MS/MS	Psilocin and psilocin glucuronide	Urine	Methanol used to deproteinize urine samples Samples evaporated using nitrogen	N/A	500–5000 ng/ml	10 min	[24]
Kamata et al. (2006)	LC/MS and -MS/MS	Psilocin and psilocin glucuronide	Serum	Liquid-liquid extraction with chloroform or deproteinization with methanol Samples evaporated using nitrogen	0.1 ml	1–100 ng/ml	10 min	[21]
del Mar Ramierez Fernandez et al. (2007)	LC-MS/MS	i.a. Psilocin	Urine	Solid-phase extraction Samples evaporated using nitrogen	0.5 ml	10–500 ng/ml	22 min	[25]
Bjornstad et al. (2009)	LC-MS/MS	i.a. Psilocin	Urine	Samples diluted with water (1:4, v/v)	0.05 ml	5–1000 ng/ml	14 min	[26]
Martin et al. (2012)	LC-MS/MS	Psilocin	Plasma	Solid-phase extraction Samples evaporated using nitrogen	0.5 ml	0.34–200 ng/ml	21 min	[23]
Brown et al. (2017)	LC-MS/MS	Psilocin	Serum	Methanol: acetonitril (1:1, v/v) used to deproteinize the samples Samples evaporated using nitrogen	N/A	0.5 ng/ml (serum) 5.0 ng/ml (urine)	N/A	[15]

(*continued on next page*)

Table 1 (*continued*)

Publication	Analytical method	Analytes quantified	Matrix examined	Sample processing	Sample volume	Quantification range	Run time	Reference
Current method	LC-MS/MS	Psilocin, 4-HIAA, and psilocin glucuronide	Plasma	Methanol used to deproteinize samples	0.05 ml	0.25–100 ng/ml (psilocin) 2.5–1000 ng/m (4-HIAA)	4.5 min	

LC, high pressure liquid chromatography; ECD, electrochemical detection; MS, mass spectrometry; GC, gas chromatography; i.a, among other analytes analysed.

the positive polarity mode was used to detect psilocin and psilocin-d_{10}. Afterwards, 4-HIAA and L-tryptophan-d_5 were ionised in the negative mode (Table 2 and Fig. 2). The analytes were detected by multiple reaction monitoring (MRM) by the following mass transitions (Q1 → Q3): psilocin; *m/z* 205.2 → 58.1, psilocin-d_{10}; *m/z* 215.2 → 66.0, 4-HIAA; *m/z* 189.9 → 130.9, and L-tryphtophan-d_5; *m/z* 208.0 → 120.0. Nitrogen was employed as ion source (gas 1; 60 l/min, gas 2; 50 l/min), curtain (10 l/min) and collision gas (4 l/min). The ion spray voltage was set at +5500 V and −4500 V in the positive and negative mode, respectively. The source temperature was 500 °C.

The LC-MS/MS system was operated with Analyst software 1.7 (AB Sciex) and data were analysed with MultiQuant software 3.0.3 (AB Sciex).

2.3. Calibration and quality control samples preparation

Psilocin and 4-HIAA were weighed in duplicate in order to obtain two separate stock solutions, one for calibration samples and the other for QC sample preparations. The analytes were dissolved in DMSO containing 0.1 M ascorbic acid (DMSO-AA) to obtain a final concentration of 10 mg/ml. A calibration and QC working solution mixture of 20 µg/ml psilocin and 200 µg/ml 4-HIAA was prepared and serially diluted in DMSO-AA up to 0.025 µg/ml and 0.25 µg/ml, respectively. Calibration and QC working solutions were mixed with blank human plasma (1:100, v/v). Calibration samples covered a range from 0.25 to 100 ng/mL for psilocin and 2.5–1000 ng/mL for 4-HIAA.

The QC samples were prepared at the lower limit of quantification (LLOQ), low concentration (QC_{LOW}), mid concentration (QC_{MID}), and high concentration (QC_{HIGH}) level corresponding to a plasma concentration of 0.25, 0.5, 10, and 50 ng/ml for psilocin and 2.5, 5.0, 100, and 500 ng/ml for 4-HIAA. All solutions were stored in light protected tubes at −20 °C.

The internal standards (IS) psilocin-d_{10} and L-tryptophan-d_5 were prepared in DMSO-AA at a final concentration of 10 mg/ml. An IS working solution containing 10 ng/ml psilocin-d_{10} and 1000 ng/ml L-tryptophan-d_5 was made in methanol and stored at −20 °C.

Table 2
The mass transitions and mass spectrometry parameters of the investigated analytes and internal standards.

Analyte	MRM (*m/z*) (Q1 → Q3)	DP (V)	EP (V)	CE (V)	CXP (V)
Psilocin	205.2 → 58.1	36	10	31	10
Psilocin-d_{10}	215.2 → 66.0	36	10	25	12
4-HIAA	189.9 → 130.9	−40	−10	−34	−19
L-tryptophan-d_5	208.0 → 120.0	−80	−10	−26	−17

MRM, Multiple reaction monitoring; *m/z*, mass to charge ratio; DP, Declustering potential; EP, Entrance potential; CE, Collision energy; CXP, Collision cell exit potential; V, voltage; 4-HIAA, 4-hydroxy-indole-3-acetic acid.

2.4. Sample extraction

Aliquots of 50 µl plasma were pipetted into 96-well autosampler plates (Matrix blank storage tubes, Thermo Fischer, Massachusetts, USA) and supplemented with 5 µl of 0.1 M ascorbic acid. Next, the samples were mixed with 150 µl IS working solution and vortexed for 30 sec. The extracts were then centrifuged for 30 min at 10 °C and 3220 × *g* to receive a clear and protein free supernatant. The samples were placed inside the autosampler at 10 °C, where 10 µl of the supernatant was injected into the LC-MS/MS system.

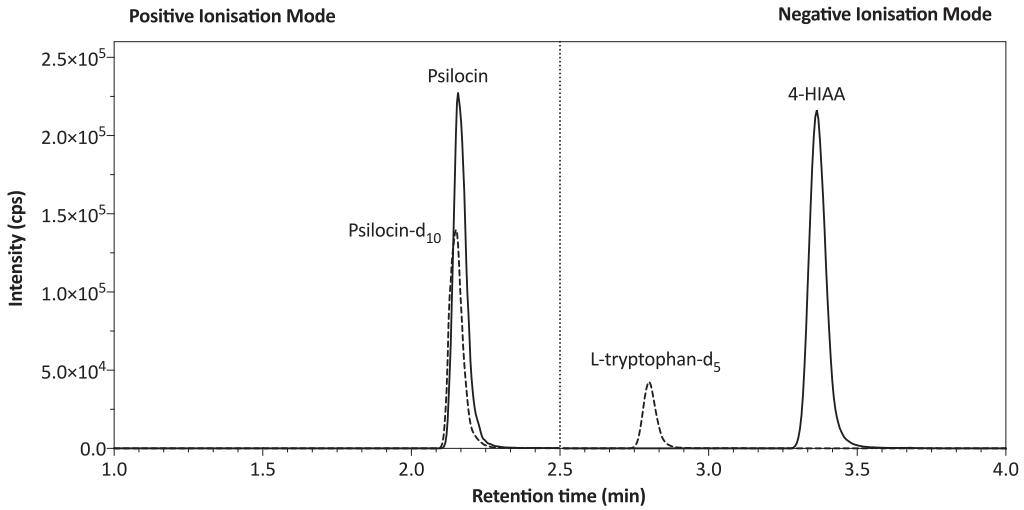

Fig. 2. The chromatographic separation of psilocin and 4-HIAA and their respective internal standards, psilocin-d_{10} and L-tryptophan-d_5, in human plasma. Psilocin (100 ng/ml, black line) and psilocin-d_{10} (10 ng/ml, dotted line) were both detected after 2.16 min in positive ionisation mode. The polarity mode was switched after 2.5 min from positive to negative electrospray ionisation in order to detect 4-HIAA (1000 ng/ml, black line) which eluted after 3.36 min. The retention time of L-tryptophan-d_5 (1000 ng/ml, dotted line), the internal standard of 4-HIAA, was at 2.81 min.

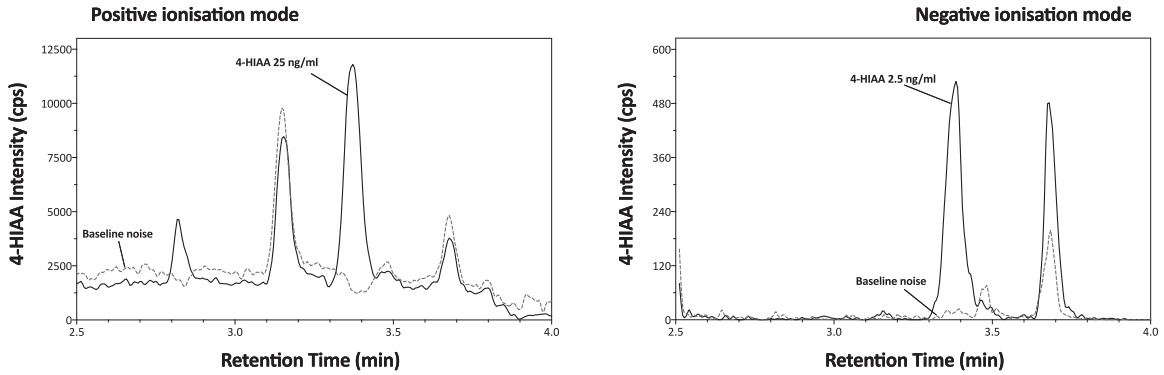

Fig. 3. 4-HIAA background noise of blank plasma recorded in positive and negative ionisation mode. A chromatogram of a plasma sample containing either 25 ng/ml (positive mode) or 2.5 ng/ml (negative mode) 4-HIAA was overlaid with a blank plasma chromatogram. In the positive ionisation mode, 4-HIAA was detected by the mass transition m/z 192.1 → 146.0. A pronounced background noise of approximately 2500 counts per seconds (cps) was observed in blank plasma samples, which interfered with the 4-HIAA signal. The mass transition m/z 189.9 → 130.9 was employed for 4-HIAA in the negative mode, resulting in a negligible background noise of < 100 cps in blank plasma.

2.5. Method validation

The analytical method was validated according to the guidelines on bioanalytical method validation of the European Medicines Agency (EMA) in terms of method linearity, accuracy and precision, selectivity and sensitivity, matrix effect and extraction recovery, and analyte stability [20].

2.5.1. Linearity

Each calibration line consisted of two sets of a blank, a double blank, and eight calibration samples. The double blank sample was extracted with pure methanol and the other samples with IS solution. Calibration samples were analysed by increasing analyte concentration, whereas the double blank sample was injected after the upper limit of quantification (ULOQ) sample to determine the analyte carry-over between the analytical runs. Analyte carry-over was calculated by comparing the peak areas of the double blank samples injected after the ULOQ samples to the peak area of the LLOQ samples (%). Carry-over below 10% (% relative to LLOQ sample) was classified as negligible.

Calibration lines were established by linear regression (weighting $1/x^2$) of the nominal analyte concentration (x) against the analyte to IS peak area (y). Psilocin-d_{10} was used as IS for psilocin, whereas L-tryptophan-d_5 was the IS of 4-HIAA. The relationship had to result in a correlation coefficient of >0.99 (R). Calibration samples with an accuracy outside of 85–115% (LLOQ: 80–120%) were excluded. However, the calibration line had to contain at least 14 determinations (>75%) including one LLOQ and one ULOQ sample.

2.5.2. Intra- and inter-assay accuracy and precision

The intra- and inter-assay accuracy and precision were examined by conducting three independent validation runs on three separate days. Each validation run consisted of two sets of calibration lines measured at the beginning and end of the assay. In-between, seven replicates of four QC levels (LLOQ, QC_{LOW}, QC_{MID}, and QC_{HIGH}) were measured. The accuracy and precision of the method was evaluated by analysing the replicas of a single run (intra-assay, n = 7) and of all three runs (inter-assay, n = 21).

The precision was determined per QC level by calculating the coefficient of variation, CV (%), which was the ratio of the standard deviation to the mean concentration. A precision of ≤15% (LLOQ: ≤20%) was acceptable. Accuracy was calculated using the following formula: $Accuracy\,\% = \frac{C_O}{C_N} \times 100$, where C_N was the nominal and C_O was the observed analyte concentration, respectively. The QC sample concentration (C_O) was calculated based on the linear equation of the two calibration sets. The mean accuracy had to be between 85 and 115% (bias < 15%) and in case of LLOQ samples between 80 and 120% (bias < 20%). At least 67% of all QC samples at each concentration level had to fall within this range (intra-assay: 5 out of 7, inter-assay: 15 out of 21).

2.5.3. Selectivity and sensitivity

The selectivity of the method was examined by analysing blank samples from seven different subjects. These samples were processed with and without IS to determine if interference was caused by components of the plasma matrix or the IS itself, respectively. Furthermore, each blank sample was spiked at the LLOQ concentration (psilocin: 0.25 ng/ml or 4-HIAA: 2.5 ng/ml) to evaluate the sensitivity of the method. The method was considered to be selective if the LLOQ signal intensity was at least five times higher than the background noise of blank plasma. To validate the method sensitivity, the LLOQ samples of seven different batches of plasma had to display a precision of ≤20% and a mean accuracy of 80–120%, where at least 67% of the samples had to lie within these limits.

2.5.4. Extraction recovery and matrix effect

The extraction recovery and matrix effect were investigated for seven different plasma batches at the LLOQ, QC_{LOW}, QC_{MID}, and QC_{HIGH} concentration levels.

The extraction recovery was estimated by spiking blank plasma (before extraction) and blank plasma supernatants (after extraction) using equal amounts of analyte. The peak area found in the spiked supernatant corresponded to 100% recovery. The extraction recovery was determined using the following formula: $Recovery = \frac{Analyte\ peak\ area\ before\ extraction}{Analyte\ peak\ area\ after\ extraction} \times 100$.

The matrix effect was determined by comparing the analyte peak area in samples with and without matrix, according to the following formula: $Matrix\ effect\ \% = \frac{Analyte\ peak\ area\ with\ matrix}{Analyte\ peak\ area\ without\ matrix} \times 100$. Therefore, pure water (without matrix) and plasma sample extracts (with matrix) were spiked with equal amounts of analyte. The CV (%) of the calculated recovery or matrix effect was determined for different plasma batches (n = 7) and for each QC level (n = 4). Overall, the recovery and matrix effect had to be consistent with a CV (%) of less than 15%.

2.5.5. Stability

The stability of psilocin and 4-HIAA in plasma were investigated under different storage conditions. Seven replicates of LLOQ, QC_{LOW}, QC_{MID}, and QC_{HIGH} samples were stored for 8 h at room temperature (bench-top stability) and for 1 month at −20 °C (one month stability).

Table 3
The intraday and interday accuracy and precision calculated for psilocin and 4-HIAA in human plasma.

Analyte	$C_{nominal}$ (ng/ml)	$C_{Ø}$ Assay 1 (ng/ml)	Accuracy ± CV (%)	$C_{Ø}$ Assay 2 (ng/ml)	Accuracy ± CV (%)	$C_{Ø}$ Assay 3 (ng/ml)	Accuracy ± CV (%)	$C_{Ø}$ Assay 1–3 (ng/ml)	Accuracy ± CV (%)
Psilocin	0.250	0.241	96.3 ± 7.7	0.249	100 ± 5.5	0.269	108 ± 9.1	0.253	101 ± 8.7
	0.500	0.502	100 ± 4.6	0.513	103 ± 3.5	0.516	103 ± 8.5	0.511	102 ± 5.8
	10.0	10.6	106 ± 3.4	10.9	109 ± 3.4	10.6	106 ± 3.7	10.7	107 ± 3.6
	50.0	51.5	103 ± 2.3	51.8	104 ± 2.3	48.9	97.9 ± 3.9	50.8	102 ± 3.8
4-HIAA	2.5	2.68	107 ± 5.9	2.80	112 ± 3.7	2.73	109 ± 4.4	2.74	109 ± 4.9
	5.0	5.05	101 ± 2.8	5.50	110 ± 4.1	5.02	101 ± 6.5	5.19	104 ± 6.2
	100	106	106 ± 2.8	107	107 ± 4.4	104	104 ± 3.2	106	106 ± 3.5
	500	498	99.6 ± 3.5	509	102 ± 1.7	487	97.5 ± 3.2	498	100 ± 3.3

$C_{nominal}$, theoretical concentration; $C_{Ø}$, average concentration of seven samples; CV (%), Coefficient of variance; 4-HIAA, 4-hydroxy-indole-3-acetic acid.

Moreover, the stability was assessed after three consecutive freeze and thaw cycles (freeze/thaw stability), thereby freezing the QC samples at −20 °C for at least 24 h and thawing them afterwards at room temperature. The concentration of those stability test samples was calculated based on a freshly prepared calibration line. Samples were designated to be stable if the accuracy was between 85 and 115% (LLOQ: 80–120%) and the precision ≤15% (LLOQ: ≤20%).

2.6. Method application

To examine the application of the developed method, psilocin and 4-HIAA concentrations were quantified in plasma samples of three healthy volunteers receiving a single oral dose of 25 mg psilocybin. This is a moderate to high dose currently used in clinical phase 2–3 studies. The study was approved by the Ethical Committee of Northwestern and Central Switzerland (EKNZ, BASEC ID: 2019-00223), registered at ClinicalTrials.gov (ID: NCT03912974), and conducted in accordance with the Declaration of Helsinki and International Conference on Harmonization Guidelines in Good Clinical Practice. All volunteers provided written informed consent prior to study participation.

To establish concentration time profiles, blood samples were collected in lithium heparin coated tubes at the following time points: 2 h before and 0, 15, 30, 45, 60, 90, 120, 150, 180, 240, 300, 360, and 420 min after treatment. Blood samples were centrifuged for 10 min at 1811*g* to obtain plasma, which was transferred into cryotubes. All samples were stored −80 °C until analysis.

Study calibration and QC samples were processed as described before. In addition, the total amount of glucuronide conjugated psilocin and 4-HIAA was determined according to the protocol of Kamata et al. [21]. In brief, 5 µl of *Escherichia coli* β-glucuronidase (3000 units/ml in water) was mixed with 50 µl plasma sample. An aliquot of 100 µl acetate buffer (0.1 M, pH 5.0) was added to the mixture. The samples were incubated for 3 h at 37 °C in a thermomixer (Eppendorf, Hamburg, Germany). Enzymatic reaction was terminated and samples extracted by the addition of 150 µl IS. The samples were vortexed and centrifuged as outlined above. In order to determine the levels of the conjugated analytes, the plasma samples were analysed twice. First in the absence and afterwards in the presence of β-glucuronidase. The difference between the two measurements accounted for the concentration of the conjugated analyte.

For each analytical run, a calibration line was analysed at the beginning and at the end of the measurements. In between, the study samples of the three volunteers were measured as well as triplicates of LLOQ, QC_{LOW}, QC_{MID}, and QC_{HIGH} samples. Samples with a concentration below the LLOQ were marked as blq (below limit of quantification) and samples with concentrations above the ULOQ were diluted with blank plasma into the calibration range.

The concentration–time profile was plotted and the maximal plasma concentration (C_{max}) and time to reach it (T_{max}) were obtained graphically from the plots. Pharmacokinetic parameters were calculated using non-compartmental methods in Phoenix WinNonlin 8.3 (Certara, New Jersey, USA). The area under the plasma concentration time profile was calculated by using the linear trapezoidal rule from 0 to 420 min (AUC_{LAST}). The elimination half-life ($t_{1/2}$) was calculated by the equation $t_{1/2} = \frac{0.693}{\lambda}$, where the elimination rate constant (λ) was the slope of log (C_t) versus t determined in the terminal elimination phase.

3. Results and discussion

3.1. Method development

Currently over 40 clinical studies are investigating the pharmacological properties of psilocybin [22]. Psilocybin research was greatly halted when it became a scheduled substance in 1968. Consequently, drug metabolism and pharmacokinetic (DMPK) studies are scarce, which accordingly demands reliable bioanalytical methods to quantify drugs and their metabolites in plasma. For this purpose, we developed and fully validated an LC-MS/MS method, prioritising on a simple and fast sample analysis work-flow. We focused on psilocin, as it is psychoactive, and on its main metabolite 4-HIAA. Furthermore, we quantified to what extent these two metabolites are glucuronidated.

First, the ionisation and fragmentation parameters of psilocin, psilocin-d_{10}, 4-HIAA, 4-HTP, and L-tryptophan-d_5 were optimized by infusing the analytes into the mass spectrometer (Table 2). Positive and negative polarity ionisation were tested for 4-HIAA considering that it possesses an amine and carboxylic acid functional group, whereas psilocin and 4-HTP were tuned only in the positive mode. A screening of the most abundant fragments was performed to allow quantification by multiple reaction monitoring. As illustrated in Supplementary Fig. 1, psilocin (*m/z* 205.2) broke down most abundantly to the fragments *m/z* 58.1 and 160.0, while psilocin-d_{10} fragmented into *m/z* 66.0 and 164.0 retaining eight and four deuterium atoms, respectively. Importantly, both fragments were also reported and used as quantifier ions by others [21,23–26]. 4-HIAA and 4-HTP were to our best knowledge not yet detected by tandem mass spectrometry. Fragment *m/z* 146.0 and 130.9 were most abundant for 4-HIAA in the positive and negative mode, respectively. 4-HTP fragmented predominantly into *m/z* 160.1 as observed for psilocin. The validation was initially launched by ionising all analytes positively to avoid polarity switching. However, the employed mass transition of 4-HIAA (*m/z* 192.1 → 146) resulted in pronounced interferences with endogenous plasma components, which were difficult to separate from the analyte signal. In the negative mode, however, the baseline noise was negligible for 4-HIAA compared to positive mode and persistent interferences (e.g. at retention time 3.7 min) could be separated from 4-HIAA signal (Fig. 3). Thus, polarity switching was unavoidable and L-tryptophan-d_5 was incorporated into the method as IS of 4-HIAA.

Next, the chromatography of the analytes was optimized in order to concentrate and separate them on the analytical column. A large variety of columns were screened showing that pentafluorophenyl (PFP) and

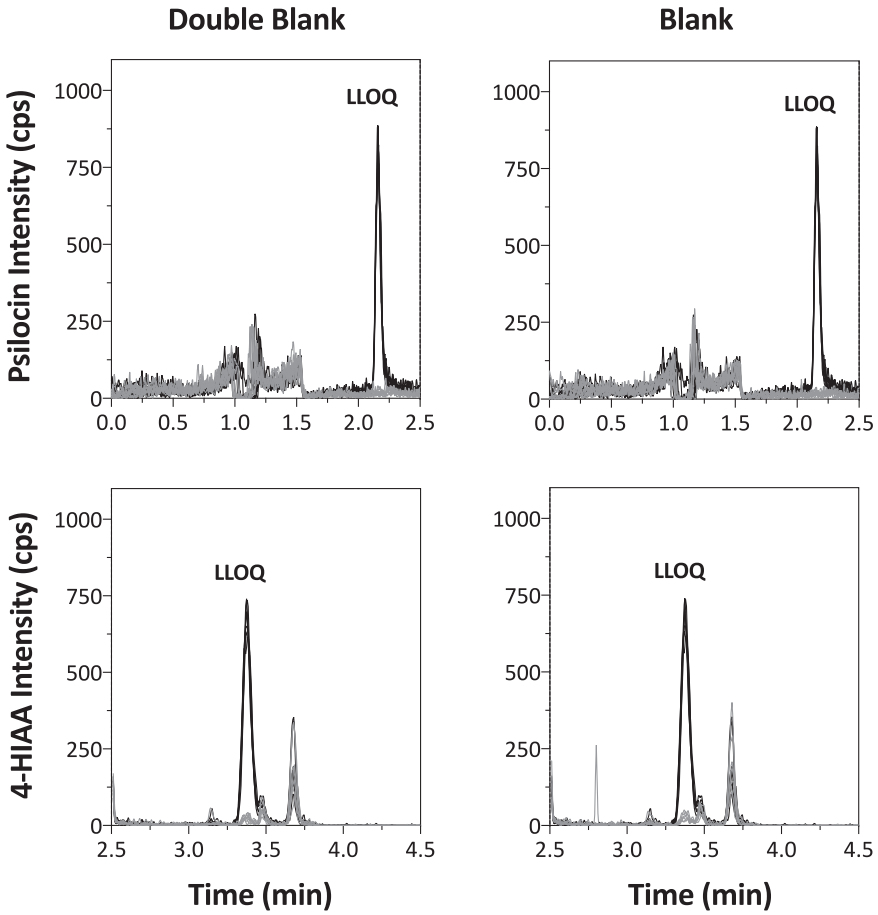

Fig. 4. The selectivity of psilocin and 4-HIAA in blank plasma from seven different individuals. Seven double blank, blank and lower limit of quantification (LLOQ) samples (psilocin: 0.25 ng/mL, 4-HIAA: 2.5 ng/ml) were prepared using different batches of plasma. The LLOQ chromatograms (black lines) were overlaid with double blank (left) and blank (right) chromatograms (grey lines). Top two panels correspond to psilocin while the bottom panels relate to 4-HIAA. The background noise determined in double blank samples did not interfere with the detection of psilocin or 4-HIAA as it accounted only for ≤4.1% and ≤5.5%, respectively of the observed LLOQ peak area. In addition, the internal standards, psilocin-d_{10} and L-tryptophan-d_5, did not affect the selectivity of the analysis regarding the baseline noise recorded for blank samples.

biphenyl phases were able to retain the relatively polar and aromatic analytes. In addition, good analyte retention and symmetric peak shapes was achieved with C_{18} columns, which facilitated analyte interaction with the alkyl ligand but also with the polar silanol surface functionalities (e.g. Symmetry C_{18}). Using methanol and acetonitrile as mobile phase B resulted in similar analyte peak intensities, whereas the acetonitrile eluted the analytes faster. Several MS compatible modifiers (formic and acetic acid, or ammonium formate, acetate and fluoride) were investigated. The addition of ammonium fluoride to both mobile phases enlarged the linear range of the method for biphenyl phase columns. However, the additive reduced also the durability of some columns (e.g. Symmetry C_{18}), as a result formic acid, a standard MS compatible modifier, was used instead. 4-HIAA eluted considerably later than psilocin, though at the same time with 4-HTP making polarity switching difficult. Lastly, 4-HTP was not included in the method, because it was not detectable at a limit of detection of 2.5 ng/ml in plasma of volunteers who received 25 mg psilocybin. Thus, 4-HTP is a minor metabolite of psilocin in humans.

Finally, different plasma protein precipitation solvents were investigated for a simple sample extraction. Methanol, acetonitrile, and ethanol yielded comparable signal intensities. However, the peak shape of the analytes was poor because the injected sample consisted of mainly organic solvent. Evaporating the extracts and resuspending the residuals in mobile phase A solved the problem. In addition, protein precipitation with perchloric acid (1 M) was evaluated because it could be expected that the hydrophilic analytes are efficiently extracted by the aqueous character of the extraction solvent. Moreover, the extract was compatible with the initial condition of the gradient program, which was composed of a high percentage of water. Indeed, the extraction with perchloric acid was promising, however the supernatant of the extract had still to be transferred into another tube to neutralize the pH to prevent the column from damage.

Table 4
The recovery and matrix effect of psilocin and 4-HIAA determined in human plasma.

Analyte	$C_{nominal}$ (ng/ml)	RRE ± CV (%)	Mean ± CV (%)	ME ± CV (%)	Mean ± CV (%)
Psilocin	0.25	94.1 ± 5.9	**96.5 ± 2.2**	129 ± 7.8	**114 ± 13**
	0.5	95.9 ± 4.5		96.2 ± 7.4	
	10	99.2 ± 5.4		106 ± 5.8	
	50	96.6 ± 7.2		122 ± 5.9	
4-HIAA	2.5	89.5 ± 8.2	**94.7 ± 4.1**	78.6 ± 3.0	**69.5 ± 7.8**
	5.0	95.0 ± 9.9		62.7 ± 6.3	
	100	98.9 ± 9.3		67.3 ± 6.1	
	500	95.3 ± 10		69.8 ± 6.8	

$C_{nominal}$, theoretical concentration; RRE, relative recovery; ME, matrix effect; CV (%), Coefficient of variance; 4-HIAA, 4-hydroxy-indole-3-acetic acid.

Finally, we extracted plasma samples with methanol, centrifuged plasma proteins to the bottom of the tubes, and injected an aliquot of the supernatant into the LC-MS/MS system. Sharp and symmetric peaks were obtained by extensively mixing the injected sample with water within a T-union, which was installed in front of the analytical column. Without this procedure, the peak shape of the analytes would have been compromised leading to peak broadening and tailing. This semi-

Table 5
The stability of psilocin and 4-HIAA in human plasma were determined following three freeze–thaw cycles (freeze/thaw), storage at room temperature for 8 h (bench-top), and one month at −20 °C.

Storage stability	$C_{nominal}$ (ng/ml)	Psilocin		$C_{nominal}$ (ng/ml)	4-HIAA	
		$C_{Ø}$ (ng/ml)	Accuracy ± CV (%)		$C_{Ø}$ (ng/ml)	Accuracy ± CV (%)
Freeze/thaw	0.25	0.269	108 ± 8.2	2.5	2.72	109 ± 2.8
	0.50	0.533	107 ± 3.5	5.0	5.00	100 ± 5.4
	10	10.8	108 ± 4.5	100	106	106 ± 5.3
	50	52.6	105 ± 3.3	500	524	105 ± 2.1
Bench-top	0.25	0.258	103 ± 7.1	2.5	2.45	98.2 ± 4.9
	0.50	0.534	107 ± 3.6	5.0	4.74	94.8 ± 4.3
	10	10.7	107 ± 3.5	100	110	110 ± 3.3
	50	49.0	98.1 ± 3.6	500	481	96.1 ± 1.8
One month at −20 °C	0.25	0.259	104 ± 5.9	2.5	2.69	108 ± 6.6
	0.50	0.542	108 ± 3.9	5.0	5.16	103 ± 5.6
	10	10.6	106 ± 4.0	100	107	107 ± 3.7
	50	49.4	98.7 ± 4.2	500	487	97.5 ± 5.6

$C_{nominal}$, theoretical concentration; $C_{Ø}$, average concentration of seven samples; CV (%), Coefficient of variance; 4-HIAA, 4-hydroxy-indole-3-acetic acid.

automated workflow allowed to extract and analyse the plasma samples in single tubes or 96 well plate format and facilitates the analysis of large amounts of samples.

3.2. Method validation

3.2.1. Method linearity, accuracy, and precision

Three validation runs were performed including four QC levels (LLOQ, QC_{LOW}, QC_{MID}, and QC_{HIGH}) with seven replicates and two calibration lines per run. In total, 54 calibration and 84 QC samples were analysed per analyte.

The method was linear over a range of 0.25–100 ng/ml for psilocin and 2.5–1000 ng/ml for 4-HIAA with a correlation coefficient of >0.998. All 4-HIAA calibrators passed the inclusion criteria, whereas only one psilocin calibrator exhibited an accuracy bias of more than 15%. The calibration range chosen for both analytes was suitable to quantify clinical samples. It encompassed concentrations that were approximately five times above the expected maximal plasma concentrations but also low concentration samples observed during early drug absorption and in late elimination phase [14,15,19].

The intra-assay precision of psilocin was ≤9.1% and of 4-HIAA ≤6.5%, while the inter-assay precision was ≤8.7% (Table 3). Furthermore, the mean intra-assay accuracy observed for psilocin was between 96.3 and 109% and for 4-HIAA between 97.5 and 109%, whereas the inter-assay accuracy bias was ≤9.0%. None of the psilocin QC samples were outside 85–115% accuracy (LLOQ: 80–120%) and only two out of 84 QC samples did not pass the acceptance criteria in case of 4-HIAA. The observed mean psilocin carry-over was 0.95% (SD: 0.42%) while for 4-HIAA the carry-over was 1.37% (SD: 0.68%). The analyte carry-over was below 10%, indicating that carry-over was negligible between the analytical runs.

Overall, the analytical method was reliable to analyse both analytes in human plasma samples.

3.2.2. Selectivity and sensitivity

The selectivity and sensitivity of psilocin and 4-HIAA were assessed by comparing the LLOQ signal intensity of seven different batches of plasma to the respective baseline signal in blank and double blank samples. As shown in Fig. 4, endogenous plasma components did not interfere with detection of psilocin and 4-HIAA. More precisely, psilocin and 4-HIAA background noise accounted for ≤4.1% and ≤5.5% of the LLOQ peak area, respectively (Supplementary Table S1 and S2).

Blank plasma samples of seven different donors were spiked at the LLOQ level to evaluate if the analytes can be reliably quantified irrespective of the employed plasma source. A mean accuracy of 102% (95.3–110%) and 84.7% (82.6–87.1%) was determined for psilocin and 4-HIAA, respectively. None of the LLOQ samples were outside 80–120% accuracy and the precision was ≤4.9% for all seven batches of plasma.

These findings show that the method is selective for the quantification of psilocin and 4-HIAA in human plasma and that the plasma matrix does not affect the sensitivity of the analysis.

3.2.3. Recovery and matrix effect

The recovery and matrix effect of psilocin and 4-HIAA were examined after deproteinization of seven different plasma batches including four QC concentration levels (Table 4).

The protein precipitation extraction was almost complete yielding a mean recovery of 96.5% for psilocin and 94.7% for 4-HIAA. Importantly, the bias between different plasma batches was smaller than 10.1% and consistent over all QC levels (CV ≤ 4.1%).

The psilocin signal in plasma extracts was on average 14% larger than in pure water. In contrast, the 4-HIAA signal was suppressed by the plasma matrix by approximately 30%. Importantly, the seven plasma batches resulted in very similar matrix effect (CV ≤ 7.8%), which were independent from the utilized analyte concentration (CV ≤ 13%).

In summary, the employed extraction method recovered almost all psilocin and 4-HIAA from plasma and resulted in consistent and negligible matrix effects.

3.2.4. Stability

The stability of psilocin and 4-HIAA were examined after three freeze and thaw cycles as well as after 8 h storage at room temperature and one month at −20 °C (Table 5).

Three repetitive freeze and thaw cycles did not decrease the stability of the analytes, because the accuracy of the QC samples was between 105 and 108% (CV ≤ 8.2%) for psilocin and between 100 and 109% (CV ≤ 5.4%) for 4-HIAA. Moreover, plasma samples that were stored for 8 h at room temperature or for one month at −20 °C contained similar amounts of psilocin and 4-HIAA in comparison to fresh samples (accuracy: 94.8–110%, CV ≤ 7.1%).

These results indicate that psilocin and 4-HIAA are stable under various conditions encountered in a laboratory, in support of previous studies evaluating the short term and freeze/thaw stability of psilocin [23].

3.2.5. Clinical application

The application of the method was assessed by analysing the PK of psilocin and 4-HIAA in three healthy volunteers treated with an oral dose of 25 mg psilocybin (Fig. 5 and Table 6).

The maximal plasma level of psilocin and 4-HIAA was on average 19.2 ng/ml (SD: 4.0 ng/ml) and 137.3 ng/ml (SD: 22.0 ng/ml), respectively. Psilocin and 4-HIAA reached T_{max} approximately after

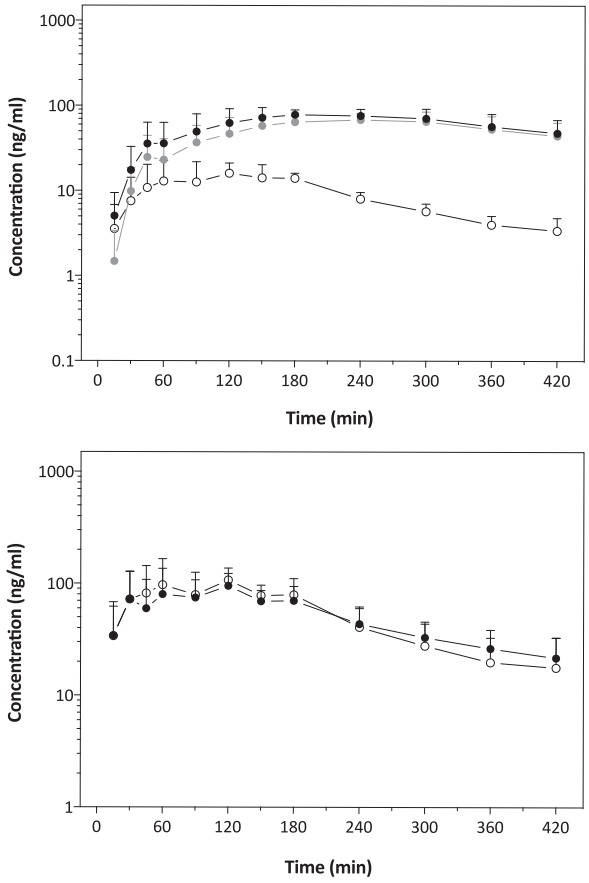

Fig. 5. Pharmacokinetic profile of psilocin, psilocin glucuronide, and 4-HIAA determined in human plasma. An oral dose of 25 mg psilocybin was administered to three healthy volunteers. Plasma concentrations of psilocin and 4-HIAA were quantified before- and up to seven hours post-treatment. All samples were reanalysed after deglucuronidation with *Escherichia coli* β-glucuronidase. The top panel shows the concentration–time profile of psilocin, while the bottom panel depicts the profile of 4-HIAA. White symbols correspond to unconjugated psilocin and 4-HIAA. The total amount of the conjugated and unconjugated metabolites is illustrated in black. The grey symbols show the difference between samples that were incubated with and without glucuronidase corresponding to the total amount of conjugated metabolites. A large proportion of psilocin underwent glucuronidation, whereas 4-HIAA was not conjugated. Mean values and the standard error of the mean are illustrated.

Table 6
The pharmacokinetic parameters of psilocin, psilocin glucuronide, and 4-HIAA of three healthy volunteers treated with an oral dose of 25 mg psilocybin.

Analyte	$C_{max} \pm SD$ (ng * ml^{-1})	$T_{max} \pm SD$ (min)	AUC_{last} (ng * min * ml^{-1})	$t_{1/2}$ (min)
Psilocin	19.2 ± 4.0	140 ± 46	3670 ± 780	127 ± 18
Psilocin glucuronide	78.3 ± 7.9	220 ± 92	20631 ± 552	215 ± 72
4-HIAA	137 ± 22	120 ± 60	22330 ± 992	139 ± 63

4-HIAA, 4-hydroxy-indole-3-acetic acid; C_{max}, maximal plasma concentration; T_{max}, time to reach maximal plasma concentration; AUC_{last}, area under the plasma concentration time curve until 7 h post treatment; $t_{1/2}$, drug half-life.

120–140 min post-treatment. The $t_{1/2}$ of psilocin and 4-HIAA were estimated to be 127 min (SD: 18 min) and 139 min (SD: 63 min), respectively. Overall, the amount of 4-HIAA as depicted by the AUC_{LAST} was about 5 times larger than that of psilocin, which is in line with observations made by Hasler and colleagues [14].

In contrast to 4-HIAA, psilocin underwent extensive O-glucuronidation. The psilocin glucuronide reached on average a C_{max} of 78.3 ng/ml (SD: 7.9 ng/ml) after roughly 220 min. The calculated AUC_{LAST} of psilocin glucuronide was 20631 ng·min·ml^{-1} (SD: 552 ng·min·ml^{-1}) and thus 5–6 fold higher compared to the AUC_{LAST} of psilocin. This result is in agreement with previous studies, which reported that the majority of psilocin is conjugated by glucuronidation [16,21,27].

Importantly, the accuracy of the QC samples was between 93.6 and 113% and the precision ≤8.1% showing that the analytical run passed the acceptance criteria. Moreover, psilocin and 4-HIAA could always be quantified within the sampling period, as the observed concentrations were between 0.36–94.1 ng/ml for psilocin and 7.2–156.7 ng/ml for 4-HIAA. The herein presented method is therefore suitable for quantification of the clinical samples.

4. Conclusion

Compared to other bioanalytical methods that measure psilocybin in human plasma, the current method is at least 8-times more sensitive, uses small amounts of sample, and includes a short run time. Furthermore, an uncomplicated extraction protocol was developed including online sample dilution, which enabled a semi-automated workflow to extract and analyse samples in 96-well plate format. The extraction protocol resulted in an almost complete analyte recovery (≥94.1%). Consistent matrix effects were observed among various plasma batches (CV ≤ 7.8%). Moreover the matrix did not interfere with the analysis of psilocin or 4-HIAA. The quantification of both analytes was accurate (bias: ≤12%) and precise (CV = 9.1%) within the chosen calibration range and compatible with observed levels in humans dosed with psilocybin. The developed method was able to determine the pharmacokinetic properties of psilocin, 4-HIAA and psilocin glucuronide in humans. Furthermore, for drug screening analysis, our method can also be adjusted to determine the metabolites of psilocybin in non-invasive biological matrices such as urine.

Overall, the current bioanalytical method will be an important tool to further progress the development of psilocybin as a therapeutic agent.

CRediT authorship contribution statement

Karolina E. Kolaczynska: Methodology, Formal analysis, Investigation, Validation, Visualization, Writing - original draft. **Matthias E. Liechti:** Conceptualization, Supervision, Funding acquisition, Writing - review & editing. **Urs Duthaler:** Conceptualization, Methodology, Investigation, Supervision, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: 'MEL is a consultant for Mind Medicine, Inc. All other authors do not have any conflicts of interest to declare for this work. Knowhow and data associated with this work and owned by the University Hospital Basel were licensed by Mind Medicine Inc. Mind Medicine Inc. had no role in planning, or conducting the present study or the present publication'.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2020.122486.

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Chapter 5: Discussion and Outlook

5.1 Discussion

The present thesis focused primarily on characterizing the *in vitro* pharmacological properties of various metabolites of popular drugs of abuse (MDMA, methylone, and MDPV) (**Chapter 2.1**) and establishing the receptor interaction profiles of 2,4,5-trimethoxyamphetamine (TMA-2) related derivatives and phenethylamine congeners (**Chapter 2.2**). The compounds assessed in the former project were selected to compare the validity of *in vivo* and *in vitro* pharmacological studies in rats to *in vitro* pharmacological studies using human monoamine transporters and receptors. The compounds in the latter project were examined in order to provide a pharmacological profile of TMA-2 related derivatives, which have previously not been investigated, but may produce psychedelic-like effects in humans. Furthermore, a secondary focus of the present thesis was on the establishment and validation of a bioanalytical method for the bioanalysis of psilocybin's metabolites (psilocin, psilocin glucuronide, and 4-HIAA) in human plasma (**Chapter 4.1**). The method was developed as a bioanalytical tool to assess psilocybin's pharmacokinetics in ongoing clinical studies in healthy volunteers, furthering the therapeutic development of psilocybin.

In **Chapter 2.1**, the *N*-demethylated, *O*-demethylenated, and *O*-methylated metabolites of popular ring-substituted stimulants, were assessed for their interactions with the human monoaminergic system. All three stimulants exhibited similar metabolism patterns; however, the effect of each biotransformation step did not produce similarly active metabolites. The *N*-demethylation of MDMA to MDA, did not alter the strong NET and SERT inhibition profile observed for MDMA. However, in the case of methylone, the *N*-demethylated metabolite, MDC exhibited an overall decrease in inhibition potency at three monoamine transporters. *O*-demethylenation of the three stimulants produced catechol metabolites which exhibited similar inhibition potencies at the NET and DAT but showed lower activity at the SERT. On the other hand, the *O*-methylation of the catechol metabolites not only lowered the potency at the NET, but overall diminished any stimulant effects associated with the compounds.

Taking all the findings into account, the *N*-demethylated and *O*-demethylenated metabolites of the ring-substituted stimulants exhibit pharmacologically relevant interactions with the human monoamine transporters and receptors, much in line with previous reports from rat *in vitro* and *in vivo* studies (Escobedo, O'Shea et al. 2005, Schindler, Thorndike et al. 2014, Anizan, Concheiro et al. 2016, Elmore, Dillon-Carter et al. 2017). These results confirm the clinical relevance of *in vitro* studies using human monoamine transporters, as these can shed a light on the pharmacological activity of these metabolites and their potential role in the overall psychoactive effects and toxicity profiles associated with their parent compounds. For example, MDA the *N*-demethylated metabolite of MDMA, exhibited a similar and potent inhibition profile to MDMA, and is also found in the unconjugated form *in vivo*, which overall accounts for less than 10% of the total MDMA levels (Schmid, Vizeli et al. 2016). Therefore, MDA contributes to a minor extent to the pharmacological effects of MDMA. Furthermore, alone, MDA also produces similar psychoactive effects in healthy volunteers as MDMA, as shown recently in a clinical study of healthy volunteers ingesting oral MDA (Baggott, Garrison et al. 2019). The comparison of the *in vitro*, *in vivo*, and clinical data is therefore necessary in having the entire picture of a compound's pharmacological profile. Furthermore, it ensures that findings from *in vitro* studies are not misinterpreted or overinterpreted. *In vitro* studies alone focus on that target site at which a specific compound is acting on but do not account for the complex and interconnected machinery that is found in the human body. For example, the MDMA's and methylone's *O*-methylated metabolites (HMMA and HMMC, respectively) were the least potent metabolites at transporters *in vitro*, when compared to their parent counterparts. *In vivo* however, both metabolites are found abundantly (~22 – 48%) in plasma or urine, but mostly in the conjugated form, bound to sulfates or glucuronides thereby contributing very little to pharmacological effects produced by their parents (Schmid, Vizeli et al. 2016, Elmore, Dillon-Carter et al. 2017). Moreover, it was observed in the study, that structurally similar compounds are often metabolized in a similar fashion and mostly in the liver (de la Torre, Farre et al. 2004, Meyer, Du et al. 2010, Pedersen, Petersen et al. 2013). Therefore, investigating the metabolism of popular and novel psychoactive compounds is useful to determine whether any of the corresponding metabolites are active and found abundantly in the body. The active, unconjugated, and abundant metabolites may play a role, to varying degrees in the pharmacological profile of their corresponding parent compound. As a

consequence, identifying these metabolites helps improve the understanding associated adverse effects, which can in part be caused by the active metabolites e.g., HMMA. Additionally, developing bioanalytical methods to detect parent compounds and their most abundant and active metabolites can be useful, as sometimes the presence of the metabolites in the body can outlast the presence of the parent compound (e.g., longer half-life). Therefore, the enabling the identification of the substance that was consumed by a drug user. Overall, this and all the aforementioned factors enable clinicians to provide better care to drug users in emergency situations.

In **Chapter 2.2**, several known but pharmacologically unexplored phenethylamine and amphetamine derivatives containing different 4-alkoxy substituents and related to the psychedelic TMA-2 were assessed in terms of their interactions with the monoamine receptors and transporters. Most of the investigated derivatives interacted mainly with the serotonergic receptors, and bound to the 5-HT_{2A} receptor with affinities in the low micromolar range (≤1 μM), activating the receptor as either partial or full agonists. In particular, phenethylamine and amphetamine-based derivatives containing 4-allyl and 4-methallyl moieties (2C-O-16, MALM; 2C-O-3, MMALM) at the key 4'-position exhibited the most promising profiles at the 5-HT_{2A} receptor. These derivatives are predicted to produce psychedelic-like effects in humans, as the binding to the 5-HT_{2A} receptor *in vitro* correlates positively to *in vivo* psychedelic effects (Luethi and Liechti 2018). Moreover, it was observed that in general, extension of the 4-alkoxy group or introduction of a multiple fluorine(s) onto the structure led to increase in binding affinity at the 5-HT_{2A} and 5-HT_{2C} receptors for both the phenethylamine and amphetamine-based derivatives. All but three derivatives (2C-O-2, MALM, and MMALM) activated the 5-HT_{2B} receptor as full agonists, indicating their potential role in drug-induced valvopathies associated with the activation of this receptor. At all remaining receptor targets, including the monoamine uptake transporters, no other relevant interactions were observed.

In summary, our findings suggest that some of the investigated derivatives which exhibit similar profile to the well-explored phenethylamine psychedelic, 2C-B, may be potentially active in humans. This finding is in contrast to the previously reported preliminary data which suggested that this series of 4-alkoxy-2,5-dimethoxyphenethylamines/amphetamine (2C-O/3C-O derivatives) were fairly inactive in humans (Shulgin and Shulgin 1991). Therefore, *in vitro* pharmacological characterizations of new compound series, like the one conducted in **Chapter 2.2** are

important in predicting which derivatives out of a series may have the optimal receptor profile *in vitro*. Furthermore, screenings in *in vitro* assays are in line with the three Rs (replace, reduce, and refine) of animal testing, which means that unnecessary animal testing is replaced by *in vitro* assays where possible. Compounds which require further examination to deepen their pharmacological properties can be assessed *in vivo* paradigms like the head twitch response or drug discrimination. As these assays are more work-intensive and expensive but carry a better predictive validity then *in vitro* assays, their use should be limited (Baker 2018, Halberstadt, Chatha et al. 2019). Although, more potent and well-established psychedelics like LSD or psilocybin exist and are examined for their potential in drug-assisted psychotherapy, new psychedelic-like substances may still be useful in the field (Reiff, Richman et al. 2020). For one, these new psychedelic substances could have better pharmacological properties than the classical psychedelics (e.g., stability, intrinsic activity, duration of action), be associated with fewer negative effects (e.g., decreased anxiety and fear associated with intake) or could produce less intense psychedelic experiences which could be of clinical benefit to certain psychological disorders. For example, patients who are afraid of hugely intense psychedelic experiences could instead take a short and less intense new psychedelic substance to experience a light psychedelic event. Secondly, these new compounds can be useful in enhancing our understanding of the serotonergic system. Discovering model drugs which act on a specific receptor subtype for mechanistic studies could help in defining the specific role of each receptor subtype. This is especially important when considering that selectivity between the different receptor subtypes is low due to their high sequence homology (Boess and Martin 1994). Moreover, furthering the insights into the structure-activity relationship of the 2c-O/3C-O derivatives as well as other related trisubstituted derivatives (e.g., mescaline derivatives and psi/pseudo-derivatives) will enable synthetic chemists in the future, to modify new derivatives in such a way that they can be optimal for their intendent purpose e.g., derivative that is a pure 5-HT_{2A} receptor agonist or antagonist. Currently, most of the agonist or antagonist ligands used to study the 5-HT system receptors are only fairly selective at best (e.g. ketanserin, 5-HT_{2A} receptor antagonist, but that also binds to the 5-HT_{2c} receptor) (Nichols and Nichols 2008). These compounds could then be used in *in vitro* or *in vivo* applications to establish more definitive roles/functions of each receptor subtype in the 5-HT system, as these receptors already governs a variety of central nervous processes (Nichols and Nichols 2008).

Currently, several compounds interact with receptors to varying degrees, activating different downstream effectors and thus producing different psychoactive effects. These new compounds could uncover why similar compounds interact with the receptors in different ways. Moreover, they could uncover more functions of the 5-HT system in the body. In the future, this could aid in developing new pharmaceutical drug targets which could specifically target a receptor involved in a particular process with less adverse drug events and side effects e.g., personalized medicines.

In **Chapter 4.1,** a bioanalytical method for the bioanalysis of psilocybin's metabolites, psilocin, 4-HIAA, and psilocin glucuronide in human plasma was developed and fully validated according to EMA bioanalytical method validation guidelines. The method's accuracy, precision, sensitivity and selectivity, stability, matrix effect, and extraction efficiency were examined. Method linearity of 0.25 to 100 ng/ml for psilocin and 2.5 to 1000 ng/ml for 4-HIAA was achieved covering the clinically relevant range observed in human plasma samples from an ongoing study. Furthermore, the method was accurate and precise, exhibiting a bias of ≤12% and coefficient of variation of 9.1%. The implemented extraction procedure (simple methanolic protein precipitation) enabled almost complete recovery of both analytes (94.7%). The method was selective for both analytes of interest, as the endogenous matrix components could be easily separated from analyte peaks Overall, the matrix produce only minor but irrelevant ion suppression which was consistent across all QC levels. Both analytes were stable under various laboratory-relevant storage conditions, with mean bias of ≤9%.

Altogether, the developed and validated bioanalytical method is already a useful tool in the clinical part of our laboratory, as it has been used to quantify samples from two clinical studies investigating the acute effects of psilocybin in healthy volunteers as well as psilocybin's pharmacokinetics (ID: NCT03912974 and NCT03604744) (ClinicalTrials.gov 2021). In comparison to previously published methods since 1990s, our method stands out because it is fully validated according to industry standards, is at least 8-times more sensitive and involves both a small amount of sample necessary for quantification and a short run time per sample (Hasler, Bourquin et al. 1997, Lindenblatt, Kramer et al. 1998, Sticht and Kaferstein 2000, Grieshaber, Moore et al. 2001, Hasler, Bourquin et al. 2002, Kamata, Nishikawa et al. 2003, Kamata, Nishikawa et al. 2006, del Mar Ramirez Fernandez, Laloup et al. 2007, Bjornstad, Hulten et al. 2009, Manevski, Kurkela et al. 2010, Brown, Nicholas et al. 2017).

Additionally, the method enables users to also quantify free and/or conjugated analyte(s) (Kamata, Nishikawa et al. 2003, Kamata, Nishikawa et al. 2006). Our robust and easily adaptable method can used to investigating different properties of psilocybin (e.g., psilocybin metabolism, drug screening) in different matrices (e.g., urine). Also, the method and the produced data from our method could, in the future, potentially aid in the acceleration of psilocybin's approval by the FDA for assisted psychotherapy.

Importantly, *in vitro* pharmacological characterization (e.g. in transfected cell lines) has proven to be a useful screening tool in the field to understand the basic properties of newly appearing psychoactive compounds (Simmler, Buser et al. 2013, Simmler, Rickli et al. 2014, Rickli, Hoener et al. 2015, Rickli, Luethi et al. 2015, Rickli, Moning et al. 2016, Luethi, Kolaczynska et al. 2018, Luethi, Trachsel et al. 2018, Rickli, Kolaczynska et al. 2019). The technique enables scientists to keep up with the swift occurrence of new compounds, by examining their basic psychoactive properties (at their primary target site) compared to well-established compounds e.g., cocaine or MDMA, determining their potential for abuse and to some extent in predicting their associated adverse effects and clinical toxicity. Moreover, the experimental set-up is relatively inexpensive, ethically appropriate, non-laborious, and enables the examination of a large catalogue of compounds at once when compared to the more expensive and time-consuming use of *in vivo* models (Lynch, Nicholson et al. 2010). Therefore, *in vitro* experiments are not only good at screening compounds and predicting their associated psychoactive profiles but are also highly reproducible, and translate well with *in vivo* experiments (Luethi and Liechti 2018, Luethi and Liechti 2020). However, the main drawback of these *in vitro* techniques is that they are not able to provide an exhaustive profile of these compounds, which may be require by drug regulatory authorities who provide scientifically-based guidance to governments trying to control these compounds in order to protect their citizens. *In vivo* techniques like head twitch response, self-administration or drug discrimination studies are more complex in their set up. However, they allow scientists to investigate novel compounds in whole organisms e.g., rodents, where the molecular targets of the psychoactive substances are modulated by the widespread neuronal connections within the entire animal. This is more analogous to what occurs when the compound is ingested in humans to produce its psychoactive effects (Lynch, Nicholson et al. 2010, Belin-Rauscent and Belin 2012). Caution must be taken due to species differences, which

can occur (Baumann, Zolkowska et al. 2009). Therefore, all the interplaying factors can be examined individually *in vitro* e.g., acute toxicity, metabolism, or blood-brain barrier permeability. *In vivo* however, a better picture is presented on how a substance would potentially act in humans e.g., psychoactive effects produced, pharmacokinetics/pharmacodynamics, bioavailability, long term toxicity. As a result, an interplay of both *in vitro* and *in vivo* experiments sheds the best light on how novel compounds are behaving in recreational drug user. *In vitro* experiments indicate which substances require further investigation in terms of prevalence of use, potential therapeutic or more/less addictive. *In vivo* experiments can then be applied to provide a comprehensive pharmacology needed to appropriately control the use of certain substances with the public's safety in mind.

Overall, analytical techniques are also a very important component in the field of novel psychoactive substances. Developed methods allow for the detection of known and unknown but structurally similar compounds in cases of acute intoxication. Furthermore, detection and thus subsequent development of analytical methods enable scientists to stay ahead in a field which is constantly changing. Most often, a new drug is identified on the recreational market by its detection in new drug products or seized goods (Wagmann and Maurer 2018). With the high frequency of new substances occurring on the drug market, it is essential for scientists in the field to be able to detect incoming substances that can be reported to the regulatory bodies. Because each analytical method (HPLC-MS/MS in particular) requires both a time/cost investment to be developed adequately, keeping up with the demand of the drug market is often a immense challenge. Despite this, a bioanalytical method enables scientists in the field to accurate and reliably identify and/or quantify target compounds in many different matrices including hair (Boumba, Di Rago et al. 2017). Complex compounds which are either light sensitive or temperature sensitive can also be detected, and novel methods nowadays can screen for several compounds in a single run (Adamowicz and Tokarczyk 2016, Vaiano, Busardo et al. 2016). This is very important in the field, as recreational drug users often ingest drugs contaminated with related acting compounds as well as impurities of other substances or drug cocktails (e.g., multiple drugs in a single night out). Additionally, with time, the methods can be adapted to screen for not only parent compounds but also potentially active or toxic metabolites, and can thus be used to investigate the pharmacokinetic/dynamic and toxicokinetic properties of each substance. One important disadvantage of

bioanalytical compound identification, is that any identified compound within a method requires an adequate reference standard, which is not always available (development of standard can take between 6 – 24 months, and is initially very expensive), especially for unknown/undiscovered NPS (King and Kicman 2011). Considering all these advantages and drawbacks in NPS bioanalysis, the field continues to progress further and is an important determinant in forensic drug screening, clinical drug development, and drug control by regulatory bodies.

5.2 Outlook and future prospects

In recent years, the quantity of new psychoactive substances detected on the recreational drug market has been declining, when compared to previous years. Legislation targeting incoming NPS has been successful in protecting public health by controlling incoming substances which share similar core structure, but have various other modifications. *In vitro* and *in vivo* pharmacological and toxicological assessments of new compounds have been of huge benefit to the governmental agencies controlling drug legislature and to public health. The knowledge gained from these studies has enabled scientists and government officials to understand how these substances work both on a physiological and neurological level. Moreover, it has allowed clinicians (as well as users) to be more aware of the dangers associated with these substances in order to provide better care to drug users in life-threatening situations. Since novel substances and the interest in their use will continue among the public, the field of *in vitro* and *in vivo* characterization of these compounds and appropriate drug legislature must follow at a rapid pace.

Moreover, considerable momentum has been made in psychedelic research in regards to investigating the potential use of classical psychedelics like LSD, psilocybin, and the mixed entactogenic-psychedelic, MDMA. The focus on mental health in our society as well as the more open-minded view by public and governmental agencies has enabled the studying and application of these historically controversial substances in drug-assisted psychotherapy. Progress has been made in the field with highlights including completion of a phase 3 MDMA-assisted therapy in post-traumatic stress disorder (PTSD) study, with potential FDA licensing by 2023 (Sessa, Higbed et al. 2019, Mitchell, Bogenschutz et al. 2021). Furthermore, other psychedelics like LSD and psilocybin are being also investigated for their potential in drug-assisted psychotherapy in various psychiatric disorders, with promising results (Gasser,

Kirchner et al. 2015, Davis, Barrett et al. 2021). The application of psychedelics (including also mescaline and DMT) in drug-assisted therapy seems very promising as more studies are ongoing to understand the pharmacological potential of these compounds in therapy. As the field grows, larger clinical studies with more diverse participants will need to be conducted to reveal not only the short-term effects of these substances but also to understand their long-term therapeutic benefits.

In the future, as more classical psychedelics are examined for their potential therapeutic role in drug-assisted psychotherapy, adequate bioanalytical methods must follow in line with these clinical studies, to examine their pharmacological and toxicological properties. Understanding each psychedelic's pharmacokinetic and pharmacodynamic relationship, drug-drug interactions, as well safety profiles and treatment efficacy will enable clinical scientists to understand how best to apply these potent compounds in psychotherapy. Moreover, this data will support and speed-up the process of each psychedelic's drug application to the governing bodies.

As part of my personal future outlook, I would try to investigate the predicted psychedelic derivatives (e.g., MALM) from **Chapter 2.2** in the head-twitch response to determine whether these predicted psychedelic compounds induced hallucinogenic effects in rodents, as a means to confirm the reliability of the *in vitro* pharmacological characterization. Similarly, I would use the already developed bioanalytical method (**Chapter 4.1**) for the bioanalysis of psilocybin, to investigate the metabolism of psilocybin, as certain steps in the pathway are still undetermined. In particular, I would like to confirm the hypothesis that psilocybin's active metabolite, psilocin is enzymatically broken down to 4-HTP and 4-HIAA by the monoamine oxidase enzymes and/or aldehyde dehydrogenases. Furthermore, I would like to also determine the role of the cytochrome P450 (CYP) enzymes, as this superfamily of enzymes may also be involved in the metabolism of psilocybin (albeit to a minor extent, as it has been previously shown for other serotonergic psychedelics like LSD). Finally, I would also develop a similar bioanalytical method for the bioanalysis of DMT and its respective metabolites, for future clinical studies which are set to take place in our clinical department.

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