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„Interaction profiles of psychoactive drugs at human organic cation transporters 1-3 and human plasma membrane monoamine transporter "

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Abstract

High-affinity and low-capacity transporters for norepinephrine (NET; norepinephrine transporter; SLC6A2), dopamine (DAT; dopamine transporter; SLC6A3), and serotonin (SERT; serotonin transporter; SLC6A4), belonging to the solute carrier 6 (SLC6) family, have been heavily researched regarding their interaction with psychoactive compounds. Recent advancements in research on SLC22 human organic cation transporters (SLC22A1-3; hOCT1-3) and SLC29 human plasma membrane monoamine transporter (SLC29A4; hPMAT), indicates an overlap in the inhibitory capacities of these two families and the SLC6 family. In contrast to the high-affinity and low-capacity SLC6 family, the SLC22 and SLC29 families can be classified as low-affinity and high-capacity monoamine transporters. In this study we utilized radiotracer-based *in vitro* uptake inhibition assays in human embryonic kidney 293 cells (HEK293) stably expressing hOCT1-3 and hPMAT. The majority of investigated compounds interacted with hOCT1 and hOCT2, while some interacted with hPMAT and one interacted with hOCT3. Additionally, we found methylphenidate and ketamine to be selectively and potently interacting with hOCT1 or hOCT2, respectively. Furthermore, we explored enantiomeric effects (*R*- *vs S*-α-pyrrolidinovalerophenone and R- *vs* S-citalopram) and the impact of aromatic substituents (ketamine *vs* 2-fluorodeschloroketamin and tramadol *vs* O-desmethyltramadol) on the transport capacities of the hOCTs and hPMAT. Our results highlight the importance of investigating drug-transporter profiles, and their impact on regulation of monoamine concentrations, xenobiotic clearance, and drug-drug interactions.

Zusammenfassung

Transporter mit hoher Affinität und geringer Kapazität für Noradrenalin (NET; Noradrenalin-Transporter; SLC6A2), Dopamin (DAT; Dopamin-Transporter; SLC6A3) und Serotonin (SERT; Serotonin-Transporter; SLC6A4) aus der Solute-Carrier-6-Familie (SLC6) sind hinsichtlich ihrer Interaktion mit psychoaktiven Substanzen intensiv erforscht worden. Jüngste Fortschritte bei den Familien der Transporter für organische Kationen SLC22 (SLC22A1-3; hOCT1-3) und des Plasmamembran-Monoamine Transporters SLC29 (SLC29A4; hPMAT) deuten auf eine Überschneidung der hemmenden Kapazitäten dieser Familien und der SLC6-Familie hin. Im Gegensatz zur SLC6-Familie mit hoher Affinität und niedriger Kapazität können die SLC22- und SLC9-Familien als Monoamin-Transporter mit niedriger Affinität und hoher Kapazität klassifiziert werden. In dieser Studie wurden Radiotracer-basierte In-vitro-Aufnahmehemmungstests in humanen embryonalen Nierenzellen 293 (HEK293) durchgeführt, die hOCT1-3 und hPMAT stabil exprimieren. Die meisten der untersuchten Verbindungen interagierten mit hOCT1 und hOCT2, während nur wenige mit hPMAT und nur eine mit hOCT3 interagierten. Außerdem stellten wir fest, dass Methylphenidat und Ketamin selektiv und stark mit hOCT1 bzw. hOCT2 interagieren. Darüber hinaus untersuchten wir die enantiomeren Effekte (R- vs. S-α-Pyrrolidinovalerophenon und R- vs. S-Citalopram) und die Auswirkungen aromatischer Substituenten (Ketamin vs. 2-Fluorodeschloroketamin und Tramadol vs. O-Desmethyltramadol) auf die Transportkapazitäten der hOCTs und hPMAT. Unsere Ergebnisse unterstreichen die Bedeutung der Untersuchung von Medikamententransporter-Profilen und deren Auswirkungen auf die Regulierung der Konzertation von Monoaminen, die Ausscheidung von Xenobiotika und die Wechselwirkungen zwischen Medikamenten.

Abbreviations

DAT ..... dopamine transporter
NET ..... norepinephrine transporter
SERT ..... serotonin transporter
SLC ..... solute carriers
OCT ..... organic cation transporter
PMAT ..... plasma membrane monoamine transporter
HEK293 ..... human embryonic kidney 293 cells
OAT ..... organic anion transporters
MFS ..... major facilitator superfamily
BBB ..... blood-brain barrier
DDI ..... drug-drug interactions
CSF ..... cerebrospinal fluid
CKD ..... chronic kidney disease
AKI ..... acute kidney injury
EMT ..... extra neuronal transporter for monoamine neurotransmitters
TEA ..... tetraethylammonium
MPP+ 1-methyl-4-phenylpyridinium
CNS ..... central nervous system
DA ..... dopamine
NE ..... norepinephrine
5-HT ..... serotonin
D22 ..... decynium-22
CPP ..... conditional place preference
ADHD ..... attention deficit hyperactivity disorder
IC50 ..... half maximum inhibitory concentration
MDD ..... major depressive disorder
PTSD ..... post-traumatic stress disorder
MDMA ..... methylenedioxymethamphetamine
2-DCK 2-fluoro-deschloroketamin

I. Introduction

Neuronal networks in the brain control complex functions such as mood, motivation, learning and motoric coordination. These networks are comprised of neurons which communicate via specific neurotransmitters released onto the presynaptic terminus that are then detected by cognate receptors on the postsynaptic neuron. Early on it was already postulated that theses neurotransmitters must subsequently be taken up by transporters into intracellular vesicles for transmission termination and future neuronal release (Hertting & Axelrod, 1961). Thus, complex systems are needed to regulate this mechanism.

Transporters are transmembrane proteins and constitute approximately 27% of all human genes (Almén et al., 2009; Lander et al., 2001). The second largest family of phylogenetically related membrane proteins, with at least 384 proteins in humans, are the solute carriers (SLCs) (Fredriksson et al., 2008). These proteins mediate the flow of various compounds such as drugs, sugars, amino acids and ions across the cellular membrane by utilizing the ion gradient as driving force (Bönisch et al., 1985). One major group of the SLC transporter family consists of high-affinity and low-capacity monoamine neurotransmitter transporters belonging to the solute carrier 6 (SLC6) family, historically coined "uptake 1," including the dopamine transporter DAT (SLC6A3), the norepinephrine transporter NET (SLC6A2) and the serotonin transporter SERT (SLC6A4). These transporters are localized on cell bodies and on dendrites of their cognate neurons, but they occur predominantly on, and in close proximality to, the presynaptic plasma membrane of the synaptic cleft (Kristensen et al., 2011). On the other hand, neurotransmitter reuptake is also regulated by the "uptake 2" system. By contrast, this group consists of low-affinity and high-capacity monoamine neurotransmitter transporters of the solute carrier family 22 (SLC22) and the solute carrier family 29 (SLC29) (Koepsell, 2021). The SLC22 family falls into two major clades of OCTs (organic cation transporters) and OATs (organic anion transporters), whereas the SLC29 family consists of facilitative nucleoside/nucleotide transports (NTs). These two families are grouped under the prominent major facilitator superfamily (MFS) Pfam clan (CL0015), which is one of four major SLC superfamilies that include more than one SLC family. A recent study by Höglund et. al. (2011) performed an evolutionary analysis of the entire SLC Family and classifies the SLC22 and SLC29 families into the phylogenetic α-group, the largest of these four groups with 13 members (Höglund et al., 2011). MFSs are found in all branches of life and carry out a wide

range of functions, including absorption of intestinal nutrients, hepatic and renal clearance, and transport of nutrients and other diverse substrates across the plasmatic and intracellular membranes (Quistgaard et al., 2016). Due to their broad and crucial role, they are implicated in a plethora of diseases, ranging from metabolic disorders, e.g., cancer, to psychiatric disorders, e.g., major depressive disorder (Koepsell, 2020). Therefore, they are heavily researched as potential drug targets.

In order to transport various substances across biological membranes the MFS transporters must undergo conformational changes. One of the most comprehensively studied bacterial MFS transporter is the lactose permease which has served as a convenient model for studying the principals of transport in the MFS family (Abramson et al., 2003; Guan & Kaback, 2006; Smirnova et al., 2011). Recent structural analysis suggests twelve transmembrane helices (TM1-12) forming a core structure, further subdividing into the N-domain (TM1-6) and the C-domain (TM7-12) (Quistgaard et al., 2016; Yan, 2013). These domains can further be subdivided into three helices each. The substrate-binding site is localized in the center of the transporter, comprising of residues of both transmembrane domains. The N- and the C-terminal domain can adopt either an outward open, inward open or occluded state. In the outward open state, the substrate-binding site is accessible through a crevice for the extracellular side; and in the inward open state, the substrate binding site is instead accessible from the cytoplasmatic side. The occluded conformation obstructs entry from both sides and is thought to serve as a transitional conformation (Law et al., 2008; Quistgaard et al., 2016). The precise translocation mechanisms of the substrate and thereby the transitions between the conformations have not yet been fully confirmed. Since the first model was described in 1966, several other alternating access models for the transition between these states and the translocation of the substrate have been proposed (Colas et al., 2016; Jardetzky, 1966; Quistgaard et al., 2016). The rocker-switch model was thought to mediate substrate translocation by rigid-body rotation of the two domains, which was updated by Quistgaard et. al. in 2016 to the clamp-switch model (Hansen et al., 2014; Quistgaard et al., 2016). In addition, this model suggests a structural change in individual transmembrane helices, in particular a bend in the pore-lining helices to form the occluded state (“clamping step”) followed by rotation of the domains forming the outward-facing to inward-facing state, and vice versa. This step exposes the binding site to the other side of the membrane and is termed the “switching step” (Quistgaard et al., 2016). The conformational changes are proposed to be triggered by substrate binding and release, modifying the energy landscape of the different conformational states. Nevertheless, a precise mechanism of

substrate translocation of each transporter has not yet been confirmed. Only six members of the SLC family (SLC2A1, SLC2A3, SLC4A1, SLC6A4, SLC29A1 and SLC2A3) have a fully resolved, high-resolution crystal structure (Bai et al., 2017; Colas et al., 2016; Coleman et al., 2016; Jonathan et al., 2016; N. J. Wright & Lee, 2019). Such crystal strucures help not only to confirm the transport mechanism, but also provide a starting point to systematically generate further insight into substrate specificities and the pharmacodynamics of relevant compounds.

Due to the SLC family's important role in the maintenance of the neurotramitter equlibrium, translocation of compounds through the blood-brain barrier (BBB), and the clearance of compounds through the renal and hepatic systems, it is of great interest to predict and prevent drug-drug interactions (DDIs)(E. C. Chen et al., 2022; Y. Chen et al., 2009; Cohen et al., 1996; Geier et al., 2013; Kido et al., 2011; Kousik et al., 2012; Gayathri N. Sekhar et al., 2017; Gayathri Nair Sekhar et al., 2019; Tzvetkov et al., 2009; Zamek-Gliszczynski et al., 2018). In this regard, the SLC6 family has been heavily researched, in particular the norepinephrine tranporter (NET; SLC6A2), dopamine transporter (DAT; SLC6A3) and serotonin transporter (SERT; SLC6A4) (César-Razquin et al., 2015; Engel et al., 2004; Torres et al., 2003). Fewer compounds have been investigated concerning their interaction with the SLC22 and SLC29 families; i.e., inhibition of substrate transport, despite over 40 percent of presently used drugs being organic cations and therefore potential substrates of these transporter families (Koepsell, 2021). Nevertheless, studies focusing on compound interaction of the SLC22 and SLC29 families have shown similarities and, to some extent, functional overlaps with the SLC6 transporters (DAT, NET and SERT) (Gebauer et al., 2021; Jensen et al., 2021; Maier, Rauter, et al., 2021; Niello, Sideromenos, et al., 2021).

I.I. Tissue expression of hOCTs and hPMAT

In the human intestine, mRNA expression of hPMAT and hOCTs has been reported (Koepsell et al., 2007; Nishimura & Naito, 2005). However, the presence of hOCT2 in the intestine is still unclear. Müller et. al. has detected hOCT2 in the human intestinal cell line Caco-2 by hOCT2-spcific antibodies, however others have reported no expression (Koepsell et al., 2007; Müller et al., 2005; Nishimura & Naito, 2005). Human OCT1 was believed to be expressed in the intestinal enterocytes, but more recent studies localized hOCT1 on the apical site of human enterocytes (T. Han et al., 2013; Koepsell et al., 2007). hPMAT was reported to be expressed on the tips of the mucosal epithelial layer of the human small intestine (M. Zhou, Engel, et al., 2007).

In the human liver, hOCT1 has been found to have the highest expression levels, followed by hOCT2 and hPMAT, although at very low mRNA levels (Nishimura & Naito, 2005; J. Wang, 2016; S. Zhou et al., 2021). The presence of transporters at the protein level has yet to be confirmed. The localization of hOCT2 and hOCT3 has also been demonstrated in the sinusoidal membrane of hepatocytes (Koepsell et al., 2007; Nies et al., 2009).

In the human kidney, the presence of all transporters was reported, with hOCT2 having the highest expression levels, followed by hOCT3 and hOCT1 (Motohashi et al., 2002; Nishimura & Naito, 2005). hPMAT exhibited a value at the quantification limit (Nishimura & Naito, 2005). hOCT2 has also been shown to be expressed in human tubular kidney cells at the protein levels (Prasad et al., 2016; Yang & Han, 2019). hOCT3 exhibits low expression levels and is thought to play a minor role in the kidneys (Ivanyuk et al., 2017). Although it was reported that OCT1 plays an important role in rodent kidneys, its expression in human cell lines is low. This suggests a species dependent function of theses transporters. At protein levels, hPMAT was proposed by Xia et al. in 2007 to be located on the apical membranes of renal epithelial cells, however subsequent studies showed a specific expression in podocytes that form a critical part of the glomerular filtration barrier (Xia et al., 2009).

At the human blood-brain barrier, which is formed by micro vessel endothelial cells, and the blood-cerebrospinal fluid (CSF) barrier of the choroid plexus, hPMAT was reported to play an important role. It functions as an efflux transporter and facilitates solute exchange between the blood and the CSF (Ho et al., 2012). It was also shown that hOCT1 and hOCT2 are localized in the human brain endothelial cells (Girardin, 2006; Lin et al., 2010; L. Liu & Liu, 2019). hOCT3 was also found to be expressed in brain micro vessels, and protein levels were also detected (Geier et al., 2013). However, studies by Wu et al. in 2014 detected mRNA and protein levels of OCT1 and OCT2 in brain micro vessels in mice (K. C. Wu et al., 2015).

Regarding the whole human brain, mRNA of all OCTs and PMAT has been reported (Engel et al., 2004; Nishimura & Naito, 2005). hOCT2 were found to be localized in neurons in various brain regions and hOCT3 was also found in diverse areas, including the substantia nigra, nucleus caudatus, and other areas (Duan & Wang, 2010; Koepsell, 2020). hOCT1 was shown to be expressed in micro vessels (Koepsell, 2020). The human PMAT was found to be present in many brain regions including the cerebral cortex, hippocampus, substancia nigra, and cerebellum (J. Wang, 2016).

hOCTs were also reported to be localized in many other peripheral tissues such as lung, bladder, placenta, salivary gland, skeletal muscle, heart and nasal epithelium (Agu et al., 2011; Koepsell, 2020). hPMAT was also found to be present in peripheral tissues such as heart, small intestine, kidney, liver, pancreas and skeletal muscles (J. Wang, 2016).

I.II. The Organic cation transporter 1 (OCT1; SLC22A1)

The organic cation transporter 1 was the first member of the SLC22 family isolated by Gründemann et al. in 1994 from rat kidney cells (Dirk Gründemann et al., 1994). Shortly thereafter, homologs were found in mouse, rabbit and human cells (Gorboulev et al., 1997; Schweifer & Barlow, 1996; Terashita et al., 1998). The human OCT1 variant exhibits about 76% amino acid homology with that of the rat (HAYER et al., 1999; Maier, Niello, et al., 2021; Maier, Rauter, et al., 2021). Since it is primarily expressed in the liver and intestine, one of its major functions is the gastrointestinal absorption and hepatic elimination of various endogenous compounds and xenobiotics (Choi et al., 2008; Mato et al., 2018; Zamek-Gliszczynski et al., 2018; S. Zhou et al., 2021). *In vitro* and *in vivo* studies have reported OCT1 to be a crucial factor in the pharmacokinetics of various organic drugs, in particular regarding its polymorphisms (S. Zhou et al., 2021). Substances interacting with OCT1 as an inhibitor, for example, can reduce a drug's hepatic clearance and therefore alter its toxicity, pharmacokinetics, or pharmacodynamics (S. Zhou et al., 2021). A well-described drug-drug interaction is the effect of inhibitors of OCT1 and the resulting impact on the pharmacodynamic properties of the widely prescribed antidiabetic and cationic drug metformin (D. S. Wang et al., 2002; Zamek-Gliszczynski et al., 2018). It was shown that in OCT1 gene-knockout mice, intravenous administrated metformin was decreased in the liver and intestine (D. S. Wang et al., 2002). In addition, genetic variations in hOCT1 were found to impact the response to metformin in type-2 diabetes patients. Patients non-responsive to metformin treatment showed hOCT1 variations that opposed the structural compactness of the protein. This might lead to a compromised and aberrated protein confirmation impeding OCT1 function and therefore its metformin transport capacity (Kawoosa et al., 2021). However, Zamek-Gliszczynski et al. argue that hepatic transport does not modulate metformin clearance, but metformin is rather eliminated via the renal system and therefore hepatic OCT1 has no effect on metformin's pharmacokinetics (Zamek-Gliszczynski et al., 2018). This was also shown in the previously mentioned study where OCT1 knockout only had minimal effects on metformin distribution in the kidney as well as the urinary excretion profile

(D. S. Wang et al., 2002). Christensen et. al also found no impact of hOCT1 variants on metformin's steady-state pharmacokinetics (Christensen et al., 2015). In this regard, OCT1 has been identified as the predominant uptake pathway and impacting the pharmacokinetics of fenoterol, β2-adrenergic agonist used in treatment of asthma due to its bronchodilating effect (Svedmyr, 1985; Zamek-Gliszczynski et al., 2018). In addition, reports have shown that the presence of reduced-function phenotypes can result in adverse reactions (Dujic et al., 2015; S. Zhou et al., 2021). Regarding metformin, studies investigating different populations, including Iran, India, Japan and Netherlands, found no association between polymorphisms and metformin response (G. Ahlin et al., 2011; Becker et al., 2009; Shikata et al., 2006; Shokri et al., 2016; Sur, 2014). Nevertheless, both co-administration of OCT1 inhibitors as well as the presence of reduced-function phenotypes can lead to adverse reactions. Like other organic cation transporters and PMAT, OCT1 was found to be expressed at the blood-brain barrier (BBB). Here it is thought to transport organic cations, such as 5-HT, in both directions and a prime target for drug-drug interaction through regulation of drug uptake (Koepsell, 2021). It was shown that OCT1 transports NE, 5-HT, DA, acetylcholine, and histamine, as do OCT2, OCT3 and PMAT (Breidert et al., 1998; A. E. Busch et al., 1996).

I.III. The organic cation transporter 2 (OCT2; SLC22A2)

The organic cation transporter 2 is primarily found in cerebral cortex and various subcortical nuclei, including the hippocampus, caudate nucleus, amygdalal, doral raphe, thalamus, median eminence, and pituitary gland (Bacq et al., 2012; Andreas E. Busch et al., 1998; Couroussé & Gautron, 2015). Further research found OCT2 to be expressed human, rat, and mouse brain micro vessels endothelial cells at the luminal and abluminal membranes (Geier et al., 2013; K. C. Wu et al., 2015). Besides humans, rats, and mouse tissue, orthologs were found in rabbit and pigs (Gorboulev et al., 1997; Dirk Gründemann et al., 1997; HAYER et al., 1999; Mooslehner & Allen, 1999; X. Zhang et al., 2002). In the periphery, OCT2 is found in the kidney and is thought to play a major role in renal secretion of organic cations and consequently is partly responsible for establishing the pharmacokinetics of many cationic drugs. It mediates the first step in renal secretion in proximal tubes, which is thought to be responsible for the clearance of approximately 40 percent of pharmaceuticals (Neuhoff et al., 2003; S. H. Wright, 2019). At the transcriptional level, it was shown that hOCT2 transcription is activated by the binding of upstream stimulation factor 1, a

member of the basic helix-loop-helix-leucine zipper family, to an upstream E-box in the hOCT2 promotor region (Asaka et al., 2007). Further investigation showed that the OCT2 proximal promotor region is hypomethylated in human kidney cells and hypermethylated in liver cells. Vice versa results were shown for the hOCT1 promotor. In vitro methylation of the OCT2 promotor dramatically reduced its activity (Aoki et al., 2008). Hypermethylation blocks the transcription factor MYCs interaction with the E-Box motif, which prevents its recruitment and activation of histone methyltransferase MLL1 to catalyze the trimethylation of lysine 4 on histone 3 at the OCT2 promotor. It is further thought that OCT2 repression in renal cell carcinoma is partially responsible for its multidrug resistance, due to role of OCT2 in the uptake of oxaliplatin, a commonly used chemotherapeutic in renal cell carcinoma (Y. Liu et al., 2016). OCT2 downregulation is also thought to be induced in chronic kidney disease (CKD) or acute kidney injury (AKI) by characteristic upregulated inflammation mediator TNFα and NF-kB (C. Han et al., 2021). Further studies also showed a epigenetic regulation of OCT2 by methylation in placental expression and imprinting (Saito et al., 2011).

I.IV. The organic cation transporter 3 (OCT3; SLC22A3)

The organic cation transporter 3 consists of 11 exons and 10 introns and is located in close proximity to the other organic cation transporters on chromosome 6 (Dirk Gründemann & Schömig, 2000). As mentioned earlier, it is broadly distributed in central and peripheral tissue, the main peripheral tissue being adrenal glands, prostate, salivary glands, and skeletal muscles; and the main central nervous system tissue being micro vessels, the blood brain barrier, substantia nigra, cerebellum, and glia cells, including astrocytes (E. C. Chen et al., 2022; Gasser, 2019; Haag et al., 2004; K. Hosoya & Tachikawa, 2011; Kekuda et al., 1998; Koepsell, 2020, 2021; Vincent Vialou et al., 2004). The first cloning of the OCT3 was done by Kekuda and colleagues in 1993 in rats (Kekuda et al., 1998). It showed high mRNA expression in placental tissue and a considerable higher expression in brain tissue compared to the other two OCTs (Kekuda et al., 1998). The first human isoform was found shortly after in 1998 by Gründemann and colleagues, termed “extra neuronal” transporter for monoamine transmitters (EMT), due to the early recognition of its synaptically distal monoamine transport (D. Gründemann et al., 1998). As a member of the SLC22A family, it is perhaps best known for its low-affinity and high-capacity transport of monoamines in the human brain (Koepsell, 2021). However, some literature highlights its

importance as a major transporter for histamine, epinephrine, and norepinephrine in the periphery (Duan & Wang, 2010). As OCT3 shares only about 50 percent sequence homology with the OCT1 and 2, it is not surprising that their compound affinities and interactions differ (Maier, Niello, et al., 2021; Sala-Rabanal et al., 2013). As in interesting peripheral function, it was found that OCT3 is primarily responsible for doxorubicin-induced cardiac injury. Doxorubicin is a commonly used anticancer drug that was found to be responsible for debilitating and irreversible cardiac injury (Huang et al., 2021). A subsequent study reported, by meta-analyzing randomized controlled trails, that the concurrent use of beta-blockers and anthracyclines reduced the risk of cardiotoxicity, due to its inhibitory potencies on hOCT3 (Ma et al., 2019). This again shows the importance of transporter specific interaction profiles of various compounds as drug-drug interactions cannot only be adverse but also in our favor. Furthermore, it was shown that OCT3 polymorphisms may play a role in use disorders. They have identified two single nucleotide polymorphisms that significantly correlated with the development of polysubstance use in patients with methamphetamine dependance (Aoyama et al., 2006).

I.V. The human plasma membrane monoamine transporter (PMAT; SLC29A4)

The human plasma membrane monoamine transporter is located on chromosome 7p22.1 and was first characterized in 2004 by Engel and colleagues (Engel et al., 2004). It was found to be widely expressed in brain, namely in the blood-brain barrier, choroid plexus, medulla oblongata, pons, cerebellum, substantia nigra, putamen, caudate nucleus, cerebral cortex, hippocampus, and NAC (Daws, 2021; J. Wang, 2016). It was also found to be expressed in the periphery including heart, small intestine, pancreas, kidney, skeletal muscle, and liver (Barnes et al., 2006; Duan & Wang, 2010; Engel et al., 2004). In human intestine and rat primary cardiomyocytes PMAT was found to be concentrated on the apical surface, although punctate intracellular staining was also observed (Duan & Wang, 2013; M. Zhou, Xia, et al., 2007). However, the expression levels were consistently the highest in brain tissue. Although PMAT has been found to be expressed in many different species, there is some evidence for species specific expressions. For example, PMAT mRNA and protein was found to be expressed in human and rat kidneys, but not in mouse kidneys (Dahlin et al., 2007; Xia et al., 2009). PMAT accepts a wide range of substrates, mainly small and polar organic cations, including biogenic amines, cationic drugs, and neurotoxins. Most well-known substrates of PMAT are known to also be substrates of OCT, such as tetraethylammonium (TEA),

1- methyl-4-phenylpyridinium (MPP^+), and metformin (Engel et al., 2004; Engel & Wang, 2005; M. Zhou, Xia, et al., 2007). Regarding neurotransmitters, PMAT shows strong kinetic preference for 5-HT and DA over norepinephrine, epinephrine and histamine (Duan & Wang, 2010).

I.VI. OCTs and PMAT in the brain

The organic cation transporters 1-3 and PMAT play a major role in maintaining the monoaminergic equilibrium in the CNS as they are located on the pre- and postsynaptic neurons as well as glia cells and are thought to act as an alternate monoamine clearance system in the brain (Amphoux et al., 2006; Couroussé & Gautron, 2015; Gasser, 2019; Koepsell, 2020; Maier, Niello, et al., 2021; Mayer et al., 2018; V. Vialou et al., 2007; J. Wang, 2016). An increasing number of research has therefore been focusing on their interaction with central nervous system active compounds, including psychoactive substances (Haenisch et al., 2012; Maier, Niello, et al., 2021; Sitte & Freissmuth, 2015; Wagner et al., 2017, 2018; X. Wu et al., 1998; Zhu et al., 2010). In addition, the monoamine transport has shown to affect the ambient concentrations of NE, DA, and 5-HT which influences ontogeny of neurons in early embryo development and regulation of neuronal properties in adults (Daws & Gould, 2011). For example, NET is associated with learning, memory, attention, mood control, motoric functions, and responses to stress (Daws, 2021). Knock-out NET mice show a decreased NE tissue concentration, reduced NE clearance after activation of noradrenergic neurons, and an increased response to behavioral despair (Xu et al., 2000). Reports also indicate OCT1-3 and PMATs expression in the blood brain barrier (BBB), although their precise role has not been fully resolved (Chaves et al., 2020; K. I. Hosoya & Tachikawa, 2011; Koepsell, 2021; Lin et al., 2010; Gayathri N. Sekhar et al., 2017; Gayathri Nair Sekhar et al., 2019). However, it has been shown that 5-HT efflux across the BBB affects the regulation of microvascular blood flow and BBB permeability (Cohen et al., 1996). OCT3 was shown to be most highly expressed OCT at the human blood brain barrier which indicates that it may play a crucial role in the transport of various substances into the CNS (E. C. Chen et al., 2022). Hypothetically, OCT3 inhibitors might reduce the permeability of these substances, including medical drugs. A recent study has found hOCT3 to be the only OCT to be expressed in human brain micro vessels (E. C. Chen et al., 2022). Studies in culture cells and *Xenopus* oocytes have shown that OCTs and PMAT accept a wide range of compounds ranging from positively charged endogenous substrates to various drugs and xenobiotics. Interestingly, the SLC22 and SLC29 transporters have wide substrate overlap with the

well-studied SLC6 transporter family, supporting their role in the maintenance of the CNS monoaminergic equilibrium (Gebauer et al., 2021). For example, dopamine is efficiently transported not only by DAT, but also by NET and OCT2, and 5-HT (serotonin) was shown to be a substrate for all mentioned transporters besides OCT2 (Gebauer et al., 2021). hPMAT, a very efficient transporter of 5-HT, is thought to be associated with depression by reduced 5-HT reuptake through inhibited PMAT expression by estradiol (Gu et al., 2022). Further studies associated OCT2 and dopamine D1 receptor (D1DR) activity, by showing that OCT2s dopamine transport is necessary for Golgi associated D1DR activation (Puri et al., 2022). Based on these studies it is not surprising that monoamine regulation by the "uptake 2" system is involved in diverse central nervous system functions. However, this is challenged by contradicting reports. For example, OCT2 knockout in mice have shown a decreased accumulation of norepinephrine and serotonin in cortical and hippocampal suspensions, although a very low uptake rate of OCT2 regarding norepinephrine has been measured (Bacq et al., 2012; Gebauer et al., 2021). Since OCT3 has been reported to be expressed in the brain and capable of transporting various compounds including neurotransmitters, it has been proposed to partake in the regulation of neuronal signal transmission. One of the first CNS function of OCT3 was reported by Vialou et al. in 2004. They have found that OCT3-deficient mice show increased levels of hypertonic saline ingestion under thirst and salt appetite. This was accompanied by alterations in neuronal repones in the subfornical organ after sodium deprivation (Vincent Vialou et al., 2004). It is well known that amphetamine and many other stimulants interact with high-affinity transporters for dopamine, serotonin, and norepinephrine (DAT, SERT, and NET, respectively). Until recently the SLC6 family was thought to be the main player in the regulation and clearance of these monoamines. However, OCT3 was also found to play a role in the methamphetamine sensitization. Sensitized rats showed not only significantly increased methamphetamine concentration in plasma and brain dialysate, but also a decreased expression of OCT3 mRNA in the kidneys, brain and heart (Kitaichi et al., 2003). This suggests that sensitization may partly occur due to the altered transport capacities resulting in increased methamphetamine concentrations. This is also supported by a different study where mice treated with antisense against OCT3 also showed a significant increase in methamphetamine induced locomotor activity (Kitaichi et al., 2005). Another study found that a substantial component of amphetamine's actions is OCT3 dependent. They have shown that amphetamine-evoked dopamine release is not only caused by reverse transport through DAT, but also OCT3. This efflux is triggered by the diffusion and active transport of amphetamine into intracellular

vesicles via DAT and VMAT2. This conclusion is based on the findings that the non-selective OCT/PMAT blocker decynium 22 (D22) inhibited amphetamine-induced DA release and locomotor activity only in OCT3 +/+ mice (Mayer et al., 2018). Another recent study has investigated gender specific mechanisms of amphetamine induced conditioned place preference (CPP) and sensitization to its locomotor stimulant effect. They were able to show that in male mice CPP is dependent on OCT3, whilst the females PMAT was necessary in the prevention of amphetamine induced CPP with the D22 (Clauss et al., 2021). Furthermore, it was shown that OCT3 partakes in the protection from dopaminergic neurodegeneration in mice astrocytes, by the efflux of the toxic organic cation 1-methyl-4-phenylpyridinium. OCT3 deletion not only impaired the removal of excess extracellular dopamine induced by methamphetamine, but also enhanced striatal dopaminergic terminal damage (Cui et al., 2009). These findings not only further solidify the role of OCT3 in the central nervous system, but also provide an opportunity in the discovery of potential treatment of psychologic and neurologic disorders. As mentioned earlier, PMAT was also found to be abundantly expressed in the central nervous system. In fact, PMAT showed higher mRNA expressions besides the caudate nucleus compared to hOCT3 (Duan & Wang, 2010). Its strong expression in various brain areas implicates important biomedical functions. One of its functions is thought to be the removal of toxic compounds such as x and MTPT through the blood brain barriers, protecting the brain from damage (Okura et al., 2011). PMAT was also found to be the major contributor to the histamine clearance of astrocytes with only minor contribution of OCT3 (Yoshikawa et al., 2013). Also, in the nucleus tractus solitarii, PMAT was found to play a major role in serotonin regulation and therefore cardiovascular homeostasis, as vagally evoked 5-HT release was not regulated by SERT, but by PMAT (Hosford et al., 2015). Another interesting finding by study by Wultsch et al. regarding OCT3s function in the CNS found that mice constitutively lacking OCT3 have decreased levels of anxiety (Wultsch et al., 2009). These findings contradict the hypothesis that OCT3 serves as an alternative reuptake mechanism for serotonin, as OCT3 mRNA is significantly upregulated in the hippocampus of serotonin transporter deficient mice, which are extensively used as genetic models for anxiety-like behavior (Holmes et al., 2003; A. V. Kalueff et al., 2007; Allan V. Kalueff et al., 2007; Schmitt et al., 2003). Another study by Vialou et al. found OCT3 knock-out mice do show subtle anxiety-like behavior such as increased levels of stress and anxiety and an increased sensitivity to psychostimulants (Vincent Vialou et al., 2008).

I.VII. Psychoactive drugs and MFS

This exciting new area of research, with the potential to uncover new targets of drugs with improved ability to modulate the amine balance and therefore augment e.g., antidepressant effects, is limited by the availably of selective modulators of the MFS system. One such modulator is corticosterone, as it was shown that the consumption of psychoactive substances causes inhibition of OCT3 via increased corticosterone levels (Baganz et al., 2010). However, withdrawal from chronic amphetamine use in rats increases the expression of OCT3 in the ventral hippocampus, resulting in an increased clearance of extracellular serotonin. As mentioned earlier, this correlates with the increased anxiety levels in the first 24 hours post-withdrawal (Barr & Forster, 2011). A follow-up study showed, that in the first 24 hours post-withdrawal rOCT3 and rSERT expressions not only increased in the ventral hippocampus, but also in the central nucleus of the amygdala and in the dorsomedial hypothalamus (Solanki et al., 2016). This elevation persisted two weeks post-withdrawal. Further studies showed that OCT3 knock-down (~25%) rats had an significantly increased extracellular dopamine levels in the nucleus accumbens and exhibited hyperlocomotion when exposed to methamphetamine, compared to controls (Kitaichi et al., 2005; Nakayama et al., 2007). This contradicts the results of Mayer and colleagues, who found no difference in locomotor response in OCT+/+ and OCT-/- mice when exposed to amphetamine (Mayer et al., 2018). However, pretreatment with D22 robustly attenuated the locomotor response to amphetamine, but only in OCT+/+ mice (Mayer et al., 2018).

In the presence one of only a handful of selective inhibitors of OCTs and PMAT is Decynium-22 with low micromolar inhibition potencies (Fraser-Spears et al., 2019; Gorboulev et al., 1997; Hayer-Zillgen et al., 2002; K. Wang et al., 2014). In addition, due to the importance of the OCTs and PMAT on the clearance and transport of drugs at the BBB and therefore impacting the pharmacokinetics of drugs, it is of great interest to further our knowledge on drug interactions with these transporters. In this study, we aimed to establish interaction profiles of a total of 17 compounds, the majority of which are known for their interplay with the high-affinity transporters SERT, DAT and NET. These compounds are part of six different chemical and medical classes, being antidepressants, antiepileptics, analgesics, new therapeutics, psychostimulants, and drugs for treatment of narcolepsy and Attention-Deficit/Hyperactivity Disorder (ADHD). Interaction profiles were investigated via radiotracer-based uptake inhibition assays, utilizing the well-known substrate of human MATs, OCTs and PMAT 1-methyl-4-phenylpyridinium (MPP^+) in its tritiated

form (Engel et al., 2004; Gorboulev et al., 1997; Johnson et al., 1998; Russ et al., 1992; Salach et al., 1984; Wall et al., 1995). In 1990 Schömig and Schönfeld found the dopaminergic neurotoxin 1-methyl-4-phenlypyridinium (MPP^+) to be an excellent OCT and PMAT substrate and cyanine derivates (i.e., decynium-22) to be a highly potent inhibitor (Schömig et al., 1993). These two compounds are up to the present day used for research of the MFS transporters.

MPP^+ is a metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and known for its neurotoxicity and its link to Parkinson's Disease (Salach et al., 1984). In order to assess the inhibitory potency of a given compound, we exposed human embryonic kidney 293 cells (HEK 293) overexpressing one of the transporters of interest to tritiated MPP^+ at a concentration of 0.05 µM in addition to various concentrations of a given compound. Uptake of the tritiated substrate MPP^+ was measured and quantified via beta-scintillation counting. Exclusion of non-specific uptake was evaluated by the previously mentioned potent inhibitor of all OCTs and PMAT 1,1'-diethyl-2,2'-cyanine (decynium-22; D22) and the subtraction of these results from the measured uptake rates (Engel et al., 2004; Engel & Wang, 2005; Gorboulev et al., 1997; Dirk Gründemann et al., 1994; Hayer-Zillgen et al., 2002; Russ et al., 1992; Schömig et al., 1993; L. Zhang et al., 1997). Interaction profiles of the respective compounds was assessed via the calculation of the compounds half maximum inhibitory concentration (IC_{50}), providing a key insight into the inhibitory capacities of the respective compound.

Bupropion, R-citalopram, and escitalopram are compound widely used antidepressants. Their main pharmacological mechanism relies on their capacity to selectively inhibit high-affinity transporters. Bupropion, sold under the brand name of Wellbutrin© or Zyban©, is a hDAT and hNET inhibitor and an antagonist of the nicotinic acetylcholine receptors (nAChR). It is used in treating depression, seasonal affective disorder, and to support smoking cessation (Foley et al., 2006). Escitalopram or citalopram, sold under to the brand name Cipralex© or Lexapro©, is a widely used selective serotonin reuptake inhibitor (SSRI) used to treat major depressive disorder (MDD) and generalized anxiety disorder (Waugh & Goa, 2003). Furthermore, we analyzed diazepam (Valium©) and phenobarbital (Luminal©), two prominent allosteric $GABA_A$ receptor modulators, both used to treat epilepsy (Brodie & Kwan, 2012; Calcaterra & Barrow, 2014). We also investigated four psychostimulants, because of the growing literature on the importance of OCTs on stress and addiction (Maier, Niello, et al., 2021). Cocaine and d-amphetamine are widely used illicit drugs due to their stimulating effects, resulting from inhibition or releasing properties at hDAT and

hNET, respectively. D-amphetamine is used to treat ADHD and narcolepsy (Kristensen et al., 2011; Luthy et al., 2015). α-pyrrolidinopentiophenone (α-PVP), commonly called "Flakka" or sold as "bath salts" is a synthetic cathinone that is scheduled by the United State Drug Enforcement Administration. In the central nervous system α-PVP acts as a norepinephrine-dopamine reuptake inhibitor. (Kaizaki et al., 2014; Katselou et al., 2016; Maier, Rauter, et al., 2021). As part of the newly emerging therapeutics, we assessed the interactions profiles of Ketamine, its relative 2-fluoro-deschloroketamin (2-DCK), methylenedioxymethamphetamine (MDMA), psilocin and tramadol. Ketamine and 2-fluoro-deschloroketamine are both used as an anesthetic while ketamine is also used as an analgesic. Although substantially less is known about 2-DCK, both ketamine and 2-DCK are non-competitive antagonists at the N-methyl d-aspartate (NMDA) receptor (Hess et al., 2022; Morgan & Curran, 2012; Tang et al., 2020). The amphetamine derivate MDMA is a popular recreational drug as it enhances the release of both serotonin and dopamine rapidly in the brain (Green et al., 2003)., recent studies have shown MDMAs effectiveness in treatment of severe post-traumatic stress disorder (PTSD) (Feduccia et al., 2018; Nicholas et al., 2022). Tramadol and its main active metabolite O-desmethyltramadol (O-DMT) are drugs used to treat acute and chronic pain, due to their activating properties at the μ-opioid receptors (Gong et al., 2014; Lassen et al., 2015). Psilocin is the active metabolite of Psylocibin, a compound commonly known for its effects of "magic mushrooms". Psilocin shows a high affinity and agonistic properties at the 5-HT_{2A} receptors, although other serotonin receptors might also be targeted. There are also results that suggest that the dopaminergic-system contributes to the psilocybin-induced psychedelic effects, although only moderately. It has been proposed for the treatment of obsessive compulsive disorder (OCD), treatment of anxiety and depression in terminal cancer patients (Kargbo, 2020, 2021; Vollenweider & Preller, 2020). We also chose two compounds, methylphenidate and modafinil, that are used to treat ADHD and narcolepsy. Methylphenidate, sold under the name Ritalin©, inhibits the DAT and NET, while modafinil only inhibits DAT.

Most of the herein screened compounds interact with the SLC6 transporters and have in this regard been heavily investigated. However, research regarding their interactions with OCTs and PMAT is scarce. This is particularly interesting due to these transporters' involvement in the maintenance of the monoaminergic equilibrium and their effects on drug-drug interactions. Furthermore, our results might lead to discovery of potential scaffolds for the development of new or adapted drugs.

In the course of this thesis, the following study was published in the International Journal of Molecular Sciences as part of a special issue on the 30th of November 2021 (Angenoorth et al., 2021).

II. Article: "*Interaction profiles of central nervous system active drugs at human organic cation transporters 1–3 and human plasma membrane monoamine transporter*"

International Journal of
Molecular Sciences

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Article

Interaction Profiles of Central Nervous System Active Drugs at Human Organic Cation Transporters 1–3 and Human Plasma Membrane Monoamine Transporter

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Abstract: Many psychoactive compounds have been shown to primarily interact with high-affinity and low-capacity solute carrier 6 (SLC6) monoamine transporters for norepinephrine (NET; norepinephrine transporter), dopamine (DAT; dopamine transporter) and serotonin (SERT; serotonin transporter). Previous studies indicate an overlap between the inhibitory capacities of substances at SLC6 and SLC22 human organic cation transporters (SLC22A1–3; hOCT1–3) and the human plasma membrane monoamine transporter (SLC29A4; hPMAT), which can be classified as high-capacity, low-affinity monoamine transporters. However, interactions between central nervous system active substances, the OCTs, and the functionally-related PMAT have largely been understudied. Herein, we report data from 17 psychoactive substances interacting with the SLC6 monoamine transporters, concerning their potential to interact with the human OCT isoforms and hPMAT by utilizing radiotracer-based in vitro uptake inhibition assays at stably expressing human embryonic kidney 293 cells (HEK293) cells. Many compounds inhibit substrate uptake by hOCT1 and hOCT2 in the low micromolar range, whereas only a few substances interact with hOCT3 and hPMAT. Interestingly, methylphenidate and ketamine selectively interact with hOCT1 or hOCT2, respectively. Additionally, 3,4-methylenedioxymethamphetamine (MDMA) is a potent inhibitor of hOCT1 and 2 and hPMAT. Enantiospecific differences of R- and S-α-pyrrolidinovalerophenone (R- and S-α-PVP) and R- and S-citalopram and the effects of aromatic substituents are explored. Our results highlight the significance of investigating drug interactions with hOCTs and hPMAT, due to their role in regulating monoamine concentrations and xenobiotic clearance.

Keywords: ketamine; psilocybin; bupropion; escitalopram; diazepam; tramadol; *O*-desmethyltramadol; cocaine; *d*-amphetamine; modafinil

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1. Introduction

Organic cation transporter subtypes 1-3 (OCT1–3; SLC22A1–3, respectively; OCTs) and the plasma membrane monoamine transporter (PMAT; SLC29A4) are poly-specific facilitative transporters which are involved in the uptake and elimination of various endogenous compounds, most notably monoamines, as well as of drugs, xenobiotics, and toxins [1]. In addition to the high-affinity, low-capacity solute carrier 6 (SLC6) neurotransmitter-sodium symporters of norepinephrine (norepinephrine transporter; NET; SLC6A2), dopamine (dopamine transporter; DAT; SLC6A3) and serotonin (serotonin transporter; SERT; SLC6A4), the low-affinity, high-capacity OCTs as well as PMAT are of paramount importance for the maintenance of monoaminergic equilibrium in the brain [2,3]. In addition, OCTs contribute

to protection against noxious compounds by intestinal absorption as well as hepatic and renal excretion [4–6]. Indeed, hOCT1 and hOCT2 transport numerous compounds in the small intestine, liver and kidneys, with OCT1 being primarily expressed in the liver and OCT2 predominantly acting in the kidney, while both are, together with hOCT3 and hPMAT, also found in the brain, albeit at a lower level [7]. By contrast, hOCT3 and hPMAT were found to be highly expressed in the brain, alongside their less ubiquitous occurrence in peripheral organs, such as the heart for hOCT3 [8]. Here, along with hOCT1 and 2, they participate in the regulation of the monoaminergic equilibrium, and their dysfunction has been associated with disturbance of monoaminergic pathways, leading to various psychiatric disorders and neurodegenerative diseases [9–13].

Due to their involvement in the excretion of drugs and metabolites, members of the OCT family have overlapping substrate and inhibitor profiles [5,14]. Substrates are classically transported into the cells by the transporter, while inhibitors bind to the transporter in the outward-facing confirmation and prevent uptake [15]. Identification of compounds interacting with OCTs and PMAT furthers knowledge of targets and effects in the central nervous system (CNS) and, additionally, allows for the explanation of substances' observed pharmacokinetic properties. Given the plethora of CNS active substances that are well-known for their interplay with SLC6 neurotransmitter-sodium symporters (summarized as monoamine transporters; MATs), there is also a growing amount of interest in their interaction with the human OCTs as well as PMAT, since they are less well-researched potential targets in the monoamingergic system [16–21]. We have recently reported that several psychoactive substances, classified primarily as inhibitors or substrates of the MATs, equipotently interact with certain OCTs [2,22].

To further extend these observations, we screened 17 substances with CNS activity that were categorized as follows: antidepressants (bupropion, S-citalopram, R-citalopram), antiepileptics (diazepam, phenobarbital), psychostimulants (cocaine, *d*-amphetamine, R- and S-*α*-pyrrolidinovalerophenone (R- and S-*α*-PVP)), emerging therapeutics (3,4-methylenedioxymethamphetamine (MDMA), psilocin, ketamine), the ketamine derivative 2-fluoro-deschloroketamine, drugs for attention deficit hyperactivity disorder (ADHD) and narcolepsy (methylphenidate, modafinil) and analgesics (tramadol and its metabolite *O*-desmethyl-tramadol). 2-fluoro-deschloroketamine and *O*-desmethyltramadol can be classified as new psychoactive substances that are not yet controlled by legislation [23]. Considering the myriad of already established interaction profiles on SLC6 transporters, we tried to start an analogous process for the low-affinity, high-capacity monoamine transporters, as doing so will help to further elucidate the pharmacological interaction of compounds with OCTs and PMAT. This approach will offer more information on these transporters' rich pharmacology and potentially lead to clinical implications for patients with OCT polymorphisms impacting substrate translocation [24]. In addition, the discovery of previously unreported interactions of well-known psychoactive substances with OCTs and PMAT might inspire the usage of compounds as potential scaffolds to develop new clinically useful drugs.

2. Results

Uptake inhibition assays were performed on human embryonic kidney 293 (HEK293) cells stably expressing hOCT1, hOCT2, hOCT3 or hPMAT to assess the compounds' potency to inhibit the uptake of 1-methyl-4-phenylpyridinium ([^{3}H]-MPP$^+$). We found several substances to be inhibitors of substrate uptake by hOCT1, and fewer interacted with hOCT2 and hPMAT, whereas interactions with hOCT3 were exceedingly rare (see Figure 1). In summary, only 1 compound (~6%) inhibited hOCT3, 5 compounds (~29%) inhibited hPMAT, 7 compounds (~41%) inhibited hOCT2, and 11 compounds (~65%) inhibited hOCT1 with an half-maximal inhibitory concentration (IC_{50}) value lower than 100 μM (see Figure 1B).

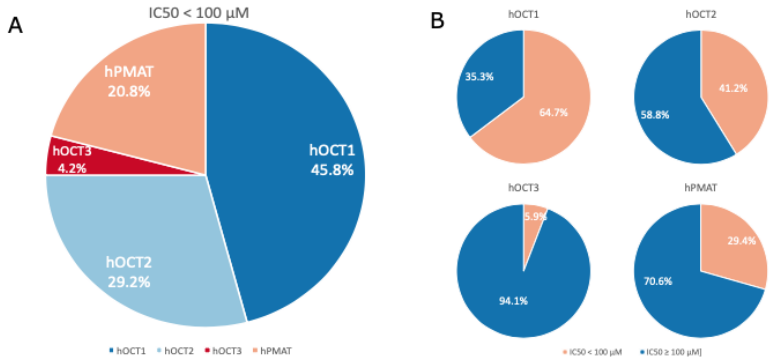

Figure 1. Graphical representation of the percentage of screened compounds inhibiting the substrate uptake of the transporters of interest with an half-maximal inhibitory concentration (IC_{50}) below 100 µM. (**A**): 45.8% of compounds inhibit hOCT1 (dark blue), 29.2% inhibit hOCT2 (light blue), 20.8% hPMAT (orange) and 4.2% inhibit hOCT3 (red). (**B**): 11 out of 17 compounds inhibit hOCT1, 7 compounds inhibit hOCT2, 1 compound inhibit hOCT3 and 5 compounds inhibit hPMAT.

The screened antidepressants had close to identical interaction profiles on all transporters (see Figure 2) with only hOCT1 uptake being inhibited at pharmacologically relevant concentrations, with IC_{50} values between 5.11 µM (95%-confidence interval (CI): 4.11–6.37) to 7.15 µM (95%-CI: 5.59–9.15) (Table 1). Interestingly, R-citalopram, S-citalopram and bupropione did not interact as potently with hOCT2 and hOCT3 (see Figure 2C,D). The examined antidepressants inhibited hPMAT with low potency at high micromolar concentrations (Figure 2E).

Table 1. Heat map portraying the inhibitory potency (in µM) of screened compounds at hOCT1–3 and hPMAT. Lower values are highlighted in red and higher in shades of blue (see legend).

Substance	Transporters			
	hOCT1	hOCT2	hOCT3	hPMAT
Bupropion	5.36			96.96
S-Citalopram	7.15			
R-Citalopram	5.11			58.32
Diazepam			44.46	29.81
Phenobarbital				
d-Amphetamine	8.39	2.21		71.77
Cocaine	6.66	27.80		
R-α-PVP	2.15	13.09		
S-α-PVP	1.07	15.02		
Ketamine		12.46		
2-Fluoro-deschloroketamin		19.18		
MDMA	1.14	2.71		7.77
Psilocin				
Tramadol	5.60			
O-Desmethyltramadol	24.16			
Metylphenidate	0.36			
Modafinil				
Decynium-22	2.66	4.56	0.16	0.35

IC_{50} [µM] 0 10 100

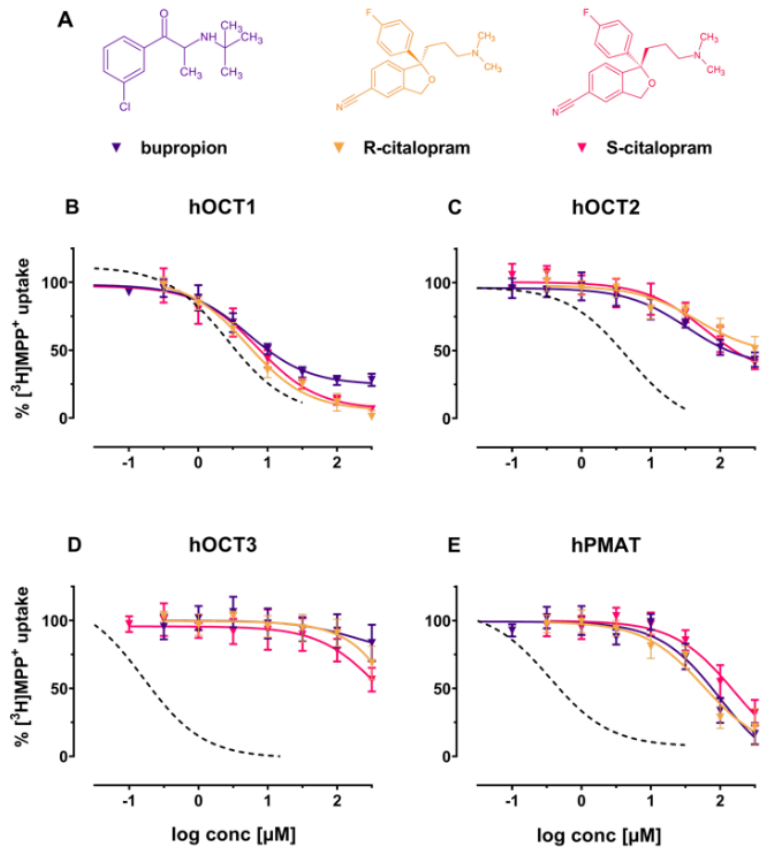

Figure 2. (**A**) Chemical structures of the herein investigated antidepressant compounds. From left to right: bupropion (violet), R-citalopram (yellow) and S-citalopram (red); Effects of the above-mentioned compounds (including decynium-22; dashed line) on (**B**) hOCT1, (**C**) hOCT2, (**D**) hOCT3 and (**E**) hPMAT of uptake of tritiated MPP^+ in HEK293 cells stably expressing the respective transporter.

Diazepam fully inhibited substrate uptake of all transporters (see Figure 3B–E), reaching half-maximal inhibition at 44.46 µM (95%–CI: 36.04–54.85) and 29.81 µM (95%-CI: 18.41–48.27) at hOCT3 and hPMAT, respectively, while weakly interacting with hOCT1 and hOCT2 ($IC_{50} > 100$ µM). Phenobarbital treatment induced increased uptake of substrate at increasing concentrations at hOCT1 and hOCT2, while not interacting with the other transporters at pharmacologically relevant concentrations.

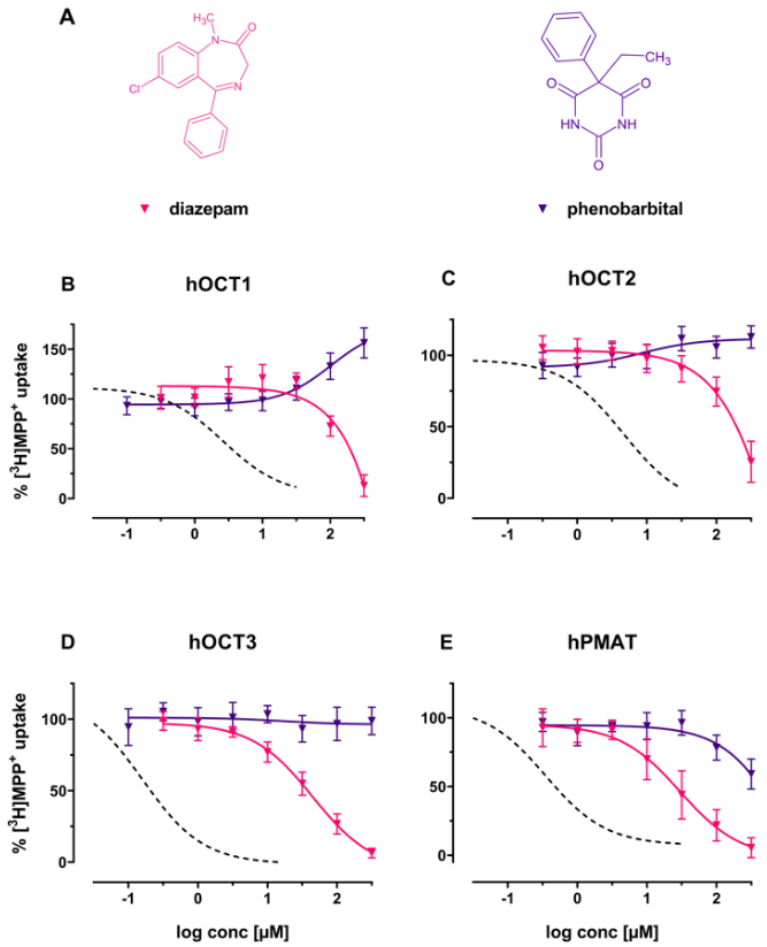

Figure 3. (**A**) Chemical structures of the herein investigated antiepileptics diazepam (red) and phenobarbital (violet); (**B**–**E**): Effects of the above-mentioned compounds (including decynium-22; dashed line) on (**B**) hOCT1, (**C**) hOCT2, (**D**) hOCT3 and (**E**) hPMAT of uptake of tritiated MPP^+ in HEK293 cells stably expressing the respective transporter.

The psychostimulants *d*-amphetamine, cocaine, R- and S-*α*-PVP showed similar inhibition profiles at hOCT1 and hOCT2 (Figure 4B,C) with IC_{50}s ranging from 1.07 (95%-CI: 0.81–1.40) to 15.02 µM (95%-CI: 11.28–20.00), with the exception of an IC_{50} of 27.80 µM (95%-CI: 19.17–40.32) of cocaine at hOCT2 (see Figure 4C). Furthermore, no pharmacologically relevant interactions with hOCT3 and hPMAT could be detected (Figure 4D,E). R- and S-*α*-PVP treatment lead to increased uptake of substrate at hPMAT (Figure 4E).

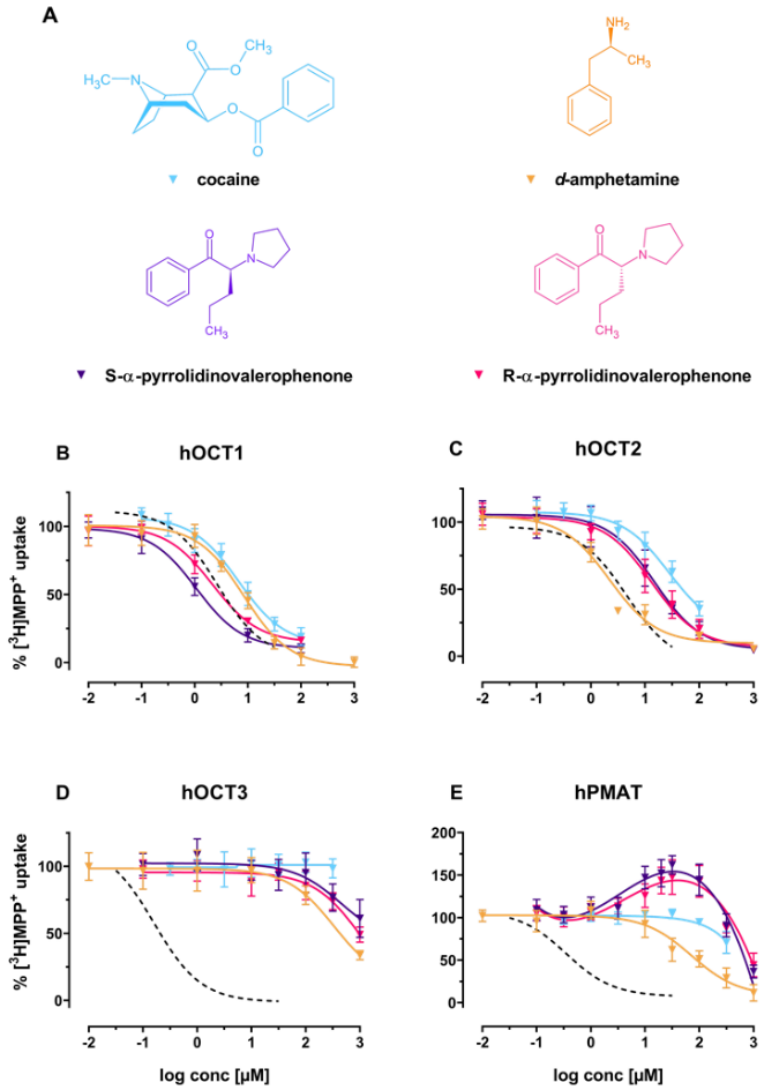

Figure 4. (**A**) Chemical structures of the herein investigated psychostimulants. From left to right: cocaine (blue), *d*-amphetamine (yellow), S-α-pyrrolidinovalerophenone (violet) and R-α-pyrrolidinovalerophenone (red); (**B**–**E**): Effects of the above-mentioned compounds (including decynium-22; dashed line) on (**B**) hOCT1, (**C**) hOCT2, (**D**) hOCT3 and (**E**) hPMAT of uptake of tritiated MPP^+ in HEK293 cells stably expressing the respective transporter.

While ketamine selectively and potently inhibited substrate uptake of hOCT2, its derivative 2-fluoro-des-chloroketamine is less potent, but similarly selective (IC_{50} of 12.46 (95%-CI: 9.71–15.98) and 19.18 µM (95%-CI: 14.89–24.70), respectively). Both compounds did not fully inhibit the other transporters at pharmacologically relevant concentrations. Psilocin, the active metabolite of psilocybin, did not inhibit any of the investigated transporters, but rather caused an increase in substrate uptake in the low micromolar range at the OCTs (see Figure 5B–D). 3,4-Methylenedioxymethamphetamine (MDMA) has high

substrate uptake inhibiting potencies at hOCT1, 2 (IC_{50} of 1.14 (95%-CI: 0.90–1.44) and 2.71 µM (95%-CI: 2.22–3.31) respectively) and hPMAT (IC_{50} of 7.77 µM; 95%-CI: 5.92–10.21), while not inhibiting uptake by hOCT3.

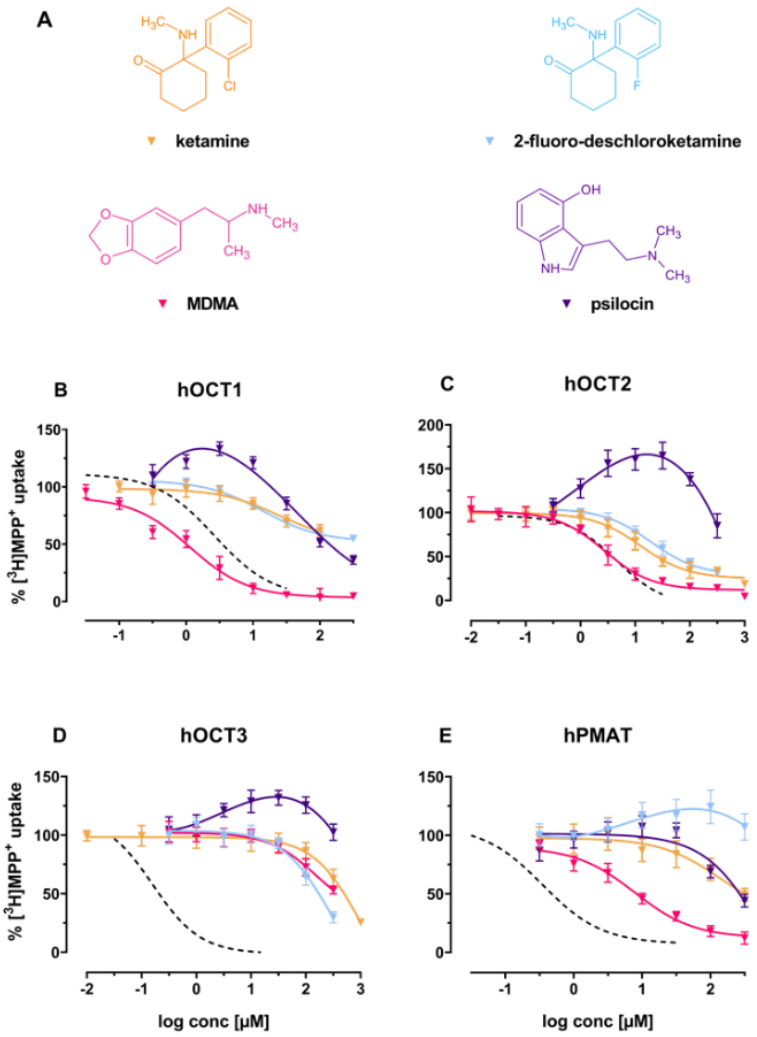

Figure 5. (**A**) Chemical structures of the herein investigated new therapeutics. From left to right: ketamine (yellow), 2-fluoro-deschloroketamine (blue; non-therapeutic derivative of ketamine), MDMA (red) and psilocin (violet): (**B**–**E**): Effects of the above-mentioned compounds (including decynium-22; dashed line) on (**B**) hOCT1, (**C**) hOCT2, (**D**) hOCT3 and (**E**) hPMAT of uptake of tritiated MPP^+ in HEK293 cells stably expressing the respective transporter.

Tramadol and its active metabolite *O*-desmethyltramadol both inhibit hOCT1 substrate uptake at low micromolar concentrations (IC_{50} of 5.60 (95%-CI: 4.65–6.75) and 24.16 µM (95%-CI: 19.12–30.54) respectively) (see Figure 6B). Tramadol also interacts with hOCT2 and hPMAT, but fails to fully inhibit the transporters (Figure 6C,E). No interaction of both tramadol and *O*-desmethylotramadol with hOCT3 was observed (Figure 6D).

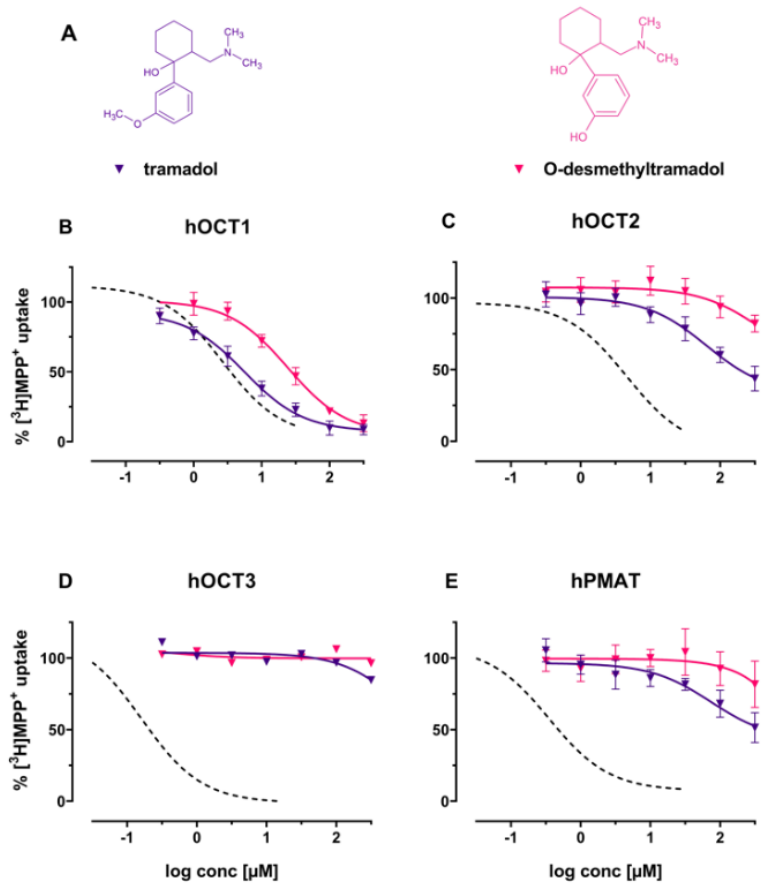

Figure 6. (**A**) Chemical structures of the herein investigated analgesics tramadol (violet) and *O*-desmethyltramadol (red); (**B**–**E**): Effects of the above-mentioned compounds (including decynium-22; dashed line) on (**B**) hOCT1, (**C**) hOCT2, (**D**) hOCT3 and (**E**) hPMAT of uptake of tritiated MPP^+ in HEK293 cells stably expressing the respective transporter.

As seen in Figure 7B–E, modafinil did not interact with hOCTs and hPMAT at pharmacologically relevant concentrations. In contrast, methylphenidate potently interacts with hOCT1 (Figure 7B) (IC_{50} of 0.36 µM; 95%-CI: 0.27–0.46), while not fully blocking any other investigated transporter (see Figure 7C–E).

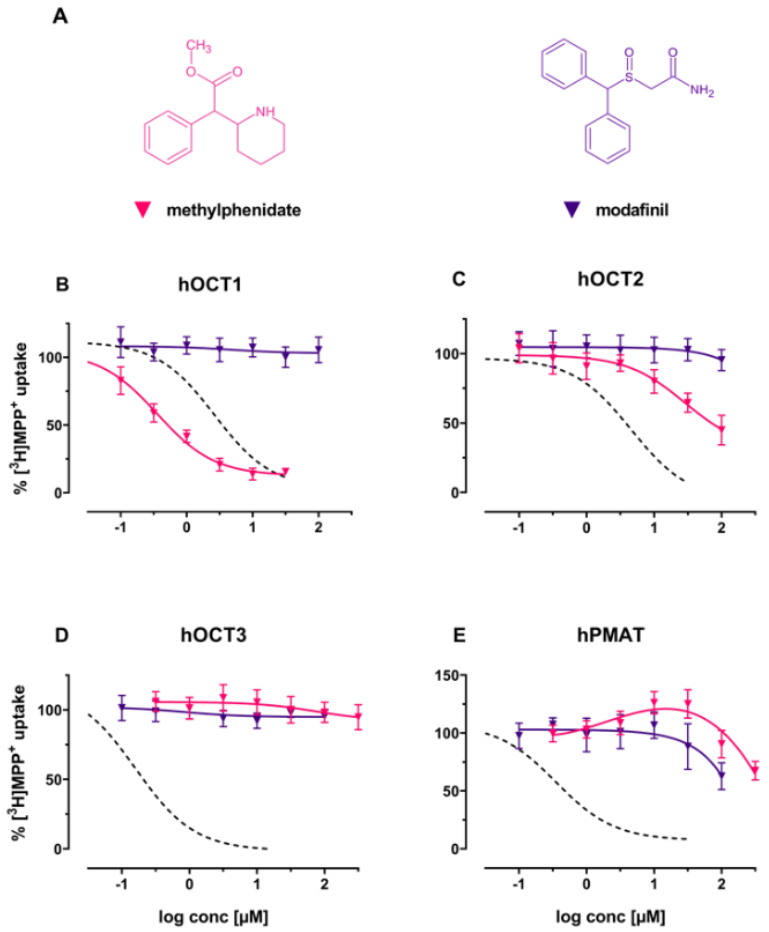

Figure 7. (**A**) Chemical structures of the herein investigated drugs to treat narcolepsy modafinil (violet) and ADHD methylphenidate (red); (**B**): Effects of the above-mentioned compounds (including decynium-22; dashed line) on (**B**) hOCT1, (**C**) hOCT2, (**D**) hOCT3 and (**E**) hPMAT of uptake of tritiated MPP^+ in HEK293 cells stably expressing the respective transporter.

3. Discussion

To date, many substances have been investigated concerning their interaction with the high-affinity, low-capacity SLC6 MATs due to their long history of use as clinically relevant drug targets, in addition to their well-established role in regulating the clearance of monoamines from the synaptic cleft and maintaining monoaminergic equilibrium in the CNS. However, a growing number of studies shows that the low-affinity, high-capacity organic cation transporters (OCT1–3), as well as the plasma membrane monoamine transporter (PMAT), are also distinctly involved in monoamine reuptake from the synaptic

cleft [11,25]. Furthermore, they transport and eliminate xenobiotics in the periphery, lending them pharmacological and clinical relevance [11,26–31].

Herein, we intended to systematically determine the interaction profiles of 17 psychoactive substances, including medical drugs, which have mostly been shown to interact with MATs, on hOCT1–3 and hPMAT [32–36]. The presented data thus fill a veritable research gap, since only a few of the compounds have previously been investigated concerning their interaction at the OCTs and PMAT (see Supplementary Table S1).

The first striking finding is that the various chemical classes of psychoactive compounds investigated herein predominantly interact with hOCT1, and less pronouncedly with hOCT2 and hPMAT. Previous studies have shown many medically relevant compounds, such as metformin, to interact with hOCT1, in turn influencing their pharmacokinetic fate [37]. Furthermore, polymorphisms of hOCT1 have been shown to influence substrate translocation and drug–drug interactions [38]. Overall, OCT1 polymorphisms seem to have potential clinical consequences, but further research needs to be undertaken [31]. In addition, FDA (Food and Drug Administration) and EMA (European Medicines Agency) recommend investigations of potential interactions with hOCT2 for drugs which are primarily renally excreted [39,40]. Thus, for compounds potently interacting with hOCTs and hPMAT, the occurrence of polymorphisms in patients, differently affecting pharmacodynamic and -kinetic fates and potentially the occurrence and severity of side-effects, must be considered in future investigations and substantiated clinically.

Currently, only few selective hOCT or hPMAT inhibitors are known, which can be explained by the high degree of sequence homology between transporters [25]. In particular, hOCT1 and 2 share approximately 70% amino acid sequence identity [2,14]. It is therefore not surprising to see an overlap of compounds interacting with hOCT1 and 2. Still, we located distinct differences between these two transporters regarding some of the compounds tested. Bupropion selectively interacts with hOCT1. This contradicts results reported in previous publications [16]. Diverging data between our results and those gathered from earlier studies might be explained by differences in experimental and laboratory conditions, as well as the established cell lines across studies [41]. In addition, tramadol, its metabolite *O*-desmethyltramadol, and both citalopram enantiomers were selective hOCT1 inhibitors. Strikingly, we found methylphenidate to potently and selectively interact with hOCT1, even more potent than the unspecific OCT and PMAT inhibitor decynium-22 [1]. Furthermore, ketamine potently interacts with hOCT2, while showing little activity at other hOCTs and hPMAT. Amphoux et al. (2006) reported similar results for the OCTs but did not investigate hPMAT [26]. This highlights the interesting finding that some compounds explicitly differ in their interactions with hOCT1 and hOCT2, despite the high degree of sequence homology between the two transporters. One possible explanation for this discrepancy could be differences between substrate binding sites. Amino acid residues that are critical for substrate specificity, which are localized in regions formed by transmembrane helices crucial for substrate binding, were found to differ between OCT1 and OCT2, and might explain variations in compound interactions with those transporters [42,43]. Still, the exact molecular basis for preference of compounds for hOCT1 or 2 has not been clearly established yet [2,14,44]. While we could not solve this gap in knowledge, our investigation led to the discovery of scaffolds that can be used for the development of selective inhibitors of OCTs, expanding pharmacological and structural understanding of this transporter family.

hOCT3 shares less sequence identity (50%) with the other OCTs and it has been previously shown that many unselective inhibitors and substrates of the organic cation transporters are least efficacious at hOCT3 [14]. In our study, diazepam not only proved to be a clearly more potent inhibitor of substrate uptake at hOCT3 when compared to hOCT1 and 2, but additionally, was the only herein tested substance to exhibit any pharmacologically relevant effect on substrate uptake by hOCT3. A previous study reported an IC_{50} of 2 µM for diazepam at hOCT3, which is much lower than the one we have measured (IC_{50} of 44.46 µM) [18]. The discrepancy may be explained by the different tracer used,

with Massmann and colleagues having employed fluorescent 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP^+), while we performed uptake inhibition assays with radioactively labelled MPP^+ [41]. In addition, diazepam relatively potently interacted with hPMAT, which was previously unreported.

In an investigation of α-pyrrolidinopropiophenone derivatives, we have previously observed that differences in aromatic ring substituents affected the hOCTs and hPMAT differently [22]. Differences in interaction with hOCT1 and 2 were marginal compared to their impact on hOCT3 and hPMAT. Here, we examined ketamine and its derivative 2-fluoro-deschloroketamine, again noticing only small differences caused by different aromatic ring substituents, although the potency to inhibit hOCT2 substrate uptake decreased for the derivative. This lack of sensitivity towards substituents of the aromatic ring differentiates hOCT1 and 2 from SLC6 MATs where, for example in the case of methcathinone analogs, fluorination of the aromatic ring improved SERT selectivity over DAT [35,45,46]. On the other hand, structural differences between tramadol and its main metabolite *O*-desmethyltramadol led to relevant changes in hOCT1 affinity, as tramadol inhibited substrate uptake of the transporter with a more than four-fold higher potency (IC_{50} of 5.60 µM) than *O*-desmethyltramadol (IC_{50} of 24.16 µM). Future studies need to focus on identified, potent chemical scaffolds and systematically investigate effects of substituents in larger-scale structure–activity relationship investigations.

To our knowledge, no previous studies have analyzed the effects of these compounds at human OCTs or hPMAT. Thus, we examined pharmacodynamic properties of S-citalopram and R-citalopram, as well as S- and R-α-PVP. Generally, we saw no distinct enantioselective differences in uptake inhibition between the two compounds on any transporter, emphasizing the robustness of OCTs and PMAT concerning drug–transporter interactions, which discerns them from MATs [23,47,48].

We observed the surprising phenomenon that treatment with higher concentrations of some compounds, most markedly phenobarbital, psilocin and α-PVP, led to elevated MPP^+ uptake at hOCT1, 2 and hPMAT. Consistent with this finding is a previous study of Ahlin and colleagues, who reported increased uptake after cell treatment with high concentrations of phenobarbital [49]. One possible explanation for this striking phenomenon might include allosteric effects on the orthosteric site of the transporter. Consequently, an allosteric ligand would change the conformational dynamics of the transporter protein and thereby regulate interaction between substrates and ligand binding site [50]. In line with this phenomenon, an earlier study detailed the existence of a high-affinity binding site at OCTs, proposing it to partake in the effectivity of MPP^+ uptake through positively affecting the transport executed by two low-affinity transporting sites [51]. However, further studies are needed to unveil the exact molecular mechanisms.

Traditionally, decyinum-22 was oftentimes used as a positive control due to its high inhibitory potency at all hOCTs and hPMAT. In the present investigation, we show multiple compounds to have similar or even lower IC_{50} values. *d*-Amphetamine proved to be a highly potent hOCT1 and 2 substrate uptake inhibitor with IC_{50} values in the low micromolar range, while not interacting with hOCT3 at concentrations lower than 100 µM, which is consistent with a previous study by Amphoux et al. (2006) [26]. Furthermore, in our experiments, MDMA was potently interacting with hOCT1 and 2, an aspect which is to some extent consistent with a previous study showing fairly low Ki values [26]. In addition, the compound potently interacted with hPMAT, an interaction which had not been previously described. The most striking finding is that methylphenidate potently and selectively inhibits substrate uptake at hOCT1 in the nanomolar range. Due to the facts that (i) similar values have been reported as IC_{50} values at the primary targets of methylphenidate, hDAT and hNET [52], and (ii) the interindividual variability concerning appropriate dosing and avoidance of toxicity is high [53], it is possible that hOCT1 polymorphisms, which have been reported to frequently occur in the general population [20], might affect the pharmacokinetic and pharmacodynamic fate of methylphenidate in vivo

in a clinically relevant manner, warranting further investigation in this particular case but also for other compounds potently interacting with hOCT1 and hOCT2 [31].

4. Materials and Methods

4.1. Chemicals and reagents

The compounds of interest were obtained from either Sigma-Aldrich (St. Louis, MO, USA) or LGC Standards (Teddington, UK). S- and R-α-PVP was graciously provided by the NIDA Drug Supply Program. 2-fluoro-deschloroketamine was kindly provided by S. Brandt. All other chemicals and cell culture supplies were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Sarstedt (Nuembrecht, Germany).

4.2. Cell Culture

HEK293 cells were transfected with a plasmid, encoding the transporter of interest and an N-terminal YFP-tag, carried out using jetPRIME© (Polyplus Transfection; (VWR International GmbH, Vienna, Austria)) reagent (for a 10 cm dish with 1–2 × 10^6 cells in 10 mL serum containing medium at 60–80% confluency: 500 µL jetPRIME© buffer, 10 µg DNA and 20 µL jetPRIME© reagent) and selection pressure maintained for two weeks [2,54,55]. Subsequently, 500,000 cells were FACS-sorted (fluorescence-activated cell sorting) according to expression level to establish cell lines stably expressing the protein of interest. Fluorescence microscopy images of cells expressing respective YFP-tagged transporters are seen in Supplementary Figure S1. Concentration dependent uptake of tritiated substrate by transiently transfected cell lines expressing hOCT1–3 and hPMAT is shown in Supplementary Figure S2. For uptake inhibition assays, cells were cultured at a subconfluent (80–90% density) state in high-glucose Dulbecco's Modified Eagle Medium (DMEM), enriched with 10% heat-inactivated Fetal Bovine Serum (FBS) and penicillin/streptomycin (PS; 100 U × 100 mL^{-1}, each). Selection pressure was maintained by the addition of geneticin (50 mg × mL^{-1}) at 37 °C and 5% CO_2 in a humidified incubator. At a density of 60,000 cells per well, HEK293 cells expressing the respective transporter were seeded onto poly-D-lysine-coated wells 24 h prior to uptake inhibition experiments in a final volume of 200 µL per well.

4.3. Uptake Inhibition Assays

Prior to the addition of the respective compounds, DMEM was removed from all wells and replaced with 200 µL of Krebs-HEPES-buffer (KHB; 10 mM HEPES, 120 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgSO_4$ and 20 mM D-glucose, pH adjusted to 7.3) at room temperature. Cells were exposed to a pre-incubation solution containing the compound of interest in the respective concentration (dissolved in Krebs-HEPES-buffer) for 10 min with a total volume of 50 µL, which was then replaced with a 50 µL uptake solution containing 0.05 µM of tritiated 1-methyl-4-phenylpyridinium ([^{3}H]MPP^+) as substrate, and the compound of interest in respective concentrations, for 10 minutes each. Finally, uptake was terminated by washing with 200 µL of ice-cold (4 °C) KHB, after which cells were lysed with 200 µL of 1% sodium dodecyl sulphate (SDS). After transferring the solution of each well into 6 mL counting vials containing 2 mL of scintillation cocktail, the vials were measured with a beta-scintillation counter for quantification of the uptake of tritiated substrate. Decynium-22 (D22) shows robust inhibitions at low micromolar concentrations at all hOCTs and at hPMAT ranging between 0.16 µM at hOCT3 and 4.56 µM at hOCT2. D22 is depicted as a reference inhibition curve (dotted line) in all Figures 2–7. The structural formula can be seen in Figure 8. To obtain specific data, non-specific uptake was assessed in the presence of 100 µM D22 and subtracted from the total data. The 100% values were defined as uptake in the absence of a tested substance, defining maximal uptake capacity. Inhibitions of the compound of interest were described as a percentage of the maximal uptake capacity. While uptake inhibition assays are an effective tool for investigating the interaction of compounds with transporters of interest, they are not able to distinguish substrates from inhibitors, per se. Thus, we cannot rule out that some of the investigated

compounds are substrates rather than inhibitors. Future studies are needed to investigate this uncertainty.

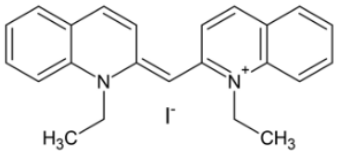

Figure 8. Chemical structure of decynium-22 (D22). D22 was used in all subsequent graphs as a highly potent transporter inhibitor for the assessment of unspecific uptake of the tritiated substrate $[^3H]$-MPP$^+$.

4.4. Data and Statistical Analysis

Half-maximal inhibitory concentration (IC_{50}) values of each substance were calculated and plotted using GraphPad Prism 8.4.3 (GraphPad Software Inc., San Diego, CA, USA). IC_{50} was determined by non-linear regression, solving the equation $Y = Bottom + (Top - Bottom)/(1 + 10^{X-LogEC_{50}})$. Data which displayed an increased uptake at higher concentrations was fit as a third-order polynomial, solving the equation: $Y = B0 + B1 * X + B2 * X^2 + B3 * X^3$. All data stem from a minimum of three separate experiments ($n \geq 3$) executed in triplicates and are shown as mean ± SD. All colors were chosen to be color-blind-friendly using a color brewer [56].

5. Conclusions

The investigation of the inhibitory potential of substances acting on the central nervous system has revealed some of them to inhibit the substrate uptake of human low-affinity organic cation transporters (OCT1–3) and PMAT in sub-micromolar or a low micromolar concentration. Bupropion and methylphenidate were found to be potent selective inhibitors of substrate uptake at hOCT1, with the latter interacting with hOCT1 more potently than the well-established OCT blocker decynium-22. MDMA was found to interact with hOCT1 and 2 more potently than decynium-22, as was *d*-amphetamine at hOCT2. Ketamine showed interaction selectivity and high uptake inhibitory potential at hOCT2. Further, tramadol, its main metabolite *O*-desmethyltramadol, and both citalopram enantiomers are selective inhibitors of the substrate uptake at hOCT1. Diazepam was the only tested compound to interact with hOCT3. We have found only small differences in the interaction caused by different aromatic ring substituents, as exemplified by ketamine and its derivative, 2-fluoro-deschloroketamine. Notably, we saw no enantioselective effect of S-citalopram and R-citalopram, or S- and R-α-PVP, accentuating the robustness of human OCTs and hPMAT. Considering the growing interest in pharmacological interaction of compounds with human OCTs and PMAT, our study provides important information regarding the complex interaction between a range of CNS-active substances and low-affinity transporters, suggesting some clinically relevant drugs as lead structures for the development of more selective inhibitors of these relatively understudied transporters [2,57].

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms222312995/s1.

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Supplementary Material

Interaction Profiles of Central Nervous System Active Drugs at Human Organic Cation Transporters 1–3 and Human Plasma Membrane Monoamine Transporter

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Supplementary Materials

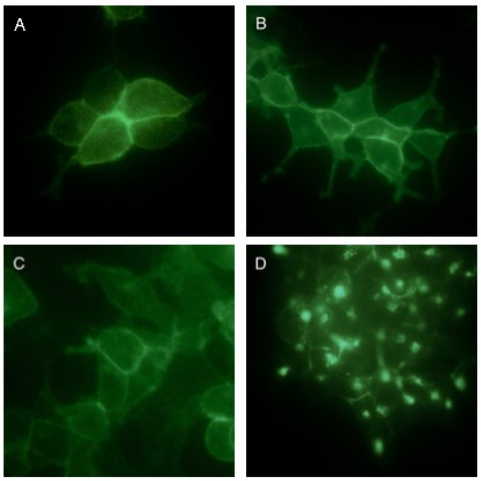

Figure S1. Microscopy images depicting cells expressing YFP-tagged transporters of interest: (**A**): hOCT1, (**B**): hOCT2, (**C**): hOCT3 and (**D**): hPMAT.

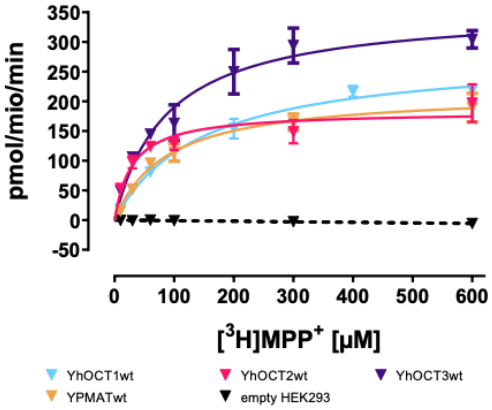

Figure S2. Representative experiments depicting concentration dependant uptake of tritiated MPP^+ by the transporters (hOCT1 light blue, hOCT2 red, hOCT3 violet and hPMAT orange, all transiently transfected) of interest and empty HEK293 cells (dashed line) as control.

Table S1. IC_{50} comparison table. Red boxes indicate that no respective data for comparison was found. n.s. = non-significant. Red text color: experiments were performed with ASP^+ as substrate. Orange text color: values show the percentage of inhibition at 100 µM of the respective compound. Blue text color: Ki (µM) values were measured.

	Substance	hOCT1		hOCT2		hOCT3		hPMAT	
		IC_{50} literature	IC_{50} [µM] (95%-CI)	IC_{50} literature	IC_{50} [µM] (95%-CI)	IC_{50} literature	IC_{50} [µM] (95%-CI)	IC_{50} literature	IC_{50} [µM] (95%-CI)
Antidepressants	Bupropion	161.00 [1]	5.36 [4.19–6.85]	28.60 [1] 32.20 [2]	32.86 [21.55–50.10]	738.00 [1]	185.60 [9.36–3681]	115.00 [3]	96.96 [56.01–167.80]
	S-Citalopram		7.15 [5.59–9.15]		59.90 [34.26–104.70]		n.s.		159.80 [83.29–306.50]
	R-Citalopram		5.11 [4.11–6.37]		46.63 [25.56–85.07]		n.s.		58.32 [37.46–90.79]
	Citalopram	13.2 [1]		115.00 [1]		188.00 [1]		116.67 [4]	
Antiepileptics	Diazepam	5.0% [5]	n.s.		917.30 [229.70–3663]	2.00 [6] 40.00 (mOCT3) [6]	44.46 [36.04–54.85]		29.81 [18.41–48.27]
	Phenobarbital	−6.0% [5]	n.s.		n.s.		n.s.		1027 [39.71–26584]
Psychostimulants	*d*-Amphetamine	202 ± 68 [7] 96.7 ± 37 [8]	8.39 [6.90–10.20]	10.5 ± 2.6 [7] 20.30 ± 16.9 [8]	2.21 [1.70–2.87]	460 ± 140 [7] 363 ± 56.4 [8] 42 ± 7 [9] 41.5 ± 7.5 (DA) 24.1 ± 70 (5-HT)[10] n.a [11]	336.6 [179.70–630.40]		71.77 [46.81–110.00]
	Cocaine	85 ± 22 [7]	6.66 [5.28–8.39]	113 ± 21 [7]	27.80 [19.17–40.32]	n.a. [7] [12]	n.s.		n.s.
	α-PVP	1.31 [13]		5.62 [13] 17.5 ± 2.5 [14]		1631 [13] >1000 [14]		13.42 [13]	
	R-α-PVP		2.15 [1.59–2.90]		13.09 [9.89–17.32]		1016 [364.90–2826]		n.s.
	S-α-PVP		1.07 [0.81–1.40]		15.02 [11.28–20.00]		456.7 [144.90–1439]		n.s
Emerging Therapeutics	Ketamine	114.5 ± 43.7 [7] 73.9 ± 15.2 (K_m) [15]	23.47 [13.34–41.37]	22.7 ± 6.3 [7] 33.5 ± 20.3 (K_m) [15]	12.46 [9.71-15.98]	225.7 ± 65 [7] 440 [6] 52.9 ± 15.0 (K_m) [15]	1028 [589.40–1792]		134.60 [54.70–331.20]
	2-Fluoro-deschloroketamine		14.07 [10.11–19.56]		19.18 [14.89–24.70]		265.5 [167.60–420.50]		n.s.
	MDMA	24.20 ± 9.20 [7]	1.14 [0.90–1.44]	1.63 ± 0.62 [7]	2.71 [2.22–3.31]	73.60 ± 50.30 [7]	153.90 [87.41–270.90]		7.77 [5.92–10.21]
	Psilocin		63.48 [44.97–89.62]		n.s.		n.s.		698.20 [88.81–5488]
Analgesics	Tramadol	60.2% [5] 30 [16]	5.60 [4.65–6.75]		64.10 [39.58–103.80]		n.s.		74.19 [32.67–168.50]
	O-Desmethyl tramadol	172 [16]	24.16 [19.12–30.54]		371.90 [44.37–3116]		n.s.		n.s.
Drugs for Narcolepsy and ADHD	Methylphenidate		0.36 [0.27–0.46]		28.80 [15.11–54.90]		n.s.		n.s.
	Modafinil		n.s.		n.s.		n.s.		n.s.
Control	Decynium-22		2.66 [2.049–3.44]		4.56 [3.786–5.496]		0.16 [0.1358–0.1904]		0.35 [0.2439–0.5042]

Experiments with MPP^+	Experiments with ASP^+	% inhbition at 100 µM	Ki (µM) Values	no data available

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III. Discussion

The high-affinity and low-capacity Na^+- and Cl^- -dependent monoamine transporters for serotonin (SERT; SLC6A4), dopamine (DAT; SLC6A3) and norepinephrine (NET; SLC6A2) are part of the solute carrier 6 (SLC6) gene family and are well known for their essential role in the regulation of neurotransmitter signaling and homeostasis by modulating uptake of released neurotransmitters in the synaptic cleft. They are widely expressed in the mammalian brain and regulate essential functions such as motivation, reward, memory and sleep (Ursin, 2002; Wise, 2004). Disturbed function can lead to a plethora of diseases, including Parkinson's disease, ADHD, Schizophrenia, and major depressive disorder (Kristensen et al., 2011). Therefore, theses transporters have been heavily investigated and are targets of a wide range of therapeutic drugs. Furthermore, they are prime targets of psychoactive drugs, as their modulation can lead to altered synaptic neurotransmitter concentrations. However, a growing number of literatures is focusing on the heterologous MFS system, which, in contrast to the SLC6 transporters, is comprised of low-affinity and high-capacity transporters. This group includes the facilitative organic cation transporters 1-3 from the SLC22 family (OCT1-3; SLC22A1-3, respectively). In addition to their role in the central nervous system, they are thought to play a major role in the elimination of peripheral endogenous compounds and xenobiotics (Amphoux et al., 2006; Goswami et al., 2014; Koepsell, 2020; Sala-Rabanal et al., 2013; J. Wang, 2016).

In the present study we chose 17 psychoactive substances, including medical drugs, that have previously been shown to interact with MATs and investigated their inhibitory properties of the substrate transport at hOCT1-3 and hPMAT. Since only few of the herein presented substances have been previously respectively investigated, our data fills a veritable research gap, incorporating the low-affinity and high-capacity transporters into the picture of the function of psychoactive substances.

In early studies, the catecholamine isoprenaline and its O-methylated metabolite were used as substrate and inhibitor, respectively, as both substances do not interact with SLC6 monoamine transporters (Bönisch, 1978). These compounds are protonated at physiological pH levels and thus behave as an organic cation. Nowadays, permanently protonated substrates are used, such as 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP^+), metformin, tetraethylammonium (TEA+), and MPP^+ (Gorboulev et al., 1997; Sandoval et al., 2018). ASP^+ is a useful and widely

used substrate as it allowes measurement via florescence (Stachon et al., 1996). However, most of the used substrates are either 3H- or 14C-labelled compounds, as is the herein used substrate MPP^+. Additionally, liquid chromatography and mass spectrometry (LS-MS/MS) can be used to measure transporter-mediated intracellular accumulation of a compound of interest. The selection of different substrates when screening for inhibitors might result in diverging IC_{50} values, as is compared in detail in the supplementary table S1. Distinct substrates might have different apparat affinities at the respective transporter resulting in potentially diverging results (Hacker et al., 2015; Thévenod et al., 2013). This can be seen for example when comparing the IC_{50} values of tramadol at hOCT1 with MPP^+ and ASP^+. The apparent affinity of MPP^+ is significantly higher than ASP^+ (Km values of 15 and 1, respectively) towards hOCT1, potentially reducing the sensitivity of the transporter to inhibitors (IC_{50} values of tramadol of 5.6 and 30 µM) (Biermann et al., 2006; Mehrens et al., 2000; Nies, Koepsell, et al., 2011; Tzvetkov et al., 2011). Other reasons for the differences in previously published results might be the usage of different cell lines aswell as diverging methodology for performing uptake inhibition assays (Ilic et al., 2020). Such methodical differences might be seen when comparing our results to those of a previous publication, that reported significantly higher IC_{50} values for bupropion at hOCT1 (5.36 and 160 µM). However, in this case the authors used a different concentration of D22 for assessing non-specific uptake (30 instead of 100 µM), had an extended pre-incubation time of 15 min, and most importantly used a reduced substrate ($^{[3H]}MPP^+$) concentration of 25 instead of 50 nM (Haenisch et al., 2012). Species specific transporters might also interact differently with a given compound, as can be seen with diazepam at the human and mouse OCT3, with IC_{50} values of 2 and 40 µM (Massmann et al., 2014). A recent study by Gorboulev at al. compared various assay conditions and parameters and found that experimental conditions have a dramatic impact on measured affinities of substrates and inhibitors (Gorboulev et al., 2018). Employing mutagenesis and modelling of the tertiary structure of the rat OCT1 several amino acids in the 4^{th}, 10^{th}, and 11^{th} transmembrane helix were identified to be involved in substrate and/or inhibitor binding (Gorboulev et al., 2005; Popp et al., 2005; Sturm et al., 2007; Volk et al., 2009). Theses amino acids are localized in central large cleft that either is in the inward or outward facing conformation and harbors multiple high- and low-affinity substrate and/or inhibitor binding sites (Gorbunov et al., 2008; Minuesa et al., 2009; Popp et al., 2005; Volk et al., 2009). Variants might not only directly interferer with the binding affinities of the substrate and/or inhibitor but might also lead to structural alteration that can change the binding affinities. Keller at al. proposed the existence of two low-affinity MPP^+ binding sites that are

involved in MPP^+ transport and one additional high-affinity MPP^+ site that participates in the transport indirectly by mediating allosteric effects on the two low-affinity MPP^+ binding sites. Inhibitors might not only bind to the central substrate binding pocket, but also to more peripheral regions. In addition, the existence of multiple substrate and inhibitor binding sites and their interactions might explain why there are often large differences in IC_{50} values while using different substrates (Nies, Koepsell, et al., 2011). Another important detail that must be addressed, is the difficulty to distinguish weather a compound that inhibits a given transporter is translocated or not. This might be due to low transport rates, diverging expression rates *in vivo*, and due to potential different affinities of the control substrate to other substrates (Nies, Koepsell, et al., 2011). Further research must be done to fully uncover the transport and inhibition mechanism of OCTs, for example by obtaining its high-resolution crystal structure.

One of the most notable findings of this study is that the majority of the herein screened psychoactive compounds interact with the hOCT1, hOCT2 and hPMAT, although the latter two to a lesser extent, and only one compound showed interactions with hOCT3. Given their broad interaction profiles, we expected the majority of compounds to interact with hOCT1 and to a lesser extend hOCT2, as previous reports have shown that the majority of clinically relevant compounds interact with these two transports (Gustav Ahlin et al., 2008; Arimany-Nardi et al., 2016). As introduced earlier, a well-studied example in this regard is metformin. This example not only emphasizes the importance of further research regarding the interactions of drugs at OCT1-3 and PMAT, but also the impact of polymorphisms. Previous studies have demonstrated population specific presence of polymorphisms of these transporters and their impact on drug absorption and distribution and drug-drug interactions (DDI) in general. For example, Arimany-Nardi et al. reported that single nucleotide polymorphisms at hOCT1 results in a reduced uptake capacity of lamivudine, a drug used in the treatment of HIV infection. Furthermore, co-administration of other antiretroviral drugs, i.e. abacavir, prompted variable inhibition of lamivudine (Arimany-Nardi et al., 2016). A different study has reported inhibited metformin uptake at hOCT1 and other OCTs by proton pump inhibitors (Nies, Hofmann, et al., 2011). An even further decreased transport capacity was reported in some hOCT1 variants, especially in M420del. This suggests that individuals carrying variants can have an increased risk for DDIs (G. Ahlin et al., 2011). Additionally, a study found that amino acid variation may participate in the binding of only some compounds (Popp et al., 2005). These are just some of many drug-drug interaction examples of OCT1-3 and PMAT, which highlight their clinical and pharmacological importance (Mato et al., 2018; Zamek-

Gliszczynski et al., 2018; S. Zhou et al., 2021). However, the majority of reported DDIs are based on *in vitro* and animal studies. The possibility of different transporters playing a role in the pharmacokinetic of compounds has yet to be evaluated (S. Zhou et al., 2021). This gap can mainly be ascribed to the limited availability of tools to probe OCT activity in humans. Thus, further studies are needed to fully elucidate and potentially eliminate DDIs in the future and to address the potential clinical consequence of OCTs and PMAT. Therefore, the investigation of putative substrates and inhibitors is of critical importance. This has also been addressed by the FDA (Food and Drug Administration) and EMA (European Medicine Agency). They have recognized the importance of hOCT2 as it is a major player in the renal elimination of drugs and therefore a potential regulator of a drug's pharmacokinetics (EMA, 2012; FDA, 2020). Additionally, interindividual differences in patience carrying polymorphisms must be further investigated in order to further understand their effect on pharmacokinetic and -dynamics, as this can lead to adverse effects (Gorbunov et al., 2008; Zolk, 2012).

Interestingly, most compounds that did not show any relevant interactions at hOCT1 are substances from the group of new therapeutics, namely ketamine, its derivate 2-fluorodeschloroketamin, and psilocin. Although MDMA, also a member of this group, shows one of the most potent interactions alongside S-alpha-PVP and methylphenidate at hOCT1, setting aside D22. All of these compounds have surprisingly shown very potent interactions at hOCT1, with IC_{50} values surpassing that of the prominently known OCT1-3 and PMAT inhibitor decynium-22 (D22) (Engel et al., 2004; Hayer-Zillgen et al., 2002). It must be noted that we refer to IC_{50} values as pharmacologically relevant if they fully inhibit the transport below a threshold of 100 µM. A compound's interaction profile may have an IC_{50} below 100 µM, but maximally inhibiting only to about 50 percent of total uptake capacity. This is the case, for example, of the antidepressants at hOCT2. All three compounds, bupropion, R-citalopram, and S-citalopram showed reduced MPP^+ uptake with increasing concentration but did not manage to fully inhibit the transport at any measured concentration.

One of the major obstacles in determining the contribution of an individual transporter to cerebral functions is the overlapping expression and selectivity. One mechanism to overcome this issue is the production of knockout strains, decreasing transport expression by antisense, and/or inhibition by the application of a selective inhibitor. Unfortunately, only a handful of such selective inhibitors are currently available. One example is corticosterone, as it shows a 8.7 fold higher inhibitory potency (IC_{50}) for hOCT3 compared to the hOCT1, hOCT2, and PMAT (Engel & Wang, 2005;

Hayer-Zillgen et al., 2002). However, corticosterone was found to have no significant difference at inhibiting the rat organic cation transporter 2 and 3 (X. Wu et al., 1998). Interestingly, the human and the rodent transporters share about 80% sequence homology (Maier, Niello, et al., 2021). Lopinavir, a HIV protease inhibitor, was found to be a selective hPMAT inhibitor (Duan et al., 2015). One possible explanation for the difficulty finding selective inhibitors might be the high degree of sequence homology between the transporters. This is especially the case for hOCT1 and hOCT2, as they share about 70 percent amino acid sequence identity. hOCT1 and hOCT2 are also about 50 percent identical to hOCT3 (Maier, Niello, et al., 2021). These overlapping interaction profiles are also to some extend reflected by this study. From the eleven compounds that interacted with hOCT1 about 60 percent also showed interaction with hOCT2, and none of the compounds investigated herein that interacted with hOCT3 also showed reducing uptake capacities at hOCT1 or hOCT2. These diverging interactions between OCT1/2 and OCT3 have been reported previously. OCT3 is thought to be more heterologous towards the other SLC22 members (Sala-Rabanal et al., 2013). Diazepam is an example of this, as it shows moderate interactions at hOCT3 with an IC_{50} value of 44.46 µM, but no interaction at hOCT1 and hOCT2. Apart from this, diazepam was the only compound tested herein that showed any pharmacologically relevant interactions with hOCT3. Previous studies have reported higher inhibitory potency of diazepam at hOCT3 with an IC_{50} of 2 µM (Massmann et al., 2014). As discussed earlier, the different substrate used in this study (ASP^+) might account for the diverging results. In this study, we found that bupropion selectively interacts with hOCT1 with an IC_{50} of 5.36 µM. Another selective hOCT1 inhibitor that we found is tramadol and its derivate O-desmethyltramadol with IC_{50} value of 5.60 and 24.16 µM, respectively. Additionally, both citalopram enantiomers, S-citalopram, and R-citalopram, were selective for hOCT1. We also report that ketamine and its derivate 2-fluorodeschloroketamin are selectively interacting with hOCT2. Despite the overlapping interaction profiles, we still found pharmacologically relevant differences between hOCT1 and hOCT2 regarding IC_{50} values. This highlights the interesting finding that explicit differences in interaction profiles between highly homologous low-affinity transporters are possible. As discussed earlier, despite the high degree of amino acid sequence homology, the complex tertiary structure of the OCTs might hold unexpected mechanisms, such as unknown substrate and/or inhibitor binding sites, that allow diverging interaction profiles between OCT1 and OCT2. (Koepsell, 2018; Nies, Koepsell, et al., 2011).

One of the most interesting findings of this study is the selectivity with which methylphenidate interacts with hOCT1, moreover it does this with an even higher affinity than decynium-22 (D22). With an IC50 of 0.36 µM, methylphenidate was the most potent substance at hOCT1 that was screened in this study. Similar low nanomolar values have been reported at methylphenidates primary high-affinity transporter targets, hDAT and hNET (Luethi et al., 2018). Additionally, when methylphenidate is used therapeutically, high interindividual variably in the dose-response relationship has to be taken into account (Kimko et al., 1999). A possible explanation for this might be the previously reported high frequency of hOCT1 polymorphisms that occur in the general population (Tzvetkov et al., 2011). This could affect the pharmacokinetic and pharmacodynamic fate of methylphenidate resulting in an increased risk of toxicity. Further investigations are essential not only in this case but also for other interactions at the low-affinity transporters.

Due to its high inhibitory potency for all hOCTs and hPMAT, D22 is often used as a positive control. However, in the present investigation we found multiple compounds that have similar or even lower IC_{50} values. hOCT1 showed interactions at low nanomolar concentrations with both PVP enantiomers, MDMA and methylphenidate, with IC_{50} values of 2.15, 1.07, 1.14 and 0.36 µM. By comparison, decynium-22 has a half-maximum inhibitory concentration at hOCT1 of 2.66 µM. We found d-amphetamine and MDMA to also inhibit MPP^+ uptake of hOCT2 with a lower IC_{50} value than D22. This is consistent with previous reports (Amphoux et al., 2006). However, we report MDMA also potently interacting with hPMAT, which to our knowledge has not been shown to date.

Previous investigations have shown that α-pyrrolidinopropiophenone (α-PVP) derivates interact differently with hOCTs and hPMAT, depending on their aromatic ring substituents (Maier, Rauter, et al., 2021). These differences had a higher impact on hOCT3 and hPMAT compared to hOCT1 and hOCT2. We here investigated ketamine and its derivate 2-fluorodeschloroketamin and found only marginal difference in the chlorine to fluorine substitution regarding their interactions with hOCT2. This lack of sensitivity differentiates hOCT1 and hOCT2 from the SLC6 transporters. For example, fluorination of the aromatic ring of methcathinone improved the selectivity of SERT over DAT (Eshleman et al., 2017; Niello et al., 2019; Rickli et al., 2015). However, we also show that tramadol and its derivate O-desmethyltramadol differ in their interaction at hOCT1, with a more than four-fold higher affinity for tramadol. This potentially indicates that methylation and extension of aromatic ring substituents might impact the affinity toward OCT1. A recent high-

throughput study tried to identify OCT3 inhibitors by screening a compound library of 2556 prescription drugs (E. C. Chen et al., 2022). As of today, only a few chemical characteristics are known to potentiate a given substance's inhibitory potency. Such criteria include the existence of at least one positive charge at physiological pH and a high degree of hydrophobicity (Bednarczyk et al., 2003; Engel & Wang, 2005; Sala-Rabanal et al., 2013). It was shown that an increasing alkyl chain length in n-tetraalkylammonium structures leads to increased potency at hOCT1, potentially due to the increased hydrophobicity (Bednarczyk et al., 2003; Sala-Rabanal et al., 2013). When investigating α-PPP and α-PVP derivates, Maier et al. were able to demonstrate that an increased carbon side chain length also led to an increased inhibition (Maier, Rauter, et al., 2021). To fully understand the mechanisms of inhibition and the compound's structural-activity relationship, further large-scale investigation must be performed. Additionally, high-resolution crystal structures of OCTs and PMAT might help in this regard.

We herein additionally investigated the enantiospecific effect of α-PVP on the interaction profile at the hOCTs and hPMAT. Furthermore, we also investigated the enantiomers of citalopram, R- and S-citalopram. In this regard we found no distinct enantiospecific effect in uptake inhibition in both cases. This highlights the robust nature of the OCTs and PMAT regarding their drug interactions, separating them from the MATs (Maier et al., 2018; Niello, Cintulová, et al., 2021; Niello, Sideromenos, et al., 2021).

Interestingly, we observed that some compounds elicited an increased MPP^+ uptake at higher concentrations. This phenomenon most markedly occurred with both α-PVP enantiomers at hPMAT, phenobarbital at both hOCT1 and hOCT2 and at all herein investigated transporters with psilocin. This increasing uptake effect was reverted at higher concentrations with all mentioned compounds, except for phenobarbital. However, increasing its concentration surpassing those assessed here might also result in a decrease in substrate uptake. This effect of an increased substrate uptake might be best explained by the possibility of allosteric binding sites that induce a change in the substrate affinity of the orthosteric site upon compound binding and therefore potentially cause an increased uptake rate (Changeux & Christopoulos, 2016; Leach et al., 2007). This possibility to modulate the function and activity of the orthosteric site by the allosteric site might be either through direct transmission of a positive or negative effect, or by modulation the physiochemical kinetics that are needed to overcome the energetic barrier in order to transport the substrate (Motlagh et al., 2014; Niello et al., 2020).

To conclude, we performed uptake inhibition assays with a set of psychoactive compounds at the low-affinity and high-capacity transporters, hOCT1, hOCT2, hOCT3 and hPMAT with MPP^+ as substrate. Our results show that none of the compounds screened herein interacted with hOCT3, except for diazepam. This potentially highlights the involvement of hOCT1, hOCT2 and hPMAT in the mechanism of action of central nervous system active drugs. However, one must keep in mind that the IC_{50} values assessed herein do not implicate the mechanism of substrate transport inhibition, such as the distinction between a competitive, non-competitive, or mixed inhibition. The drug's inhibitory potential additionally depends on the transported substrate, as this can lead to differences in IC_{50} values (Belzer et al., 2013; Gorboulev et al., 2018; Koepsell, 2020). Furthermore, to fully understand the role of the low-affinity transporters in pharmacokinetics, treatment efficacies, and potential drug toxicity, additional investigations are needed, as many questions remain unanswered. For example, how co-localization of other transporter with overlapping substrate selectivity affect the substrate's kinetics. Clinically relevant drug concentrations must be determined in addition to their passive diffusion across the plasma membrane. Future studies in computational modeling of the transporter could help understand the molecular mechanism of OCTs and PMAT transport and inhibition, as has been done with other transporter types (Matsson & Bergström, 2015). These findings, in addition to high resolution structure data for each transporter in relevant transport cycle states, with and without inhibitors, will potentially elucidate detailed transport mechanisms and inhibitor action.

This study advanced our knowledge of the pharmacological interactions between clinically relevant central nervous system active compounds at the human organic cation transporters 1-3 and the human plasma membrane monoamine transporter. The herein presented findings can lead to the identification of potential chemical scaffolds for the development of novel or improved medical drugs, perhaps customized for patients carrying certain OCT and/or PMAT polymorphisms. This research might give impetus for future investigations in the young but promising field.

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