CHARLES UNIVERSITY
FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ
DEPARTMENT OF ORGANIC AND BIOORGANIC CHEMISTRY

SORBONNE UNIVERSITY
FACULTY OF SCIENCE AND ENGINEERING
LABORATORY OF BIOMOLECULES

PREPARATION AND EVALUATION OF NEW LIGANDS TARGETING ORGANIC CATION TRANSPORTERS IN THE CENTRAL NERVOUS SYSTEM FOR THE TREATMENT OF DEPRESSION

PŘÍPRAVA A HODNOCENÍ NOVÝCH LIGANDŮ TRANSPORTÉRŮ ORGANICKÝCH KATIONTŮ V CENTRÁLNÍM NERVOVÉM SYSTÉMU PRO LÉČBU DEPRESE

DIPLOMA THESIS

SUPERVISOR: Assoc. Prof. PharmDr. Jaroslav Roh, Ph.D.

SPECIALIZED SUPERVISORS: Nicolas Pietrancosta, Ph.D.
Sophie Gautron, Ph.D.

HRADEC KRÁLOVÉ 2021 ANDREA MONTEIRO

Author’s Declaration:

“I hereby declare that this diploma thesis is my original copyrighted work. All the literature and other resources used for writing have been acknowledged using explicit references. I further affirm that I have not previously used this work, or any version of it, to obtain any other degree.”

Hradec Králové ……………………

Acknowledgements

First, I would like to thank Prof. Olivier Lequin, director of Laboratory of Biomolecule in Sorbonne University, for accepting me into the team.

My special thanks belong to Nicolas Pietrancosta, Ph.D. and Sophie Gautron, Ph.D. who both introduced me to the topic, guided me during my stay, gave me a lot of great advice and taught me critical thinking.

I am very grateful to all my colleagues who welcomed me warmly. Specifically, I would like to thank Carole Gruszczynski, Vincent Vialou Ph.D., Françoise Illien Ph.D. and members of the team of prof. Arnaud Gautier, who at some stage participated in my work.

I would also like to thank Assoc. Prof. PharmDr. Jaroslav Roh, Ph.D. for leading my work at my home faculty.

Eventually, my heartfelt thanks go to Thierry and my family for their support and understanding.

I would like to acknowledge Erasmus+ Programme for financial support.

Abstract

Charles University

Faculty of Pharmacy in Hradec Králové

Department of Organic and Bioorganic Chemistry

Student: Andrea Monteiro

Supervisor: Assoc. Prof. PharmDr. Jaroslav Roh, Ph.D.

Specialized supervisors: Nicolas Pietrancosta, Ph.D.

Sophie Gautron, Ph.D.

Title: Preparation and Evaluation of New Ligands Targeting Organic Cation Transporters in the Central Nervous System for the Treatment of Depression

Brain organic cation transporters (OCT2, OCT3) are polyspecific facilitated diffusion transporters that regulate aminergic tonus and have a complementary role to high-affinity monoamine transporters (serotonine transporter SERT, noradrenaline transporter NET and dopamine transporter DAT) in monoamine clearance in the brain. Their complementary characteristics compared to the high-affinity transporters (widespread distribution, broader pharmacological profile) and their involvement in mood-related functions make brain OCT relevant and original targets for the development of novel antidepressants.

H2-cyanome is a newly developed prodrug of cyanome that targets OCT. This prodrug showed promising antidepressant efficacy in a rodent model of chronic depression. Despite the positive impact of this prodrug on antidepressant efficacy, its limitation is its high affinity for α_1 adrenergic receptors that may confer potential cardiovascular side effects. The aim of this project was therefore to prepare designed hybrid derivative of cyanome and its analogues that would overcome the above-mentioned disadvantage while maintaining selectivity to brain OCT and antidepressant efficacy.

Interaction of two novel designed and synthesized compounds with brain OCT were predicted via molecular docking and scoring in BIOVIA Discovery Studio software using previously generated homology models of OCT2, OCT3 and α adrenoreceptors. TLC, NMR spectroscopy, MS and HRMS analysis were used for characterization of prepared compounds. One of these compounds was assessed for potential toxic effects in cell viability assays using a colorimetric CCK-8 assay, flow cytometry and a trypan blue exclusion test, and its antidepressant-like efficacy was evaluated in the forced swim test (FST) in mice.

The predicted virtual affinities of the designed and synthesized compounds showed high selectivity to OCT3 and α_{2C} adrenoreceptors and lack of binding at α_1 adrenoreceptors. The viability assays revealed some toxicity on the cell lines HEK-293T and CHO-K1. Gradual dose escalation, up to 3 mg/kg (i.p.) over four days in test mice showed no lethal toxicity. Acute administration of this compound at 0.2 mg/kg showed significant antidepressant-like effect in the FST, a classical test for antidepressant activity. It therefore appears to be a very promising compounds for further testing in a rodent model of chronic depression. In terms of predicted affinities, the second molecule as well appears suitable for chemical synthesis and further evaluation.

Abstrakt

Univerzita Karlova

Farmaceutická fakulta v Hradci Králové

Katedra organické a bioorganické chemie

Student: Andrea Monteiro

Školitel: doc. PharmDr. Jaroslav Roh, Ph.D.

Školitelé-specialisti: Nicolas Pietrancosta, Ph.D.

Sophie Gautron, Ph.D.

Název: Příprava a hodnocení nových ligandů transportérů organických kationtů v centrálním nervovém systému pro léčbu deprese

Transportéry organických kationtů v mozku (OCT2, OCT3) jsou polyspecifické přenašeče, které zajišťují facilitovanou difúzi, regulují aminergní tonus a mají komplementární roli k vysokoafinitním monoaminovým transportérům (serotoninový transportér SERT, noradrenalinový transportér NET a dopaminový transportér DAT) při zpětném vychytávání monoaminů v mozku. Jejich komplementární charakteristika ve srovnání s vysokoafinitními transportéry (rozšířená distribuce, širší farmakologický profil) a jejich zapojení do funkcí souvisejících s náladou činí z OCT v mozku relevantní a originální cíle pro vývoj nových antidepresiv.

Nově vyvinutý H2-cyanome je prekurzor cyanomu, který cílí na OCT. Toto proléčivo vykazovalo slibnou antidepresivní účinnost v myším modelu chronické deprese. Limitací v užití tohoto proléčiva je jeho vysoká afinita k α_1 adrenergním receptorům, což může mít za následek kardiovaskulární nežádoucí účinky. Cílem tohoto projektu bylo proto připravit navržený hybridní derivát cyanomu a jeho analoga, která by překonala výše uvedenou nevýhodu, se zachovalou selektivitou k OCT v mozku a zachovalou antidepresivní účinností.

Interakce dvou nově navržených sloučenin s OCT v mozku byla predikována pomocí molekulového dockingu a skórování v softwaru BIOVIA Discovery Studio s použitím již dříve vytvořených modelů OCT2, OCT3 a α adrenoreceptorů pomocí homologního modelování. K charakterizaci připravených sloučenin byla použita TLC, NMR spektroskopie, MS a HRMS analýza. U jedné z připravených látek bylo sledováno ovlivnění viability vybraných buněčných linií pomocí kolorimetrického testu CCK-8, průtokové cytometrie a testu vylučování trypanové

modři. Účinnost podobající se antidepresivům byla hodnocena v testu nuceného plavání (FST) u myší.

Predikce virtuální afinity navržených sloučenin ukázala vysokou selektivitu k OCT3 a α_{2C} a zároveň žádnou interakci s α_1 adrenoreceptory. Viabilitní testy odhalily určitou toxicitu na buněčných liniích HEK-293T a CHO-K1. Postupné zvyšování dávky až po dávku 3 mg/kg (i.p.) po dobu čtyř dnů u testovaných myší nevykazovalo žádnou letální toxicitu. Akutní podávání testované sloučeniny v dávce 0,2 mg/kg ukázalo významný účinek podobný antidepresivům v FST, který je klasickým testem pro hodnocení antidepresivní aktivity sloučenin. Mohlo by se tedy jednat o velmi slibnou sloučeninu pro další testování na myším modelu chronické deprese. I druhá sloučenina testovaná v molekulovém dokování se jeví jako vhodná pro chemickou syntézu a další hodnocení.

Table of contents

1 List of abbreviations 10
2 Introduction 12
3 Aims of the work 13
4 Theoretical section 14
4.1 Depression 14
4.2 Neurobiology of depression 14
4.2.1 Involvement of the HPA axis 15
4.2.2 Brain monoaminergic systems in depression 15
4.3 Pharmacotherapy of depression – brief history 17
4.3.1 Drugs targeting monoaminergic systems 17
4.3.2 Drugs targeting glutamatergic systems 19
4.4 Organic cation transporters 20
4.4.1 Tissular distribution 20
4.4.2 Pharmacological properties of OCT 21
4.4.3 Physiological and pharmacological importance 22
4.5 Organic cation transporters in the brain 23
4.5.1 OCT as an alternate monoamine clearance system 23
4.5.2 Roles of OCT in mood 25
4.6 Targeting brain organic cation transporters 28
4.6.1 The blood-brain barrier and chemical delivery systems 28
4.6.2 H2-cyanome and cyanome 30
4.6.3 Hybrid derivative of cyanome 31
5 Experimental section 31
5.1 Synthesis 31
5.2 Binding affinity prediction of OCT ligands via docking/scoring method 31
5.3 Effect of OCT ligand A2 on cultured cell viability 31
5.3.1 Reagents and apparatus 31
5.3.2 Methods 31
5.4 Mouse behaviour 35
5.4.1 Verification of non-toxicity of A2 prior to the forced swim test 35
5.4.2 Forced swim test (FST) 35

6 Results 37
6.1 Synthesis of OCT ligands 37
6.2 Binding affinity prediction of OCT ligands via docking/scoring method 37
6.3 Effect of OCT ligand A2 on cell viability in culture 37
6.4 Effects of A2 in mice 41
6.4.1 Verification of non-toxicity of A2 prior to the forced swim test 41
6.4.2 Anti-depressant-like effects of A2 in the forced swim test 41
7 Discussion 42
8 Conclusion 42
9 List of figures 43
10 List of tables 43
11 References 43
12 Appendix 51

1 List of abbreviations

5-HT	Serotonin
ACTH	Adrenocorticotropic hormone
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AR	Adrenoreceptors
BBB	Blood-brain barrier
CCK	Cell counting kit
CDSs	Chemical delivery systems
CHO	Chinese hamster ovary (cell line)
CNS	Central nervous system
COMT	Catechol-O-methyl transferase
CRF	Corticotropin-releasing factor
D22	Decynium 22
D24	Disprocynium 24
DA	Dopamine
DAT	Dopamine transporter
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicine Agency
FBS	Fetal bovine serum
FSC	Forward scatter light
FST	Forced swim test
GABA	γ-aminobutyric acid
H-bond	Hydrogen bond
HBSS	Hank's balanced salt solution
HEK	Human embryonic kidney (cell line)
HeLa	Human cervix adenocarcinoma (cell line)
His-Pro	Histidyl-proline
hOCT	Human OCT
HPA	Hypothalamic-pituitary-adrenocortical (axis)
IC_{50}	Half-maximal inhibitory concentration
ICD	International classification of diseases
KO	Knock-out
LBM	Laboratory of biomolecules
L-DOPA	Levodopa
MAOIs	Monoamine oxidase inhibitors
MFS	Major facilitator superfamily
mGlu2/3	Metabotropic glutamate receptors
MPP	1-methyl-4-phenylpyridinium
mRNA	Messenger ribonucleic acid

MS	Mass spectrometry
$NAD(P)^+$	Nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	Reduced nicotinamide adenine dinucleotide (phosphate)
NaSSAs	Noradrenergic and specific serotonergic antidepressants
NDRIs	Noradrenaline-dopamine reuptake inhibitors
NE	Noradrenaline
NET	Noradrenaline transporter
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
NMR	Nuclear magnetic resonance
NRIs	Noradrenaline reuptake inhibitors
NRTIs	Nucleoside/nucleotide reverse transcriptase inhibitors
NSF	Novelty-suppressed feeding
OCT	Organic cation transporters
PMAT	Plasma membrane monoamine transporter
PVN	Paraventricular nucleus
SARIs	Serotonin antagonist and reuptake inhibitors
SERT	Serotonin transporter
SLC22	Organic solute carrier 22
SNRIs	Serotonin and noradrenaline reuptake inhibitors
SSC	Side scatter light
SSRI	Selective serotonin reuptake inhibitor
TLC	Thin-layer chromatography
TST	Tail suspension test
VMAT	Vesicular monoamine transporters
WST	Water-soluble tetrazolium salt

2 Introduction

This Diploma Thesis was processed as a part of the Erasmus+ programme 2020/2021 at Sorbonne University at the Laboratory of Biomolecules in cooperation with the Neuroscience Paris-Seine. The team's research focuses on identifying novel processes underlying the emergence of depression and the action of antidepressants, on a family of atypical monoamine transporters in the brain and on developing new therapeutic strategies. Within the intellectual property, some parts of this work are included in the Attachment including the Aims described in more detail.

Among the above-mentioned atypical monoamine transporters are brain organic cation transporters (OCT). In the theoretical part of this work, I first summarized some knowledge about depression and OCT. The work then describes the preparation of new OCT ligands and their evaluation.

3 Aims of the work

The aim of this Diploma Thesis was to develop new compounds that are selective for brain organic cation transporters (OCT) and do not interact with α_1 adrenergic receptors. These compounds are expected to have antidepressant effects through their interaction with brain OCT.

The main tasks were:

- to synthesise new compounds and to characterize them by TLC, NMR spectroscopy, MS and HRMS analysis;
- to predict binding affinities of synthesized and designed compounds with brain OCT and α adrenergic receptors using grid-based molecular docking and scoring;
- to assess possible cytotoxic effects of chosen compounds on culture cell lines using CCK-8 assay, flow cytometry and a trypan blue exclusion tests;
- to verify lack of toxicity in mice and to assess the antidepressant-like efficacy of chosen compounds using the forced swim test

The aim of the theoretical section was to summarize the basic facts about depression with a focus on its neurobiological bases and pharmacotherapy, as well as the basic facts about OCT and their involvement in mood-related functions.

4 Theoretical section

4.1 Depression

Depression is a serious mental disorder whose one of the main symptoms is a persistent depressed mood. Major depressive disorder affects about 4.7% of the world population with a significant gender gap (more affecting women) (Ferrari, Somerville et al., 2013; James et al., 2018). Estimates indicate that 11 % of people experience at least one depressive episode in their lifetime (Ferrari, Charlson et al., 2013). Depression is one of the three leading causes of disability in the world, which has a negative impact on the lives of individuals and society (Ferrari, Charlson et al., 2013; James et al., 2018).

According to the International Classification of Diseases (ICD-11), depression is classified among mood disorders ("ICD-11 - ICD-11 for Mortality and Morbidity Statistics," n.d.). For diagnosis, a certain number of symptoms must be present most of the day for a period of at least two weeks. Key symptoms include persistent low mood, anhedonia, anxiety and fatigue. These symptoms can be accompanied by additional ones such as disturbed sleep, changes in appetite, indecisiveness, cognitive impairment, guilt, irritability, low self-esteem, suicidal thoughts or acts ("ICD-11 - ICD-11 for Mortality and Morbidity Statistics," n.d.). Depressive disorder is a risk factor for suicide. Once diagnosed, adequate treatment is necessary.

4.2 Neurobiology of depression

Depression is multifactorial and polygenic disorder that is associated with environmental causes (Nestler et al., 2002). Neural circuits of emotion include several brain regions and systems comprising the limbic system which are interconnected in a complex manner (LeDoux, 2000). Functional and structural abnormalities of these regions have been implicated in mood-related disorders. Patients with depression show altered metabolic activity and blood flow in various areas of the brain, along with morphometric and histological changes (Drevets, 2000; Drevets, 2001). Depression is associated with neurotransmitter dysfunction such as monoamines, glutamate and γ-aminobutyric acid (GABA), reduced brain neurotrophins and altered inflammatory and stress response system (Krishnan & Nestler, 2008; Hasler et al., 2007). The hypothalamic-pituitary-adrenal (HPA) axis seems to have a central position in the pathophysiology of depression (Pariante and Lightman, 2008).

4.2.1 Involvement of the HPA axis

The HPA axis has a prominent role in mediating the stress response; in the case of activation HPA axis neurons in paraventricular nucleus (PVN) of the hypothalamus release corticotropin-releasing factor (CRF), which stimulate synthesis and secretion of adrenocorticotropin (ACTH) from the anterior lobe of pituitary gland. ACTH subsequently induces the synthesis and release of glucocorticoids (cortisol in humans, corticosterone in rodents) from adrenal cortex. By binding to its intracellular receptors (the high-affinity mineralocorticoid and low-affinity glucocorticoid receptors), cortisol affects DNA transcription and thus metabolism as well as mood. In turn, cortisol exerts negative feedback at pituitary and hypothalamic level.

Dysregulation of the HPA axis has been associated with mood disorders. Many individuals with depression shows signs of abnormal activation of HPA axis (Hindmarch, 2002). The response to stress implicates a number of interconnected circuits in the brain (Ulrich-Lai and Herman, 2009). The stress response has an adaptative role, however chronically elevated activation of the HPA axis and thus elevated levels of cortisol may be damaging (Lupien et al., 2009). Abnormal HPA activity resulting in vulnerability to depression may be caused by environmental factors such as exposure to prenatal stressors and psychosocial stress, and genetic factors (Pariante and Lightman, 2008; Lupien et al., 2009).

4.2.2 Brain monoaminergic systems in depression

Decreased monoamine levels have been associated with the pathophysiology of depression (Hamon & Blier, 2013) but are not a direct cause, as proposed by the monoamine theory of depression. In healthy individuals there are no mood swings associated with decreases in monoamines (serotonin, noradrenaline, dopamine) (Charney, 1998; Ruhé et al., 2007). Moreover, most of common antidepressants acutely increase the levels of monoamines, which, however, do not correlate with the onset of antidepressant effect. The action of antidepressant is believed to implicate numerous adaptative processes such as the progressive disinhibition of regulatory autoreceptors (Artigas, 2013), neurotrophic effects and activation of second messengers inducing neuroplastic changes in the long term (Nestler et al., 2002; Pittenger & Duman, 2008).

Serotonin

The major area of serotonin (5-HT) production in the central nervous system is the raphe nuclei in the brainstem, where serotonin is biosynthesized from L-tryptophan. 5-HT then reaches other regions of the brain from the raphe through diffuse projections (Berger et al., 2009). Serotonin affects behaviour – mood, perception, memory, anger, aggression, fear, stress responses, appetite, addiction, sexuality (Berger et al., 2009). When serotonin is released from serotonergic nerve terminals into the synaptic cleft, it can bind to serotonin receptors. There are seven classes of serotonin receptors ($5\text{-}HT_{1\text{-}7}$) that differ in signalling mechanisms; all receptors are G-protein-coupled except the $5\text{-}HT_3$ receptor, which is a ligand-gated ion channel (Nichols & Nichols, 2008). The serotonin transporter (SERT) transports extracellular serotonin back into nerve endings. Patients with depression show changes in the levels of some 5-HT receptors and SERT (Stockmeier, 2003; Kambeitz & Howes, 2015).

Noradrenaline

In central nervous system (CNS), noradrenaline (NE) is biosynthesized from dopamine in the locus coeruleus in the pons. Noradrenaline functions include evaluation of stressful events, inhibition of irrelevant or unacceptable behaviour, the regulation of verbal and nonverbal working memory, regulation of affect, motivation and arousal, planning, decision-making, self-monitoring and self-evaluation (Moret & Briley, 2011).

Extracellular NE binds to adrenergic receptors which are G-protein-coupled receptors. Adrenoreceptors (AR) are divided into α-receptors, which are further divided into α_1 and α_2-AR, each of which includes three other subtypes, and β-adrenergic receptors which also contain three subtypes (Alexander et al., 2015). Patients with depressive disorder show altered central adrenoreceptor density (Brunello et al., 2002; Cottingham & Wang, 2012). Presynaptic α_{2A}-AR that mediates feedback inhibition of noradrenaline release (Brede et al., 2004) seems to be particularly involved in pathophysiology of depression. The noradrenaline transporter (NET) carries out the reuptake of NE from the synaptic cleft back into the presynaptic neuron and is thus one of the targets of antidepressants.

Dopamine

Dopamine (DA) is biosynthesized from L-DOPA mainly in the substantia nigra and ventral tegmental area in CNS. When released from dopaminergic neurons, dopamine binds to G-protein-coupled dopamine receptors (D_1, D_2, D_3, D_4, D_5) and is reinternalized into the aminergic terminals by the dopamine transporters (DAT) (Beaulieu & Gainetdinov, 2011). Dopamine affects voluntary movement, feeding, affect, reward, sleep, attention, working memory, and learning (Beaulieu & Gainetdinov, 2011).

In depressive disorders, alterations in dopaminergic system concerns changes in dopamine receptor expression as well as in endogenous dopamine production (Dunlop & Nemeroff, 2007). Dysregulation in dopaminergic transmission has been also associated with schizophrenia and Parkinson's disease (Beaulieu & Gainetdinov, 2011).

Current antidepressants targets are mostly the high-affinity reuptake transporters (SERT, NET, DAT) and certain monoamine receptors. Other actors in the monoaminergic system are enzymes that metabolize and thus inactivate monoamines - monoamine oxidase (inhibition of type A, which primarily metabolizes 5-HT and NE, is used clinically) and catechol-O-methyl transferase (COMT), which metabolizes catecholamines, i.e., NE and DA. Besides the high-affinity plasma membrane reuptake transporters, other transporters involved in monoamine homeostasis are vesicular monoamine transporters (VMAT), organic cation transporters (OCT) (Koepsell et al., 2007; Couroussé & Gautron, 2015) and plasma membrane monoamine transporter (PMAT) (Engel et al., 2004).

4.3 Pharmacotherapy of depression – brief history

4.3.1 Drugs targeting monoaminergic systems

The 1950s were a turning point in the treatment of depression, since until then there was no effective treatment. Tricyclic antidepressants, specifically imipramine, were discovered by chance in the development of antihistamines and antipsychotics (Pereira & Hiroaki, 2018). Around the same time, the now obsolete iproniazid, a monoamine oxidase inhibitor, was discovered during the development of new antituberculotics (Hillhouse & Porter, 2015). The common mechanism of action of these drugs, which in both cases leads to an increase of monoamine concentration in the synaptic cleft, contributed to the

development of the “monoamine” theory of depression – which stipulates that the underlying pathophysiologic basis of depression is a deficiency in monoamines (especially serotonin and noradrenaline) in the central nervous system (Hirschfeld, 2000).

The subsequent development of more selective antidepressants, based on this theory, was aimed at reducing side effects. Since the first tricyclic antidepressants, new antidepressant classes have been created. Among the ones developed next, were selective serotonin reuptake inhibitors, SSRIs, which are now the first line for the treatment of depression. Most antidepressants used today modulate the amounts of monoamines, preferentially 5-HT and NE at synapses by blocking high-affinity monoamine reuptake transporters in the nerve terminals (Tab. 1). More recent drugs are vortioxetine targeting monoaminergic systems and agomelatine, melatonin M1/M2 agonist (Mathew et al., 2008; Mandrioli et al., 2018).

The general drawbacks of these drugs are the onset of an antidepressant effect only after weeks of administration, and the occurrence of resistance to treatment in approximately one third of patients with major depressive disorder (Rush et al., 2006). Studies show that 40-85% of individuals who recover from a depressive episode experience at least one more episode (Hughes & Cohen, 2009).

Type of antidepressant	Representatives
Tricyclic and tetracyclic antidepressants	imipramine, amitriptyline, nortriptyline, desipramine, dosulepine, dibenzepine, clomipramine, maproptiline, amoxapine, dothiepine, doxepine, lofepramine
Monoamine oxidase inhibitors (MAOIs)	tranylcypromine, isocarboxazid, phenelzine, moclobemide
Selective serotonin reuptake inhibitors (SSRIs)	citalopram, escitalopram, fluoxetine, paroxetine, sertraline
Serotonin and noradrenaline reuptake inhibitors (SNRIs)	venlafaxine, duloxetine, desvenlafaxine, milnacipran
Noradrenergic and specific serotonergic antidepressants (NaSSAs)	mirtazapine, mianserine
Serotonin antagonist and reuptake inhibitors (SARIs)	trazodone, nefazodone
Noradrenaline-dopamine reuptake inhibitors (NDRIs)	bupropion
Noradrenaline reuptake inhibitors (NRIs)	reboxetine, viloxazine
Melatonergic antidepressant	agomelatine
Multi-modal antidepressant	vortioxetine, vilazodone
Other	tianeptine, Hypericum perforatum

Tab. 1 List of antidepressants.
Adapted from Švihovec et al., 2018

4.3.2 Drugs targeting glutamatergic systems

A recent turning point in the field of the pharmacotherapy and neurobiology of depression was the discovery of the antidepressant effects of ketamine in 2000 (Berman et al., 2000). Compared to substances affecting monoaminergic neurotransmission, the onset of the antidepressant effect of ketamine was rapid (within hours) and long-lasting (within days) (Berman et al., 2000; Zarate et al., 2006). Ketamine is a non-selective antagonist of glutamatergic NMDA receptors (NMDAR), used to induce dissociative anaesthesia. At low doses, NMDAR blockade releases glutamate, which subsequently binds to AMPA receptors, while ketamine also affects other receptors outside the glutamatergic system (Pereira & Hiroaki, 2018).

Although the clinical use of ketamine in the treatment of depression was limited by its psychomimetic effects, these findings opened up a new therapeutic pathway. Compounds that show clinical or preclinical fast antidepressant onset act on NMDA receptors, AMPA receptors, α_5-containing $GABA_A$ receptors or mGlu2/3 receptors. (Witkin et al., 2019). The currently approved substances targeting the glutamatergic system for the therapy of depression system are esketamine (Mahase, 2019) and brexanolone (Azhar & Din, 2021). However, the administration mode remains complicated (Commissioner, 2020; Wilkinson & Sanacora, 2019). These fast-acting antidepressants targeting NMDA or mGlu2/3 receptors have recently raised high hopes in clinical studies for the management of depression associated with suicidal risk but concerns relating to their efficacy in large patient groups, the persistence of their effects and potential side-effects still have to be addressed (Witkin et al., 2019).

4.4 Organic cation transporters

4.4.1 Tissular distribution

Organic cation transporters (OCT) are polyspecific membrane proteins of the SLC22 (solute carrier) transporter family which belong to the major facilitator superfamily (MFS) (Gründemann et al., 1994; Koepsell et al., 2007). OCT exist as three subtypes - OCT1 (SLC22A1), OCT2 (SLC22A2) and OCT3 (SLC22A3). These transporters mediate the transport of various xenobiotics and physiological compounds in the organism, where their location and expression levels vary depending on the OCT subtype (Tab. 2). In humans, OCT1 is highly expressed in the liver (Gorboulev et al., 1997) where it is located in the sinusoidal membrane of hepatocytes (Nies et al., 2008), but is also moderately expressed in other organs. OCT2, with a more limited expression pattern, is abundantly expressed in the kidney (Gorboulev et al., 1997; Nies et al., 2008). OCT3 shows a broader expression pattern, with high abundance in placenta, but also expression in lung, heart, liver, kidney, intestine, skeletal muscle, salivary gland and adipose tissue (Koepsell et al., 2007, Koepsell et al., 2020). Both OCT2 and OCT3 are expressed in the brain, in neurons and in some cases glial cells (Couroussé & Gautron, 2015).

Organs, Tissue, or Cells	OCT1	OCT2	OCT3
Adipose tissue	+	n.d.	++,*
Adrenal gland	+	n.d.	+++
Brain	+	+	+
Heart	+	n.d.	++
Kidney	++,*	+++,*	+
Liver	++++,*	n.d.	++,*
Lung	+,*	+,*	++,*
Placenta	+	+	++,*
Prostate	+	+	+++
Salivary glands	+	n.d.	+++,*
Skeletal muscle	+	n.d.	++++
Small intestine	+,*	n.d	+,*

Tab. 2 Abundance of mRNA and protein of human organic cation transporters in chosen organs, tissue and cells.
*++++, very high expression of mRNA; +++, high expression of mRNA; ++, expression of mRNA; +, low but significant expression of mRNA; n.d., no expression of mRNA detected; *, detection of protein by Western blotting, immunochemistry, or proteomics. Adapted from Koepsell et al., 2020*

4.4.2 Pharmacological properties of OCT

Substrates

OCT mediate the sodium and proton-independent transport of substrates across the plasmatic membrane in both directions according to their concentration gradient and membrane potential, with low affinity and high capacity (Gründemann et al., 1998; Koepsell et al., 2020). These transporters exhibit broadly overlapping substrate (and inhibitor) specificities with some divergences. In general, OCT transport mostly small cations, weak bases that are positively charged at physiological pH, and some noncharged compounds. To name a few, endogenous substances (Tab. 3) that are transported by OCT are acetylcholine, choline, dopamine, epinephrine, histamine, serotonin. Transported xenobiotics include model cations such as 1-methyl-4-phenylpyridinium (MPP) and various drugs for example cisplatin and metformin (Koepsell et al., 2020).

Compounds	OCT subtype	References
Acetylcholine	OCT1, OCT2	Lips et al., 2005
Choline	OCT2	Gorboulev et al., 1997
Creatinine	OCT2	Urakami et al., 2004
Cyclo (His-Pro)	OCT1, OCT2, OCT3	Taubert et al., 2007
Dopamine	OCT1, OCT2, OCT3	Busch et al., 1998; Amphoux et al. 2006
Epinephrine	OCT1, OCT2, OCT3	Amphoux et al., 2006
Histamine	OCT2, OCT3	Busch et al., 1998; Amphoux et al. 2006
Noradrenaline	OCT1, OCT2, OCT3	Busch et al., 1998; Amphoux et al. 2006
Salsolinol	OCT1, OCT2, OCT3	Taubert et al., 2007
Serotonin	OCT1, OCT2, OCT3	Busch et al., 1998; Amphoux et al. 2006

Tab. 3 Chosen endogenous substrate of human organic cation transporters (OCT).

Inhibitors

Polyspecific OCT are inhibited by a broad spectrum of compounds including numerous organic cations, organic anions and neutral compounds, with varied efficacy and often low selectivity (Koepsell et al., 2007; Couroussé & Gautron, 2015). Although the three OCT subtypes show broadly an overlapping range of structurally distinct inhibitors, there are differences in selectivity between individual OCT subtypes (Koepsell et al., 2020).

Many OCT inhibitors can be found among prescribed drugs. Moderately to highly potent inhibitors (IC_{50} less than 10 μM) can be found among proton pump inhibitors (Nies et al., 2011), antineoplastic tyrosine kinase inhibitors (Minematsu and Giacomini, 2011), antiretroviral drugs (Minuesa et al., 2009) and certain α-adrenoceptor ligands as antihypertensive drugs and cyanine dye derivatives (Hayer-Zillgen et al., 2002; Amphoux et al., 2010), of which disprocynium 24 (D24) is the most potent drug, with IC_{50} in nanomolar range for OCT (Amphoux et al., 2010). Other cyanine dyes such as decynium 22 (D22) are also OCT inhibitors, with overall less affinity than D24 (Hayer-Zillgen et al., 2002). D22 was often used as a pharmacological tool to study the OCT properties (Couroussé & Gautron, 2015).

4.4.3 Physiological and pharmacological importance

The polyspecificity and wide distribution of OCT result in their involvement in many physiological functions, in particular excretion and clearance (Koepsell et al., 2007). Experiments using knock out mice also showed the implication of OCT1 and OCT3 in lipid and glucose metabolism (Liang et al., 2018; Song et al., 2019). OCT2 and OCT3 in brain regulate

aminergic tonus and thus contribute to the modulation of mood-related functions such as anxiety, response to stress and antidepressant efficacy (Couroussé & Gautron, 2015). OCT2 also transport a neurotoxic metabolite of DA, salsolinol and its derivative histidyl-proline diketopiperazine [Cyclo (His-Pro)], which have been implicated in the pathogenesis of Parkinson disease (Taubert et al., 2007). OCT contribute to absorption and/or excretion of an essential choline in small intestine and are involved in its renal secretion/reabsorption (Gorboulev et al., 1997; Dantzler et al., 1998). In the kidney, OCT are also actively implicated in the secretion of creatinine, the product of muscle creatine catabolism (Urakami et al., 2004).

A large number of drugs are transported by OCT and/or inhibit OCT, which implies a high risk of potential drug-drug interaction. OCT affect the pharmacokinetics of metformin (Wang et al., 2002; Koepsell et al., 2020) and so which may interfere with the transporter's inhibition. Studies with OCT1 and OCT2 deficient mice showed their implication in cisplatin-induced nephrotoxicity (Filipski et al., 2009). Guideline on the investigation of drug interactions from European Medicine Agency (EMA) recommends preclinical in vitro testing of novel compounds for inhibition of human OCT1 and OCT2 due to their clinical relevance (European Medicine Agency, 2012).

4.5 Organic cation transporters in the brain

4.5.1 OCT as an alternate monoamine clearance system

Two subtypes of the OCT family, OCT2 and OCT3, are expressed in the central nervous system and in sympathetically-innervated tissues where they are implicated in aminergic neurotransmission (Couroussé & Gautron, 2015). As mentioned previously, OCT have the capacity to transport monoamine neurotransmitters. Although in vitro studies on transfected cells have shown that OCT2 and OCT3 have similar preferences for individual monoamines (Amphoux et al. 2006), knock-out mice studies revealed fine differences in low-affinity monoamine transport in brain. OCT2 and OCT3 genetic deletion in mice led to a significant decrease of monoamine concentrations in brain tissue extracts. According to these studies, OCT2 seems to have a preference for 5-HT and NE (Bacq et al., 2012) while OCT3 deletion had higher impact on DA concentration (Vialou et al., 2008).

Use of OCT2 mutant mice and decynium22, a selective OCT inhibitor (Russ et al., 1993) demonstrated the participation of OCT2 in 5-HT and NE clearance in the brain. The transport properties of endogenous OCT2 were assessed ex vivo in fresh cell suspensions from two regions with high OCT2 expression, hippocampus and cortex, in the presence of high-affinity monoamine transporter inhibitors. Decreased D22-sensitive 5-HT and NE uptake was found in OCT2 knock-out mice while DA uptake was not significantly impaired in these mice devoid of OCT2 (Bacq et al., 2012). Furthermore, in this study, the in vivo clearance of NE and 5-HT was evaluated using microionophoretic and electrophysiological approaches; clearance was reduced in mutant mice compared to wild type in the presence of venlafaxine, a serotonin and noradrenaline reuptake inhibitor. Hence, OCT2 has a key role in the modulation of postsynaptic neuronal activity when the NET and SERT are saturated or inhibited. Another study using OCT3-antisense oligonucleotides confirmed the implication of OCT3 in DA homeostasis (Nakayama et al., 2007).

In contrast to the classical monoamine uptake transporters, brain OCT are located in postsynaptic neurons, and also astrocytes for OCT3 (Vialou et al., 2008; Couroussé & Gautron, 2015). Furthermore, they are also expressed in areas of lower high-affinity transporter density at a distance from the aminergic varicosities. Thus, OCT operate as an alternate monoamine clearance system in case of high-affinity transporters saturation or inhibition, as well as in areas with lower density of these classical transporters (Fig. 1). They regulate aminergic tonus and have a complementary role to high-affinity monoamine transporters in monoamine clearance in the brain.

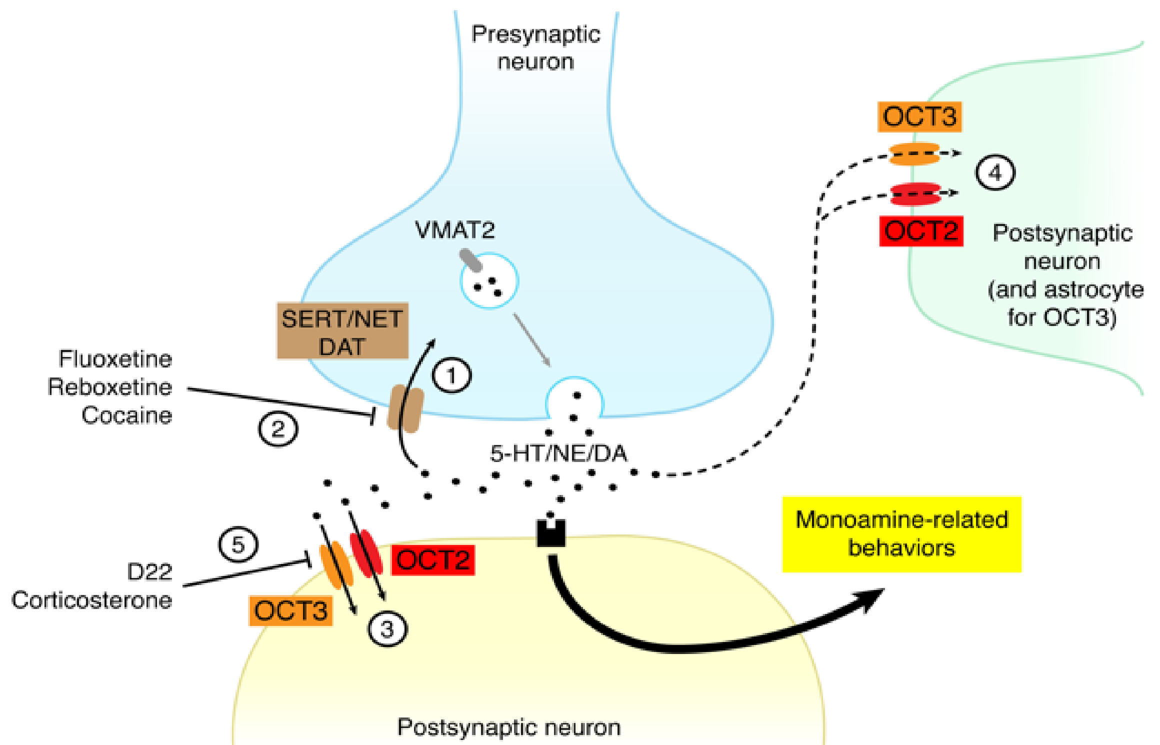

Fig. 1 Schematic representation of the OCT involvement in monoamine clearance in central nervous system.

After release of monoamines (5-HT, NE and DA) in the synaptic cleft, high affinity SERT, NET and DAT transporters transport the monoamines back into the presynaptic neuron (1). Pharmacological inhibition of these transporters for instance by antidepressants (2) increases the amount monoamines available in the synapse. Brain OCT located at proximal postsynaptic sites (3) contribute to monoamine clearance, in the event of inhibition or saturation of the high affinity transporters. Furthermore, they are also expressed in areas of lower high-affinity transporter density at a distance from the aminergic varicosities (4) where they contribute to clearance. OCT blockers (5) potentiate the neurochemical and behavioural effects of high-affinity monoamine reuptake inhibitors (2). Taken from: Couroussé & Gautron, 2015

4.5.2 Roles of OCT in mood

Immunostaining and in situ hybridization in rodent brain revealed significant expression of OCT in main aminergic projections such as the cortex, hippocampus, thalamus, hypothalamus, amygdala and hindbrain, as well as in some aminergic nuclei, raphe, locus coeruleus and tuberomammillary nucleus (Busch et al., 1998; Vialou et al., 2008; Gasser et al., 2009; Bacq et al., 2012; Couroussé & Gautron, 2015). Based on their implication in aminergic clearance, it is not surprising that OCT2 and OCT3 contribute to mood-related functions such as anxiety, immobility in resignation tests, response to stress and antidepressant efficacy (Couroussé & Gautron, 2015), assessed in specific rodent behavioural tests.

Mood related behaviours and depression models

Anxiety

To assess anxiety level, tests in which a rodent is exposed to a stressful environment (an open-field, an elevated plus-maze, an elevated O-maze) and conflict-based test, novelty-suppressed feeding (NSF) were used (Couroussé & Gautron, 2015). The open field is used to evaluate both general locomotor activity and anxiety level by measuring the activity and time spent in the inner (anxiogenic) and outer zone (Bacq et al., 2012). Elevated plus and O-maze consists of closed and open (anxiogenic) sectors where the time spent and number of sector entries are measured (Bacq et al., 2012). The NSF paradigm evaluates the latency to begin eating in a novel stressful environment (David et al., 2007; Bacq et al., 2012); anxiolytic drugs act on these parameters.

Invalidation of either OCT2 or OCT3 were associated with anomalies in behavioural responses to anxiety. Consistent with the OCT2 presence in the limbic system, OCT2-deficient mice showed alterations in anxiety-related behaviour, concretely a decreased anxiety level in the elevated O-maze, in the NSF test and in the open-field without altered locomotor activity compared to wild-type mice (Bacq et al., 2012). Mice lacking OCT3 presented a similar anxiolytic-like phenotype in the elevated-plus maze and open-field test (Wultsch et al., 2009). However, in another study, results from the open-field test of OCT3-deficient animals indicated an increased anxiety level (Vialou et al., 2008). The reason for this discrepancy is not known and might be related to genetic background (Couroussé & Gautron, 2015).

Behavioural despair

Typical tests for assessing behavioural despair are the forced swim test (FST) and the tail suspension test (TST) (Fig. 2). An animal is exposed to an inescapable stressful situation i.e., plunged into a beaker filled with water (Porsolt et al., 1977) or hung on the hook by the tail with an adhesive tape (Steru et al., 1985). The time the animal spends motionless is measured. Escape attempt is thought to reflect an antidepressant-like state, whereas the immobility reflects a state of behavioural despair, or resignation. Most antidepressants induce robust antiimobility effect in these tests even though it does not reproduce symptoms of depression (Porsolt et al., 1977, Steru et al., 1985).

Fig. 2 Representation of behavioural despair tests.
Adapted from: Planchez et al., 2019

Antisense knock-down of OCT3 in mice was shown to decrease immobility time in the FST (Kitaichi et al., 2005). D22, a potent OCT inhibitor, showed antidepressant like effects only in SERT KO mice and not in wild type mice (Baganz et al., 2008), suggesting that one or both OCT may modulate this behaviour. This result may be skewed by inhibition of adrenergic receptors or PMAT, another low affinity monoamine transporter blocked by D22 (Engel & Wang, 2005; Amphoux et al., 2010; Couroussé & Gautron, 2015). In a recent study, H2-cyanome, a newly developed OCT2-selective prodrug, had a moderate antidepressant-like effect on immobility in the forced-swim test (Orrico-Sanchez et al., 2020).

Antidepressant efficacy

FST and TST assessing behavioural despair are classically used for the screening of the antidepressant potential of new compounds, as most known antidepressants induce robust effects in this test. OCT2 and OCT3 are involved in the response to 5-HT/NE antidepressants in the behavioural despair paradigms (Bacq et al., 2012; Kitaichi et al., 2005). However, these tests are not a paradigm of depression since they lack symptomatic dimensions of depression and naive "non-depressed "animals are used.

To induce depression-like behaviours, chronic corticosterone exposure which is a validated model of depression was used to evaluate implication of OCT2 in the long-term response to common antidepressants and in the antidepressant efficacy of H2-cyanome, a novel prodrug (Bacq et al., 2012; Orrico-Sanchez et al., 2020). Chronic corticosterone

treatment via drinking water induces behaviours mimicking symptoms of depression such as anhedonia, increased anxiety, social aversion and resignation (Bacq et al., 2012). Further resembling human depression, these depression-like alterations can be improved by long-term antidepressant treatment, contrarily to behavioural despair paradigms.

The use of this model of chronic depression and OCT2 mutant mice has shown a critical role for OCT2 in the long-term efficacy of antidepressants (Bacq et al., 2012). Long-term administration of the prodrug H2-cyanome induced rapid positive effect on several depression-like symptoms, such as anhedonia, anxiety, social withdrawal, and memory impairment; H2-cyanome showed in particular a faster onset of action on behaviours reflecting anhedonia, a main symptom of depression, and better anxiolytic effects compared to fluoxetine (Orrico-Sanchez et al., 2020).

4.6 Targeting brain organic cation transporters

The complementary characteristics of brain OCT compared to the high-affinity transporters (widespread distribution, broader pharmacological profile) and their involvement in mood-related functions make brain OCT relevant and original targets for the development of novel antidepressants. Devising improved more selective OCT ligands is necessary as no selective agents have been identified. OCT inhibitors and substrates include a wide range of compounds with varied efficacy and often low selectivity whose primary target is frequently another transporter or receptor (Couroussé & Gautron, 2015; Koepsell et al., 2020). Furthermore, for molecules to interact with the brain transporters, they must first cross the blood-brain barrier (BBB).

4.6.1 The blood-brain barrier and chemical delivery systems

The blood-brain barrier (BBB) is a highly selective semi-permeable barrier formed by endothelial cells with tight junctions associated with perivascular elements such as astrocytes and pericytes (Fig. 3). It regulates the entry of molecules from the blood into the brain tissue and extracellular fluid in the central nervous system (CNS) and thus maintains homeostasis of the brain. This precise control of CNS homeostasis ensures proper function and protection of the brain from toxins and pathogens circulating in blood (Daneman & Prat, 2015).

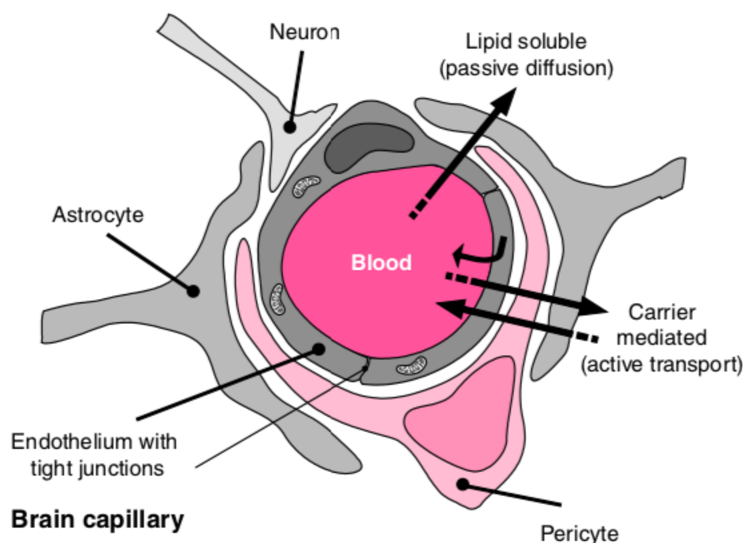

Fig. 3 Schematic representation of a brain capillary.
The capillary wall is formed by endothelial cells with tight junctions, which thus form a semipermeable blood-brain barrier (BBB). Only lipid soluble molecules can cross the BBB by passive diffusion. Furthermore, the molecules can be also transposed by carriers. Taken from: Bodor & Buchwald, 2003

In the development of CNS-acting therapeutics, it should be borne in mind that these structures and controlling permeability may prevent the entry of therapeutic molecules. The cerebral capillaries, which comprise more than 95% of the total surface area of the BBB, represent the principal route by which chemicals enter the brain (Smith, 1989). The molecules can either enter the brain by passive diffusion according to their concentration gradient, or the transport can be facilitated by passive and active transporters located in by endothelial cells of the capillaries (Fig. 3). There is a correlation between passive diffusion and chemical-physical properties of molecules such as lipid solubility, polar surface area, molecular weight, H-bond donors and acceptors and rotatable bonds (Lipinski, 2004). However, there are many CNS-active agents that do not comply with the general rules for crossing the BBB and use a different pathway than passive diffusion (Abbott et al., 2010; Bodor & Buchwald, 2003). Brain delivery of therapeutic agents that do not meet the rules for passive diffusion into the brain requires some strategy to overcome the BBB (Bodor & Buchwald, 2003).

Chemical delivery systems (CDSs) are one of the alternative approaches (Bodor et al., 1992; Bodor & Buchwald, 2003). CDSs are inactive prodrugs providing the site-specific or site-enhanced delivery of a drug through multistep enzymatic and/or chemical transformations (Bodor & Buchwald, 2003). This strategy, specifically a redox chemical drug-delivery system,

has been used for the targeting of cyanome, a selective OCT ligand, through generation of a prodrug H2-cyanome (Orrico-Sanchez et al., 2020). The lipophilic dihydro form (the prodrug H2-cyanome) enters more easily the brain where is converted to the hydrophilic (charged cyanome) form. This conversion is closely related to the ubiquitous NAD(P)H ↔ NAD(P)$^{+}$ coenzyme system (Bodor & Buchwald, 2003; Orrico-Sanchez et al., 2020).

4.6.2 H2-cyanome and cyanome

Cyanome, a positively charged isocyanine derivative, is a newly developed potent ligand of OCT2 with increased selectivity. Cyanome is derived from dysprocynium 24 (D24) and shows similar IC_{50} for OCT2 compared to D24 (in the nanomolar range) and reduced affinity for α_2 adrenergic receptors compared to other isocyanine derivatives (Amphoux 2010; Orrico-Sanchez et al., 2020). Virtually cyanome showed similar interaction pattern as D24 with OCT – presence of ionic interaction. Position 6 of D24 was substituted with a methoxy group in the cyanome (Fig. 4), thus hindering interaction with the fourth helix of the α_{2C} adrenoceptor (Orrico-Sanchez et al., 2020). Transport across the BBB was increased by modification of cyanome into a neutral prodrug, H2-cyanome (Fig. 4), that is metabolized in vivo into the active cyanome via a redox brain system. This prodrug showed promising antidepressant efficacy in a rodent model of chronic depression compared with a classical antidepressant, fluoxetine, a serotonin reuptake inhibitor (SSRI). Despite the positive impact of this prodrug on antidepressant efficacy, its limitation was its high affinity for α_1 adrenergic receptors (unpublished data), conferring potential cardiovascular side effects.

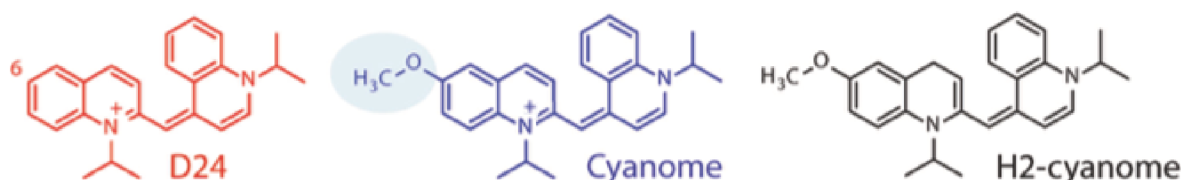

Fig. 4 Chemical structure of D24 (red), cyanome (blue), and H2-cyanome (black). Adapted from Orrico-Sanchez et al., 2020

4.6.3 Hybrid derivative of cyanome

Hybride derivative of cyanome has been included into Attachment due to intellectual property reasons.

5 Experimental section

5.1 Synthesis

Synthesis has been included into Attachment due to intellectual property reasons.

5.2 Binding affinity prediction of OCT ligands via docking/scoring method

Binding affinity prediction of OCT ligands via docking/scoring method has been included into Attachment due to intellectual property reasons.

5.3 Effect of OCT ligand A2 on cultured cell viability

5.3.1 Reagents and apparatus

Cell culture reagents used were Dulbecco’s modified Eagle’s medium (DMEM), Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12), fetal bovine serum (FBS), Dulbecco’s phosphate-buffered saline (DPBS), Hank's balanced salt solution (HBSS), trypan blue stain 0.4%, trypsin 0,25% (Trypsin-EDTA) from Thermo Fisher Scientific (Massachusetts, USA). Poly-L-Lysine solution (0.01%), triton X-100 and DMSO (dimethyl sulfoxide) were supplied from Sigma-Aldrich (Missouri, USA) and Cell Counting Kit-8 (CCK-8) was from Dojindo Molecular Technologies (Japan).

Apparatus used were Biological safety cabinet Safemate ECO Class II (LAF Technologies Pty Ltd, Australia), Microscope Primo Vert Binocular (Zeiss, Germany), Automated Cell Counter Countess II FL (The Thermo Fisher Scientific, Massachusetts, USA), Microplate reader POLARstar OPTIMA (BMG Labtech, Germany) and Flow cytometer FACSCalibur (BD Bioscience, New Jersey, USA).

5.3.2 Methods

Cell Culture

New cell cultures were prepared by members of Prof. Arnaud Gautier’s team at the Laboratory of Biomolecules (LBM). The HeLa cell line derived from human cervical cancer cells

and the human embryonic kidney 293T (HEK-293T) cell line were maintained in DMEM containing 10% FBS and the Chinese Hamster Ovary (CHO-K1) cell line in DMEM/F-12 containing 10% FBS at 37 °C in a humidified atmosphere (5% CO_2). Medium was replaced every 1 to 3 days. Cells were passaged when they reached 80 – 90% confluency.

For cell subcultures, cell culture reagents were pre-warmed in the water bath (37 °C). The original medium was aspirated from the side of the flask. Cells were gently washed two times with 10 ml DPBS. 2 ml of trypsin in DPBS (1:10) were added (for T-75 flask) and incubated for 5 minutes (37 °C, 5% CO_2) to harvest the cells. Trypsinization was neutralized by adding 10 ml of media with FBS. Cells were resuspended in the fresh medium and transferred to a Falcon tube. According to the number of cells per ml (see Trypan blue exclusion test) and the subsequent experiment, the cell suspension was diluted with medium and transferred to a flask or wells. Procedures were performed under sterile conditions.

A2 solution

The stock solution of A2 consisted of dissolved A2 in DMSO. This solution was a thousand times more concentrated than the highest concentration used in the experiment. This ensured that the cells would be exposed to a maximum of 0.1% DMSO, a concentration that is non-toxic to the cells. The stock solution was then added to the medium and other solutions were prepared by serial dilutions.

Cell viability assays used

- Trypan blue exclusion test

The method is based on the membrane integrity. Living cells do not take up trypan blue, whereas this dye passes the membrane of dead cells and stains the nucleus.

10 µl of cell suspension was mixed with 10 µl of trypan blue in Eppendorf tube. 10 µl of the sample with the trypan blue was pipetted into a chamber slide (Countess, The Thermo Fisher Scientific, Massachusetts, USA). Chamber slide was inserted into the counter which counted the number of live and dead cells. Measures were done twice and averaged. Trypan blue staining was used during cell subculture preparation and for estimation of A2 effect on cells.

For A2 effect, a cell suspension of HEK-293T was seeded into 6-well plate in a volume of 1 ml/well (20 x 104 cells/well and 15 x 104 cells/well) and incubated for 24 hours (37 °C, 5% CO_2) until 95% confluence. The original medium was removed, and cells were rinsed with DPBS. Different concentrations of pre-prepared solutions of A2 were added (1 µM, 3 µM, 10 µM, 30 µM, 100 µM). Cells with medium and DMSO (0.1%) served as a negative control. The cells were incubated for 24 hours (37 °C, 5% CO_2). The cells were resuspended after DPBS washing and trypsinization to count viability.

- Flow cytometry assay

Flow cytometer measurement was performed by Dr. Françoise Illien at the Laboratory of Biomolecules (LBM). The method is based on the different morphology of living, apoptotic and necrotic cells, resulting in a different scattering of light. The assay was made with HeLa cells with three duplicate wells in each group.

HeLa cell suspension was inoculated into 24-wells plates in a volume of 500 µL/well (25 x 104 cells/well) and incubated for 24 hours (37 °C, 5% CO_2) until they reached 95% confluence. The original medium was removed, and cells were rinsed with HBSS. Different concentration of pre-prepared solution of A2 were added (10 µM, 30 µM, 100 µM, 200 µM). Negative controls were untreated cells i.e., with only medium added, and untreated cells i.e., with medium plus DMSO (0.1%). A positive control was carried out with cells lysed with 0.1% detergent triton. The cells were incubated for one hour (37 °C, 5% CO_2). The original medium was removed, and cells were rinsed two times with HBSS. 100 µl of trypsin in DPBS (1:10) were added per well and incubated for 5 minutes (37 °C, 5% CO_2) to harvest the cells. Trypsin was neutralized by adding 1 ml of HBSS and 200 µl of FBS. Cell suspensions were transferred from the wells to flow cytometry tubes and wells were rinsed with 500 µl HBSS, that was added to the cell suspension. Cells were centrifuged at 300 g at 4 °C for 5 min, the supernatant was removed and placed into the ice. 500 µl HBSS were added by tube and cells were resuspended. BD CellQuest software was used for analysis. 20 000 cells were measured per tube at the wavelength of 488 nm. Cells were analysed from scattered light. Forward scatter light (FSC) refers to cell size and Side scatter light (SSC) refers to cellular granularity.

- CCK-8 assay

The colorimetric assay is based on reduction of water-soluble tetrazolium salt (WST-8) by dehydrogenase activities in cells to give a yellow-colour formazan dye. The amount of generated formazan dye is directly proportional to the number of living cells.

Cytotoxicity of A2 was estimated in CHO-K1 and HEK-293T cells. The assay was made with three duplicate wells in each group.

Cell suspension of CHO-K1 was inoculated into 96-wells plates (Nunc MicroWell, The Thermo Fisher Scientific, Massachusetts, USA) in a volume of 100 µL/well (2 x 104 cells/well) and incubated for 24 hours (37 °C, 5% CO2) so they reached 80% confluence. After rinsing cells with buffer, different concentration of pre-prepared solution of A2 were added (1 µM, 3 µM , 10 µM, 30 µM, 100 µM, 200 µM) with two negative controls (medium and 0.1% DMSO) and two positive controls – blank (medium with no cell and for the second positive control, detergent triton 0.1% for lysing cells). After 24-hour incubation and subsequent removal of original medium, cells were rinsed two times with FBS-free DMEM/F-12 and 10% CCK-8 in FBS-free DMEM/F-12 was added (100 µL/well). The absorbance was measured at 450 nm with a microplate reader after 3 hours of incubation.

In the case of HEK-293T, the microplates were treated with poly-L-Lysine 3 hours before the cell inoculation to enhance cell adhesion. DMEM was used instead of DMEM/F-12. Cells (1 x 104 cells/well) were incubated for 48h to reach 80% confluence before adding A2 solution. The next procedure was the same as for CHO-K1 cells with addition of 50 µM A2.

Cell viability was determined from the mean optical density (OD, absorbance). IC_{50} (half-maximal inhibition concentration; A2 concentration that modified the absorbance to 50% in comparison with the control) values were determined from the nonlinear dose-response curve.

$$\text{Cell viability (\%)} = \frac{OD\ (expetiment) - OD\ (blank)}{OD\ (control) - OD\ (blank)} \times 100$$

PRISM (GraphPad Software, California, USA) was used for the data analysis of cytotoxicity on HEK and CHO-K1 cells (mean ± SEM).

5.4 Mouse behaviour

Carole Gruszczynski or Dr. Vincent Vialou in the team manipulated directly the animals. Male C57BL/6JRj mice were provided from Janvier Labs and from another project. Mice were housed in cages in groups of four or five and maintained under controlled environmental conditions (23±1°C, 40±5%, 12-h light/dark cycle) with food and water freely available. For the behavioural test, the experimenters were blinded to the treatment of mice during the evaluation. The behavioural experiments were performed with age-matched (8-13 weeks) mice during the inactive phase (i.e., between 9 a.m. and 1 p.m.) to avoid the circadian rhythm influence. All experiments and animal treatment were in compliance with the European Communities Council Directive for the Care and the Use of Laboratory Animals (2010/63/EU) and approved by the French ethical committee (#5786-2016062207032685).

5.4.1 Verification of non-toxicity of A2 prior to the forced swim test

Day	Dose (mg/kg)
1	0.01
2	0.10
3	0.50
4	3.00

Tab. 4 Increasing doses of A2 administered i.p. to mice (n = 5) for four days.

Male mice (n = 5) were administered daily with increasing doses of A2 i.p. using 1 ml syringe (Terumo, Japan) with 26-gauge needle (0.45x12mm) (Henke-Sass Wolf GmbH, Germany) at a volume of 10 ml/kg over four days (Tab. 4). The corresponding concentration of A2 was prepared on the day of testing by dissolving in saline (0.9 % [wt/vol]) and heating 50°C for 5 minutes. Mice were observed for 1 hour after injection and the next day. Mice were also observed periodically during two weeks after treatment.

5.4.2 Forced swim test (FST)

The FST was carried out to evaluate the antidepressant-like efficacy of A2. The tested mice are placed individually in a transparent beaker (30x10 cm) filled with tap water at room temperature (23-25 °C) to a depth of 20 cm. The time spent immobile is considered a sign of

behavioural despair (or resignation) and most current antidepressants reduce immobility time in this paradigm. A mouse was considered motionless when floating in an upright position with only small movements to keep its head above the water. Active behaviour was swimming or climbing (Fig. 5). The mice were administered saline (0.9% [wt/vol]) for the control group or fluoxetine (15 mg/kg, 18 mg/kg, 20 mg/kg) dissolved in saline using 1 ml syringe (Terumo, Japan) with 26-gauge needle (0.45x12mm) to validate the method. During the experiments, different brands of fluoxetine hydrochloride were tested to validate the method – fluoxetine hydrochloride, Sigma (Missouri, USA), LKT Labs (Minnesota, USA) and Biotrend (Germany), that was used for the final experiments. After this validation, A2 (0.2mg/kg; 10 ml/kg; i.p.) was tested. The 6-minute test session was recorded by a camera (Camcorder FDR-AX53 4K Handycam (Sony, Japan) and the immobility time was measured for 4 minutes after the first 2 minutes of habituation by the same observer.

Certain measures and care for animal manipulation were taken. Sterilized material was used. Mice were injected in a different room than the test room and quickly processed. Untested mice were separated from those tested. Non-transparent barriers were placed between the beakers. The experimenters were not present in the room of the test session.

Each group was represented in every FST session. Immobility time measurements were performed by two independent observers. Data analysis was performed with PRISM (GraphPad Software, California, USA) and unpaired Mann-Whitney test was used for statistical analysis.

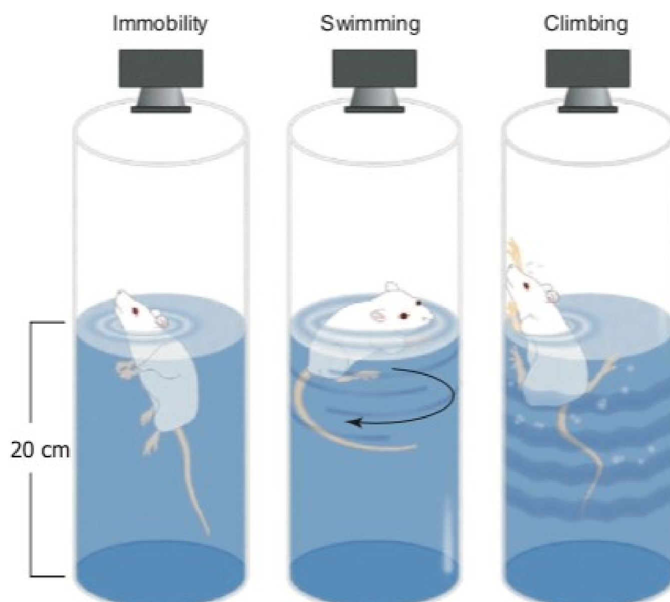

Fig. 5 Schematic representation of immobility, swimming and climbing in forced swim test. Adapted from Cryan et al. (2002)

6 Results

6.1 Synthesis of OCT ligands

Synthesis of OCT ligands has been included into Attachment due to intellectual property reasons.

6.2 Binding affinity prediction of OCT ligands via docking/scoring method

Binding affinity prediction of OCT ligands has been included into Attachment due to intellectual property reasons

6.3 Effect of OCT ligand A2 on cell viability in culture

To get insight on the potential toxicity of A2, a number of experiments assessing cell viability in presence of this compound of various cells lines were carried out.

A first experiment was performed to evaluate the effect of various doses of A2 on HEK-293T cells by trypan blue staining of the dead cells. This experiment showed a reduced number of living cells per ml after treatment with A2 at a concentration of 100 µM and to some extent 3 µM (Fig. 6), suggesting toxicity of A2 on this cell line. The trypan blue positive cells were detached or did not have a typical shape. However, a definite conclusion could not be drawn,

and statistical analysis was not possible because the experiment did not include duplicates or triplicates, nor was replicated.

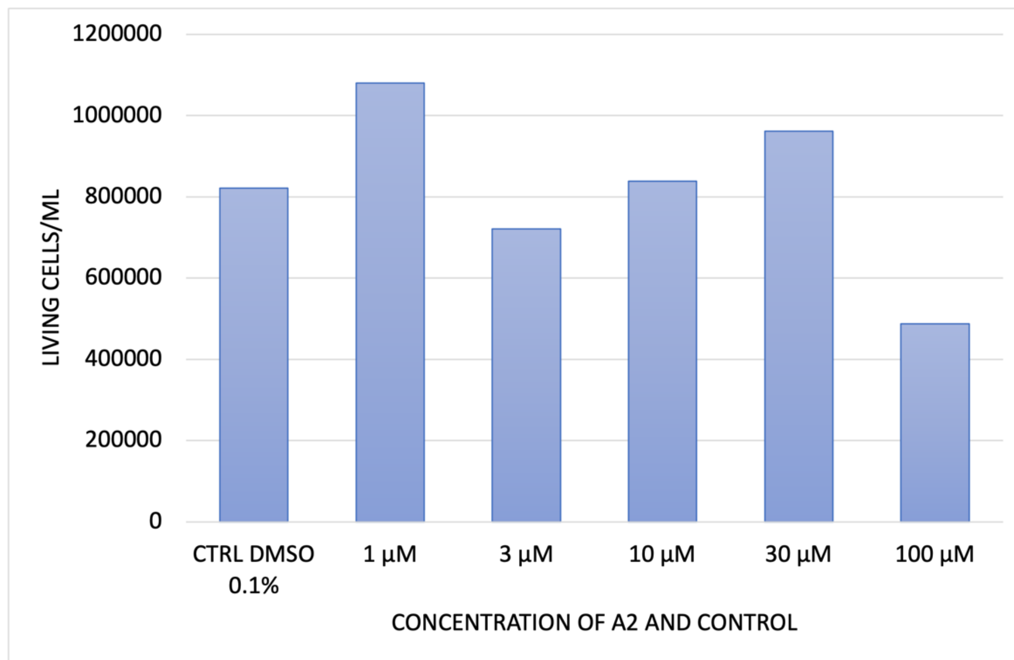

Fig. 6 Number of living HEK-293T cells per ml after a 24h incubation with increasing concentrations of A2 or control (CTRL) DMSO 0.1%.
Trypan blue exclusion test was used to count dead and live cells with an Automated Cell Counter Countess II FL apparatus

To confirm this finding, in a second experiment, HEK-293T cells, and another cell line, CHO-K1 were exposed for 24 hours to various concentrations of A2, and cytotoxicity was evaluated by a CCK-8 assay. The IC_{50} for CHO-K1 was evaluated at 78.62 µM, and for HEK-293T IC_{50} at 8.67 µM (Fig. 7).

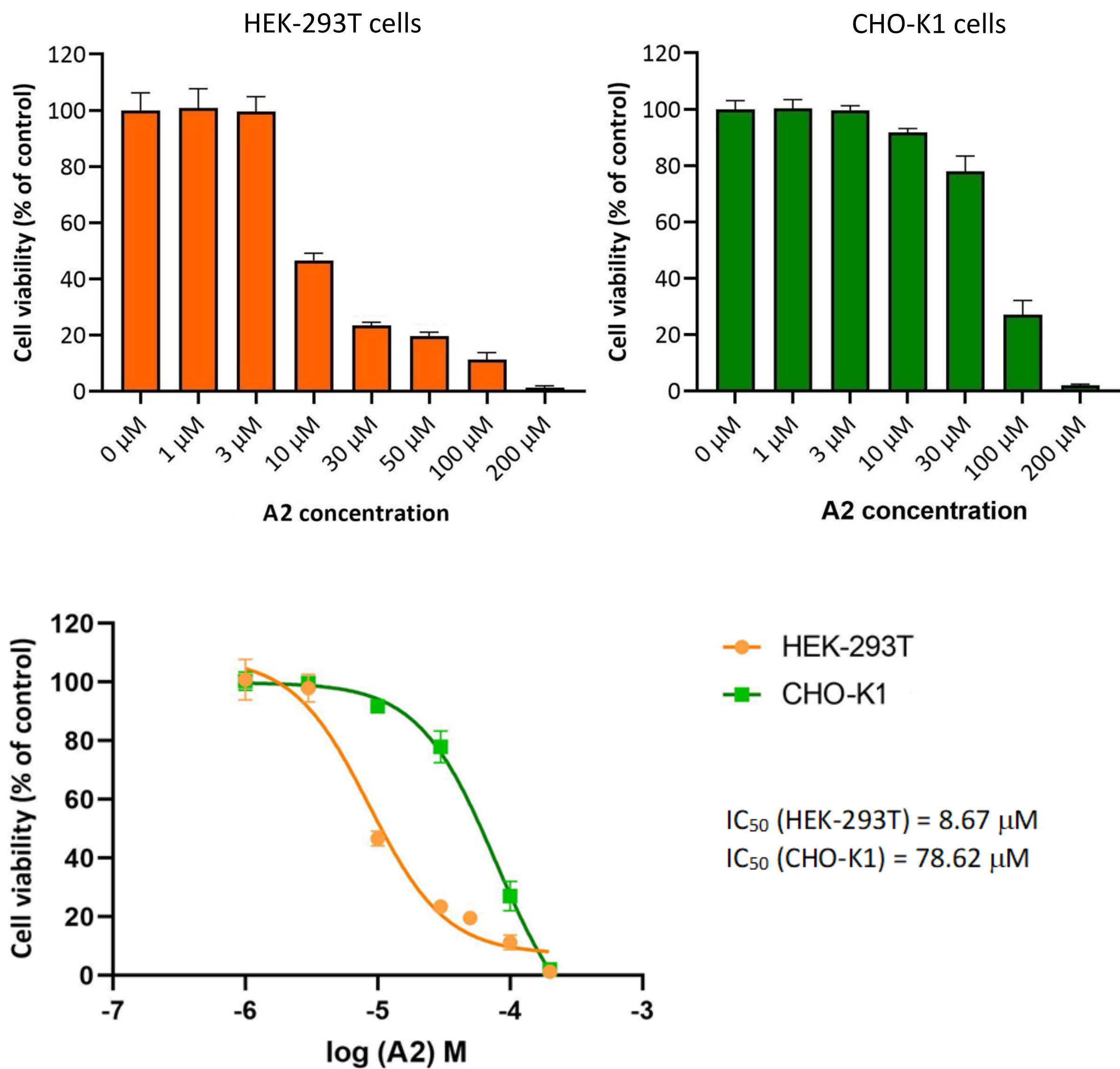

Fig. 7 Cell viability and IC_{50}.

CCK8-assay showing the viability of HEK-293T and CHO-K1 cells after exposure to increasing concentrations of A2 for 24 h. IC_{50} was 8.67 µM for HEK-293T and 78.62 µM for CHO-K1 cells. Data are presented as mean ± SEM for a single experiment.

In a last experiment, short-term (1 h) exposure of HeLa cells to A2 did not affect their viability, as quantified using a flow cytometer. This experiment showed no change in the proportion of subpopulations, with a majority of live cells after treatment with up to 200 µM A2 (Fig. 8). No toxicity was detected after 1 hour of exposure.

This latter manipulation suggests that this Hela line is less sensitive than other cell lines or there is no acute A2 toxicity within one hour compared to triton 0.1%. It would be advisable to expose the HeLa cell line to A2 for a longer period of time and also to test the sensitivity of other cell lines within one hour in order to compare individual cell lines.

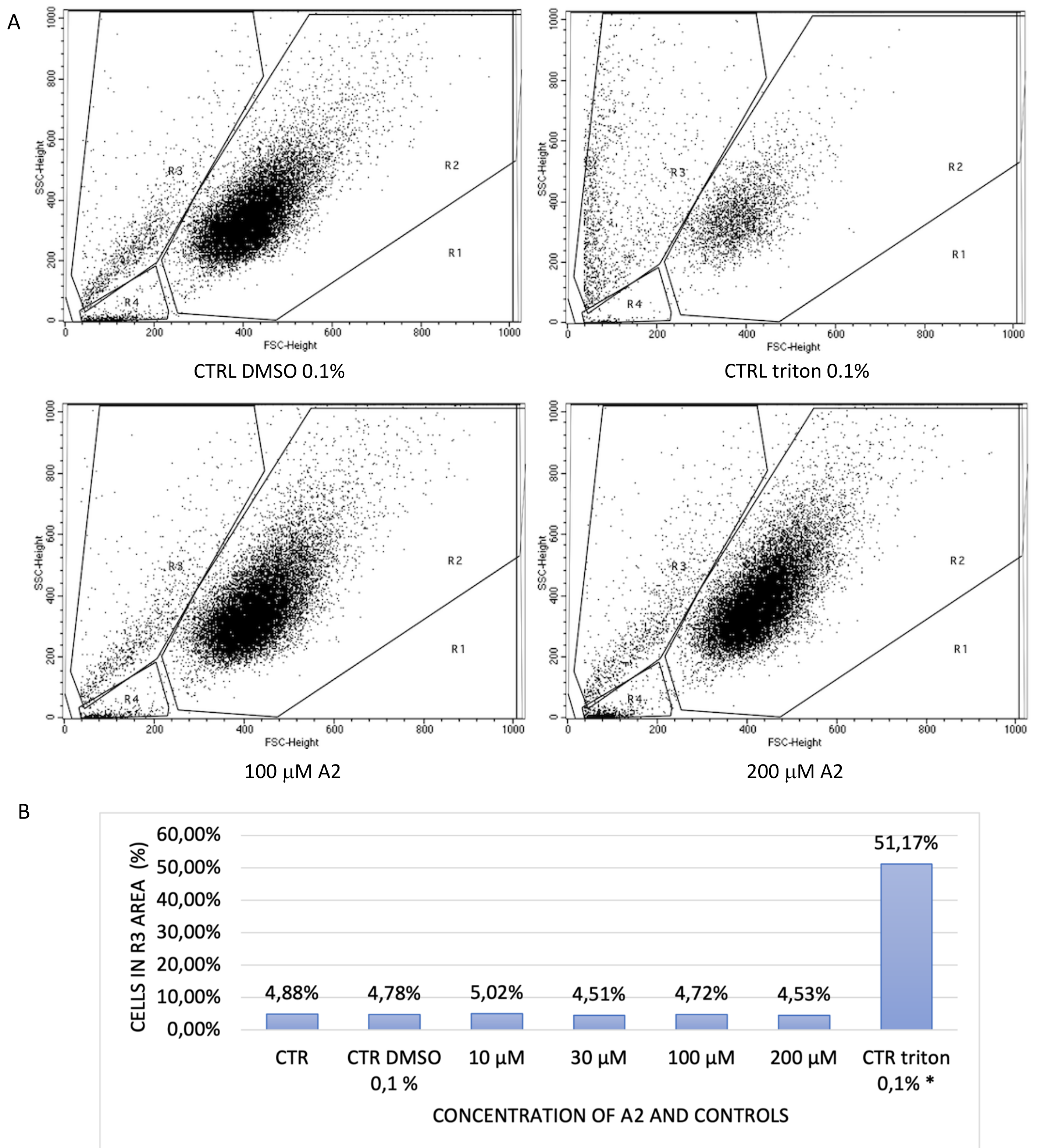

Fig. 8 HeLa cells analyzed by flow cytometry.

*(A) HeLa cells were sorted by flow cytometry – R1 area represents all measured cells. R2 represents living cells. Dead cells are included in R3 area and R4 area represents debris. Chosen dot plots display sorted cells of control (CTRL) DMSO 0.1% and triton 0.1% (4200 events) and of 100 µM and 200 µM A2 after 1 hour of incubation. (B) Percentage of cells included in R3 area in the portion of R1. 20,000 events were recorded in one experimental session. The result was calculated as a mean of triplets. *Less than 5000 events were recorded due to detachment of cells caused by triton*

6.4 Effects of A2 in mice

6.4.1 Verification of non-toxicity of A2 prior to the forced swim test

To verify the innocuity of A2 before planning the FST experiments, we administered mice daily with increasing doses of A2 up to 3 mg/kg. Gradual dose escalation over four days in test mice (n = 4) showed no lethal toxicity. After the injection, some mice licked the injection site. Few minutes after injection, they would start cleaning themselves and one hour later they would usually sleep.

6.4.2 Anti-depressant-like effects of A2 in the forced swim test

A FST was carried out to evaluate antidepressant-like efficacy of A2. A preliminary validation of the experimental conditions was carried with various fluoxetine brands and delay between injection and testing. The most significant anti-immobility effect compared to the control saline group was achieved with 18 mg/kg fluoxetine (i.p.; Biotrend) administered one hour before testing (Fig. 9).

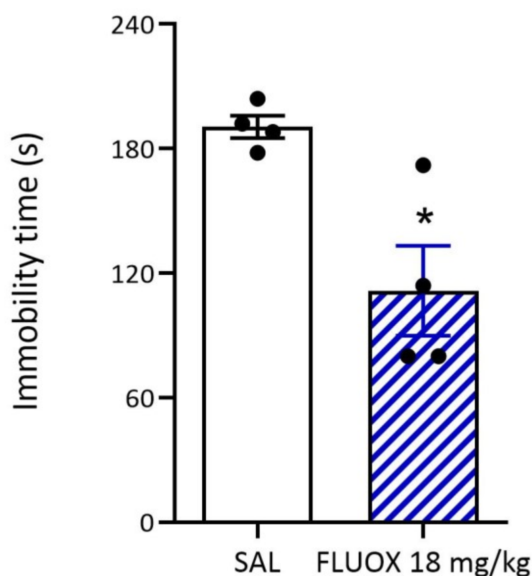

Fig. 9 Fluoxetine reduced immobility time in forced swim test.
*Fluoxetine (FLUOX) administrated (i.p.) one hour before testing reduced immobility time in forced swim test compared to saline (SAL) group (*P < 0.05, unpaired Mann-Whitney test).*
Data are presented as mean ± SEM.

The same scheme was followed for testing of A2. A2 (0.2 mg/kg), fluoxetine (18 mg/kg) or saline were administered to naïve mice one hour before the FST. The dose of A2 tested was established based on the comparison of the virtual affinities for OCT2 of cyanome and A2, and the preliminary tests showing no lethal toxicity at 0.2 mg/kg. From this experiment, we deduced that A2 (0.2 mg/kg) showed a significant antidepressant-like efficacy in FST (Fig. 10).

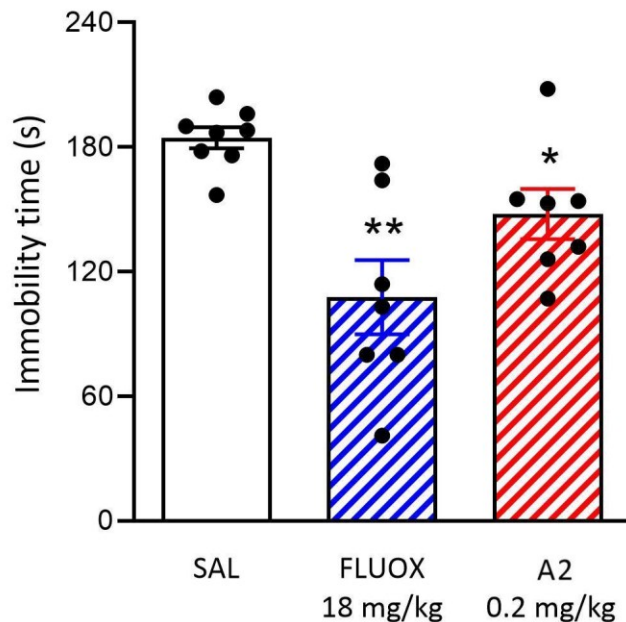

Fig. 10 Antidepressant-like efficacy of A2 in the forced swim test.

*A2 (i.p.) significantly decreased immobility time compared to saline (SAL) group (*P <0.05, unpaired Mann-Whitney test), although less strongly than fluoxetine (FLUOX) (**P < 0.01, unpaired Mann-Whitney test). Pooled data are presented as mean ± SEM.*

7 Discussion

Discussion has been included into Attachment due to intellectual property reasons.

8 Conclusion

Conclusion has been included into Attachment due to intellectual property reasons.

9 List of figures

Fig. 1 Schematic representation of the OCT involvement in monoamine clearance in central nervous system. _ 25
Fig. 2 Representation of behavioural despair tests. ____ 27
Fig. 3 Schematic representation of a brain capillary. ____ 29
Fig. 4 Chemical structure of D24 (red), cyanome (blue), and H2-cyanome (black). ____ 30
Fig. 5 Schematic representation of immobility, swimming and climbing in forced swim test. ____ 37
Fig. 6 Number of living HEK-293T cells per ml after a 24h incubation with increasing concentrations of A2 or control (CTRL) DMSO 0.1%. ____ 38
Fig. 7 Cell viability and IC_{50}. ____ 39
Fig. 8 HeLa cells analyzed by flow cytometry. ____ 40
Fig. 9 Fluoxetine reduced immobility time in forced swim test. ____ 41
Fig. 10 Antidepressant-like efficacy of A2 in the forced swim test. ____ 42

10 List of tables

Tab. 1 List of antidepressants. ____ 19
Tab. 2 Abundance of mRNA and protein of human organic cation transporters in chosen organs, tissue and cells. ____ 21
Tab. 3 Chosen endogenous substrate of human organic cation transporters (OCT). ____ 22
Tab. 4 Increasing doses of A2 administered i.p. to mice (n = 5) for four days. ____ 35

11 References

Abbott, N. J., Patabendige, A. A. K., Dolman, D. E. M., Yusof, S. R., & Begley, D. J. (2010). Structure and function of the blood–brain barrier. *Neurobiology of Disease*, *37*(1), 13–25. https://doi.org/10.1016/j.nbd.2009.07.030

Alexander, S. P., Davenport, A. P., Kelly, E., Marrion, N., Peters, J. A., Benson, H. E., Faccenda, E., Pawson, A. J., Sharman, J. L., Southan, C., Davies, J. A., & CGTP Collaborators. (2015). The Concise Guide to PHARMACOLOGY 2015/16: G protein-coupled receptors. *British Journal of Pharmacology*, *172*(24), 5744–5869. https://doi.org/10.1111/bph.13348

Amphoux, A., Millan, M. J., Cordi, A., Bönisch, H., Vialou, V., Mannoury la Cour, C., Dupuis, D. S., Giros, B., & Gautron, S. (2010). Inhibitory and facilitory actions of isocyanine derivatives at human and rat organic cation transporters 1, 2 and 3: A comparison to human α1- and α2-adrenoceptor subtypes. *European Journal of Pharmacology*, *634*(1), 1–9. https://doi.org/10.1016/j.ejphar.2010.02.012

Amphoux, A., Vialou, V., Drescher, E., Brüss, M., Mannoury La Cour, C., Rochat, C., Millan, M. J., Giros, B., Bönisch, H., & Gautron, S. (2006). Differential pharmacological in vitro properties of organic cation transporters and regional distribution in rat brain. *Neuropharmacology*, *50*(8), 941–952. https://doi.org/10.1016/j.neuropharm.2006.01.005

Artigas, F. (2013). Serotonin receptors involved in antidepressant effects. *Pharmacology & Therapeutics*, *137*(1), 119–131. https://doi.org/10.1016/j.pharmthera.2012.09.006

Azhar, Y., & Din, A. U. (2021). Brexanolone. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK541054/

Bacq, A., Balasse, L., Biala, G., Guiard, B., Gardier, A. M., Schinkel, A., Louis, F., Vialou, V., Martres, M.-P., Chevarin, C., Hamon, M., Giros, B., & Gautron, S. (2012). Organic cation transporter 2

controls brain norepinephrine and serotonin clearance and antidepressant response. *Molecular Psychiatry*, *17*(9), 926–939. https://doi.org/10.1038/mp.2011.87

Baganz, N. L., Horton, R. E., Calderon, A. S., Owens, W. A., Munn, J. L., Watts, L. T., Koldzic-Zivanovic, N., Jeske, N. A., Koek, W., Toney, G. M., & Daws, L. C. (2008). Organic cation transporter 3: Keeping the brake on extracellular serotonin in serotonin-transporter-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(48), 18976–18981. https://doi.org/10.1073/pnas.0800466105

Beaulieu, J.-M., & Gainetdinov, R. R. (2011). The Physiology, Signaling, and Pharmacology of Dopamine Receptors. *Pharmacological Reviews*, *63*(1), 182–217. https://doi.org/10.1124/pr.110.002642

Berger, M., Gray, J. A., & Roth, B. L. (2009). The Expanded Biology of Serotonin. *Annual Review of Medicine*, *60*, 355–366. https://doi.org/10.1146/annurev.med.60.042307.110802

Berman, R. M., Cappiello, A., Anand, A., Oren, D. A., Heninger, G. R., Charney, D. S., & Krystal, J. H. (2000). Antidepressant effects of ketamine in depressed patients. *Biological Psychiatry*, *47*(4), 351–354. https://doi.org/10.1016/S0006-3223(99)00230-9

Berton, O., & Nestler, E. J. (2006). New approaches to antidepressant drug discovery: Beyond monoamines. *Nature Reviews Neuroscience*, *7*(2), 137–151. https://doi.org/10.1038/nrn1846

Bodor, N., & Buchwald, P. (2003). Brain-Targeted Drug Delivery. *American Journal of Drug Delivery*, *1*(1), 13–26. https://doi.org/10.2165/00137696-200301010-00002

Bodor, N., Prokai, L., Wu, W., Farag, H., Jonalagadda, S., Kawamura, M., & Simpkins, J. (1992). A strategy for delivering peptides into the central nervous system by sequential metabolism. *Science*, *257*(5077), 1698–1700. https://doi.org/10.1126/science.1529356

Brede, M., Philipp, M., Knaus, A., Muthig, V., & Hein, L. (2004). alpha2-adrenergic receptor subtypes—Novel functions uncovered in gene-targeted mouse models. *Biology of the Cell*, *96*(5), 343–348. https://doi.org/10.1016/j.biolcel.2004.03.006

Brunello, N., Mendlewicz, J., Kasper, S., Leonard, B., Montgomery, S., Nelson, J. C., Paykel, E., Versiani, M., & Racagni, G. (2002). The role of noradrenaline and selective noradrenaline reuptake inhibition in depression. *European Neuropsychopharmacology*, *12*(5), 461–475. https://doi.org/10.1016/S0924-977X(02)00057-3

Busch, A. E., Karbach, U., Miska, D., Gorboulev, V., Akhoundova, A., Volk, C., Arndt, P., Ulzheimer, J. C., Sonders, M. S., Baumann, C., Waldegger, S., Lang, F., & Koepsell, H. (1998). Human Neurons Express the Polyspecific Cation Transporter hOCT2, Which Translocates Monoamine Neurotransmitters, Amantadine, and Memantine. *Molecular Pharmacology*, *54*(2), 342–352. https://doi.org/10.1124/mol.54.2.342

Charney, D. S. (1998). Monoamine dysfunction and the pathophysiology and treatment of depression. *The Journal of Clinical Psychiatry*, *59 Suppl 14*, 11–14.

Commissioner, O. (2020, March 24). *FDA approves new nasal spray medication for treatment-resistant depression; available only at a certified doctor's office or clinic*. FDA; FDA. https://www.fda.gov/news-events/press-announcements/fda-approves-new-nasal-spray-medication-treatment-resistant-depression-available-only-certified

Cottingham, C., & Wang, Q. (2012). α2 adrenergic receptor dysregulation in depressive disorders: Implications for the neurobiology of depression and antidepressant therapy. *Neuroscience & Biobehavioral Reviews*, *36*(10), 2214–2225. https://doi.org/10.1016/j.neubiorev.2012.07.011

Couroussé, T., & Gautron, S. (2015). Role of organic cation transporters (OCTs) in the brain. *Pharmacology and Therapeutics*, *146*, 94–103. https://doi.org/10.1016/j.pharmthera.2014.09.008

Cryan, J. F., Markou, A., & Lucki, I. (2002). Assessing antidepressant activity in rodents: Recent developments and future needs. *Trends in Pharmacological Sciences*, *23*(5), 238–245. https://doi.org/10.1016/s0165-6147(02)02017-5

Daneman, R., & Prat, A. (2015). The blood-brain barrier. *Cold Spring Harbor Perspectives in Biology*, *7*(1), a020412. https://doi.org/10.1101/cshperspect.a020412

Dantzler, W. H., Evans, K. K., & Wright, S. H. (1998). Basolateral choline transport in isolated rabbit renal proximal tubules. *Pflügers Archiv*, *436*(6), 899–905. https://doi.org/10.1007/s004240050721

David, D. J., Klemenhagen, K. C., Holick, K. A., Saxe, M. D., Mendez, I., Santarelli, L., Craig, D. A., Zhong, H., Swanson, C. J., Hegde, L. G., Ping, X. I., Dong, D., Marzabadi, M. R., Gerald, C. P., & Hen, R. (2007). Efficacy of the MCHR1 Antagonist N-[3-(1-{[4-(3,4-Difluorophenoxy)phenyl]methyl}(4-piperidyl))-4-methylphenyl]-2-methylpropanamide (SNAP 94847) in Mouse Models of Anxiety and Depression following Acute and Chronic Administration Is Independent of Hippocampal Neurogenesis. *Journal of Pharmacology and Experimental Therapeutics*, *321*(1), 237–248. https://doi.org/10.1124/jpet.106.109678

Drevets, W. C. (2000). Neuroimaging studies of mood disorders. *Biological Psychiatry*, *48*(8), 813–829. https://doi.org/10.1016/S0006-3223(00)01020-9

Drevets, W. C. (2001). Neuroimaging and neuropathological studies of depression: Implications for the cognitive-emotional features of mood disorders. *Current Opinion in Neurobiology*, *11*(2), 240–249. https://doi.org/10.1016/S0959-4388(00)00203-8

Dunlop, B. W., & Nemeroff, C. B. (2007). The Role of Dopamine in the Pathophysiology of Depression. *Archives of General Psychiatry*, *64*(3), 327. https://doi.org/10.1001/archpsyc.64.3.327

Engel, K., & Wang, J. (2005). Interaction of Organic Cations with a Newly Identified Plasma Membrane Monoamine Transporter. *Molecular Pharmacology*, *68*(5), 1397–1407. https://doi.org/10.1124/mol.105.016832

Engel, K., Zhou, M., & Wang, J. (2004). Identification and Characterization of a Novel Monoamine Transporter in the Human Brain *. *Journal of Biological Chemistry*, *279*(48), 50042–50049. https://doi.org/10.1074/jbc.M407913200

European Medicine Agency. „Guideline on the Investigation of Drug Interactions", 2012. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-investigation-drug-interactions-revision-1_en.pdf.

Ferrari, A. J., Charlson, F. J., Norman, R. E., Patten, S. B., Freedman, G., Murray, C. J. L., Vos, T., & Whiteford, H. A. (2013). Burden of Depressive Disorders by Country, Sex, Age, and Year:

Findings from the Global Burden of Disease Study 2010. *PLOS Medicine*, *10*(11), 12. https://doi.org/10.1371/journal.pmed.1001547

Ferrari, A. J., Somerville, A. J., Baxter, A. J., Norman, R., Patten, S. B., Vos, T., & Whiteford, H. A. (2013). Global variation in the prevalence and incidence of major depressive disorder: A systematic review of the epidemiological literature. *Psychological Medicine*, *43*(3), 471–481. https://doi.org/10.1017/S0033291712001511

Filipski, K. K., Mathijssen, R. H., Mikkelsen, T. S., Schinkel, A. H., & Sparreboom, A. (2009). Contribution of Organic Cation Transporter 2 (OCT2) to Cisplatin-Induced Nephrotoxicity. *Clinical Pharmacology & Therapeutics*, *86*(4), 396–402. https://doi.org/10.1038/clpt.2009.139

Gasser, P. J., Orchinik, M., Raju, I., & Lowry, C. A. (2009). Distribution of organic cation transporter 3, a corticosterone-sensitive monoamine transporter, in the rat brain. *The Journal of Comparative Neurology*, *512*(4), 529–555. https://doi.org/10.1002/cne.21921

Gorboulev, V., Ulzheimer, J. C., Akhoundova, A., Ulzheimer-Teuber, I., Karbach, U., Quester, S., Baumann, C., Lang, F., Busch, A. E., & Koepsell, H. (1997). Cloning and characterization of two human polyspecific organic cation transporters. *DNA and Cell Biology*, *16*(7), 871–881. https://doi.org/10.1089/dna.1997.16.871

Gorbunov, D., Gorboulev, V., Shatskaya, N., Mueller, T., Bamberg, E., Friedrich, T., & Koepsell, H. (2008). High-affinity cation binding to organic cation transporter 1 induces movement of helix 11 and blocks transport after mutations in a modeled interaction domain between two helices. *Molecular Pharmacology*, *73*(1), 50–61. https://doi.org/10.1124/mol.107.040170

Gründemann, D., Gorboulev, V., Gambaryan, S., Veyhl, M., & Koepsell, H. (1994). Drug excretion mediated by a new prototype of polyspecific transporter. *Nature*, *372*(6506), 549–552. https://doi.org/10.1038/372549a0

Gründemann, D., Schechinger, B., Rappold, G. A., & Schömig, E. (1998). Molecular identification of the corticosterone-sensitive extraneuronal catecholamine transporter. *Nature Neuroscience*, *1*(5), 349–351. https://doi.org/10.1038/1557

Hamon, M., & Blier, P. (2013). Monoamine neurocircuitry in depression and strategies for new treatments. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *45*, 54–63. https://doi.org/10.1016/j.pnpbp.2013.04.009

Hasler, G., van der Veen, J. W., Tumonis, T., Meyers, N., Shen, J., & Drevets, W. C. (2007). Reduced Prefrontal Glutamate/Glutamine and γ-Aminobutyric Acid Levels in Major Depression Determined Using Proton Magnetic Resonance Spectroscopy. *Archives of General Psychiatry*, *64*(2), 193–200. https://doi.org/10.1001/archpsyc.64.2.193

Hayer-Zillgen, M., Brüss, M., & Bönisch, H. (2002). Expression and pharmacological profile of the human organic cation transporters hOCT1, hOCT2 and hOCT3. *British Journal of Pharmacology*, *136*(6), 829–836. https://doi.org/10.1038/sj.bjp.0704785

Hillhouse, T. M., & Porter, J. H. (2015). A brief history of the development of antidepressant drugs: From monoamines to glutamate. *Experimental and Clinical Psychopharmacology*, *23*(1), 1–21. https://doi.org/10.1037/a0038550

Hindmarch, I. (2002). Beyond the monoamine hypothesis: Mechanisms, molecules and methods. *European Psychiatry*, *17*, 294–299. https://doi.org/10.1016/S0924-9338(02)00653-3

Hirschfeld, R. M. A. (2000). History and evolution of the monoamine hypothesis of depression. *Journal of Clinical Psychiatry*, *61* (SUPPL. 6), 4–6.

Hughes, S., & Cohen, D. (2009). A systematic review of long-term studies of drug treated and non-drug treated depression. *Journal of Affective Disorders*, *118*(1), 9–18. https://doi.org/10.1016/j.jad.2009.01.027

ICD-11—ICD-11 for Mortality and Morbidity Statistics. (n.d.). Retrieved 28 June 2021, from https://icd.who.int/browse11/l-m/en#/http%3a%2f%2fid.who.int%2ficd%2fentity%2f1563440232

James, S. L., Abate, D., Abate, K. H., Abay, S. M., Abbafati, C., Abbasi, N., Abbastabar, H., Abd-Allah, F., Abdela, J., Abdelalim, A., Abdollahpour, I., Abdulkader, R. S., Abebe, Z., Abera, S. F., Abil, O. Z., Abraha, H. N., Abu-Raddad, L. J., Abu-Rmeileh, N. M. E., Accrombessi, M. M. K., … Murray, C. J. L. (2018). Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990–2017: A systematic analysis for the Global Burden of Disease Study 2017. *The Lancet*, *392*(10159), 1789–1858. https://doi.org/10.1016/S0140-6736(18)32279-7

Kambeitz, J. P., & Howes, O. D. (2015). The serotonin transporter in depression: Meta-analysis of in vivo and post mortem findings and implications for understanding and treating depression. *Journal of Affective Disorders*, *186*, 358–366. https://doi.org/10.1016/j.jad.2015.07.034

Kitaichi, K., Fukuda, M., Nakayama, H., Aoyama, N., Ito, Y., Fujimoto, Y., Takagi, K., Takagi, K., & Hasegawa, T. (2005). Behavioral changes following antisense oligonucleotide-induced reduction of organic cation transporter-3 in mice. *Neuroscience Letters*, *382*(1–2), 195–200. https://doi.org/10.1016/j.neulet.2005.03.014

Koepsell, H. (2020). Organic Cation Transporters in Health and Disease. *Pharmacological Reviews*, *72*(1), 253–319. https://doi.org/10.1124/pr.118.015578

Koepsell, H., Lips, K., & Volk, C. (2007). Polyspecific Organic Cation Transporters: Structure, Function, Physiological Roles, and Biopharmaceutical Implications. *Pharmaceutical Research*, *24*(7), 1227–1251. https://doi.org/10.1007/s11095-007-9254-z

Krishnan, V., & Nestler, E. J. (2008). The molecular neurobiology of depression. *Nature*, *455*(7215), 894–902. https://doi.org/10.1038/nature07455

LeDoux, J. E. (2000). Emotion circuits in the brain. *Annual Review of Neuroscience*, *23*, 155–184. https://doi.org/10.1146/annurev.neuro.23.1.155

Liang, X., Yee, S. W., Chien, H.-C., Chen, E. C., Luo, Q., Zou, L., Piao, M., Mifune, A., Chen, L., Calvert, M. E., King, S., Norheim, F., Abad, J., Krauss, R. M., & Giacomini, K. M. (2018). Organic cation transporter 1 (OCT1) modulates multiple cardiometabolic traits through effects on hepatic thiamine content. *PLOS Biology*, *16*(4), e2002907. https://doi.org/10.1371/journal.pbio.2002907

Lipinski, C. A. (2004). Lead- and drug-like compounds: The rule-of-five revolution. *Drug Discovery Today: Technologies*, *1*(4), 337–341. https://doi.org/10.1016/j.ddtec.2004.11.007

Lips, K. S., Volk, C., Schmitt, B. M., Pfeil, U., Arndt, P., Miska, D., Ermert, L., Kummer, W., & Koepsell, H. (2005). Polyspecific Cation Transporters Mediate Luminal Release of Acetylcholine from Bronchial Epithelium. *American Journal of Respiratory Cell and Molecular Biology*, *33*(1), 79–88. https://doi.org/10.1165/rcmb.2004-0363OC

Lupien, S. J., McEwen, B. S., Gunnar, M. R., & Heim, C. (2009). Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nature Reviews Neuroscience*, *10*(6), 434–445. https://doi.org/10.1038/nrn2639

Mahase, E. (2019). Esketamine is approved in Europe for treating resistant major depressive disorder. *BMJ*, l7069. https://doi.org/10.1136/bmj.l7069

Mandrioli, R., Protti, M., & Mercolini, L. (2018). New-Generation, Non-SSRI Antidepressants: Therapeutic Drug Monitoring and Pharmacological Interactions. Part 1: SNRIs, SMSs, SARIs. *Current Medicinal Chemistry*, *25*(7), 772–792. https://doi.org/10.2174/0929867324666170712165042

Mathew, S. J., Manji, H. K., & Charney, D. S. (2008). Novel Drugs and Therapeutic Targets for Severe Mood Disorders. *Neuropsychopharmacology*, *33*(9), 2080–2092. https://doi.org/10.1038/sj.npp.1301652

Minematsu, T., & Giacomini, K. M. (2011). Interactions of Tyrosine Kinase Inhibitors with Organic Cation Transporters, OCTs, and Multidrug and Toxic Compound Extrusion Proteins, MATEs. *Molecular Cancer Therapeutics*, *10*(3), 531–539. https://doi.org/10.1158/1535-7163.MCT-10-0731

Minuesa, G., Volk, C., Molina-Arcas, M., Gorboulev, V., Erkizia, I., Arndt, P., Clotet, B., Pastor-Anglada, M., Koepsell, H., & Martinez-Picado, J. (2009). Transport of Lamivudine [(-)-β-l-2′,3′-Dideoxy-3′-thiacytidine] and High-Affinity Interaction of Nucleoside Reverse Transcriptase Inhibitors with Human Organic Cation Transporters 1, 2, and 3. *Journal of Pharmacology and Experimental Therapeutics*, *329*(1), 252–261. https://doi.org/10.1124/jpet.108.146225

Moret, C., & Briley, M. (2011). The importance of norepinephrine in depression. *Neuropsychiatric Disease and Treatment*, *7*(Suppl 1), 9–13. https://doi.org/10.2147/NDT.S19619

Nakayama, H., Kitaichi, K., Ito, Y., Hashimoto, K., Takagi, K., Yokoi, T., Takagi, K., Ozaki, N., Yamamoto, T., & Hasegawa, T. (2007). The role of organic cation transporter-3 in methamphetamine disposition and its behavioral response in rats. *Brain Research*, *1184*, 260–269. https://doi.org/10.1016/j.brainres.2007.09.072

Nestler, E. J., Barrot, M., DiLeone, R. J., Eisch, A. J., Gold, S. J., & Monteggia, L. M. (2002). Neurobiology of Depression. *Neuron*, *34*(1), 13–25. https://doi.org/10.1016/S0896-6273(02)00653-0

Nichols, D. E., & Nichols, C. D. (2008). Serotonin Receptors. *Chemical Reviews*, *108*(5), 1614–1641. https://doi.org/10.1021/cr078224o

Nies, A. T., Herrmann, E., Brom, M., & Keppler, D. (2008). Vectorial transport of the plant alkaloid berberine by double-transfected cells expressing the human organic cation transporter 1 (OCT1, SLC22A1) and the efflux pump MDR1 P-glycoprotein (ABCB1). *Naunyn-Schmiedeberg's Archives of Pharmacology*, *376*(6), 449–461. https://doi.org/10.1007/s00210-007-0219-x

Nies, A. T., Hofmann, U., Resch, C., Schaeffeler, E., Rius, M., & Schwab, M. (2011). Proton Pump Inhibitors Inhibit Metformin Uptake by Organic Cation Transporters (OCTs). *PLoS ONE*, *6*(7), e22163. https://doi.org/10.1371/journal.pone.0022163

Orrico-Sanchez, A., Chausset-Boissarie, L., Alves de Sousa, R., Coutens, B., Rezai Amin, S., Vialou, V., Louis, F., Hessani, A., Dansette, P. M., Zornoza, T., Gruszczynski, C., Giros, B., Guiard, B. P., Acher, F., Pietrancosta, N., & Gautron, S. (2020). Antidepressant efficacy of a selective organic cation transporter blocker in a mouse model of depression. *Molecular Psychiatry*, *25*(6), 1245–1259. https://doi.org/10.1038/s41380-019-0548-4

Pariante, C. M., & Lightman, S. L. (2008). The HPA axis in major depression: Classical theories and new developments. *Trends in Neurosciences*, *31*(9), 464–468. https://doi.org/10.1016/j.tins.2008.06.006

Pereira, V. S., & Hiroaki-Sato, V. A. (2018). A brief history of antidepressant drug development: From tricyclics to beyond ketamine. *Acta Neuropsychiatrica*, *30*(6), 307–322. https://doi.org/10.1017/neu.2017.39

Pittenger, C., & Duman, R. S. (2008). Stress, Depression, and Neuroplasticity: A Convergence of Mechanisms. *Neuropsychopharmacology*, *33*(1), 88–109. https://doi.org/10.1038/sj.npp.1301574

Planchez, B., Surget, A., & Belzung, C. (2019). Animal models of major depression: Drawbacks and challenges. *Journal of Neural Transmission (Vienna, Austria: 1996)*, *126*(11), 1383–1408. https://doi.org/10.1007/s00702-019-02084-y

Porsolt, R. D., Le Pichon, M., & Jalfre, M. (1977). Depression: A new animal model sensitive to antidepressant treatments. *Nature*, *266*(5604), 730–732. https://doi.org/10.1038/266730a0

Ruhé, H. G., Mason, N. S., & Schene, A. H. (2007). Mood is indirectly related to serotonin, norepinephrine and dopamine levels in humans: A meta-analysis of monoamine depletion studies. *Molecular Psychiatry*, *12*(4), 331–359. https://doi.org/10.1038/sj.mp.4001949

Rush, A. J., Trivedi, M. H., Wisniewski, S. R., Nierenberg, A. A., Stewart, J. W., Warden, D., Niederehe, G., Thase, M. E., Lavori, P. W., Lebowitz, B. D., McGrath, P. J., Rosenbaum, J. F., Sackeim, H. A., Kupfer, D. J., Luther, J., & Fava, M. (2006). Acute and Longer-Term Outcomes in Depressed Outpatients Requiring One or Several Treatment Steps: A STAR*D Report. *American Journal of Psychiatry*, *163*(11), 1905–1917. https://doi.org/10.1176/ajp.2006.163.11.1905

Russ, H., Engel, W., & Schoemig, E. (1993). Isocyanines and pseudoisocyanines as a novel class of potent noradrenaline-transport inhibitors: Synthesis, detection, and biological activity. *Journal of Medicinal Chemistry*, *36*(26), 4208–4213. https://doi.org/10.1021/jm00078a010

Schramm, N. L., McDonald, M. P., & Limbird, L. E. (2001). The alpha(2a)-adrenergic receptor plays a protective role in mouse behavioral models of depression and anxiety. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *21*(13), 4875–4882.

Smith, Q. R. (1989). Quantitation of Blood-Brain Barrier Permeability. In E. A. Neuwelt (Ed.), *Implications of the Blood-Brain Barrier and Its Manipulation: Volume 1 Basic Science Aspects* (pp. 85–118). Springer US. https://doi.org/10.1007/978-1-4613-0701-3_4

Song, W., Luo, Q., Zhang, Y., Zhou, L., Liu, Y., Ma, Z., Guo, J., Huang, Y., Cheng, L., Meng, Z., Li, Z., Zhang, B., Li, S., Yee, S. W., Fan, H., Li, P., Giacomini, K. M., & Chen, L. (2019). Organic cation transporter 3 (Oct3) is a distinct catecholamines clearance route in adipocytes mediating the beiging of white adipose tissue. *PLoS Biology*, *17*(1), e2006571. https://doi.org/10.1371/journal.pbio.2006571

Steru, L., Chermat, R., Thierry, B., & Simon, P. (1985). The tail suspension test: A new method for screening antidepressants in mice. *Psychopharmacology*, *85*(3), 367–370. https://doi.org/10.1007/BF00428203

Stockmeier, C. A. (2003). Involvement of serotonin in depression: Evidence from postmortem and imaging studies of serotonin receptors and the serotonin transporter. *Journal of Psychiatric Research*, *37*(5), 357–373. https://doi.org/10.1016/S0022-3956(03)00050-5

Švihovec, Jan, Jan Bultas, Pavel Anzenbacher, Jaroslav Chládek, Jan Příborský, Jiří Slíva, a Martin Votava. „Antidepresiva". In *Farmakologie*, 2018: s. 258. Praha: Grada Publishing, ISBN 978-80-247-5558-8.

Taubert, D., Grimberg, G., Stenzel, W., & Schömig, E. (2007). Identification of the endogenous key substrates of the human organic cation transporter OCT2 and their implication in function of dopaminergic neurons. *PloS One*, *2*(4), e385. https://doi.org/10.1371/journal.pone.0000385

Ulrich-Lai, Y. M., & Herman, J. P. (2009). Neural regulation of endocrine and autonomic stress responses. *Nature Reviews. Neuroscience*, *10*(6), 397–409. https://doi.org/10.1038/nrn2647

Urakami, Y., Kimura, N., Okuda, M., & Inui, K. (2004). Creatinine transport by basolateral organic cation transporter hOCT2 in the human kidney. *Pharmaceutical Research*, *21*(6), 976–981. https://doi.org/10.1023/b:pham.0000029286.45788.ad

Vialou, V., Balasse, L., Callebert, J., Launay, J.-M., Giros, B., & Gautron, S. (2008). Altered aminergic neurotransmission in the brain of organic cation transporter 3-deficient mice. *Journal of Neurochemistry*, *106*(3), 1471–1482. https://doi.org/10.1111/j.1471-4159.2008.05506.x

Wang, B., Wang, Y., Wu, Q., Huang, H., & Li, S. (2017). Effects of α2A Adrenoceptors on Norepinephrine Secretion from the Locus Coeruleus during Chronic Stress-Induced Depression. *Frontiers in Neuroscience*, *0*. https://doi.org/10.3389/fnins.2017.00243

Wang, D.-S., Jonker, J. W., Kato, Y., Kusuhara, H., Schinkel, A. H., & Sugiyama, Y. (2002). Involvement of organic cation transporter 1 in hepatic and intestinal distribution of metformin. *The Journal of Pharmacology and Experimental Therapeutics*, *302*(2), 510–515. https://doi.org/10.1124/jpet.102.034140

Wilkinson, S. T., & Sanacora, G. (2019). A new generation of antidepressants: An update on the pharmaceutical pipeline for novel and rapid-acting therapeutics in mood disorders based on glutamate/GABA neurotransmitter systems. *Drug Discovery Today*, *24*(2), 606–615. https://doi.org/10.1016/j.drudis.2018.11.007

Witkin, J. M., Martin, A. E., Golani, L. K., Xu, N. Z., & Smith, J. L. (2019). Rapid-acting antidepressants. In *Advances in Pharmacology* (Vol. 86, pp. 47–96). Elsevier. https://doi.org/10.1016/bs.apha.2019.03.002

Wultsch, T., Grimberg, G., Schmitt, A., Painsipp, E., Wetzstein, H., Breitenkamp, A. F. S., Gründemann, D., Schömig, E., Lesch, K.-P., Gerlach, M., & Reif, A. (2009). Decreased anxiety in mice lacking the organic cation transporter 3. *Journal of Neural Transmission*, *116*(6), 689–697. https://doi.org/10.1007/s00702-009-0205-1

Yanpallewar, S. U., Fernandes, K., Marathe, S. V., Vadodaria, K. C., Jhaveri, D., Rommelfanger, K., Ladiwala, U., Jha, S., Muthig, V., Hein, L., Bartlett, P., Weinshenker, D., & Vaidya, V. A. (2010). Alpha2-adrenoceptor blockade accelerates the neurogenic, neurotrophic, and behavioral effects of chronic antidepressant treatment. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *30*(3), 1096–1109. https://doi.org/10.1523/JNEUROSCI.2309-09.2010

Zarate, C. A., Singh, J. B., Carlson, P. J., Brutsche, N. E., Ameli, R., Luckenbaugh, D. A., Charney, D. S., & Manji, H. K. (2006). A randomized trial of an N-methyl-D-aspartate antagonist in treatment-resistant major depression. *Archives of General Psychiatry*, *63*(8), 856–864. https://doi.org/10.1001/archpsyc.63.8.856

12 Appendix

Appendix has been included into Attachment due to intellectual property reasons.