Action des récepteurs 5-HT2A sur les systèmes de neurotransmetteurs dans le cerveau de souris : application aux psychédéliques

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THÈSE PRÉSENTÉE
POUR OBTENIR LE GRADE DE

DOCTEUR DE
L'UNIVERSITÉ DE BORDEAUX

ÉCOLE DOCTORALE : [154]

SPÉCIALITÉ : [NEUROSCIENCES]

Par Jasmine J Butler

Action des récepteurs 5-HT_{2A} sur les systèmes de neurotransmetteurs dans le cerveau de souris : application aux psychédéliques

Action of 5-HT_{2A} receptors on neurotransmitter systems in the mouse brain: application to Psychedelics

Sous la direction de : Prof. Dr. Philippe De Deurwaerdere

Soutenue le 7/3/2025

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Résumés en français et en anglais

Action des récepteurs 5-HT_{2A} sur les systèmes de neurotransmetteurs dans le cerveau de la souris : application aux psychédéliques

Résumé

Le sous-type de récepteur de la sérotonine 2A (5-HT_{2A}R) suscite un grand intérêt suite aux recherches clinique et préclinique sur les psychédéliques sérotoninergiques. Ces composés ont une action agoniste convergente sur le 5-HT2AR et ont des propriétés antidépressives et anxiolytiques. Cet intérêt s'ajoute à des données antérieures montrant que l'antagonisme du récepteur 5-HT2AR, qui fait partie du profil pharmacologique des antipsychotiques atypiques, pourrait avoir des effets bénéfiques dans plusieurs pathologies dont la schizophrénie. Néanmoins, l'impact des 5-HT2AR sur le fonctionnement cérébral, en particulier sur la neurotransmission, est peu connu. Les réseaux cérébraux fonctionnels ont été conceptualisés en corrélant des signaux électriques ou métaboliques entre les régions du cerveau grâce à l'imagerie par résonance magnétique fonctionnelle ou l'encéphalographie. Ces études de neuroimagerie ont montré que les psychédéliques modifient la connectivité de réseaux cérébraux mais elles ne peuvent pas évaluer la neurochimie des systèmes de neurotransmetteurs et leur interaction. Cette thèse aborde l'hypothèse selon laquelle les agonistes 5-HT2AR psychédéliques perturbent l'organisation fonctionnelle des systèmes de neurotransmetteurs à l'échelle du cerveau. Le contenu tissulaire des neurotransmetteurs classiques (glutamate et GABA) et monoaminergiques (sérotonine, dopamine et noradrénaline) et de leurs métabolites a été mesuré dans 28 régions du cerveau de souris après l'administration d'un agoniste et d'un antagoniste 5-HT2AR de haute affinité, le TCB-2 et le MDL100,907, respectivement. Pour promouvoir une organisation cohérente des systèmes de neurotransmetteurs, les souris ont été placées dans un paradigme d'exploration forcée et leur comportement a été filmé avant la quantification neurochimique post-mortem. Un défi important de cette thèse fut la manipulation d'un large nombre de données neurochimiques qui, au-delà de l'aspect quantitatif, permet une approche corrélative incorporant la théorie des graphes pour construire des réseaux de connectivité neurochimique. Cette nouvelle analyse a entraîné le développement d'un code accompagnant le lancement d'une base de données neurochimiques pour rendre accessible ces données.

Mots clés: Neurochimie, neurotransmetteurs, réseaux neurochimiques, comportement, pharmacologie, récepteur de la sérotonine 2A

Action of 5-HT$_{2A}$ receptors on neurotransmitter systems in the mouse brain: application to Psychedelics

Abstract

The serotonin 2A receptor subtype (5-HT2AR) has gained interest following a resurgence of clinical and pre-clinical research on serotonergic psychedelics, compounds with converging agonist action on the 5-HT2AR. Psychedelics have antidepressant and anxiolytic properties, particularly when paired with therapy. Antagonism at the 5-HT2AR, as part of the pharmacological profile of atypical antipsychotics, may have benefits for schizophrenia and bipolar disorder. Despite their clinical relevance, the known impact of 5-HT2ARs on brain function, particularly neurotransmission, is limited. Functional brain networks have been conceptualised by correlating electrical or metabolic neuroimaging signals between brain regions using functional magnetic resonance imaging or encephalography. These studies have shown that psychedelic 5-HT2AR agonists alter the connectivity of these brain-wide networks. However, neuroimaging is currently unable to assess the neurochemistry of neurotransmitter systems and their interplay. This thesis addresses the hypothesis that psychedelic 5-HT2AR agonists disrupt the functional organization of brain-wide neurotransmitter systems. Tissue content of both classical (glutamate and GABA) and monoaminergic (serotonin, dopamine and noradrenaline) neurotransmitters and their metabolites were measured in 28 regions of the mouse brain following high-affinity 5-HT2AR agonist and antagonist, TCB-2 and MDL100,907 respectively. To promote a coherent organisation of neurotransmitter systems mice were placed in a forced exploration paradigm and their behaviour was filmed before post-mortem neurochemical quantification. A significant challenge of this thesis lies in manipulating such a large neurochemical dataset that, beyond quantitative modulation, allows for a correlative approach incorporating graph theory to build networks of neurochemical connectivity. This novel analysis prompted the development of code to accompany the launch of a neurochemical database, including this dataset, making analysis using this new approach accessible.

The obtained results demonstrate that a variety of compounds across the 28 brain regions form distinct neurobiological networks that can be monitored with high-pressure liquid chromatography coupled to electrochemical detection. A striking density of neurochemical correlations between brain regions in vehicle-treated animals was observed, with a distinct regional organisation for dopamine and noradrenaline. The 5-HT2AR agonist TCB-2 (0.3, 3, and 10 mg/kg) as well as the 5-HT2AR antagonist MDL-100,907 (0.2 mg/kg) reduced the number of correlations and disrupted the organisation of correlations for all neurotransmitters across the brain. Some effects of TCB-2, notably on serotonergic parameters were independent of 5-HT2ARs in several brain regions. Other effects including behavioural parameters such as head twitches or components of the exploratory behaviour, as well as the levels of serotonin, dopamine, and noradrenaline in the anterior cingulate cortex were reduced by MDL-100,907 pretreatment. MDL-100,907 alone had very few effects on the quantity of neurochemicals across brain regions. Overall, this thesis highlights that 5-HT2ARs likely play an important role in

organising the coherence of neurotransmitter systems in response to a forced exploratory behaviour whether or not it is associated with quantitative changes. The thesis offers a new paradigm to address the function of neurotransmitter systems. It enlarges the understanding of the mechanism of psychedelic action in the brain including vast brain territories (sensory, motor, cognitive) with some lateralized effects, and altered connectivity of neurotransmission systems.

Keywords: Neurochemistry, neurotransmitters, neurochemical networks, behaviour, pharmacology, serotonin 2A receptor

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Acknowledgments

Firstly, I thank Prof. Muriel Darnaudery, Dr. Carine Bécamel, Prof. Mickael Naassila, and Dr. Nasser Haddjeri for being part of the jury for my PhD defence, with special acknowledgments to Dr. Bécamel and Prof. Naassila for kindly agreeing to review the thesis.

I would like to express my deepest gratitude to my supervisor, Prof. Dr. Philippe De Deurwaerdere, for his continued support, guidance, and education throughout the duration of this thesis. His expertise and diligence have been invaluable to my development as a researcher and the work of thesis. I would also like to thank Dr. Anna Beyeler for her support and scientific discussion throughout the thesis. I extend my gratitude to the students who contributed to the experimental work, without who a project of this scale is not possible: Margherita Virgili, Ginalie Samji, Chloe Aman, and Clement Laffont. Regarding the development of the code, I am deeply grateful to Shiva Lindi for her support in building the initial conception of the code. I also wish to thank Remi Corn PhD for his continued contributions and improvements to its development since, without which the analytical development of this kind of neurochemical data would not have been possible.

I would like to thank Dr. Timothy Zolnik and Dr. Matthew Larkum for hosting me in the Larkum lab, Humboldt University of Berlin, during 9 months of my PhD for an ongoing electrophysiological study. Lastly, I would like to thank those who have helped in the development of my scientific writing and figure design, as well as for their fruitful discussions on the scientific literature: Sarah Duverdin, Chloe Aman, and Luna Soso Zdravkovic.

This thesis was made possible through the generous funding and support of the University of Bordeaux, France, the Centre National de la Recherche Scientifique (CNRS), the Institut des Neurosciences Intégratives et Cognitives d'Aquitaine (INCIA), the Neurocampus graduate school, and Adapsy subgroup of the GPR (University of Bordeaux) for their financial support of this project.

To everyone who has contributed to this journey, directly or indirectly, I extend my sincere gratitude.

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Abbreviations

5-HT: 5-hydroxytryptamine (serotonin), 5-HT1AR: Serotonin 1A receptor, 5-HT2AR: Serotonin 2A receptor, 5-HT2BR: Serotonin 2B receptor, 5-HT2CR: Serotonin 2C receptor, 5-HT3R: Serotonin 3 receptor, 5-HTR: Serotonin receptor, AC: Adenylyl cyclase, aCC: Anterior cingulate cortex, APZ-OAV: Abnormal Mental States/Altered States of Consciousness questionnaire, ASL: Arterial spin labelling, BOLD-fMRI: Blood oxygen level-dependent functional magnetic resonance imaging, CNS: Central nervous system, DAT: Dopamine transporter, DMN: Default mode network, DMT: N,N-Dimethyltryptamine, DOB: 2,5-Dimethoxy-4-bromoamphetamine, DOM: 2,5-Dimethoxy-4-methylamphetamine, DOI: 2,5-Dimethoxy-4-iodoamphetamine, DR: Dorsal raphe, EEG: Electroencephalography, fMRI: Functional magnetic resonance imaging, GABA: Gamma-aminobutyric acid, GLU: Glutamate, HPLC: High-pressure liquid chromatography, IEG: Immediate early gene, IT: Intra-telencephalic, L5: Layer 5, LSD: Lysergic acid diethylamide, MAO: Monoamine oxidase, MDMA: 3,4-Methylenedioxymethamphetamine, MEG: Magnetoencephalography, MRS: Magnetic resonance spectroscopy, NA: Noradrenaline, NET: Noradrenaline transporter, NMDA: N-methyl-D-aspartate, OCD: Obsessive-compulsive disorder, PET: Positron emission tomography, PFC: Prefrontal cortex, PN: Pyramidal neuron, pCC: Posterior cingulate cortex, PPI: Pre-pulse inhibition, PT: Pyramidal tract, PTSD: Post-traumatic stress disorder, REBUS: Relaxed beliefs under psychedelics, sEPSPs/sEPSCs: Spontaneous excitatory synaptic potentials/currents, SNORD: Small nucleolar RNA, SERT: Serotonin transporter, SSRIs: Selective serotonin reuptake inhibitors, TCB-2: 4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide, TBG: Tabernanthalog, VMAT: Vesicular monoamine transporter, 25I-NBOMe: 2C-I-NBOMe (2,5-dimethoxy-4-iodo-N-[(2-methoxyphenyl)methyl]phenethylamine).

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Publications

Published

Butler, J. J. *, Parrot, S. *, Aman, C., & De Deurwaerdère, P. (2024). Neurochemical Databases: Purpose and Expectations. ACS Chemical Neuroscience, acschemneuro.4c00812. https://doi.org/10.1021/acschemneuro.4c00812

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Butler JJ*, Aman C*, Rivalan M, Parrot S, Dellu-Hagedorn F, De Deurwaerdère P. Neurochemical assessment of tissue levels of neurotransmitters: from quantification to correlative networks. Submitted January 24th to ACS Chemical Neuroscience.

In preparation

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Butler J. J. The 5HT2A receptor agonist TCB-2 disrupts the correlative links between numerous classical neurotransmitters in the mouse brain.9th Meeting of the MNS, Symposium S31.3 , Tunisia, Carthage, Oct 14th- 18th, 2023.

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Preamble

The serotonergic psychedelics are a chemical class apart in psychopharmacology. Renewed interest in their potential to treat major depressive disorder resistant to traditional antidepressants, general anxiety, drug addiction, and possibly post-traumatic stress disorder has brought them back to the forefront. But the question remains: how do they work?

The initial, and still prominent, interest in these drugs stems from their ability to profoundly alter perception and consciousness. These compounds were first used in experimental psychology and psychiatry to probe the mind, and later by neurobiologists to probe the brain. Yet, after years of neuropharmacological studies, their mechanism of action, to promote the alteration of consciousness, or to be efficacious in certain neuropsychiatric conditions is still not well understood. In classical reviews on these drugs (Murnane, 2018; D. E. Nichols, 2004, 2016), it is commonly noted that there are two families of compounds, distinguished by their chemical classes: phenylalkylamines and tryptamines. This classification is reminiscent of an older system for categorizing antipsychotic drugs based on their chemical structures, at a time when their mechanisms of action were still unclear. Regarding psychedelics, we continue to use the classification of phenylalkylamines and tryptamines compounds in 2025.

Most famous class of central nervous system (CNS) medication (antidepressants, antipsychotics, and antiparkinsonian drugs), have seen considerable progress on the understanding of their mechanism of action over the last 70 years. In fact, the serendipitous discovery of LSD by Albert Hoffmann in Sandoz preceded the discovery of all these classes of modern medicines, originating in the 50's or 60's. The mechanism of action of LSD was better known at that time than that the other pharmacological classes, as it was determined during the 50's that LSD bound at serotonergic sites, reported in 1953 by Twarog and Page to be expressed in the brain (Twarog & Page, 1953). Woolley and Shaw (1954) even proposed the first hypothesis of schizophrenia as a defect of the serotonergic system, combining knowledge of the psychotomimetic properties of LSD with its ability to target brain 5-HT receptors (Woolley & Shaw, 1954). Could this be considered as a syllogism from our contemporary perspective? 35 years later, it has been proposed that antagonism at 5-HT_2 receptors (5-HT_{2A}Rs) as a factor which, combined

with the blockade of dopamine D_2 receptors (D_2R) could explain the therapeutic superiority of the so-called atypical antipsychotics (Meltzer et al., 2003). Nowadays, all atypical antipsychotics have dual actions at DA and 5-HT receptors, the latter being antagonism at 2A/2B subtypes, and/or agonism at 5-$HT_{1A}R$. Speaking of 5-$HT_{1A}R$, George Aghajanian was the first to reveal a strong inhibitory effect of LSD on the electrical activity of 5-HT neurons in the dorsal raphe nucleus (G. K. Aghajanian et al., 1968). He discovered the 5-HT autoreceptors, a key mechanism indirectly targeted by classical selective serotonin reuptake inhibitors (SSRIs). LSD, or later the phenylalkylamine DOI used for its “selective” agonism at 5-HT_2R subtypes, led to considerable progresses in the mechanism of action of the CNS medications of today, and in the understanding of 5-HT neurons function, alone or in interaction with other neuronal systems.

Psychopharmacological researches, particularly those dealing with monoaminergic systems, kept in mind the existence of psychedelic compounds without being able to indicate how they worked. Their complexity starts from the very beginning, the pharmacological profile. It is often assumed that classical serotonergic psychedelics are agonists at 5-$HT_{2A}R$, yet the situation even for perhaps the most well described member of its class, LSD, has never been clear. In the past, LSD was considered as an antagonist of 5-HT function (Glennon, 1990; Pierce & Peroutka, 1990). In terms of pharmacological responses in heterologous system of expression, the data are incongruent, often reporting low efficacy agonism. Meanwhile, some psychedelic compounds like 5-MeO-DMT, which display higher affinity for 5-$HT_{1A}R$ compared to 5-$HT_{2A}R$, can trigger psychedelic-induced responses in rodents (increased locomotion) via 5-$HT_{1A}R$, but not 5-$HT_{2A}R$ (Halberstadt & Geyer, 2011). Recently, positron emission tomography studies in rats failed to detect 5-$HT_{2A}R$ occupancy of DMT during the psychedelic effects of the serotonergic psychedelic DMT (Egger et al., 2023).

Phenylalkylamies and tryptamines, why not? We might consider that the serotonergic psychedelic class of compounds is more heterogeneous than previously conceived. Even if their mechanisms trigger psychedelic effects, the nature of the experience differs between the compounds, at least from the human perspective, also supported by their behavioural actions in animals. In contrast to various classes of CNS medication for which we know at least one essential target (D_2R antagonism - antipsychotics) we can only propose for psychedelics a heteroclite pharmacological profile made of 5-$HT_{1A}R$, 5-

$HT_{2A/2B/2C}R$, and even more if ibogaine or salvinorin A are considered. Common mechanisms if any could belong to the field of integrative neurosciences. For more than 20 years, it has been documented in humans using functional magnetic resonance imaging, electrical or magnetic encephalography that psychedelics alter brain function, perhaps differently between psychedelics. These techniques allow for monitoring cerebral blood flow or electrical activity in superficial cortical layers. They do not inform on the action of drugs on chemical transmitters in the brain, a field that remains a black box. The data collected in animals using techniques such as intracerebral microdialysis further suggest that there is no consistent pattern of effects or clear signature induced by psychedelics on neurotransmission (Butler, Ricci, et al., 2024).

The growing number of pre-clinical and clinical studies published on psychedelics, and psychoplastogens in general, will surely result in better understanding of the brain actions triggered by these compounds. Their legacy in neuroscience is undeniable and continues to expand into psychiatry, offering new perspectives on therapeutic strategies for antidepressant, anxiolytic, and anti-addictive treatments.

One of the mechanisms that is acknowledged to produce psychedelic effects is the stimulation of 5-$HT_{2A}R$. Studying the psychedelic mechanism remains a challenge in neuropsychopharmacology. Considering the methods that could provide valuable sources of information to understand their wider CNS effects, I pursued two distinct approaches during this PhD. The first approach was initiated during the master 2 in Berlin, in the lab of Matthew Larkum and prolonged 9 months during the PhD. This involved studding the electrophysiological responses of neurons in layers 5 and 6 of the mouse somatosensory cortex to the bath application of different psychedelics. The second approach constitutes this thesis. The initial angle of the thesis was to describe the hypothesized alteration of neurotransmitter systems alone and in interaction across the brain in response to the stimulation of central 5-$HT_{2A}Rs$. This corresponds to a new approach in the field of neurochemistry that could offer a new perspective in the field of psychedelics.

Introduction

The term *psychedelic* was coined in 1957 by psychiatrist Humphry Osmond during correspondence with writer Aldous Huxley (Osmond, 1957). Derived from the Greek words *psychḗ* ("soul, mind") and *dēlos* ("to manifest"), the term was intended to describe these substances capable of "manifesting the mind." At the time, Osmond was one of the most prominent researchers studying the therapeutic potential of psychedelics working predominantly with mescaline and with LSD.

This era also saw prominent figures such as writer Aldous Huxley, who became an influential advocate for the responsible exploration of altered states of consciousness. Huxley's own experiences with mescaline, as documented in *The Doors of Perception*, captivated the public imagination and highlighted the cultural and philosophical potential of these substances (Huxley, 1954). In parallel, the Harvard Psilocybin Project, led by Timothy Leary and Richard Alpert (later Ram Dass), began as psychological research but soon escalated into a controversial public experiment (Lattin, 2010). Following chemists such as Albert Hofmann, Alexander Shulgin synthesized and documented the effects of hundreds of novel psychoactive compounds, greatly expanding the chemical and experiential understanding of psychedelics (Hofmann, 1980; Shulgin & Shulgin, 1991). However, the growing recreational use of these substances in the 1960s provoked a backlash from government authorities, culminating in their criminalization by the end of the decade, which marked the end of an era of open psychedelic research. Today, in 2025, we still face challenges regarding the scheduling of these compounds and their availability for research (Sessa, 2005).

Chemical classes of psychedelics

There are four broad classes of psychedelics generally acknowledged: classical serotonergic psychedelics, empathogens, dissociative psychedelics, and atypical psychedelics. The focus of this work is on the action of classical serotonergic psychedelics; however, a brief description of each class will be included to provide a comprehensive framework for understanding their distinct mechanisms of action and effects.

Classical serotonergic psychedelics

Classical serotonergic psychedelics primarily act as agonists or partial agonists on the serotonin 2A receptor (5-HT_{2A}R) and can be divided into two structural classes, indolamines (tryptamines) and phenethylamines, as indicated in Figure 1 (Halberstadt, 2015; Kwan et al., 2022; D. E. Nichols, 2016).

Indolamines (tryptamines) typically possess an indole moiety bearing resemblance to serotonin. Classic examples include psilocin, N,N-dimethyltryptamine (DMT), and lysergic acid diethylamide (LSD). Indolamines can be found naturally, for instance psilocin (4-MeO-DMT) and ergot from in the fungal genus' *Psilocybe* and *Claviceps purpurea* respectively, or 5-MeO-DMT in the venom of the Bufo *Alvarius* toad. DMT is found in a variety of plants and animals, it has been proposed to be the only endogenous psychedelic in humans. They can also be synthesized, such as LSD from ergot. While all these compounds have considerable affinity for the 5-HT_{2A}R, they each have unique profiles beyond it.

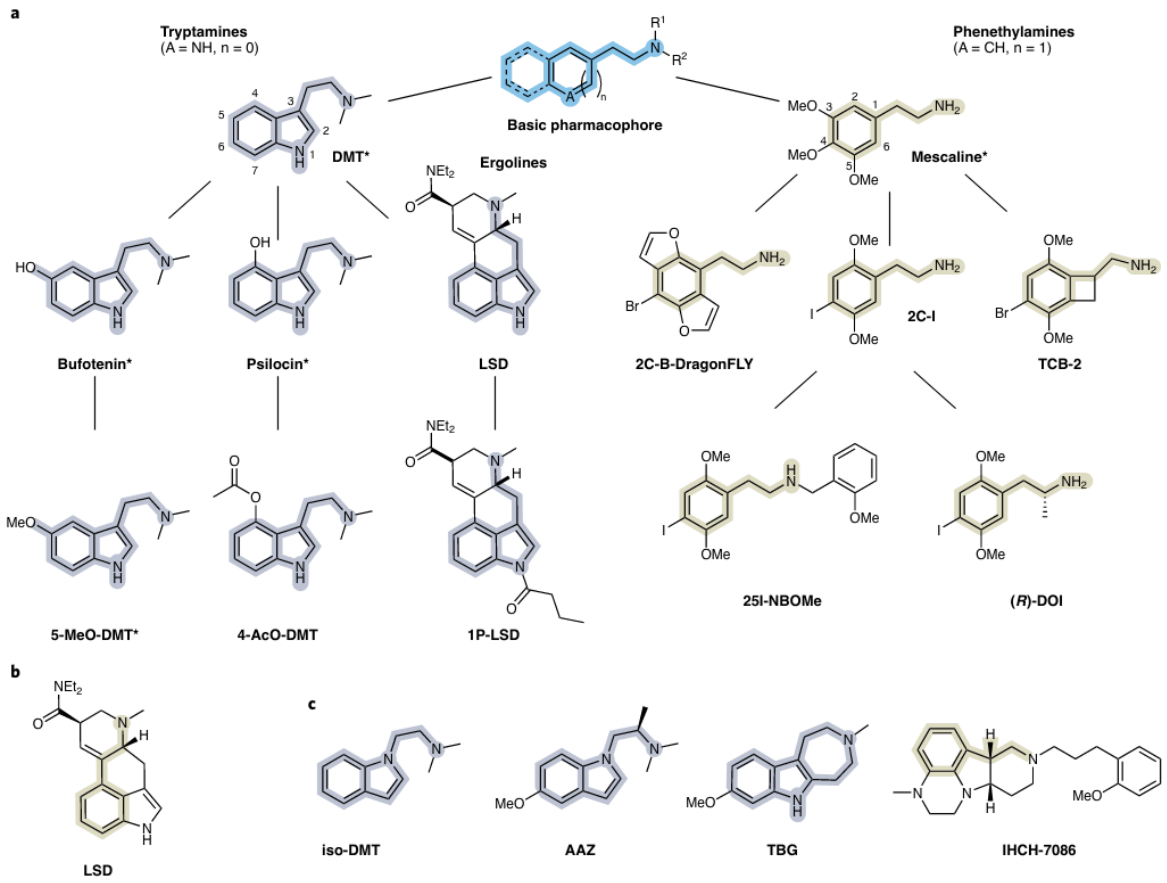

Figure 1 – Chemical Phylogeny of Psychedelics.

*(**a**) The core psychedelic pharmacophore is highlighted in blue, with tryptamine and phenethylamine pharmacophores in grey and yellow, respectively. Ergolines (e.g., LSD and 1-propionyl-d-LSD (1P-LSD)) are specialized tryptamines. Branches indicate structurally related compounds; natural products are marked with asterisks. (**b**) LSD incorporates the phenethylamine substructure (yellow), combining elements of both psychedelic families.(**c**) Structures of non-hallucinogenic psychedelic analogs with therapeutic potential, featuring tryptamine-like (grey) or phenethylamine-like (yellow) pharmacophores. TBG, tabernanthalog. (Kwan et al., 2022)*

The second class of classical psychedelics are Phenethylamines, generally derived from either mescaline or amphetamines. Classic examples include mescaline itself (3,4,5-trimethoxyphenethylamine), well as synthetic derivatives like 4-bromo-2,5-dimethoxyphenethylamine (2C-B), 2,5-Dimethoxy-4-methylamphetamine (DOM), 2,5-dimethoxy-4-bromoamphetamine (DOB), 2,5-dimethoxy-4-iodoamphetamine (DOI) and 4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide (TCB-2) (Figure 1). Naturally, mescaline is found in the *Peyote* cactus, *San Pedro* cactus, and *Peruvian torch*, additional compounds have been synthesised with modifications on the aromatic ring, typically introducing methoxy or halogen groups, altering their

potency and effects. Phenethylamines share a significant affinity for the 5-HT_{2A}R, but their effects go beyond this receptor interaction, with some compounds also engaging dopamine (DA) and noradrenaline (NA) receptors, leading to a combination of psychedelic and stimulant effects.

Empathogens/ entactogens

The primary compound in this class is 3,4-methylenedioxymethamphetamine (MDMA). It is also a phenethylamine amphetamine derivative although is not considered a classical psychedelic. While MDMA has weak affinity for the 5-HT_{2A}R, it's activity at serotonin (SERT), dopamine (DAT), and noradrenaline (NET) transporters gives it stimulant-like properties, which phenethylamine psychedelics lack (Verrico et al., 2006). Additionally, MDMA and its active metabolite MDA are serotonergic neurotoxins (De La Torre et al., 2004; Ricaurte et al., 1988).

Dissociative aesthetics

Dissociate anaesthetics are generally N-methyl-D-aspartate receptor (NMDAR) antagonists, and thereby disrupting glutamate transmission in the brain. Dissociative anaesthetics produce a sense of detachment from reality and self at lower doses and analgesia at high doses. This class contains Ketamine, phencyclidine (PCP) and nitrous oxide (NO_2) (Kalmoe et al., 2020).

Atypical Psychedelics

The final class includes substances that are psychedelic by experience, although their mechanisms of action are complex and differ from those described above. Salvinorin A, derived from the plant *Salvia divinorum*, is a potent psychoactive compound with a strong affinity for kappa-opioid receptors and no apparent affinity for the 5-HT_{2A}R (Roth et al., 2002). Another substance in this class is ibogaine, found in the root of the plant *Tabernanthe iboga*. Ibogaine does exhibit some affinity for the 5-HT_{2A}R, but its effects are largely attributed to its inhibition of SERT and the 5-HT_{2B}R, as well as its interactions with other receptor systems, including sigma receptors and NMDARs. Both substances induce profound alterations in perception, cognition, and self-awareness, but their distinct receptor profiles set them apart from classical psychedelics, dissociatives, and empathogens.

Relevance Psychedelic drugs in humans

The importance of psychedelic compounds as described by Osmond in 1957 included their capacity to mimic psychosis, a place in psychotherapy, a tool to explore “the normal mind under unusual circumstances”, and the social philosophical and religions implications in the discoveries made by means of these agents. Today, 2025, the primary interest fuelling psychedelic research is their clinical and therapeutic potential, perhaps even separate from psychotherapy or the experiential effects after which they were named. Although this profound alteration in conscious experience has captivated the interest of researchers and lay persons alike since the 1950’s, offering a reproducible method to study and explore non-ordinary states of consciousness.

The study of consciousness has shifted from philosophical inquiries, like Nagel's exploration of subjective experience, to theories viewing it as an emergent property of neurophysiological and environmental interactions, as proposed by Dennett and Varela et al. (Dennett, 1991; Nagel, 1974; Varela et al., 1991). These foundational ideas laid the groundwork for 21st-century models such as Tononi’s Integrated Information Theory, a proposed mathematical quantification of consciousness (Tononi, 2004; Tononi et al., 2016). With foundations in the neural correlates of the psychedelic state, Carhart-Harris et al. (2014) proposed the Entropic Brain Hypothesis, which describes consciousness in terms of brain entropy under psychedelics, providing a bridge to predictive coding approaches such as the Relaxed Beliefs Under Psychedelics (REBUS) model (Carhart-Harris et al., 2014; Carhart-Harris & Friston, 2019).

In the following section we briefly contextualise psychedelics within a larger anthropological scope and describe the clinical evidence for their use in the treatment of neuropsychiatric disorders.

Anthropological history

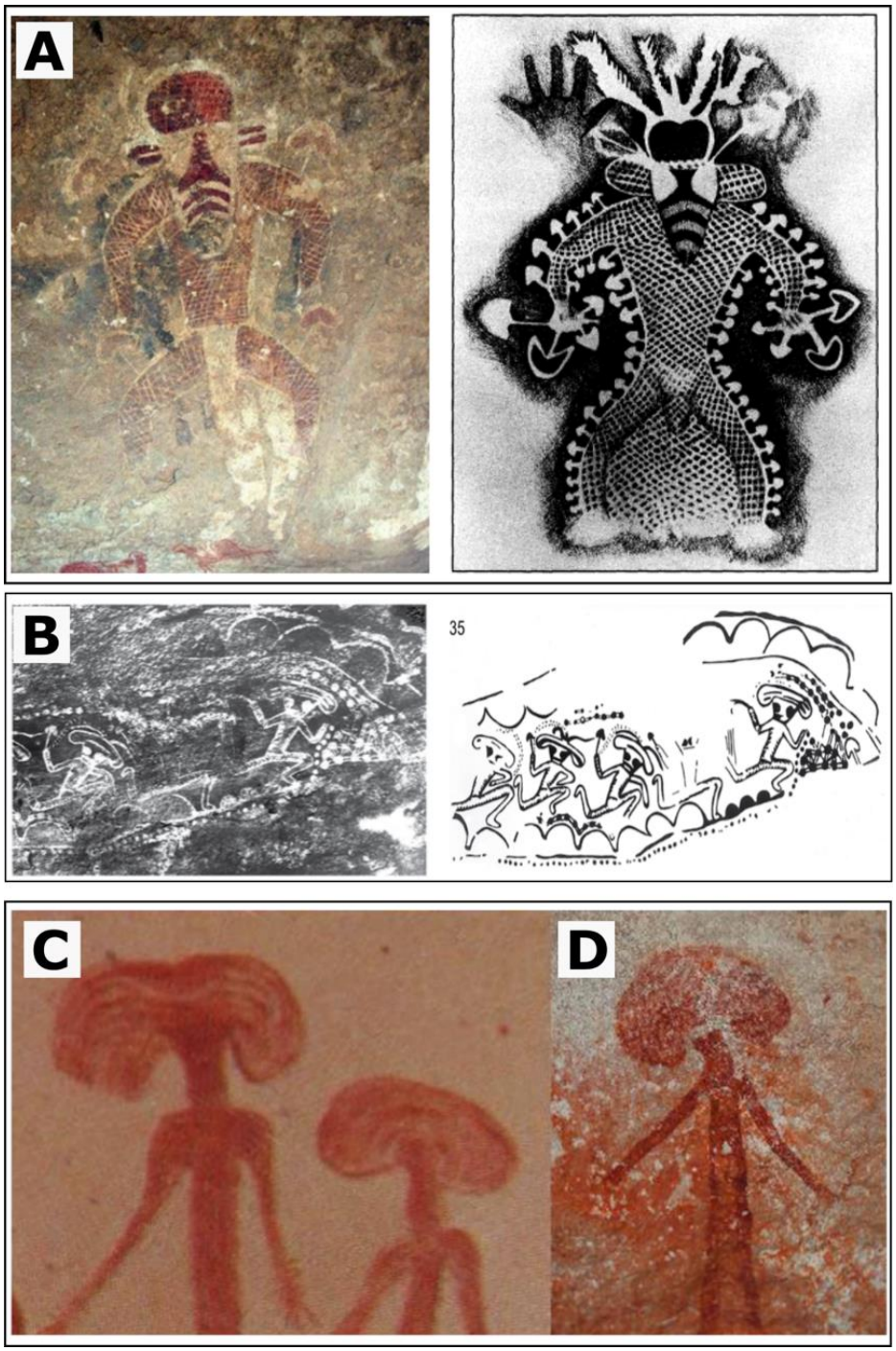

Figure 2 - Anthropological findings related to psychedelic use.

A-B Tassili prehistorical murals of **A** **‘**Shaman covered by mushrooms’, and **B** ‘Running men’. Depictions by Samorini (2001) on the right and photographed on the left (Guzmán, 2008). These murals were found in Algeria, dating back to approximately 8,000-10,000 BCE. **C-D.** ‘Mushroom head’ depictions from **C** the Sandawe and **D** Bradshaw cultures. Murals found in Tanzania (Sandawe) and Australia (Bradshaw), artwork dating to approximately 6,000-10,000 years ago (Pettigrew, 2011).

Psychedelic plants cover the globe and evidence of their use in human cultures dates back millennia (Schultes et al., 1979). The focus of this work is not anthropological and therefore this is a brief review of some of the literature surrounding this topic, with the intention only to establish a historical context of the use of these compounds. Some of the oldest evidence of psychedelic use comes from mushroom depictions in cave art, such as in Tassili n'Ajjer, Algeria, dating to ~7,000-9,000 BCE (Figure 2A-B) (Guzmán, 2008; McKenna, 1993), in the Kolo region of Eastern Tanzania (Figure 2C) and in the Kimberly region of Australia (Figure 2D) (Pettigrew, 2011).

The two most prevalent compounds described are psilocybin and mescaline. In Mesoamerica, psilocybin-containing mushrooms, referred to as *teonanacatl* ("god's flesh") by the Aztecs, were central to healing, religious, and divinatory rituals. These practices are documented in both Aztec and pre-Columbian societies, including the Olmec, Zapotec, and Maya cultures (Carod-Artal, 2015; D. E. Nichols, 2016; Schultes et al., 1979). Evidence of the ritual use of mescaline-containing cacti was found in Peruvian ceremonial sites dating back to 2,000 BCE, where the San Pedro cactus (*Echinopsis pachanoi*) was used. Additionally, dried peyote buttons (*Lophophora williamsii*) were found in Shumla Cave No. 5 in Texas, radiocarbon-dated to 3,700 BCE (El-Seedi et al., 2005; D. E. Nichols, 2016). Other references to ancient psychedelics include *Soma*, a revered substance frequently mentioned in the Rigveda of ancient India, praised in several Vedic hymns for its divine and psychoactive qualities (Wasson & Ingalls, 1971). Similarly, the *kykeon*, a hallucinogenic brew, was central to the Eleusinian Mysteries of ancient Greece, a secret ceremony held annually near Athens for over 2,000 years (Wasson et al., 1978). In the Amazon, the plant brew Ayahuasca, has a long history of use among indigenous groups. The combination of DMT containing, *Psychotria viridis*, and monoamine oxidase (MAO)-inhibiting *Banisteriopsis caapi* allows the brew to be orally psychoactive (Dobkin De Rios, 1971; Schultes et al., 1979).

The use of psychedelic compounds across cultures has almost exclusively occurred in ceremonial or ritual contexts, typically for purposes of divination or healing (Majić et al., 2015; D. E. Nichols, 2016; Schultes et al., 1979; Wasson et al., 1978). This tradition continues today, with modern examples such as the use of peyote in the Native American Church (Halpern et al., 2005) and the ritual use of ayahuasca in traditional

Amazonian practices, as well as in syncretic churches in Brazil, including União do Vegetal and Santo Daime (Tupper, 2009).

Clinical relevance

Despite the reverential anthropological history of psychedelics, their scientific exploration started in the clinic, as a tool in psychedelic-assisted psychotherapy (Osmond, 1957; Pahnke, 1970). Following a hiatus, research into the therapeutic potential of psychedelic drugs has recommenced demonstrating substantial clinical value in the treatment of a variety of psychiatric disorders. The early work on the clinical efficacy of psychedelics is of limited use due to a general lack of controls. However, this early work provided a solid ground for tolerability, demonstrating that classical psychedelic are non-addictive and not physiologically toxic (D. E. Nichols, 2004; Rucker et al., 2016).

Table 1 provides a non-exhaustive list of the psychiatric illness for which classical psychedelic treatment is currently being assessed, including the psychedelic used, dose and reference of the study. Although beyond the scope of this thesis, it should be noted that dissociative-anaesthetic ketamine is a rapid acting antidepressant in humans and rodents and further research into its application in other psychiatric condition is being conducted (Matveychuk et al., 2020; Walsh et al., 2022). MDMA, particularly MDMA assisted psychotherapy for the treatment of post-traumatic stress disorder (PTSD), is currently the most widely used psychedelic in the clinic, for this reason a single reference has been included despite it being an entheogen (Feduccia et al., 2019; A. N. Siegel et al., 2021). Other non-classical psychedelics are also being investigated for therapeutic use in including atypical psychedelic salvinorin-A (Braida et al., 2009; Harden et al., 2012) and ibogaine (Mash et al., 2018).

Apart from those including MDMA, all clinical trials cited in the review by Siegel et al. have been included, alongside additional double-blind placebo-controlled studies (A. N. Siegel et al., 2021). Earlier studies from the 1960s and 1970s were excluded as they did not meet these criteria, except for the meta-analysis by Krebs and Johnson (Krebs & Johansen, 2012). Classical psychedelics have been repeatedly demonstrated as effective in the treatment of depression, end of life anxiety and substance used disorder. Psilocybin (4-MeO-DMT), DMT and 5-MeO-DMT all act as

rapid acting antidepressants. Additionally, LSD and psilocybin have demonstrated anxiolytic and anti-addictive properties in humans (Table 1). These studies primarily use a single high dose in a controlled environment.

While initial investigations into the clinical benefits of psychedelics were in a context of psychotherapy, current clinical trials vary significantly in their inclusion and emphasis on therapeutic frameworks. The psychedelic-psychotherapy that emerged in the 1960's consisted of intensive preparative psychotherapy, a single high-dose psychedelic session, and follow-up integration sessions, akin to modern MDMA-assisted therapy. This procedure has been followed in some studies administering the psychedelic within a psychotherapeutic framework (indicated + in Table 1). However, most modern studies include a less rigorous psychotherapeutic approach comprised of a preparatory session with a therapist, the same therapist present during the session and an integration session following the session (indicated ~ in the table). Some studies aiming to isolate the pharmacological effects had no psychological intervention (no sign indicated in table 1). Efficacy of psychedelics has been demonstrated in all three settings. Most modern studies include a psychological screening, with an exclusion criteria of psychosis, and presence of facilitators during the treatment session, the same facilitators who conducted preparatory sessions when relevant. Most studies administer psychedelics in environments with soft lighting and in the presence of therapists who encourage internal reflection. During the session, patients may be offered eye shades and music of their preference, usually with minimal therapist interaction to encourage eyes-closed awareness (Nutt & Carhart-Harris, 2021).

This focus on clinical setting, distinct from other pharmacological clinical trials, is primarily due to the susceptibility of the psychedelic-experience to psychological and environmental context (Studerus et al., 2012). As the nature of the psychedelic-experience, discussed below, has been proposed to mediate their therapeutic effects the clinical setting and the use of therapy may confer additional benefit (Barrett & Griffiths, 2017). Although, it remains open whether these compounds will be equally as efficacious, with and without psychotherapeutic intervention.

Table 1 - Publications on the clinical application of psychedelics.

Ailment	Dose/s	Psychedelic/s	Reference
Depression (major depressive disorder,	2: 25mg (3 weeks) 2: 10, 25mg (1 week)	Psilocybin ~ Psilocybin ~	(Carhart-Harris et al., 2021) (Carhart-Harris, Bolstridge, et al., 2016; Carhart-Harris et al., 2017, 2018)
treatment resistant depression)	1: 96-160 mg 1: 96-160 mg 3: 6, 12, 18mg (3 hours) 1: 25mg 1: 25mg 1: 0.36 mg/kg 2: 15, 60 mg (single day)	DMT (Ayahuasca) DMT (Ayahuasca) 5-MeO-DMT Psilocybin ~+ Psilocybin ~ DMT (Ayahuasca) DMT (vaporised)	(Sanches et al., 2016) (Osório et al., 2015) (J. T. Reckweg et al., 2023) (Goodwin et al., 2022) (Raison et al., 2023) (Palhano-Fontes et al., 2019) (Falchi-Carvalho et al., 2024)
Post traumatic stress disorder (PTSD)	3: 80-120mg	MDMA +	(Mitchell et al., 2023)
Anxiety – life threatening illness **cancer*	1: 0.2 mg/kg 2: 0.04, 0.3-0.4 mg/kg (5 weeks) 1: 0.3 mg/kg 2: 200ug (6±2 weeks) 1: 200μg	Psilocybin ~ Psilocybin + Psilocybin+ LSD + LSD +	(Grob et al., 2011)* (Griffiths et al., 2016) * (Ross et al., 2016) * (Holze et al., 2023, 2024) (Gasser et al., 2014)
Substance abuse disorders **Tabacco* *#Alcohol* *$Heroin/Cocaine*	2-3: 0.35, 0.4, 0.4 mg (7 weeks) 2: 0.35, 0.57 mg/kg (4 weeks) 1: 6-12 mg/kg 1: 210-800 μg	Psilocybin + Psilocybin + Ibogaine LSD ~	(Johnson et al., 2014, 2016)* (Bogenschutz et al., 2015, 2022) # (Mash et al., 2018) $ (Krebs & Johansen, 2012) # *(metanalysis of 6 studies from 1966-1970)*
Obsessive Compulsive Disorder (OCD)	3: 0.1, 0.2, 0.3 mg/kg	Psilocybin	(F. A. Moreno et al., 2006)
Demoralisation	1: 0.3-0.36 mg/kg	Psilocybin +	(Anderson et al., 2020)

'+' indicates the inclusion of psychotherapy or therapy-like interventions, and a '~' represents a single preparatory, dosing, and integration session. If no symbol is indicated, this signifies the absence of psychological intervention.

Psychedelic effect

Experiential

The effect of psychedelic drugs on conscious perception are profound, variable and ineffable, making a definitive scientific definition of the psychedelic-effect challenging. They may “alter one’s concepts of reality, may change one’s views on life and death, and can provoke and challenge one’s most cherished beliefs” (D. E. Nichols, 2004). Psychedelics seem to induce states “…that are rarely experienced except in dreams, naturally occurring mystical experiences, and acute psychosis” (Barrett & Griffiths, 2017).

Pahnke describes 5 major kinds of psychedelic experience: psychotic- (fear, panic, isolation), cognitive- (lucid thought, novel perspective), aesthetic- (synesthesia, eyes-closed visions), psychodynamic- (emergence of previously unconscious or preconscious material) and peak-/cosmic-/transcendental-/mystical- psychedelic experiences (Pahnke, 1970). The peak- or mystical- experience induced by psychedelics, is characterised by a sense of unity and oneness, transcendence of time and space, deeply felt positive mood, a sense of awe reverence and wonder, meaningfulness of insight and ineffability. Note, that peak-, transcendent- and mystical- experiences are not by necessity psychedelic induced and occur naturally, although sporadically, in the human condition (Maslow, 1959).

“Besides general epistemic limitations on the assessment of qualitative experiences, additional difficulty is created by the fact that these experiences often go beyond the previously experienced epistemic range” (Majić et al., 2015). Consequently, assessing these altered states of consciousness is one of the first challenges in characterising the psychedelic effect. Without a sufficient understanding of the neurophysiology of consciousness, in any state, this assessment is currently done using questionnaires. The most widely used psychedelic questionnaires are listed in Table 2, note that the metrics that are used often bare correspondence to the 5 psychedelic-experiences described by Pahnke, particularly peak-psychedelic experiences.

These questionnaires are continually being assessed and developed (Dittrich, 1998; Nour et al., 2016; Studerus et al., 2010). In clinical studies, questionnaires are typically

administered during or immediately after the experience, as acute drug effects may disrupt the evaluation, and introspective questions could disrupt the experience. Of note, the subjective effects of psychedelics also limit the validity of double-blind placebo-controlled studies (Oram, 2014).

These questionnaires allow comparison of qualitative states within- and between-individuals and compounds. Curiously, the same compound in the same individual does not necessitate the same qualitative state. Since the initial investigations with psychedelics, it was observable that both psychological and environmental context, influenced the psychedelic-experience. These two factors, often referred to as 'set and setting', include psychological factors of personality, preparation, expectation and intention, in addition to environmental factors, such as physical, social and cultural setting (Hartogsohn, 2016; Pahnke, 1970; Studerus et al., 2012).

The influence of these non-pharmaceutical factors likely contributes to the variable and contradictory description of these compounds as psychotomimetic, anxiogenic, anxiolytic, or antidepressant. For this reason, modern clinical trials with psychedelics control and document the setting in which the compound was administered, and often engage the patient in some kind of psychological assessment or psychotherapy before and during the trial, Table 1.

Table 2 - The main questionnaires used in the assessment of the psychedelic effect.

Questionnaire	Metrics	References
APZ-OAV (Abnormal Mental States/Altered States of Consciousness)	Altered state of consciousness, oceanic boundlessness, dread of ego dissolution, visionary restructuralization	(Dittrich, 1998; Studerus et al., 2010; Vollenweider et al., 1998; Vollenweider & Preller, 2020)
5D-ASC (5-Dimensional Altered States of Consciousness)	oceanic boundlessness, dread of ego dissolution, visionary restructuralization, reduced vigilance, auditory alterations,	(Carhart-Harris et al., 2012; Grob et al., 2011; Majić et al., 2015; Vollenweider et al., 2007)
11D-ASC (11-Dimensional Altered States of Consciousness)	ego dissolution, altered meaning of experiences, visual hallucinations, synaesthesia, impaired cognition, auditory distortions, increased emotionality, spiritual experiences, time perception, body sensations and distortions, anxiety and fear	(Carhart-Harris, Bolstridge, et al., 2016; Mason et al., 2020; Roseman et al., 2018)
MEQ-30 / *MEQ-43* (Mystical Experiences Questionnaire)	unity, positive mood, transcendence of time/space, ineffability, noetic quality, sacredness	(Barrett & Griffiths, 2017; Bogenschutz et al., 2015; Griffiths et al., 2006; Madsen et al., 2019)
CEQ (Challenging Experiences Questionnaire)	fear, grief, insanity, isolation, death, physical distress, paranoia	(Barrett et al., 2016; Carbonaro et al., 2016; Ly et al., 2018; J. Reckweg et al., 2021; J. T. Reckweg et al., 2022)
EDI (Ego-Dissolution Inventory)	ego dissolution, ego inflation	(Millière et al., 2018; Nour et al., 2016)
PEQ (Persisting Effects Questionnaire)	positive changes, negative changes, spiritual significance	(Barrett et al., 2020; Griffiths et al., 2006; Kiraga et al., 2021; Richards et al., 1977; Y. Schmid & Liechti, 2018)
SOCQ (States of Consciousness Questionnaire)	mysticism, transpersonal states, insight, positive mood	(MacLean et al., 2011)
HRS (Hallucinogen Rating Scale)	somesthesia, affect, volition, cognition, perception, intensity	(Pallavicini et al., 2021; Riba et al., 2001; Strassman, 1994)

Quantifying metrics of qualitative psychedelic-experiences allows us to assess its relation to therapeutic outcomes. The extent to which psychedelics induce peak- or mystical-experiences has been suggested to mediate their therapeutic effect. Higher doses tend to facilitate mystical-psychedelic experiences, however, as discussed above is not the only factor. In patients with life threatening illness, depression and anxiety scores decrease proportionally to mystical-experience scores (Barrett & Griffiths, 2017; Griffiths et al., 2016; Richards et al., 1977; Romeo et al., 2021; Roseman et al., 2018; Ross et al., 2016). Psychedelic efficacy on alcohol and tobacco dependence has also been correlated to mystical-experience scores (Barrett & Griffiths, 2017; Bogenschutz et al., 2015; Garcia-Romeu et al., 2015; Johnson et al., 2016; Romeo et al., 2021). These newer works corroborate preliminary findings from the 1970's, assessing the value of psychedelic-peak experience in LSD in psychotherapy (Pahnke, 1970).

From the evidence presented here, the clinical efficacy of psychedelics appears dependent on the psychedelic-experience, which in turn is influenced by a variety of factors including dose, mindset and setting. However, whether a psychedelic-effect is required for the clinical benefits of psychedelics remains a point of contention within the field. While much remains unknow about the neural underpinnings of these experiential effects, they are reduced by pretreatment with 5-HT_2R antagonist Ketanserin, at least for LSD- and psilocybin- induced experiences (Becker et al., 2023; Kraehenmann et al., 2017; Preller et al., 2017; Vollenweider et al., 1998).

Neuroimaging to "Connectivity"

The neural mechanisms of psychedelics may be studied independently from the qualitative experience. The development of neuroimaging has been particularly useful in this field. Non-invasive imaging techniques such as arterial spin labelling (ASL; cerebral blood-flow), blood oxygen level dependent functional magnetic resonance imaging (BOLD-fMRI), magnetoencephalography (MEG; electrical activity), positron emission tomography (PET; metabolism, blood flow, receptor-binding), and electro encephalography (EEG) have been used in the study of acute and post-acute psychedelic effect in humans.

MEG and EEG, or PET and BOLD fMRI are used as measures of electrical activity and blood oxygenation respectively, providing proxies for neural activity in specific

brain regions. Functional connectivity uses the correlations between these signals in different regions to describe network activity, capturing inter-region synchronous activity *in vivo* in humans. Resting-state networks are the regions that correlate in the absence of an explicit task, a resting state. The default mode network (DMN) is a resting-state network involving the ventromedial prefrontal cortex, the posterior cingulate cortex (pCC), angular gyrus, hippocampus and parietal lobe. The DMN is thought to be involved in self-referential thought and its activity is downregulated when performing non-self-referential tasks (Raichle, 2015). MEG and fMRI data demonstrate reduced activity or connectivity of the DMN during psychedelic-experiences, (Carhart-Harris et al., 2012, 2014; Carhart-Harris, Muthukumaraswamy, et al., 2016; Muthukumaraswamy et al., 2013; Palhano-Fontes et al., 2015; Tagliazucchi et al., 2014). This disruption has been attributed to ego disillusion, heightened sensory awareness and altered time perception in the psychedelic-experience. Another notable resting-state network influenced by psychedelics is the salience network, comprising the anterior insular cortex, anterior cingulate cortex (aCC), and amygdala. Psychedelics have been shown to modulate activity within these regions, with particular emphasis on the aCC and amygdala (Castelhano et al., 2021; Davoudian et al., 2023; Golden & Chadderton, 2022; Gresch et al., 2007; Pasquini et al., 2020). The salience network plays a critical role in attributing significance to external stimuli proposedly complementing the DMN in its assignment of emotional valence to internally generated stimuli (Goulden et al., 2014; Hamilton et al., 2015; Seeley et al., 2007).

Structural evidence further highlights the involvement of these regions. Regular ayahuasca users, >50 sessions in the last 2 years (Santo Daime church members), were found to have thinner pCC and thicker aCC compared to psychedelic-naive controls (Bouso et al., 2015). PET studies of 5-HT_{2A}R occupancy, one important receptor target of classical psychedelics, found the highest occupancy brain regions included the bilateral angular gyrus (part of the DMN) and subgenual aCC (part of the salience network) (Barrett et al., 2022). Whether psychedelic alteration in these networks is linked to their clinical or experiential outcomes remains unknown. However, the altered appearance of resting-state networks in pathological conditions, such as the DMN in major depressive disorder (MDD) has been suggested (Hamilton et al., 2015).

The study of psychedelic-induced neural dynamics can be correlated with metrics of qualitative experience. Work that aims to further the understanding of the neural underpinnings of altered states of qualitative experience, or consciousness, as has been the interest of many a psychedelic researcher. LSD-induced ego disillusion and psilocybin-indued mystical experience correlate with the extent of altered DMN activity (Carhart-Harris, Muthukumaraswamy, et al., 2016; Griffiths et al., 2016). Similarly, the vivid psychedelic visual phenomena induced by Ayahuasca and LSD have been associated with increased activity in the visual cortex (De Araujo et al., 2012; Kraehenmann et al., 2017). Pasquini et al. (2020) found increased aCC connectivity within the salience network correlated with somesthesia scores, while decreased pCC connectivity with the DMN was linked to altered volition following Ayahuasca (Pasquini et al., 2020).

Inter-network dynamics are also modified by psychedelics. The connectivity between the salience network and DMN has been shown to be increased following psychedelics (Carhart-Harris et al., 2013; Pasquini et al., 2020). It has been recurrently proposed that psychedelics cause a desynchronisation of network activity or reduced network integrity as assessed by MEG, PET and fMRI in humans (Madsen et al., 2021; J. S. Siegel et al., 2024).

All current evidence of the neurological-effect of psychedelics in humans suggests brain-wide alterations in activity (metabolic or electrical), although a wider range of function-anatomical measurements, such as neurotransmission, will be curtal to completing this picture. In humans, a single PET study on the binding of [11C]raclopride to dopamine D_2 autoreceptors following psilocybin, found decreased binding in the ventral striatum, suggesting increased extracellular DA (Vollenweider et al., 1999). PET is the only method to indirectly study neurotransmission, and is not without caveat, however, is perhaps the only tool we have in humans to begin to characterise the neurochemical environment of the human brain.

In summary, psychedelics alter the functional connectivity in the brain, decrease connectivity in the DMN and increase sensory and thalamocortical connectivity. Both within and between network dynamics are sensitive to psychedelics and may correlate

with the experiential effects (Yu et al., 2024). The study of brain networks and functional connectivity is a relatively recent advent in neuroscience, and it should be kept in mind that what is being measured is the correlation of a single, often transformed, signal between any two areas of the brain, and the causal underpinnings of such a correlation are beyond current interpretation.

Psychedelic models of action: REBUS

Although beyond the scope of this thesis, it feels relevant to mention the psychedelic model of action proposed by Carhart-Harris and Friston (2019), which suggests that psychedelics act by disrupting the brain's hierarchical predictive coding system. Under normal conditions, the brain uses top-down processing to generate predictions about the world, with higher-level cognitive models constraining sensory input. Authors propose that psychedelics reduce these top-down constraints, increasing neural entropy and allowing for more bottom-up processing. This leads to a higher degree of prediction error and a shift toward a more anarchic brain state, where sensory experiences become less constrained, resulting in altered states of consciousness such as ego dissolution and enhanced perception (Carhart-Harris & Friston, 2019).

Molecular pharmacology of classical serotonergic psychedelics

Molecular targets

The action of classical psychedelics on the 5-HT system was initially demonstrated by LSD application on isolated organs, resulting in a similar effect to 5-HT application (Shaw & Woolley, 1956). Since then, there has been tremendous development in our understanding of the serotonergic system and its role in the central nervous system, identifying 7 distinct 5-HT receptor (5-$HT_{1\text{-}7}$R) families. Classical psychedelics have varying affinity for the 5-HT_{1A}, 5-HT_{2B}, and 5-HT_{2C}R in addition to consistent affinity for the 5-HT_{2A}R. Table 3 shows the affinity of psychedelic and non-psychedelic (lisuride, Ro60-0175, mCPP, quipazine) 5-HT_{2A}R agonists and antagonists for the 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}R families (Butler et al., 2024). Of note, the ergot derivatives LSD or lisuride are somehow apart because they exhibit nM affinity for 5-$HT_{5\text{-}7}$R (Table 3) (Passie et al., 2008).

There are several lines of evidence supporting a central role of the 5-HT_{2A}R in classical psychedelic action. In humans ketanserin (5-$HT_{2A/2C}$R antagonist) reduces the subjective effects of LSD and psilocybin (Becker et al., 2023; Kraehenmann et al., 2017; Preller et al., 2017; Vollenweider et al., 1998). 5-HT_{2A}R occupancy following psilocybin correlates with the intensity of subjective effects (Madsen et al., 2019) and the potency in humans, and ED50 in animal drug discrimination, correlates with 5-HT_2R affinity (Glennon et al., 1984). Furthermore in animals, the use of 5-HT_{2A}R knockout mice supports the involvement of 5-HT_{2A}R to elicit a behavioural effect corresponding to psychedelics, the head twitch response (HTR) (González-Maeso et al., 2003, 2007).

Table 3 - Affinities of various 5-HT ligands and hallucinogenic drugs.

	5-HT Receptor subtype				Other sites <300 nM	References
	1 A	2 A	2B	2 C		
5-HT_{2A}R agonists						
5-HT	3.32 (av)	9.84 (av)	8.71 (av)	9.09 (av)	5-HT_{1B} (av 4.3), 5-HT_{1D} (3.4), 5-HT_{1E} (av 7.5), 5-HT_{1F} (10), 5-HT_3 (269.2)[m], 5-HT_4 (125.9), 5-HT_{5A} (126), 5-HT_{5B} (7.8)[r], 5-HT_6 (av 87.3), 5-HT_7 (av 8.1), SERT (22)[r]	From PDSP Ki Database (n=13)
TCB−2		0.73				(McLean et al., 2006)
LSD	2.29 (av)	0.73 (av)	0.98	2.91	5-HT_{1B} (3.9)[r], 5-HT_{1D} (14), 5-HT_{1E} (93)[r], 5-HT_{5A} (9)[r], 5-HT_{5B} (av. 3.2)[r], 5-HT_6 (2.3), 5-HT_7 (av. 104.5)[r], D1 (av 291.5), D2 (20), D3 (96), α_{2A} (12), β_1 (140)[r], TrkB (3.38)[m]	From PDSP Ki Database (n=10) (Moliner et al., 2023)
DOI	2355[r]	0.68 (av)	20.03 (av)	2.38 (av)		From PDSP Ki Database (n= 4)
DOM	>10,000 (av)	3.18 (av)	nd	25.7		From PDSP Ki Database (n= 2)
DOB	6326	0.89 (av)	35.28 (av)	6.31		From PDSP Ki Database (n=3)
Psilocybin	>10,000	13[r]	98.7	>10,000[r]	5-HT_{1E} (194.8)	From PDSP Ki Database (n= 1); PDSP certified Data;
Psilocin	49	25[r]	nd	10[r]	TrkB (6.73)[m]	From PDSP Ki Database (n= 1) (Moliner et al., 2023)
Mescaline	4600	150[r]	nd	>10,000		From PDSP Ki Database (n= 1)
Lisuride	0.32 (av)	1.4	1.07	6.31	5-HT_{1B} (18.6); 5-HT_{1D} (1), 5-HT_{5A} (3.1), 5-HT_6 (7.3), 5-HT_7 (6.8); α_{1A} (5.5), α_{1B} (16.6), α_{1D} (3), α_{2A} (0.1), α_{2B} (0.1); α_{2D} (0.8)[r], β_1 (67.6), β_2 (8), D1 (64.6), D2 (1.4), D3 (av 1), D4 (4.57), D5 (3.6), H2 (114.3)	From PDSP Ki Database (n= 8)
m-CPP	43.9	54.48(av)	30.28 (av)	13.04 (av)	5-HT_{1B} (av 251. 9), 5-HT_3 (50)[r], 5-HT_7 (162.5), α_{2B} (105.8), α^{2C} (123.5), SERT (av 265.1)	From PDSP Ki Database (n= 8); PDSP certified Data
Quipazine	1697	128.82	66.91 (av)	53.70	5-HT_3 (1.23)	From PDSP Ki Database (n= 4)
Ro60–0175	6 680	29.14 (av)	4.29 (av)	7.30		From PDSP Ki Database (n= 4)
Lorcaserin	700	112	174	15		(Thomsen *et al.*, 2008)
5-MeO-DMT	1.9	14[r]	3884	538	5-HT_{1B} (74), 5-HT_{1D} (6.3), 5-HT_{5A} (276.6), 5-HT_6 (35.5), 5-HT_7 (3.9), D1 (79.5)	From PDSP Ki Database (n= 2)
DMT	450	131.50 (av)	107.6	166	5-HT_{1D} (93), 5-HT_6 (av 277.7), 5-HT_7 (87.5), α_{2B} (257.7), α_{2C} (258.6), D1 (271.1)	From PDSP Ki Database (n= 3)
25I-NBOMe	1800	0.2	nd	4.6	H1 (90)	(Rickli *et al.*, 2015)
5-HT_{2A}R antagonists						
MDL100, 907	>10 000 (av)	0.64 (av)	1 023.29	30.20	α_{2B} (270), H1 (66.7), σ (87)[r]	From PDSP Ki Database (n= 4); PDSP certified Data
Ketanserin	5373.22 (av)	4.77 (av)	911.74 (av)	146.33 (av)	5-HT_{1D} (111), α_{2B} (199), H1 (1.79)	From PDSP Ki Database (n= 13)

Beyond action on serotonergic receptors classical psychedelics can have affinity at a variety of other receptors, LSD is a notoriously 'dirty' drug, with affinities <300nM at several DA, alpha-2A and beta-1 adrenergic (α_{2A}, β_1) receptors (Passie et al., 2008), and tropomyosin receptor kinase B (TrkB). Action on DA receptors is not exclusive to LSD, both DMT and 5-MeO-DMT have substantial affinity for DA D_1 receptors (D_1R). 5-MeO-DMT is also peculiar for its several-fold higher affinity for the 5-HT_{1A}R than the 5-HT_{2A}R, which has been proposed to underlie some of its behavioural effects (see *Animal Behavioural Pharmacology*). TrkB affinity has also been identified for psilocin (4-MeO-DMT), which is the active metabolite of psilocybin found in the mushroom genus *Psilocybe*. Both psilocin and psilocybin have affinity for the 5-HT_{2A}R however, only the metabolite psilocin has considerable affinity for the 5-HT_{2C}R and TrkB (Moliner et al., 2023). It should therefore be kept in mind that the molecular targets of

a parent compound do not necessarily describe all relevant targets in the mechanism of action.

Classical serotonergic psychedelics act on 5-HT_{2A}Rs, however they do so to varying extents and with variable additional targets. 5-HT_{2A}R affinity is often a concern in the pharmaceutical industry due to the propensity of 5-HT_{2A}R agonists to have psychotomimetic effects, but not all 5-HT_{2A}R agonists are psychedelic.

Intracellular pathways and biased agonism

Ergoline lisuride shares significant structural similarities with LSD, including nanomolar affinity for the 5-HT_{2A}R, as well as other 5-HT, DA, and adrenergic receptors. Despite this similarity LSD is a potent psychedelic in humans, inducing a vivid psychedelic-experience, whereas lisuride has no such effect. This variability has been hypothesized to result from biased agonism (Kenakin, 1995), where different ligands stabilize distinct receptor conformations, leading to selective activation of intracellular signalling pathways.

In favour of this Gonzalez-Maeso et al. showed that lisuride and LSD have distinct transcriptome activation profiles in the mouse somatosensory cortex. They found that LSD robustly activated ERK1/2, a kinase associated with cellular plasticity and transcriptional regulation, while lisuride did not. Additionally, they showed that LSD uniquely increased the expression of early growth response genes (*egr-1*, *egr-2*), consistent with neuroplastic changes (González-Maeso et al., 2007).

The conical signalling pathway of the 5-HT_{2A}R is via $G_{q/11}$ protein signalling, which activates of phospholipase C (PLC) stimulating inositol trisphosphate (IP3) production, leading to calcium release from intracellular stores (Figure 3A). Inhibition of this pathway eliminated the gene response of both LSD and lisuride, suggesting it is not a unique pathway of psychedelic compounds. However, inhibition of $G_{i/o}$ protein signalling, also implicated in 5-HT_{2A}R mediated responses, greatly attenuated LSD-induced gene responses, not lisuride. $G_{i/o}$ protein-regulated phosphorylation networks often involve $G_{\beta\gamma}$ subunit-mediated activation of Src kinase (Figure 3A). Authors additionally show that only LSD-induced responses were sensitive to Src inhibition.

These results suggest that hallucinogenic 5-HT_{2A}R agonist LSD causes a unique regulation of $G_{i/o}$ protein and Src kinase relative to non-psychedelic lisuride (González-Maeso et al., 2007).

Beyond G-protein signalling, the 5-HT_{2A}R can also engage β-arrestin pathways (Figure 3). The 5-HT_{2A}R binds both β-arrestin 1 (βArr1) and β-arrestin 2 (βArr2) *in vitro* and forms complexes with these proteins in cortical neurons *in vivo* (Gelber et al., 1999). LSD-induced psychedelic behaviour (HTR) is absent in βArr2 knockout mice (Rodriguiz et al., 2021). However, DOI-induced HTR is conserved in βArr2 knockout mice (C. L. Schmid et al., 2008). While βArr2 is critical for LSD induced HTR and likely ERK1/2 phosphorylation, DOI can activate ERK1/2 in the absence of βArr2. DOI-induced HTR and anxiolytic-like behaviour is attenuated in $G\alpha_q$ knockout mice, suggesting its action through $G\alpha_q$ rather than βArr2 mediated pathways (Garcia et al., 2007). This data suggests that different psychedelic 5-HT_{2A}R agonists act preferentially via different intracellular pathways, in addition to differences between psychedelic and non-psychedelic agonists.

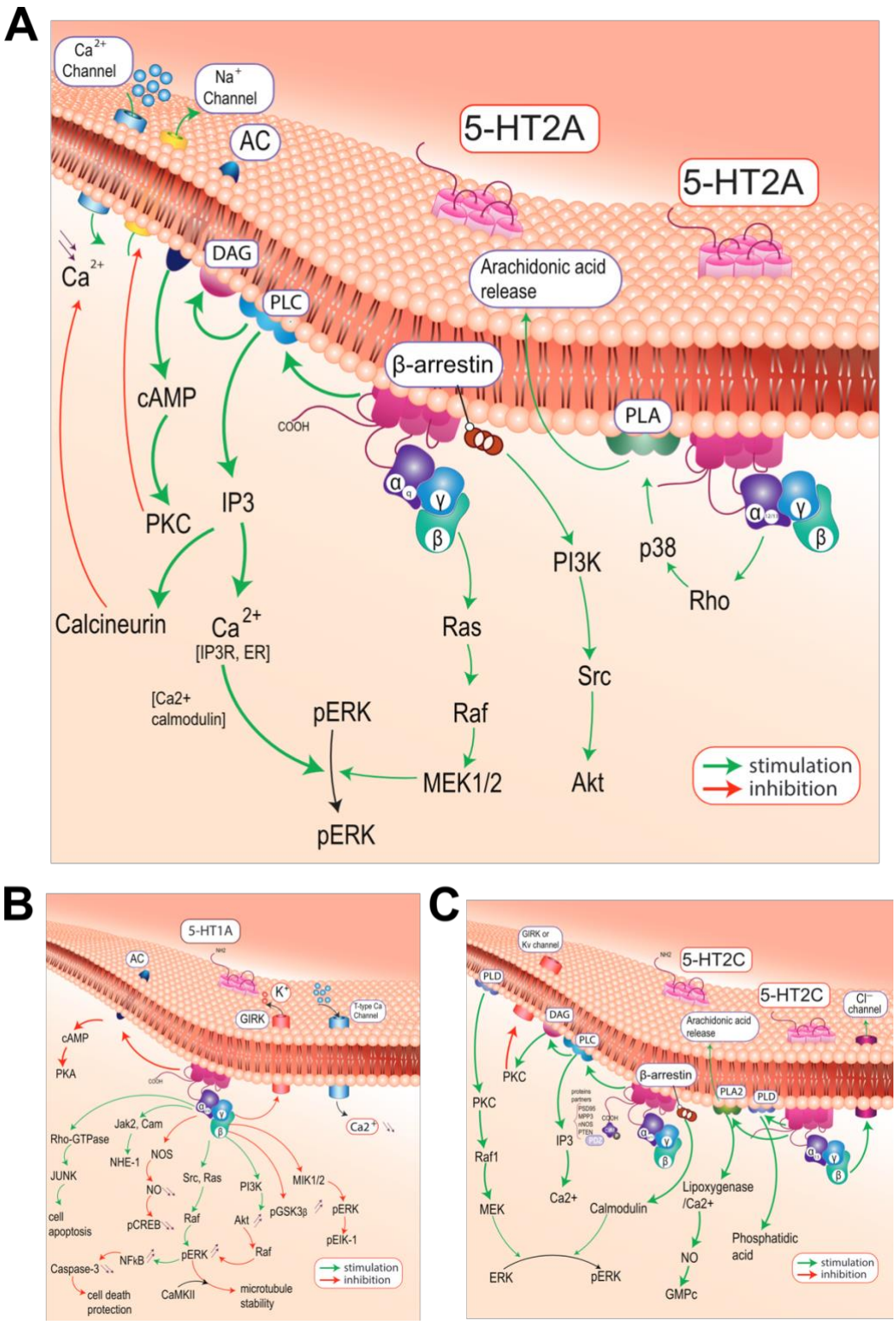

Figure 3 - 5-HTR signalling pathways.

A 5-HT$_{2A}$R stimulation stimulates Phospholipase C (PLC) which catalyses hydrolysis of phosphadytilinositol-4,5-biphosphate, leading to inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). Intracellular accumulation of IP3 stimulate the release of calcium (Ca2+) from the endoplasmic reticulum, in turn, stimulate L-type Ca2+ channels and PKC. Through Gα12/13, 5-HT2AR-mediated activation of the PLA2 releases of arachidonic acid (AA). 5-HT$_{2A}$R also mediates phosphorylation of ERK1,2 and p38. Stimulation of 5-HT$_{2A}$R in pyramidal neurons (prefrontal cortex) appears to

have an inhibitory effect of Cav1.2 L type Ca2+ currents through Gαq-mediated PLCβ/IP3/calcineurin pathway and an increase in the intracellular Ca2+ concentration. 5-HT_{2A}R can also diminish the amplitude of persistent Na+ currents without affecting their voltage dependency. These effects involve PLC via Gαq activation. Activation of a β-arrestin2/PI3K/Src/Akt cascade promotes Akt phosphorylation via 5-HT_{2A}R signalling. ERK activation from 5-HT_{2A}R stimulation involves a Gαq/PKC/raf- pathway. **B** 5-HT_{1A}R, through Gαi/o proteins, is negatively coupled to AC/PKA/cAMP signaling leading to a reduction of cAMP and PKA. 5-HT_{1A}R stimulation increases K+ conductance requiring no second messenger (but direct control of K+ channels via the Gi/Go protein). This results in a reduction of action potential frequency, which indirectly reduces Ca2+ influx in nerve terminals. The ERK (or MAPK) signaling pathways are differentially regulated according to the tissues studied. Through Gα13 protein coupling, in hippocampal primary cultures, 5- HT_{1A}R stimulation activates Akt and PI3Kdependent pathway. In transfected CHO cells, 5- HT_{1A}R regulates Na+/H+ exchanger (NHE) through Gαi2 and Gαi3. Other intracellular effects mediated by 5-HT_{1A}R activation include suppression of neuronal nitric oxide synthase (NOS) expression in the hippocampus resulting in CREB activation. 5-HT_{1A}R can also inactivate GSK3β via Ser9-phosphorylation of this kinase. Other effects observed by 5- HT_{1A}R activation include Ca2+/CaM-dependent protein kinase II (CaMKII) or MEK-dependent reduction of microtubule stability. **C** Stimulation of 5-HT_{2C}R activates PLC via Gαq subunit. 5-HT_{2C}R also activates PLD via the involvement of both Gα13 and Gβγ subunits. Stimulation of both PLA2 and adenylyl cyclase (AC) through 5- HT_{2C}R has been reported in transfected cells. In Xenopus oocytes expressing exogenous 5-HT_{2C}R as well as in the choroid plexus cells, stimulation of 5-HT_{2C}R closes K+ channels. Moreover, 5-HT_{2C}R-mediated activation of PKC activity inhibits the Kv1.1 and Kv1.3 channels which contributes to K+ efflux at the apical side of choroid plexus epithelial cells. In addition, 5-HT_{2C}R stimulation has been reported to activate an apical Cl- conductance resulting in an apical Cl- efflux. Stimulation of NO and cGMP production in the choroid plexus appear to require PLA2, lipoxygenase activity, and Ca2+. ERK1/2 phosphorylation mediated by 5-HT_{2C}R occurs through a pathway that requires PKC, PLD, and activation of the Raf/MEK/ERK sequence. In CHO cell, ERK1/2 phosphorylation mediated by 5-HT_{2C}R appears to occur through a pathway that requires PKC, PLD, and activation of the Raf/MEK/ERK sequence. In choroid plexus epithelial cells and cultured cortical neurons, ERK 1/2 signaling has been reported to be mediated via interaction CaM-5-HT_{2C}R and G protein-independent, arrestin-dependent receptor signalling. Taken from (De Deurwaerdère, Bharatiya, et al., 2020)

Biased agonism between psychedelic and non-psychedelic 5-HT_{2A}R agonists fostered the proposition that the therapeutic effects might be dissociated from the psychoactive effects of psychedelics. Cao et al. (2022) used high-resolution cryo-electron microscopy (cryo-EM) to investigate the structure of 5-HT_{2A}R bound LSD, a conformation that promotes both G-protein signalling and β-arrestin recruitment. Authors identified the tyrosine residue (Y3707.43) in transmembrane domain 7 (TM7) of the 5-HT_{2A}R to be crucial in β-arrestin signalling activation. Modifying LSD's pharmacophore authors designed low efficacy β-arrestin-biased agonists IHCH-7079

and IHCH-7086. These compounds have low transduction efficiency relative to psychoactive compounds DOI, LSD, and psilocin and are biased towards β-arrestin recruitment (Figure 4A). Furthermore, these low efficacy β-arrestin-biased agonists had antidepressant-like effects in mice without producing the HTR (Cao et al., 2022).

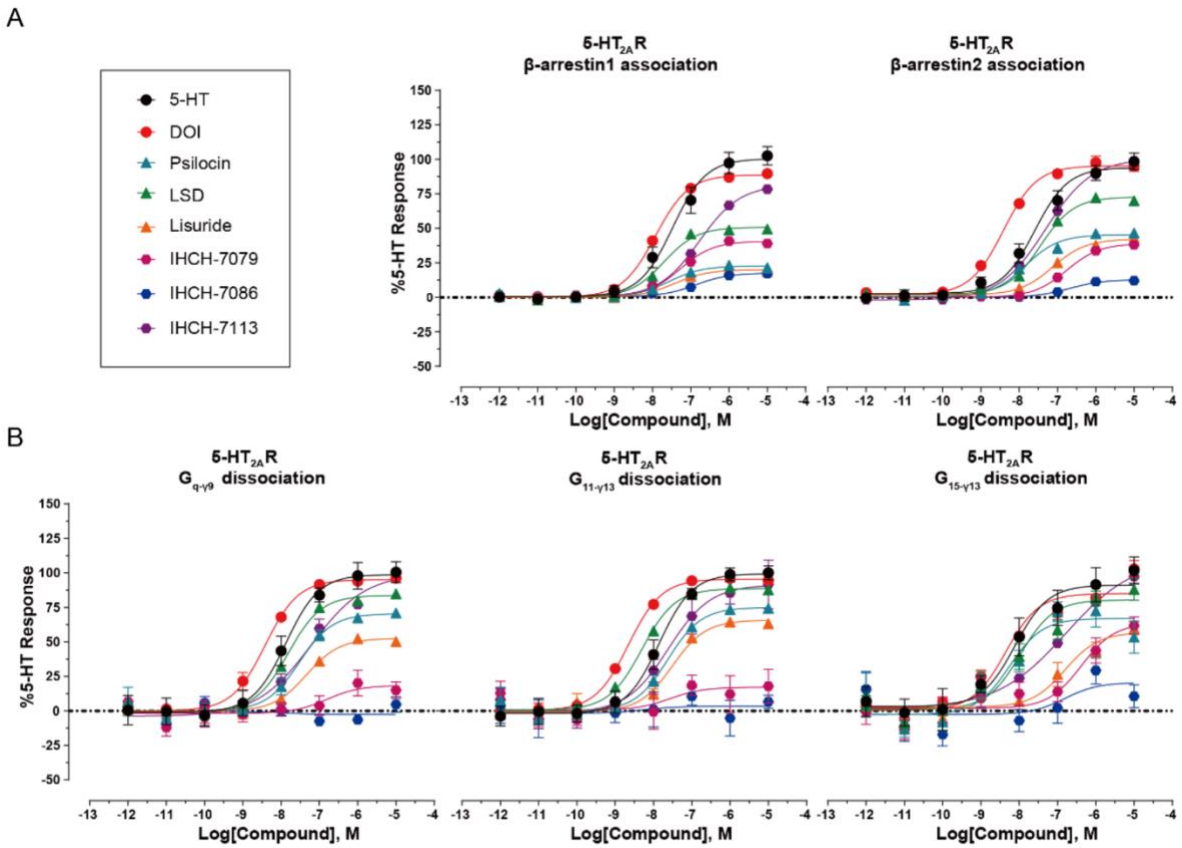

Figure 4 - Biased agonism at the 5-HT$_{2A}$R.

Profiling of psychedelics and non-hallucinogenic psychedelic analogs for **A** β-arrestin1/2 association activity and **B** Gq-γ, G11-γ, and G15-γ dissociation (BRET assay). Error bars represent SEM (n = 3)(Cao et al., 2022).

In summary, biased agonism at the 5-HT$_{2A}$R is evident between psychedelic and non-psychedelic agonists, and within classes of psychedelic agonists. The current understanding of intracellular mechanisms is not sufficient to characterise the experiential or therapeutic effects of 5-HT$_{2A}$R agonists. For the 5-HT$_{2A}$R specifically, it has also been proposed that the differential psychedelic effects observed with some 5-HT$_{2A}$R agonists originate from their action on intracellular 5-HT$_{2A}$R (Vargas et al., 2023). Other psychedelic targets such as the 5HT$_{2C}$R and 5HT$_{1A}$R also have complex intracellular mechanisms subject to biased agonism (Figure 3B-C).

Receptor interactions

The 5-HT_{2A}R can form receptor complexes with various partners, acting as a potential biased agonist to modulate its intracellular signalling pathways. Notably, 5-HT_{2A}R has been shown to interact with several GPCRs to form stable homo- or heteromeric complexes, including D_2R, metabotropic glutamate receptors (mGluR), and cannabinoid CBR (Guiard & Di Giovanni, 2018). Functional crosstalk between 5-HT_{2A}R and D_2R has been described, where D_2R activation increases the affinity of hallucinogenic agonists for 5-HT_{2A}R while simultaneously reducing 5-HT_{2A}R-induced IP3 production (Albizu et al., 2011). Heterodimerization of 5-HT_{2A}R and mGluR has also been described and implicated in DOI-induced HTR (J. L. Moreno et al., 2012). Similarly behavioural evidence for 5-HT_{2A}R and μ-opioid receptor complexes in DOI-induced HTR in mice has been found (Marek, 2003).

Brain localisation

The biased agonism property of ligands and/or the heterodimerization are molecular mechanisms dependent on the availability of cellular partners, and therefore dependent on the phenotype of the cell, and the cellular and sub-cellular location of the expressed receptor (dendrite, soma, axons, terminals, endoplasmic reticulum). As far as 5-HTRs are concerned, it has been elegantly shown that the 5-HT_6R changes its cell location in pyramidal cells during the development from the cilium to the soma, thereby modifying molecular partners and activity (Chaumont-Dubel et al., 2023) . It is important to examine the distribution of 5-HT_{2A}R not only at the regional level but also in terms of the different cell types in which it is expressed and its subcellular localization.

Initial human studies recurrently describe 5-HT_{2A}R expression throughout the neocortex. Quantitative autoradiography with 5-HT_{2A}R antagonist [3H]ketanserin showed high 5-HT_{2A}R expression localized in layers III-V of several cortical areas, including the frontal, parietal, temporal, and occipital lobes, the anterogenual cortex, and the entorhinal area (Pazos et al., 1987). Outside the neocortex 5-HT_{2A}Rs were labelled in the hypothalamus (mammillary bodies), claustrum, and the amygdala (nucleus lateralis). *In situ* hybridization histochemistry revealed extensive 5-HT_{2A}R

mRNAs expression in post-mortem human neocortex, minimal levels in the hippocampus and an absence in the raphe, cerebellum, substantia nigra, and striatum (Burnet et al., 1995). Labelled cells in the neocortex were predominantly pyramidal in layer V, and to a lesser extent layer III, non-pyramidal cells (putative interneurons) were also labelled. These findings were corroborated by Pasqualetti et al., describing the highest 5-HT_{2A}R density in layers III-V of the neocortex, with regional differences across the prefrontal, parietal, temporal and occipital lobes, as well as in the insular and limbic cortex (Pasqualetti et al., 1996).

Cortical 5-HT_{2A}Rs are also described in primates (*macaques*), mice, and rats, particularly in frontal and parietal cortex (Cornea-Hebert et al., 1999; Jakab & Goldman-Rakic, 2000; Weber & Andrade, 2010). Consistent with the human data cortical layer V has the highest density of 5-HT_{2A}Rs, although 5-HT_{2A}R immunoreactivity was found in every layer, expressing predominantly on the apical dendrites of pyramidal neurons (Figure 5) (Cornea-Hebert et al., 1999; Jakab & Goldman-Rakic, 1998; Pazos et al., 1985; Weber & Andrade, 2010; Willins et al., 1997).

While the majority of 5-HT_{2A}R expressing neurons are pyramidal, a secondary population of non-pyramidal 5-HT_{2A}R expressing neurons is scattered through cortical layers II-VI, generally characterised as GABAergic interneurons (Figure 5A-B). In the mouse three subpopulations of 5-HT_{2A}R-mRNA expressing cells have been defined, layer V pyramidal cells, parvalbumin (PV) expressing fast spiking GABAergic interneurons in layers II-V, and non-pyramidal cells in layer Vb (Weber & Andrade, 2010). In the rat, both PV positive and negative 5-HT_{2A}R positive interneurons were described (Willins et al., 1997). In the *macaque* 5-HT_{2A}R/GABA double labelled neurons were of two types. Large, and to a lesser extent medium, PV interneurons colocalised with the 5-HT_{2A}R labelling, particularly marked at their proximal dendrites, neurons were located within layers IV-VI and reminiscent of basket cells. The second population of 5-HT_{2A}R-positive interneurons were calbindin-positive (CB) and distributed throughout layers II-VI, again the 5-HT_{2A}R labelling was usually concentrated at proximal dendrites (Jakab & Goldman-Rakic, 1998, 2000). These results suggest that 5-HT_{2A}Rs are associated with both PV and CB perisomatic interneurons in the cortex. Double *in situ* hybridization in both humans and monkey

(*Macaca fascicularis*) frontal cortex, show that the majority (86–100%) of layer II-V glutaminergic cells express 5-HT_{2A}R-mRNA while only a subpopulation (13-46%) of GABAergic interneurons do. Furthermore 45–69% of PV- and in 61–87% of CB-positive cells express the 5-HT_{2A}R-mRNA (Willins et al., 1997). In the rat prefrontal cortex (PFC) 23% of PV-expressing cells co express the 5-HT_{2A}R-mRNA (Puig et al., 2010).

The presence of both pre- and post-synaptic 5-HT_{2A}R was posited during the initial characterisation of frontal cortical 5-HT_{2A}R (Jakab & Goldman-Rakic, 1998). This was later confirmed by Miner et al. using immunoperoxidase 5-HT_{2A}R labelling. In the frontal cortex of rats the 5-HT_{2A}R is predominantly postsynaptic (73%) on dendritic shafts and spines, congruent with previously reported 5-HT_{2A}R labelling (Miner et al., 2003). A predominantly postsynaptic location of cortical 5-HT_{2A}R is consistent with the asymmetric junctions of 5-HT terminals on dendritic shafts and spines in the frontal cortex (Blue et al., 1988; Séguéla et al., 1989). Presynaptic 5-HT_{2A}R profiles were found to a lesser extent (24%), on axons and varicosities with morphological features characteristic of monoamine axons (Miner et al., 2003). Authors propose DA axons as a site for presynaptic 5-HT_{2A}R, given the regulatory control of 5-HT_{2A}R on DA systems (Pehek et al., 2001). This seems a plausible hypothesis given the distinct localisation of 5-HT_{2A}R immunolabeling and 5-HT terminal like varicosities (Jansson et al., 2001). The remaining 4% of 5-HT_{2A}R labelled profiles were glial processes (Miner et al., 2003).

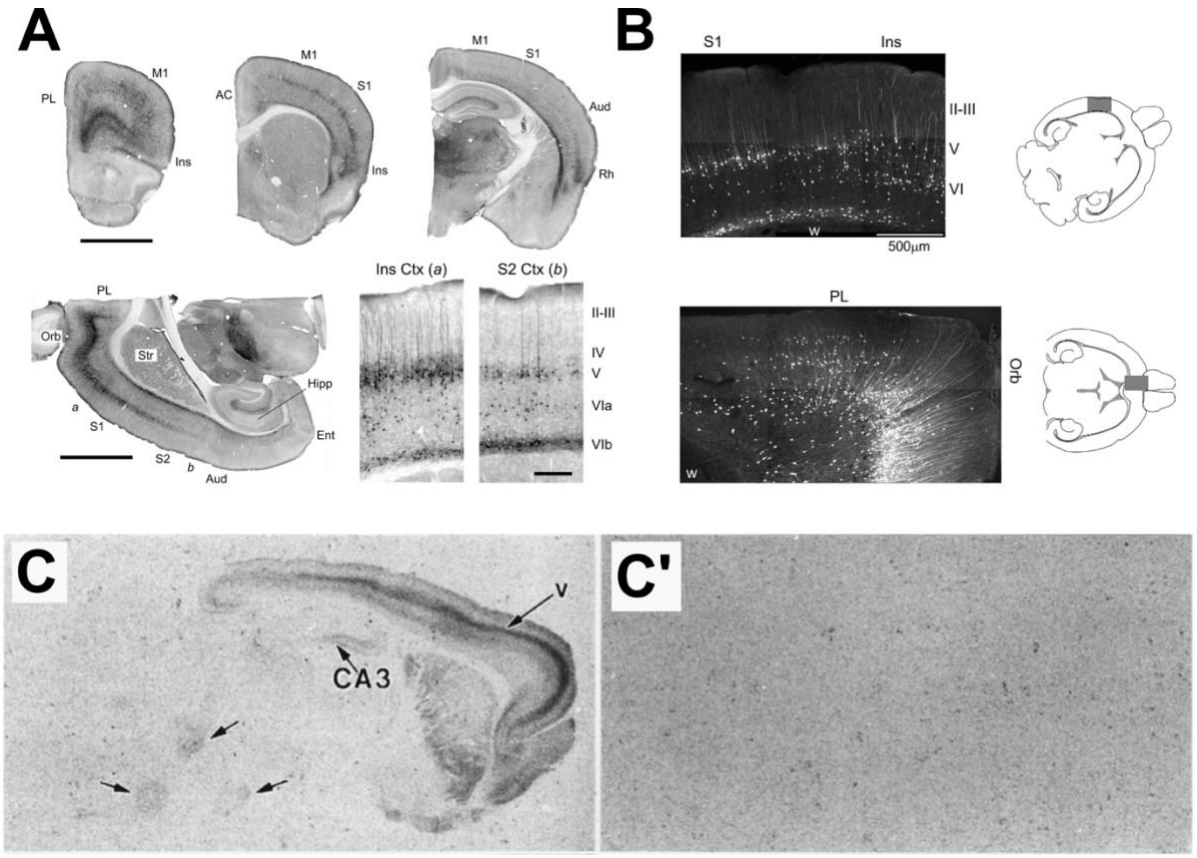

Figure 5 - Expression of the 5-HT$_{2A}$R in mouse and rat.

A-B Cellular expression of EGFP in 5-HT$_{2A}$R-EGFP mice. **A** Low magnification images illustrating the distribution of EGFP-expressing cells in coronal and horizontal sections. Calibration bar = 2 mm. Higher magnification images depicting the EGFP immunoreactivity in the insular (Ins) and secondary somatosensory (S2) cortex. **B** Confocal image illustrating the distribution of EGFP-immunoreactive cells in the primary sensory (S1) and adjacent insular cortex (Ins), as well as the medial prefrontal (PL) and medial orbital (Orb) cortices. AC: anterior cingulate cortex; Aud: auditory cortex; Ent: entorhinal cortex; Hipp: hippocampus; Ins: insular cortex; M1: primary motor cortex; Orb: orbital cortex; PL: prelimbic cortex; Rh: rhinal cortex; S1: primary somatosensory cortex; S2: secondary somatosensory cortex; Str: striatum (Weber & Andrade, 2010). Radiolabelled 5HT$_{2A}$Rs in the rat brain with **C** [3H]MDL100,907 (0.4 nM), **C'** non-specific binding determined with 10–5 M spiperone (López-Giménez et al., 1997).

The profound effects on perception and cognition, for instance synaesthesia, lend themselves toward a cortical action of psychedelics. Cortical 5-HT$_{2A}$Rs have been implicated in psychedelic associated animal behaviours, HTR (González-Maeso et al., 2007) and reduced density following chronic psychedelic exposure (Dougherty & Aloyo, 2011; D. E. Nichols, 2016). Human PET studies report cortical 5-HT$_{2A}$R binding with N1-[11C]-methyl)-2-bromo-LSD, specifically in the frontal and temporal cortices (Wong et al., 1987), and psilocin displacing [11C]MDL 100,907, greatest in the angular

gyrus, intraparietal sulcus, precentral gyrus, and subgenual anterior cingulate cortex (Barrett et al., 2022).

The 5-HT_{2A}R is also region-specifically expressed subcortically (Doherty & Pickel, 2000; López-Giménez et al., 1997; Wright et al., 1995). Autoradiographic studies using different ligands such as ^{3}H-M100907 or ^{3}H-ketanserin report that the density of 5-HT_{2A}R in basal ganglia, a group of subcortical structures involved in the control of motor behaviours, is low compared to cortical fields (López-Giménez et al., 1997). A low density was also reported in the subthalamic nucleus, the substantia nigra (SN), the globus pallidus and the caudate-putamen of primates and rodents (Figure 5) (López-Giménez et al., 1997).

Conversely, 5-HT_{2A}R exhibit modest to high density in the hippocampus, and the amygdala. In the amygdala and hippocampus, like in the cortex, 5-HT_{2A}R are expressed by glutamatergic neurons and GABAergic interneurons (Bombardi, 2011, 2012). 5-HT_2AR expression thalamic regions, specifically the reticular nucleus, lateral geniculate nucleus, and zona incerta, has been described, regions involved in sensory processing and the regulation of arousal states as also described (Pompeiano et al., 1994; Rodríguez et al., 2011).

It is important to address the distribution of 5-HT_{2A}R receptors with respect to monoaminergic neurons in the context of this thesis (see below). Immunohistochemical studies in rodents have reported their presence on dendrites of DA cell bodies in the substantia nigra pars compacta (SNc) (Nedergaard, 1988), although the whole labelling of the receptor in the SN and VTA is lower compared to forebrain regions (Morilak & Ciaranello, 1993). DA neurons in both the parabrachial and paranigral subdivisions of the VTA, mainly innervating the PFC and nucleus accumbens (NAc), respectively, express somatodendritic 5-HT_{2A}R (Doherty & Pickel, 2000; Nocjar et al., 2002). The 5-HT_{2A}R immunolabelling is also detected on non-DA cells of the VTA. These anatomical findings suggest that 5-HT_{2A}Rs could regulate the activity of DA neurons in the VTA by acting through 5-HT_{2A}Rs present on non-DA (presumably GABAergic) neurons or directly on DA cells (Doherty & Pickel, 2000; Nocjar et al., 2002). Their expression by DA neurons could justify the hypothesis that 5-HT_{2A}R found on monoaminergic like axons as mentioned above are in DA fibers.

However, their presence in the terminal fields of DA neurons has never been demonstrated. Rather, the 5-HT_{2A}R labelling in the regions receiving DA innervations would be mainly due to local neurons, including striatofugal GABAergic neurons (Laprade et al., 1996; Ward & Dorsa, 1996).

In summary, even considering only the distribution of 5-HT_{2A}R, a brain-wide effect of classical serotonergic psychedelics would be expected. Acknowledging the diverse pharmacological profiles of these compounds, it is reasonable to anticipate that they exert effects on systems throughout nearly the entire CNS.

Psychedelic mechanism of action

A unified psychedelic mechanism of action would be particularly satisfying to characterize. However, given the heterogeneity of this class of compounds—ranging from their receptor profiles to their behavioural and experiential effects—we should acknowledge that this may not be the case. It seems entirely possible at this point that these compounds, which have been grouped based on the nature of the non-ordinary experiences they induce, may induce several integrative brain mechanisms leading to similar states. Alternatively, our experience with non-ordinary states may be sufficiently limited that we are unable to distinguish between what are, in fact, different non-ordinary states. Nonetheless, the class of serotonergic psychedelics does share the action of the 5-HT_{2A}R. For this reason, the next section will focus on the electrophysiological, neurocircuitry, genetic, and neuroanatomical (plasticity) effects influenced by serotonergic psychedelics.

Electrophysiology

Electrophysiology, *in vivo* and *ex vivo*, has been used to further characterise the mechanisms of psychedelic action through the 5-HT_{2A}R. Given the predominantly cortical expression of the 5-HT_{2A}R across species (*see Brain localisation*) and their action on a subset of 5-HTergic receptor subtypes, this section will focus on the effect of psychedelics on monoaminergic systems and in the cortex. Monoaminergic neurons have cell bodies located in nuclei in the brainstem (DR, LC, VTA), although project across the entire brain (Matsuda et al., 2009; Ren et al., 2019). They have been historically studied *in vivo* since they can be identified by their homogeneous tonic activity during wakefulness. On the other hand, cortical neurons are often studied in

ex vivo slices, given their smaller size, more heterogeneous responses, and lack of reliable tonic activity.

Subcortical

DRN 5-HT neuron activity

Since their discovery psychedelics have been associated with 5-HT-like action (Shaw & Woolley, 1956; Woolley & Shaw, 1954), later their specific affinities for 5-HTR subtypes were described (*see Molecular Targets*). It is therefore unsurprising that the largest amount of electrophysiological data on subcortical responses to psychedelics is the dorsal raphe nucleus (DRN) , housing 5-HT neurons.

Most psychedelics alter DRN activity, often inhibition, although to varying extents. LSD, both systemically or microiontophoretically applied in the DRN, consistently inhibits DRN 5-HT neurons (G. K. Aghajanian et al., 1968; De Gregorio et al., 2016; Haigler & Aghajanian, 1973). This finding has been replicated in freely moving cats where systemic LSD dose dependently decreased DRN unit activity, as did 5-MeO-DMT, and psilocin (Trulson et al., 1981; Trulson & Jacobs, 1979a). LSD, 5-MeO-DMT, and psilocin, have good affinity for the 5-HT_{1A}R, an autoreceptor on 5-HT DRN neurons, which is a likely site of action, although LSD's vast pharmacological profile allows for several other sites of action, such as D_2Rs (De Gregorio et al., 2016).

Conversely mescaline administered systemically or microiontophoretically in the DRN inhibits only a subpopulation of 5-HT neurons (Haigler & Aghajanian, 1973). Authors note that inhibition seen after microiontophoretic mescaline was associated with decreased action potential (AP) size, suggesting both global and local effects contribute the their DRN action (Haigler & Aghajanian, 1973). Supporting a heterogeneous action on 5-HT DRN neurons, systemic DOM and mescaline in freely moving cats did not have a net effect on DRN unit activity. Rather, some neurons showed a significant increase, while others showed a significant decrease (Trulson et al., 1981). Systemic DOI has also been shown to suppress the firing rate of a subset of DRN 5-HT-neurons, an effect blocked by both 5-HT_{2A}R and $GABA_A$ antagonists (Martín-Ruiz et al., 2001). Mescaline, DOM and DOI all have relatively weak affinity for the 5-HT_{1A}R, perhaps corresponding to their lack of homogeneous DRN inhibition, as seen with psychedelics with 5-HT_{1A}R affinity (LSD, 5-MeO-DMT and psilocin). Even without 5-HT_{1A}R affinity they still induce neuron specific increases and decreases in

DRN activity. This may be a consequence of 5-HT_{2A}Rs, as $5HT_{2A}$R antagonists block both DOI and LSD induced in inhibition of DRN 5-HT neurons.

While psychedelics alter DRN 5-HT neuron activity, they do so in different manners, via different mechanisms as evidenced by the differential response of systemic and local administration of psychedelics. For instance, cortical application of DOI increases the firing rate of DRN neurons, and extracellular 5-HT in the cortex (Martín-Ruiz et al., 2001; Puig et al., 2003). This may be one of the mechanisms at play in the heterogeneous response seen following systemic DOI administration. The psychedelic effects on the electrical activity of DRN neurons are complex and likely not unique to psychedelics. Lisuride, which is devoid of psychedelic-effects, also suppresses DRN firing comparably to LSD (Rogawski & Aghajanian, 1979). In fact, the dissociation between psychedelic behavioural effects and altered DRN unit activity was made by Trulson et al. in the 80's (Trulson et al., 1981). Although the activity of 5-HT neurons in the DRN is altered by psychedelics this does not seem to be a unique feature of psychedelic action (Butler, Ricci et al., 2024).

LC NA neuron activity

The locus coeruleus (LC) houses the primary site of NA innervation, *in vivo* electrophysiological recordings provided evidence that it may be involved in the psychedelic mechanism of action. Mescaline, DOM and LSD suppress the spontaneous activity of LC neurons while increasing their reactivity to the excitatory influences of peripheral stimuli (G. K. Aghajanian, 1980; Rasmussen & Aghajanian, 1986). In the case of DOM, systemic but not local LC application of a 5-HT_2R antagonist blocked this effect, suggesting a role of 5-HT_2Rs outside the LC (Rasmussen & Aghajanian, 1986). The increased reactivity and reduced spontaneous activity of NA neurons can be interpreted as an increase in signal-to-noise in the LC, a site of convergence of sensory information. As opposed to psychedelics, lisuride, lacking any subjective psychedelic-experience, increased spontaneous firing in the LC (Rogawski & Aghajanian, 1979), hence, supporting the importance of NA in the experiential effect of psychedelics.

VTA DA neuron activity

Although the 5-HT_{2A}R is expressed in the VTA (Doherty & Pickel, 2000; Nocjar et al., 2002) and DAergic systems can be modulated by the 5-HTRs targeted by psychedelics (Butler, Ricci, et al., 2024), electrophysiological studies have not revealed a consistent action of psychedelics on VTA DA neurons. DOI does not alter the electrical activity of VTA DA neurons (Di Matteo et al., 2000; Shi et al., 1995), nor does LSD at moderate-low doses (5-20 µg/kg i.v.). However, higher doses of LSD (50–120 µg/kg i.v.) decrease VTA DA activity, leading to a decrease in cortical DA release attributed to several mechanisms including the direct stimulation of DA receptors (De Gregorio et al., 2016). It should also be noted here that a strong central action on DA would parallel the rewarding properties we see with substances of abuse (cocaine, amphetamine), of which serotonergic psychedelics are not. Rather the influence of psychedelics on DAergic systems are likely region- and state- dependent (Butler, Ricci, et al., 2024).

The subcortical effects of psychedelics on monoaminergic nuclei result from both local and global actions. Two receptors implicated in this global action are, the 5-HT_{2A}R, expressed densely in the neocortex, and the $GABA_A$ receptor, implicating GABAergic interneurons (IN) (Martín-Ruiz et al., 2001). In the neocortex, the 5-HT_{2A}R is expressed in both glutamatergic pyramidal neurons (PN) and INs. Their roles have typically been studied independently using whole-cell patch-clamp ex vivo electrophysiology.

Cortical interneurons

It is known that 5-HT_{2A}R are expressed on interneurons across the cortex in several species (*see Brain localisation*). It has been proposed that they play an important role in the altered cortical activity observed following psychedelics.

Using whole-cell patch-clamp 5-HT has been demonstrated to excite interneurons in the piriform cortex in a 5-HT_{2A}R dependent manner (Marek & Aghajanian, 1994). Concurrently layer II PNs in the piriform cortex have increased IPSPs following 5-HT bath application (Gellman & Aghajanian, 1993). 5-HT-induced sIPSPs in PNs were sensitive to tetrodotoxin (TTX) and a 5-HT_{2A}R antagonist, supporting a role of 5-HT_{2A}R expressing GABAergic Ins (Marek & Aghajanian, 1994; Sheldon & Aghajanian, 1990).

There are several lines of indirect evidence implicating INs in the psychedelic mechanism of action. The blockade of DOI-induced reduced 5-HT DRN neuron with $GABA_A$ antagonist (Martín-Ruiz et al., 2001). Additionally, Martin and Nichols characterised the cortical cells activated by DOI, which included PNs, INs (PV+ and SOM+), glia, and astrocytes (Martin & Nichols, 2016).

Before concluding with a discussion on cortical circuitry, it's important to note that most of this research has been conducted in the frontal regions, which have the highest 5-HT_{2A}R density, as well as in the piriform cortex. Similar patterns of 5-HT_{2A}R distribution are also observed in other cortical areas. Therefore, further investigation into the effects of cortical 5-HT_{2A}R agonists, particularly in regions like the primary somatosensory cortex, would be valuable.

Cortical pyramidal neurons

Pyramidal neurons (PN) in layer V (L5) of the prefrontal cortex (PFC) express 5-HT_{2A}Rs, primarily localized on their apical dendrites (see *Brain localisation*). Whole-cell patch-clamp of L5 PNs in the PFC show increased frequency and amplitude of spontaneous excitatory synaptic potentials/currents (sEPSPs/sEPSCs) in response to bath application of 5-HT (G. K. Aghajanian & Marek, 1997). 5-HT-induced increase in sEPSCs were blocked by 5-HT_{2A}R antagonist MDL-100,907 (20 μM), and by AMPA-selective excitatory amino acid antagonist, confirming that the 5-HT-induced currents are glutamate mediated EPSPs rather than IPSPs. Microiontophoretic application of 5-HT, adjacent to the apical dendrite of the L5 PNs induced EPSCs, supporting 5-HT excitatory action on their apical dendrites (G. K. Aghajanian & Marek, 1997). To determine whether GLU release induced by 5-HT is synchronous release (coupled to action potentials) or asynchronous, authors substituted Ca+ for Sr+, and found that Sr+ was effective in enabling 5-HT-induced EPSCs in the absence of Ca+, indicative of asynchronous release (Marek & Aghajanian, 1998b).

Congruent with 5-HT_{2A}Rs action on PFC L5 PNs, DOI increases the frequency and amplitude of sEPSCs, an effect blocked by 5-HT_{2A}R antagonists. This enhancement persists in the presence of tetrodotoxin (TTX), consistent with an action potential-independent mechanism. The findings implicate calcium-dependent presynaptic

mechanisms driven by 5-HT_{2A}R activation, promoting asynchronous glutamate release as depicted in Figure 6A (G. K. Aghajanian & Marek, 1999).

Kłodzinska et al. (2002) replicated this result and further showed that DOI-induced sEPSCs were attenuated by mGlu2/3 agonists (Kłodzinska et al., 2002). mGlu2/3 has also been recurrently implicated in the mechanism of 5-HT-induced increased sEPSCs (D. E. Nichols, 2016). Supporting the hypothesis that mGlu2/3 receptors function as inhibitory autoreceptors on cortical glutamatergic terminals and are positively regulated by activation of 5-HT_{2A}R. This action is apparently shared by μ-opiate receptors, as agonists supress 5-HT-induced sEPSCs (Marek & Aghajanian, 1998a). Finaly, tryptamines and LSD additionally have affinity for the 5-HT_{1A}R, which are colocalized with 5-HT_{2A}Rs in cortical PNs (Amargos-Bosch, 2004; Martín-Ruiz et al., 2001), with opposing functional effects (Araneda & Andrade, 1991). The consequences of this dual activation remain poorly understood.

Cortical circuitry

Psychedelic action in L5 of the PFC is interesting as it is generally considered the 'output' layer of the cortex. The PFC receives subcortical and thalamic inputs in upper L5, where the apical dendrites of the PNs hosting $5HT_{2A}$Rs are localized. With their soma lying deep in the cortex, and their apical dendrites spanning up to Layer I, L5 PNs are a site of integration for both the cortico-cortical and thalamo-cortical loops (Aru et al., 2019). Two subpopulations of L5 PNs can be classified by their different axonal projections: L5 intra-telencephalic (IT) neurons, in upper L5 mainly involved in cortico-cortical loops, projecting their axon in other cortical regions and L5 pyramidal tract (PT) neurons, in deeper L5 mainly involved in cortico-thalamic loops, projecting axons outside of the telencephalon (Molnár & Cheung, 2006). Some PT neurons express 5-HT_{2A}R, with subcortical projections to the DRN, VTA and NAc (Mengod et al., 2015). Electrophysiological data suggests that both L5 IT and PT neurons are responsive to 5-HT application (Avesar & Gulledge, 2012; Elliott et al., 2018; Stephens et al., 2014). The 5-HT_{2A}R has be implicated differently between IT and PT neurons in both the rat and mouse. Further characterisation of the 5-HT_{2A}R expressing PNs in the PFC may help understand their circuit and brain wide effects. The distribution of $5HT_{2A}$Rs positions them ideally to mediate cortico-cortical and cortico-thalamic loops,

as well as broader cortical-subcortical circuitry, it has been proposed that psychedelics may induce their profound experiential alterations through actions on these circuits.

As seen in the previous section, several electrophysiological works consistently showed increased GLU in the cortex following 5-HT_{2A}R agonism (G. K. Aghajanian & Marek, 1997, 1999; Marek & Aghajanian, 1998a) . Several hypothesis were posited for this, initially that GLU is released from terminals arising from the mediodorsal thalamus (G. K. Aghajanian & Marek, 1997; Marek & Aghajanian, 1998b; Scruggs et al., 2000). However, lesions of mPFC-projecting thalamic nuclei did not affect systemic effects of DOI. Both DOI-induced increased firing of mPFC PNs, or DOI-reduced amplitude of low frequency oscillations (Celada et al., 2008; Puig et al., 2003). Furthermore, a thalamic origin of the increase in cortical GLU following $5HT_{2A}$Rs agonism is incongruent with the fact that $5HT_{2A}$Rs are localised primarily on postsynaptic neurons and their presynaptic location corresponds with unmyelinated axons (Miner et al., 2003).

Recalling that psychedelics can modulate unit activity in the DRN and LC, and that the presynaptic location of 5-HT_{2A}R is on unmyelinated axons (Miner et al., 2003), Aghajanian & Marek propose a role of 5-HT_{2A}R on 5-HT and NA cortical projections to L5 (Figure 6A). Hypothesising that increased cortical 5-HT or NA would act on cortical glutaminergic neurons increasing GLU release, via α1 or 5-HT_{2A}R (G. Aghajanian & Marek, 1999). Inhibitory modulators of 5-HT_{2A}R-induced glutamate release include μ-opioid receptors, mGluR2/3, and at the time unidentified though suspected 5-HT_{1A}R (G. Aghajanian & Marek, 1999). This early work identified many of the receptors involved in the cortical dynamics that might be induced by psychedelics; however, the identity of the cells involved was not clear, nor their projections.

Finaly, Beique et al. identified a subpopulation of cortical neurons in deep layers that depolarised sufficiently to fire action potentials in response to 5-HT (10–30 μM) and 5-HT_{2A}R agonist application (Béïque et al., 2007). This 5-HT_{2A}R depolarization was blocked by a μ-opioid agonist, which had previously been shown to block the 5-HT_{2A}R induced sEPSCs in L5 PNs (Marek & Aghajanian, 1998a). Authors therefore posited

that the enhanced sEPSCs recurrently observed in PFC PNs results from a subpopulation of highly responsive PN neurons in the deep PFC, not the activation of thalamocortical axon terminals or the increased PFC NA or 5-HT. An interesting candidate for these highly responsive neurons are layer VI glutaminergic PNs, which both express the $5HT_{2A}R$ and project directly onto L5 PNs (J. Kim et al., 2014).

While some of the early work, particularly in the piriform cortex implicated interneurons (Gellman & Aghajanian, 1993; Marek & Aghajanian, 1994; Sheldon & Aghajanian, 1990), little attention had been placed on their role in modulating these circuit dynamics. Figure 6B, depicts the Localization of 5-HT receptors within the PFC microcircuit (Puig & Gulledge, 2011). The inclusion of $5\text{-}HT_{2A}R$ and $5\text{-}HT_{1A}R$ expressing INs is of relevance, although their modulation of this circuitry under psychedelics remains to be characterised. It has been proposed that fast spiking interneurons generate gamma oscillations, and the subpopulation which express the $5\text{-}HT_{2A}R$ has been implicated in the altered gamma oscillations following DRN stimulation (Puig et al., 2010). More work on the role of $5\text{-}HT_{2A}$ and $5HT_{1A}$ expressing cortical interneurons is warranted to better understand the action of psychedelics on these circuits, particularly as several tryptamines have affinity for both receptors which likely exert opposing effects.

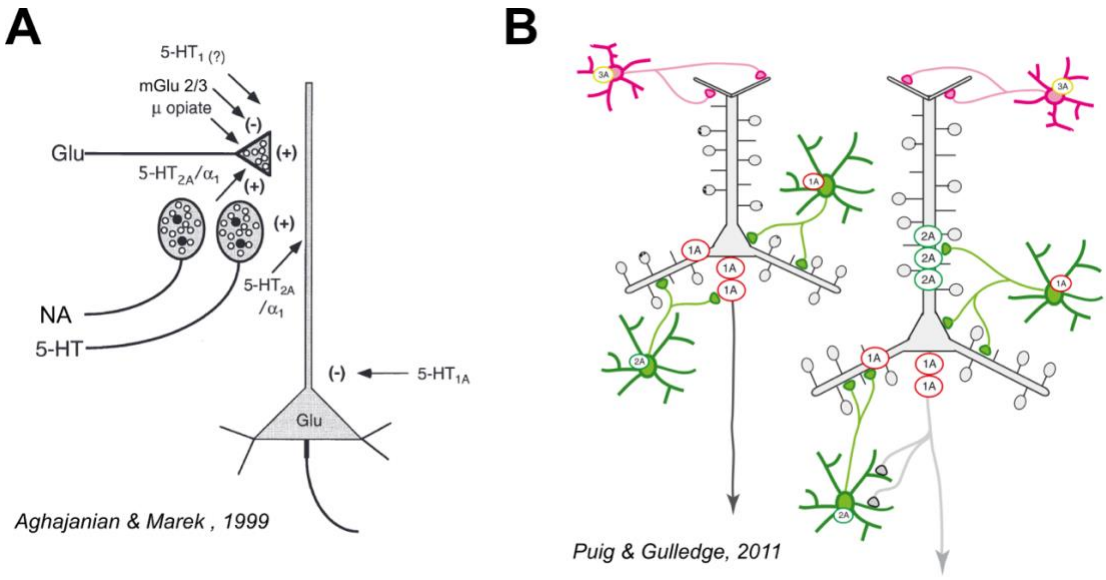

Figure 6 - Proposed mechanisms of psychedelic action in the cortex.

A layer V pyramidal neuron under the regulation 5-HT and NA, via the 5-HT$_{1A}$R, 5-HT$_{2A}$R, metabotropic glutamate receptor 2/3 (mGlu2/3), adrenergic α_1 receptor and μ-opiate receptor (G. Aghajanian & Marek, 1999). **B** location of 5-HTRs on cortical layer V pyramidal neurons and layer I-VI interneurons (Puig & Gulledge, 2011).

Psychedelic action involves complex and integrated mechanisms, from modulation of brain-wide activity, synchrony (*see Neuroimaging*), and neurotransmission (*see Psychedelics' action on neuromodulation*) to regional circuit dynamics, and down to the modulation within individual neurons (*see Molecular pharmacology*). The co-expression of the 5-HT$_{2A}$R and 5HT$_{1A}$R on PFC L5 PNs (Amargos-Bosch, 2004), and their excitatory and inhibitory action, respectively, suggests that they may play specific localised roles in the integration of information within a single PN. It has also been described that the modulation of cortical L5 PN apical dendrites plays a role in perception (Takahashi et al., 2016). Cortical PNs and their immediate circuitry are a very plausible site of psychedelic action on perception and cognition and warrant further characterisation. However, the electrophysiological response of neurons to psychedelics provides only a partial understanding of their effects. For example, in monoaminergic nuclei, changes in neuronal activity do not necessarily correspond to neurotransmitter release. Predicting the consequences of these altered cortical network dynamics induced by psychedelics remains challenging, although such effects are likely encapsulated in the altered connectivity observed in neuroimaging studies (*see Neuroimaging to 'Connectivity'*). Beyond excitation, inhibition, the activation of specific receptors can lead to intracellular changes such as gene expression.

Immediate Early Gene Expression (IEG)

Immediate early genes (IEGs) are a class of genes that are rapidly and transiently expressed in response to various stimuli without requiring *de novo* protein synthesis. Serotonergic psychedelics have been shown to robustly induce IEG expression region-specifically in the brain. Nichols and Sanders-Bush (2002) reported LSD administration in rats upregulated 5 genes, including nuclear transcription factor *early growth response protein 2* (*erg-2/Krox-20*), required for brain development and maintenance of long-term potentiation (LTP). Increased *erg-2* expression following both LSD and DOI corroborate this initial finding, although non-psychedelic 5-HT_{2A}R agonist lisuride did not enhance *erg-2* expression, nor did any compound in 5-HT_{2A}R knockout mice (González-Maeso et al., 2003). Expression of *activity-regulated cytoskeleton-associated protein* (*arc*), localized to dendrites and involved in synaptic plasticity, was also increased following LSD and DOI (C. D. Nichols & Sanders-Bush, 2002; D. E. Nichols, 2016).

The unaltered transcription of *erg-2* and *arc* following DOI and LSD in the PFC of 5-HT_{2A}R knockout mice supports the involvement of the 5-HT_{2A}R. This was verified with 5-HT_{2A}R antagonist pretreatment, attenuating the increase in both *erg-2* and *arc* expression, not 5-HT_{1A}R antagonist (C. D. Nichols et al., 2003). Most LSD-induced gene expression alterations were maximal at 90 minutes and returned to baseline within 3-5 hours, apart from *nor1* which remained elevated 5 hours post LSD (C. D. Nichols et al., 2003). LSD enhanced activity- and neurotransmitter-induced early gene 3 (*Ania3)* expression, implicated in metabotropic glutamate receptor (mGluR)–mediated plasticity (C. D. Nichols & Sanders-Bush, 2002). Its increase is blocked by MDL-100,907, implicating the 5-HT2AR. The neuronal orphan receptor 1 (*Nor1*), a nuclear receptor involved in synaptic plasticity and *Ania3* have been linked to synaptic plasticity mediated by mGlu2/3Rs.The neuronal orphan receptor 1 (*Nor1*), has also been linked to synaptic plasticity mediated by mGlu2/3Rs. It is noteworthy that MDL-100,907 alone increased *ania3, arc, c-Fos, Nor1* but the peripheral doses of 0.5 mg/kg and 1 mg/kg do not guaranty the sole action of 5-HT_{2A}R blockade (C. D. Nichols et al., 2003).

Neuroplasticity

Several of the IEGs upregulated by psychedelics are associated with neural plasticity. Furthermore, the alerted functional connectivity following psychedelics may be consistent with enhanced neuroplasticity allowing for remodelling of neural circuits and perhaps enhanced cognitive flexibility (Doss et al., 2021). It is also pertinent to note the dissociate-aesthetic and antidepressant ketamine promotes dendritic spine growth and synthesis of synaptic proteins which has been linked to its therapeutic actions (Ly et al., 2018). Ly et al. proposed in 2018 that the therapeutic effects of classical psychedelics may also by due to their ability to induce structural and functional neuroplasticity. The authors show that psychedelics LSD, DMT and DOI increased dendritic arbour complexity, spine growth and stimulate synapse formation in cortical neurons *in vitro* and *in vivo*, comparably to ketamine (Ly et al., 2018). Additionally, psilocin and nor-ibogaine promoted neuritogenesis, measured by number of crossings. These effects were blocked with ketanserin (100 μM), rapamycin (100 nM), and ANA-12 (10 μM) pretreatment leading authors to implicate TrkB, mTOR, and 5-HT_{2A}R signalling in the psychoplastogenic effects of psychedelics (Ly et al., 2018).

Brain derived neurotrophic factor (BDNF) was implicated in early studies showing region-dependant modulation of BDNF mRNA following DOI (Vaidya et al., 1997). BDNF primarily acts on TrkBRs mediating neuroplasticity, survival, and synaptic function. While the TrkBR had been implicated in the mechanism of psychedelic-induced neuroplasticity by Ly et al. (2018), the direct action of classical psychedelics, psilocin and LSD, on the TrkBR wasn't established until the seminal paper by Moliner et al. (Moliner et al., 2023). These two psychedelics act as positive allosteric modulators of TrkB, facilitating its dimerization and enhancing the binding of endogenous BDNF released at active synapses. Contrary to previous findings, LSD-induced spinogenesis and dendritogenesis were not blocked by a 5-HT_{2A}R antagonist but were inhibited by TrkB.Fc, a fusion protein disrupting TrkB signalling, implicating a direct action of LSD and psilocin on TrkB in its psychoplastic effects (Moliner et al., 2023).

This work reinforced a sort of division in the literature between the authors pushing towards or against the role of 5-HT_{2A}R in the therapeutic effects of psychedelics. The work by Moliner et al. (2023) put a name on one mechanism involved in neuroplasticity

that could occur away from the 5-HT_{2A}R: TrkBR. Yet, it concerned only two drugs, LSD and psilocin, and it does not discard a role for 5-HT_{2A}R in acting on neuroplasticity or as antidepressants (Sekssaoui et al., 2024).

Animal Behavioural Pharmacology

Animal studies have allowed a more in-depth investigation into the neural correlates of psychedelic action. For this reason, their behavioural responses to psychedelics are of particular interest, especially those that might parallel the profound experiential effects observed in humans. The following is a characterisation of the behavioural and neuro-physiological response of animals to psychedelics, acknowledging that the experiential nature of psychedelic-effects in animals is well beyond our grasp. That said, instances of animal use of psychedelic plants have been documented, as described by Samorini (Samorini, 2002).

Head twitch response

The most established unconditioned behaviour characteristic of psychedelic action in animals is the head twitch response (HTR), a paroxysmal rotation of the head. The HTR is a naturally occurring phenomenon that is markedly increased by serotonergic psychedelics. One reason for its wide use it its apparent selectivity for hallucinogenic or psychedelic compounds in humans. Psychedelic compounds LSD, DMT, psilocin, 5-MeO-DMT, DOI, mescaline, 2C-I and 25I-NBOMe all elicit HTR in rodents. However, non-psychedelic 5-HT_2R agonists such as lisuride, do not (Halberstadt, 2017; Halberstadt et al., 2020; Halberstadt & Geyer, 2011). Additionally, HTR correlates with the potency of a given psychedelic, increasing relative to the human reports and ED50 for drug discrimination in rodents (Halberstadt et al., 2020).

This behaviour is most distinct in the mouse; however, a comparable movement occurs in rats often called wet dog shake (WDS), akin to a dog emerging from water. Similar responses have been observed in cats, pigs, shrews, *Stumptail* macaque monkeys and a ‘head bob’ response described in rabbits (Halberstadt & Geyer, 2013, 2018). However, there are clear species differences in HTR response to psychedelics. For instance, lisuride, which does not induce HTR in rodents, does induce HTR in the shrew, a species reported to be highly sensitive to 5-HT_{2A}R agonists (Halberstadt & Geyer, 2013). We also see different responses between rodents, rat and mouse, and

even strains of mice (Canal et al., 2010; Halberstadt & Geyer, 2018). The remaining discussion will focus on mice, the species for which the most information is available, and the primary animal model used in the work presented in this thesis.

Early work documented HTR following administration of 5-HT precursor 5-hydroxytryptophan (5-HTP), suggesting a role of 5-HTR activation (Corne et al., 1963). With increasingly specific 5-HTR antagonists a primary role of the 5-HT_{2A}R was established. This assertion is most consistently validated by the blockade of psychedelic-induced HTR with pretreatment with 5-HT_{2A}R antagonists, most commonly MDL-100,907 and MDL-11,939 (Halberstadt & Geyer, 2011; D. E. Nichols, 2016). Additionally, 5-HT_{2A}R knockout mice do not display psychedelic-induced HTR, and restoration of cortical 5-HT_{2A}R expression rescues psychedelic-induced HTR (González-Maeso et al., 2003, 2007; Halberstadt & Geyer, 2010). Tolerance has also been demonstrated, with reduced HTR following repeated psychedelic-administration, consistent with the decreased cortical density of 5-HT_{2A}Rs (Darmani et al., 1992; Vickers et al., 2001).

Beyond a clear involvement of 5-HT_{2A}R activation in the HTR, other 5-HTRs have been shown to modulate psychedelic-induced HTR, particularly the 5-HT_{2C}R for which many psychedelics also have considerable affinity (Table 3). Vickers et al. suggest that the activation of 5-HT_{2C}Rs mitigate 5-HT_{2A}R-mediated HTR (Vickers et al., 2001). Phenylethylamine psychedelics, with affinity for both 5-HT_{2A} and 5-HT_{2C}Rs, produce a biphasic HTR with increasing doses. Fantegrossi et al. show that in mice 5-HT_{2C}R agonist (Ro-60-0175) pretreatment blunted DOI-induced HTR, flattening the dose-response curve, while 5-HT_{2C}R antagonist (SB242084) pretreatment caused a rightward shift of the descending limb of the curve only (Fantegrossi et al., 2010). Therefore, it was proposed that the descending limb of the curve is modulated by 5-HT_{2C}Rs, activated at higher doses of DOI.

In contrast Canal et al. has shown an attenuation of DOI-induced HTR both following 5-HT_{2C}R antagonists (SB242084 and SB206553), and in 5-HT_{2C}R knockout mice (Canal et al., 2010). There is a clear consensus that 5-HT_{2C}Rs play a modulatory role in psychedelic-induced HTR, although contradictory findings suggest further research is required to understand nature of its involvement. Despite less research there has

also been some suggestion as to the involvement of the 5-HT_{2B}R and the 5-HT_{1A}R in the modulation of the HTR (Canal & Morgan, 2012).

Additionally there is evidence for the role of glutamate in the HTR as mGluR2 knockout mice, one of the eight subtypes of metabotropic glutamate receptors, lack DOI- or LSD-induced HTR (J. L. Moreno et al., 2011). Furthermore, pretreatment with mGluR2/3 agonists has been reported to attenuate DOI-induced HTR, while mGluR2/3 antagonists perpetuate DOI-induced HTR (Gewirtz & Marek, 2000; Kłodzinska et al., 2002; D. E. Nichols, 2016). These findings suggest a role of mGluR2/3 systems in the psychedelic-induced HTR. Additionally, there is evidence of D_1R involvement in the HTR in rats, as DOI-induced HTR is attenuate by D_1 antagonists (Halberstadt & Geyer, 2013).

Given the heteroclite pharmacological profile of psychedelics it isn't surprising that several receptors may modulate their effects. Even μ-opioid receptors have been implicated in DOI-induced HTR a (Marek, 2003), congruent with electrophysiological studies (Béïque et al., 2007; Marek & Aghajanian, 1998a). Despite modulation by several mechanisms, it is widely accepted and consistently replicated that the HTR is primarily mediated via psychoactive 5HT_{2A}R agonists.

Pre-pulse Inhibition (PPI) of startle

The startle reflex is a transient motor response to a startling stimulus. It can be attenuated by giving a weak pre-stimulus prior to the startle pulse, called a pre-pulse. This effect is called pre-pulse inhibition (PPI) of startle reflex and is used as a measure of sensorimotor gating. Psychedelics are proposed to disrupt sensory filtering mechanisms. PPI is applicable to both human and animal studies. In rodents however, there has been considerably more research. In rats psychedelics DOI, DOB, LSD, mescaline, 2C-B and 25CN-NBOH reduce PPI (Halberstadt & Geyer, 2018). This is likely a 5-HT_{2A}R effect as pretreatment with 5-HT_{2A}R antagonist blocks it. PPI reduction is also seen following 5-MeO-DMT however this is blocked by pretreatment with 5-HT_{1A} and 5-HT_{2C}R antagonists (Krebs-Thomson et al., 2006). The research on PPI in mice has been less congruent, with many studies not observing a disruption of PPI in mice following DOM or DOI, with variability between strains. Tryptamine

psychedelics, DMT, psilocin and 5-MeO-DMT, increase PPI in mice. Additionally, LSD has been shown to disrupt PPI in mice (Rodriguiz et al., 2021).

Psychedelics alter PPI in humans depending on the inter-stimulus intervals (ISI). Psilocybin has been shown to reduce PPI for short ISI (30ms), likely 5-HT_{2A}R dependent, and increase PPI for long (120-2000ms) ISI (Gouzoulis-Mayfrank et al., 1998; Vollenweider et al., 2007). LSD (200 µg) also reduces short ISI (30–120 ms) PPI (Y. Schmid et al., 2015). However, DMT was not found to alter PPI, although it did affect the P50 auditory evoked potential (Halberstadt & Geyer, 2018). Further study is warranted to characterise the disturbance of sensorimotor gating by psychedelics, the current literature in humans and rodents suggest that the 5-HT_{2A}R plays a role in its regulation. This could be associated with the localisation of 5-HT_{2A}R on subcortically projecting cortical layer 5 pyramidal neurons, modulating thalamocortical loops (Yu et al., 2024).

Exploratory behaviour and locomotion

Locomotor activity, particularly in a novel environment, is a classical metric for pharmacological classification. However, tests such as an open field do not allow the distinction of psychedelics from other classes of drugs. Multivariate assessment of exploratory behaviour in paradigms such as the Behavioural Pattern Monitor (BPM), an activity and hole-board chamber in which locomotor activity, spaciotemporal activity, rearings, and hole pokes can be quantified, do provide a characteristic profile of psychedelic-behaviours (Adams & Geyer, 1985a; Halberstadt, 2015; Halberstadt & Geyer, 2011).

Rats in an unfamiliar BPM following psychedelic administration (mescaline, DOM, DOI, LSD, DMT, 5-MeO-DMT, and psilocin) demonstrate reduced locomotor activity, rearings, time in the centre and hole pokes, in the first 30 minutes (Adams & Geyer, 1985a, 1985b; Krebs-Thomson et al., 2006; Mittman & Geyer, 1991; Wing et al., 1990). Analysing spatial patterns of locomotion showed LSD and DMT reduced the repetition of old roots and increased random direction changes (Adams & Geyer, 1985a, 1985b, 1985c). It was postulated that psychedelics induce neophobia in rats as these effects are diminished by habituation to the BPM chamber, unlike the

response seen to 5-HT_{1A}R agonist 8-OH-DPAT which reduced exploration and locomotion independently of environment novelty, likely reflecting sedation (Mittman & Geyer, 1989). Exploration is also distinct between psychedelic 5-HT_{2A}R agonist LSD and non-psychedelic lisuride (Adams & Geyer, 1985c). The reduction in exploration following DOM, DOI and mescaline administration is blocked by 5-HT_{2A}R antagonists (ritanserin, ketanserin and MDL-100,907), but not 5-$HT_{2B/2C}$R antagonist SER-082 (Krebs-Thomson, 1998; Wing et al., 1990). However, the effect of LSD on behavioural organisation was sensitive to both 5-HT_{2A} and 5-HT_{2C}R antagonist pre-treatment (Krebs-Thomson, 1998). Psychedelic 5-MeO-DMT, with considerable 5-HT_{1A}R affinity, shows reduced exploration even in a familiar BMP chamber (Krebs-Thomson et al., 2006). The reduction in exploratory behaviour by both indolamines LSD and 5-MeO-DMT is more complicated. 5-MeO-DMT's behavioural effects are attenuated by 5-HT_{1A}R antagonist (WAY-100,635), but not 5-HT_{2A}R antagonist (MDL-100,907). LSD-induced decrease in locomotion was attenuated by 5-HT_{1A}R antagonism, and decreased investigation by 5-HT_{2A}R antagonism, and altered locomotor patterns by 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C}R antagonism (Krebs-Thomson et al., 2006).

The BMP chamber can also be used in mice, although there seems to be a distinction in response observed with phenylalkylamine and indolamine psychedelics. Phenylalkylamines, DOI, DOM, mescaline, and TCB-2, inhibit investigatory behaviour (rearings and hole-pokes) and alter locomotor activity dose dependently, increased at low doses and reduced at high doses (Halberstadt et al., 2009). Conversely Chen et al., (2023) report increased rearing following psilocin, DOM, and 25CN-NBOH administration, the latter two blocked by MDL-100,907 pretreatment (Chen et al., 2023). These contradictory results in rearing behaviour have been ascribed to the sensitivity of this parameter to environmental context, however phenylalkylamine-induced locomotion changes were consistent in both studies.

The increased locomotor activity at lower doses has been attributed to the 5-HT_{2A}R, as it is absent in 5-HT_{2A}R knockout mice, while the reduced locomotor activity at higher dose is potentiated in 5-HT_{2A}R knockout mice and blocked by 5-$HT_{2B/2C}$R antagonist SER-082 (Halberstadt et al., 2009). The increased locomotor activity induced by mescaline and TCB-2 is also absent in 5-HT_{2A}R knockout mice and blocked by 5-HT_{2A}R antagonist MDL-100,907 (Halberstadt & Geyer, 2013). These findings suggest

that 5-HT_{2A}R and 5-HT_{2C}R have opposing effects on locomotion, with 5-HT_{2A}R agonism increasing and 5-HT_{2C} R reducing locomotor activity, consistent with reduced locomotor activity following 5-HT_{2C}R agonists (Di Giovanni & De Deurwaerdère, 2016; Halberstadt et al., 2009). Indolamines psilocin and 5-MeO-DMT reduce locomotor activity and exploratory behaviour in mice, effects blocked by 5-HT_{1A}R antagonist WAY-100,635, but not 5-HT_{2C}R antagonist or 5-HT_{2A}R gene deletion (Halberstadt et al., 2011; Halberstadt & Geyer, 2011). This suggests that behavioural modification induced by these indolamines are mediated by 5-HT_{1A}Rs, consistent with a lack of effect of 5-MeO-DMT in 5-HT_{1A}R knockout mice (Van Den Buuse et al., 2011).

In rodents the BPM paradigm generally shows a reduction in exploratory behaviour and disorganised patterns of movement following psychedelics sensitive to familiarity with the BPM chamber. There are marked difference in receptor involvement both between species and compounds, demonstrating the complexity in describing a general 'psychedelic' effect. The BPM is a paradigm that is also applicable to human studies although it has not been used in the context of psychedelics (Halberstadt & Geyer, 2018). The neural underpinnings of exploratory behaviour are likely integrative across several neurological systems. How psychedelics act to alter this exploration behaviour is unknown, although from the work presented above it can involve several distinct targets, depending on the psychedelic. Exploration and novelty is associated with mobilised monoaminergic systems, particularly DA and NA (Le Moal & Simon, 1991; Vankov et al., 1995). It is therefore conceivable that modulation of monoaminergic systems may contribute to altered exploration in rodents after psychedelic administration.

Monoaminergic systems

Rational to look at monoamines

Monoaminergic systems, involving DA, 5-HT, NA play a role in virtually all cerebral functions. As a preamble to justify a focus on monoaminergic systems in the mechanism of action of psychedelics, one may recall that these compounds target monoaminergic targets, mostly 5-HTR (see above). This is extremely challenging for elaborating scenarios because the 5-HT system does not play an executive role in the CNS and is considered as the prototypical neuromodulator system in the CNS, quite

conserved in the phylum (Bacqué-Cazenave et al., 2020; Soubrié, 1986). Trulson and Jacobs made an analogy between psychedelic action and rapid eye movement (REM) sleep, marked by vivid dreams and the decrease in the activity of DRN neurons (Trulson & Jacobs, 1979b). However, the uniform decrease in 5-HT DRN neuron activity concerned some (LSD, 5-MeO-DMT) but not all (mescaline, psilocybin) psychedelics. Soon after this analogy, they provided evidence that the mechanism of action of psychedelics was due to post-synaptic effects, i.e. the responses of non-5-HT cells in the CNS expressing 5-HTRs (Trulson et al., 1981).

How can psychedelics hijack elementary CNS processes to produce these powerful effects on consciousness? Do we know pathophysiological situations where 5-HT could dramatically interfere with cognition and/or consciousness? The psychotomimetic property of these drugs led indeed to consider the participation of the 5-HT system in schizophrenia, but the nature of the hallucinations (rare with psychedelics) is different from schizophrenia and psychedelics do not reproduce the core delirium. Although, from an external observation, the distinction between a psychotic state and a psychedelic may not be clear. It is noteworthy that the 5-HT precursor 5-hydroxytryptophan (5-HTP), administered at high doses in mice, induces HTR, which can be mitigated by 5-HT_{2A}R antagonists (Corne et al., 1963; Shahar et al., 2022). 5-HTP promotes an excessive release of 5-HT from catecholaminergic neurons (Stamford et al., 1990) in a non-regulated manner as well described for the DA precursor L-DOPA releasing DA from 5-HT neurons (De Deurwaerdère et al., 2017). This implies that 5-HTP creates a massive disequilibrium of 5-HT transmission in the CNS relative to the homeostasis maintained in physiological conditions. However, in addition to possible psychotic episodes, the main risk with 5-HTP is to develop serotonergic toxicity and serotonin syndrome, which can also occur with some psychedelics, and different medications including selective serotonin reuptake inhibitor (SSRI) (Malcolm & Thomas, 2022).

Irrespective of their acute impact on monoaminergic systems activity, the evidence that psychedelics could promote fast-acting antidepressant, anxiolytic, and anti-addictive effects indirectly place a focus on monoaminergic systems. Indeed, the current first-line strategies for anxiety and depression are based on SSRIs or compounds that share their mechanisms of action (Di Giovanni et al., 2016).

Brief overview of current monoaminergic-based medicines

Antipsychotic drugs

The antipsychotic drugs correspond to a complex family of compounds, all of them able to bind the D_2R (Table 4), and reduce DA transmission via D_2Rs (Seeman et al., 1976). The extrapyramidal side effects (dystonia, parkinsonism, and tardive dyskinesia) and vegetative effects induced by these compounds have been a serious concern. Some antipsychotics, the atypical antipsychotics, reduce the positive symptoms of schizophrenia (hallucinations, delusion, agitation) with fewer extrapyramidal and vegetative side effects. This led to a reappraisal of their mechanism of action towards the 5-HT/DA interaction. Indeed, atypical antipsychotics exhibited a higher affinity at 5-$HT_{2A}R$ compared to D_2R (Meltzer, 1999). This hypothesis led to extensive pre-clinical studies aimed at understanding how blockade of 5-$HT_{2A}R$ could protect against extrapyramidal side effects, notably along the nigrostriatal DA tract. Yet, clinical studies combined with PET evaluation of the occupancy of D_2R and 5-$HT_{2A}R$ strongly suggested that the blockade of 5-$HT_{2A}R$ does not protect against extrapyramidal side effects as olanzapine or risperidone can induce extrapyramidal side effects, even at high doses with maximal occupancy of 5-$HT_{2A}R$ (Kapur & Seeman, 2001). At the same time, newer antipsychotics came out, notably aripiprazole, which retained a D_2R mitigating effect (partial agonism with low efficacy) with D_3R antagonism, 5-$HT_{1A}R$ agonism, and 5-$HT_{2B}R$ antagonism properties (Table 4). This newer pharmacological “recipe” led to the development of new compounds such as cariprazine (De Deurwaerdere, P., 2016). Note that 5-$HT_{2A}R$ antagonism is always present, although at lower affinity relative to 5-$HT_{1A}R$ agonism, both may have similar outcomes. The neuropharmacological views of the mechanism of action of antipsychotics changed over the years as the understanding of the disease progressed. It is acknowledged that schizophrenia is a syndrome encompassing multiple dimensions: positive symptoms, negative symptoms, cognitive defects, mood disorders. While the D_2R antagonism would preferentially target the positive symptoms, the other mechanisms could have benefit on other dimensions of the disease.

As far as we know, psychedelics are not concerned yet in the treatment of schizophrenia, but several of them target the 5-$HT_{2A}R$, 5-$HT_{1A}R$, 5-$HT_{2B}R$, and/or 5-$HT_{2C}R$. The 5-$HT_{2A}R$ antagonist MDL-100,907 was not efficacious in schizophrenic

patients in monotherapy. However, the 5-HT_{2C}R agonist vabicanserin was as efficacious as olanzapine (Shen et al., 2014), but the project has been discontinued. Thus, it is too early to exclude the possibility to develop psychoplastogen compounds devoid of psychotomimetic action, targeting the 5-HTRs in the treatment of schizophrenia.

Table 4 - Receptor binding affinities of typical and atypical antipsychotic drugs. (Di Giovanni et al., 2016)

Antipsychotics	D_2	D_3	5-HT_{1A}	5-HT_{2A}	Other targets*
TYPICAL ANTIPSYCHOTICS					
Haloperidol	8.84	8.56	6.29	7.28	D_4 (8.83); α_{1A} (7.9); σ_1 (8.52)
flupentixol	9.46	8.76	5.1	7.06	D_1 (8.46); H_1 (9.07)
Thioridazine	9.4	8.82	6.84	7.56	α_{1A} (8.5); α_{1B} (8.62); M_1 (7.89); M_5 (7.9); H_1 (7.78)
Pimozide	9.48	9.6	6.19	7.32	5-HT_7 (9.3); D_4 (8.74)
Perphenazine	8.47	9.89	6.38	8.25	5-HT_6 (7.77); 5-HT_7 (7.64); D_1 (7.52); D_4 (7.77); α_{1A} (8); H1 (8.1); σ_1 (7.73)
Loxapine	7.96	7.71	5.61	8.18	5-HT_{2C} (7.88); 5-HT_6 (7.51); 5-HT_7 (7.06); D_1 (7.27); D_4 (8.08); D_5 (7.12); α_{1A} (7.51); α_{1B} (7.28); α_{2A} (6.82); α_{2B} (6.97); α_{2C} (7.1); M_1 (6.92); H_1 (8.3)
ATYPICAL ANTIPSYCHOTICS					
Clozapine	6.87	6.66	7.06	8.39	5-HT_{1B} (6.28)b; 5-HT_{2B} (8.79); 5-HT_{2C} (8.56 - IA); 5-HT_3 (6.62); 5-HT_6 (7.87); 5-HT_7 (7.75); D_1 (7.64); D_4 (7.33); D5 (6.63); α_{1A} (8.79); α_{1B} (8.15); α_{2A} (7.43); α_{2B} (7.58); α_{2C} (8.22); M_1 (8.21); M_2 (7.44); M_3 (7.72); M_4 (7.81); M_5 (7.81); H_1(8.95); H_2 (6.82); H_4 (6.18)
Risperidone	8.21	8.16	6.75	9.69	5-HT_{1B} (7.83); 5-HT_{1D} (7.07); 5-HT_{2B} (7.8); 5-HT_{2C} (8.17); 5-HT_7 (8.18); D_4 (8.21); D_5 (7.8); α_{1A} (8.3); α_{1B} (8.04); α_{2A} (7.78); α_{2C} (8.89); H_1 (7.7)
Olanzapine	7.67	7.46	5.82	8.88	5-HT_{2B} (8.41); 5-HT_{2C} (8.41); 5-HT_3 (6.69); 5-HT_6 (8.09); 5-HT_7 (6.98); D_4 (7.75); D_5 (7.04); α_{1A} (6.95); α_{1B} (6.58); α_{2A} (6.5); α_{2B} (7.09); α_{2C} (7.54); M_1 (7.58); M_2 (7.2); M_3 (7.28); M_4 (7.61); M_5 (8.12); H_1 (8.66); H_2 (7.36)
Ziprasidone	8.09	8.35	9.01	9.51	5-HT_{1B} (8.4); 5-HT_{1D} (8.64); 5-HT_{2B} (9.08); 5-HT_{2C} (9.01); 5-HT_6 (7.21); 5-HT_7 (8.22); D_1 (8.45); D_4 (7.33); α_{1A} (7.74); H_1 (7.2); SERT (7.26); NET (7.32)
Quetiapine	6.38	6.41	6.78	6.81	5-HT_{2B} (7.33); 5-HT_{2C} (5.98); 5-HT_6 (6.02); 5-HT_7 (6.51); D_1 (6.71); D_4 (5.85); D_5 (7.8); α_{1A} (7.66); α_{1B} (7.84); α_{2A} (5.44); α_{2C} (7.65); H_1 (8.16); H_2 (7.38); M_1 (489); M_3 (5.79)
Brexpiprazole	9.48	8.9	9.21	9.67	α_{1A} (8.42); α_{1B} (9.23); α_{1D} (8.58); α_{2C} (9.77); 5-HT_{2B} (8.72); 5-HT_7 (8.43)
Aripiprazole	8.9	8.85	8.57	8.02	5-HT_{2B} (9.59)
Cariprazine	9.31	10.07	8.59	7.73	5-HT_{2B} (9.24)
Amisulpiride	8.89	8.62	5.8	5.08	5-HT_{2B} (7.89); 5-HT7 (7.94)
Blonaserin	9.84	9.3	6.09	9.09	

*The pK_i values shown are taken from several sources, including mean values from the PDSP K_i database (Roth and Driscol, 2016). The values for the receptors: D_2, D_3, 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} with atypical antipsychotics were from Leggio et al. (2016) or Citrome (2015). The colors indicate: Blue, drugs with higher affinity for D_2 receptors; Orange, drugs with a 5-HT/DA binding profile; Violet, drugs with a D2/3 effects; Green, drugs with partial agonist activities at D_2 receptors. 5-HT_{2B} receptors are a recurrent target of several atypical antipsychotic drugs. Conversely, none of the agents presented above bind at 5-HT_4 receptors. M1-M5, muscarinic receptors; σ1, sigma 1 receptors. *in the range of 1 order of magnitude compared to the affinity at D_2 receptors.*

Antidepressant/anxiolytic drugs

Like antipsychotic drugs, the serendipitous discovery of antidepressant compounds led to an extensive characterization of their mechanism of action. Both monoamine oxidase inhibitors (iproniazid) or monoamine uptake blockers (imipramine) lead to an increase in monoamine transmission. This progressively evolved towards the selective blockade of 5-HT reuptake sites. Escitalopram is probably the most selective compounds targeting the serotonin reuptake transporter (SERT), and often used as the first line treatment of major depressive disorders or generalized anxiety. Other newer compounds (vortioxetine) or older compounds (sertraline, paroxetine) have similar issues: the onset of efficacy can be reached only after 2 or 3 weeks, there is a

large percentage of patients resistant to treatment, and side effects such as sexual dysfunction, emotional blunting can limit their use (Sharp & Collins, 2023).

SSRIs first induces a desensitization of 5-HT autoreceptors. After some days, the auto-inhibitory break being less effective, extracellular levels of 5-HT are increased, acting more on post-synaptic receptors. The latter steps are less known and include desensitization of some (5-HT_{2A}R, 5-HT_{2C}R), but not all (5-HT_{1A}R), 5-HTRs at least in the hippocampus and the cortex (Sharp & Collins, 2023). This remodelling of monoaminergic transmission is associated with the increase in BDNF (Ampuero et al., 2024). Thus, the mechanism of action of SSRIs corresponds to several steps possibly culminating in enhanced neuroplasticity. There are also atypical antidepressant drugs like mirtazapine, mianserin or more recently, agomelatine, which block 5-HT_2Rs, indirectly suggesting that reduced tone at 5-HT_2Rs could achieve antidepressant effects (Millan, 2006). Nonetheless, the pharmacological profile of these agents is much more complex than a blockade of 5-HT_2Rs.

In this context, it is difficult to extrapolate on the mechanism of action of psychedelics which might correspond to their therapeutic effects. Additionally, the neural mechanisms underlying depression or general anxiety are numerous although sensitive to monoaminergic intervention. Despite clear monoaminergic disturbances in patients with Alzheimer's disease, addiction, and epilepsy, no approved monoamine-based treatments are yet available (Di Giovanni et al., 2016). The causal implication of monoaminergic disturbance in any given pathology is a classical shortcoming shared by the scientific and medical communities. Ultimately, we still don't exactly understand the function of monoamine systems.

Monoamine system anatomy: functional considerations

In this part, the monoaminergic systems will be briefly described, and some functional or pathophysiological facts will be mentioned. In general, monoamine systems operate through volume transmission and strike a balance between neuron-like and gland-like functions. Monoaminergic neurons have extensive axonal networks projecting throughout the CNS with *en-passant* synapses along them, facilitating the regulation of dynamic regional neurochemical environments. Matsuda et al. labelled a single DA neuron from the substantia nigra projecting to the striatum, with axons crossing

different territories of the striatum to innervate different functional sub-regions (Matsuda et al., 2009). The equivalent for 5-HT neurons has been published some years ago a single neuron from the DRN being able to innervate several brain regions (Ren et al., 2019).

Serotonin (5HT)

Almost all parts of the brain receive 5-HT innervation arising from cell bodies located in the medial and dorsal raphe nucleus (MRN, DRN) (Azmitia & Segal, 1978). The DRN nucleus contains 8,000 to 91,000 5-HT neurons in mice and humans, respectively. It represents 30-56% of 5-HT neurons in the CNS depending on the species (Jacobs & Azmitia, 1992). Some 5-HT neurons may co-express and release other neurotransmitters such as GABA, glutamate and nitric oxide and neuropeptides such as corticotropin-releasing factor or substance P (De Deurwaerdère & Di Giovanni, 2017; Jacobs & Azmitia, 1992; Ren et al., 2019).

Based on their anatomy and functional topography, six parts of the DRN have been described (Hale & Lowry, 2011). The ventral part of the DRN innervates the sensorimotor cortex and the caudate putamen. The dorsal part of the DRN innervates the medial prefrontal cortex, the central and basolateral nuclei of amygdala, and the dorsal hypothalamic area (Lowry et al., 2008). The lateral part projects mainly to subcortical regions including superior colliculus or the lateral hypothalamus. The rostral parts project to the caudate putamen and the SN, whilst the caudal part sends projections to the amygdala, ventral hippocampus and thalamic nuclei. In mice, the 5-HT neurons innervating adjacent territories such as prelimbic and infralimbic cortices or substantia nigra pars compacta (SNpc) and reticulata (SNpr) are distinct from an ontogenetic point of view (De Deurwaerdère & Di Giovanni, 2017). The DRN also receives several afferences from the lateral habenula, the lateral dorsal and posterior hypothalamic nuclei, bed nucleus of the stria terminalis, amygdala, cingulate cortex, and prefrontal cortex. In central and caudal levels, the DRN receives projections from the substantia nigra pars reticulata. These projections are organized and show synaptic specialization (Valentino et al., 2001).

The MRN contains less 5-HT cell bodies, comprising approximately 5% of the neurons in the nucleus that project in hippocampus. Although the MRN innervates several brain regions, some of the projections from this nucleus are presumably not releasing 5-HT including the basal ganglia (De Deurwaerdère & Di Giovanni, 2017; Jacobs & Azmitia, 1992). The fact that projections of 5-HT neurons from both the DRN and MRN reach same brain regions such as the ventral hippocampus or the amygdala has always been puzzling. The prefrontal cortex or the basal ganglia are selectively innervated by the DRN, whereas the dorsal hippocampus and the septum are mainly innervated by the MRN. Nonetheless, other 5-HT projections from pontic raphe nuclei could also send 5-HT projections, notably to the medial septum (Köhler et al., 1982).

Due to its widespread distribution in the brain, 5-HT is associated with virtually all CNS functions, including thermoregulation, feeding, sexual behaviour, cognition, awareness, sleep-wake cycles, arousal, vigilance, and motor behaviour (Bacqué-Cazenave et al., 2020; Jacobs & Azmitia, 1992; Soubrié, 1986) .

Dopamine (DA)

DA neurons are a heterogeneous group of cells that are localized in the mesencephalic, diencephalic and the olfactory bulb regions of the brain. Most DA cells reside in the ventral part of the mesencephalon. The total neuronal population of DA neurons in the brain accounts for less than 1%, ranging from 40,000 in rats and 600,000 in humans (Björklund & Dunnett, 2007). The major DAergic pathways in terms of fibre quantity are the nigrostriatal and the mesolimbic pathways. They originate from mesodiencephalic DA neurons which correspond to A8, A9 and A10 group of cells. The nigrostriatal pathway includes DA neurons originating from the more lateral SNpc (A9 cell group) and projects its fibers into the striatum (caudate-putamen nucleus) and are the most studied due to their involvement in the pathogenesis of Parkinson's disease (Grace & Bunney, 1985). Some DA cell bodies of the nigrostriatal tract are also present in the caudal, lateral part of the SNpr (Matsuda et al., 2009). The mesolimbic and the mesocortical pathways comprise of DA neurons originating from VTA (A10 cell group) and extend towards structures closely associated with the limbic system and different cortices (Björklund & Dunnett, 2007; White, 1996). The mesocorticolimbic DA systems appear critically involved in the modulation of emotion-related behaviour and cognitive function (Le Moal & Simon, 1991). Drugs of abuse,

and natural reward stimuli such as sexual stimuli and feeding behaviours exert their effects through actions in the mesolimbic system and DA contributes at least partially to their reinforcing effects (Di Chiara & Imperato, 1988; Koob, 1992). The mesocortical system projection to the medial prefrontal cortex (mPFC) is generally associated with cognitive functions including working memory, planning and execution of behaviour, inhibitory response control and maintenance of focused attention (Le Moal & Simon, 1991).

Mesencephalic DA neurons have been shown to respond to exploration, novelty, reward stimuli and are involved in the prediction error of rewarding stimuli. This property concerns VTA and SNpc DA neurons (Düzel et al., 2010; Lisman & Grace, 2005; Schultz, 1998, 2000; Schultz et al., 1993). Also, within the VTA, electrophysiological recordings in both non-human primates and rodents showed that both reward and aversive stimuli can excite two different subpopulations of VTA DA neurons (Brischoux et al., 2009; Bromberg-Martin et al., 2010; Eddine et al., 2015; Matsumoto & Hikosaka, 2009; Schultz, 2010; Ungless et al., 2010). Additionally, there is anatomical evidence that the shell of the NAc is indirectly connected to the dorsolateral striatum via striatonigral-nigrostriatal loops (Haber et al., 2000).

DA are also located in the hypothalamus (A12 nucleus), involved in neuroendocrine regulation through their projections to the pituitary gland, forming the tuberoinfundibular pathway. Additionally, some DA neurons have been reported in the DRN (De Deurwaerdère & Di Giovanni, 2017). There are also some less defined DA neurons in the A11 nucleus, responsible for DA innervation of the spinal cord (Sharples et al., 2014).

Noradrenaline (NA)

The adrenergic cell bodies are dispersed across 7 distinct nuclei (A1 to A7) in the brainstem and innervate in almost all regions of the brain. The NA system corresponds mainly to the locus coeruleus (LC; A6). A1, A5 and A7 clusters are responsible for some ascending and ventral NA projections to the septum, the bed nucleus of the stria terminalis, or the hypothalamus. The LC, in which 50% of neurons are adrenergic, is present in all mammalian species and is located dorsally in the pons. It is mainly responsible for the adrenergic innervation of the telencephalon and represents the main source of NA within CNS (Aston-Jones, 2004; Aston-Jones & Cohen, 2005). The

widespread network of LC-derived adrenergic projections exerts a powerful modulatory influence on cortical and sub-cortical circuit. The adrenergic innervation of the striatum and globus pallidus is very sparse (Aston-Jones, 2004; Fitoussi et al., 2013).

The LC NA system plays a central role in modulating arousal, attention, vigilance, and stress responses. Recent findings suggest it also dynamically regulates behaviour through phasic and tonic activity modes, balancing focused task performance (exploitation) and disengagement to explore alternative behaviours (exploration) (Aston-Jones & Cohen, 2005).

Metabolism of monoamines

In this section, the metabolism of monoamines is briefly described because some of the reported metabolic compounds were monitored in our conditions. Figure 7 reports a general organization of the anabolic and catabolic pathways of monoaminergic neurons. The metabolism involves both monoaminergic neurons and cells (neurons and/or glial cells) at vicinity. Catecholamines DA and NA share the initial involvement of cytosolic tyrosine hydroxylase (TH) in their biosynthesis to give endogenous L-DOPA. L-DOPA is decarboxylated into DA by aromatic amino acid decarboxylase (AADC) in the cytosol of catecholaminergic neurons. DA is entering the vesicles of exocytosis via the vesicular monoamine transporter type 2 (VMAT2) thanks to the acidic gradient generated by an ATPase pump (Justice, 1993). The pH of the vesicles being acidic, it also protects catecholamines from auto-oxidation (Black et al., 2021). In NA neurons, the cytosolic synthesis of NA would be minimal via cytosolic, soluble DA-β-hydroxylase (DBH) (Lewis & Asnani, 1992). The synthesis of NA mainly occurs in the vesicles of exocytosis themselves due to the presence of DBH in higher quantity compared to the cytosol and the presence of DA. Thus, NA neurons could release DA as exemplified in numerous in vivo studies (Devoto et al., 2005, 2014, 2019; Kempadoo et al., 2016). The synthesis of 5-HT occurs from the mono-oxygenation of tryptophan into 5-HTP by tryptophan hydroxylase 2 (TPH2) and its decarboxylation into 5-HT by AADC, all steps occurring in the cytosol. 5-HT is then transported into the vesicles of exocytosis via VMAT2. The anabolic pathways of monoamines show that monoaminergic neurons do not have strong biochemical identity because they share the AADC and VMAT2 steps. Meanwhile, as previously evoked, the exogenous administration of the precursors 5-

HTP or L-DOPA, which are aromatic amino acids virtually entering CNS and all cells via the transporter of aromatic amino acid LAT1, promotes synthesis and release of 5-HT and DA from catecholaminergic and 5-HT neurons, respectively (De Deurwaerdère et al., 2017; Navailles et al., 2010; Stamford et al., 1990).

The degradation of biogenic amines is a complex mechanism because it involves several enzymes and cell types. The MAO exists in two isoforms: MAO-A and MAO-B, which catalyses the oxidative deamination and produce hydrogen peroxide, ammonia and the corresponding aldehyde. The catecholaminergic neurons mainly contain MAO-A, whereas serotonergic neurons are rich in MAO-B. DA, NA and 5-HT can be degraded by MAO-A (Di Giovanni et al., 2016).

The other enzyme catechol-O-methyltransferase (COMT) can also metabolize DA and NA (Eisenhofer et al., 2004). COMT is not present in DA terminals (Schendzielorz et al., 2013), implying that glial cells or other neurons reuptake extracellular DA in tissues where the clearance of DA is low. COMT is also involved in the degradation of metabolites generated by MAO activities. The final product from these two distinct catabolic pathways is homovanillic acid (HVA) for DA metabolism and either vanillyl mandelic acid (VMA) for NA metabolism (Di Giovanni et al., 2016; Eisenhofer et al., 2004).

Hence, DA is metabolized to 3,4-dihydroxyphenylacetic acid (DOPAC) by the activity of MAO-A and aldehyde dehydrogenase enzymes which is further converted to HVA by COMT. Likewise, NA is metabolized to VMA by the activity of MAO-A and COMT. The 5-HT is metabolized to 5-hydroxyindole acetic acid (5-HIAA) by MAO-A and aldehyde dehydrogenase.

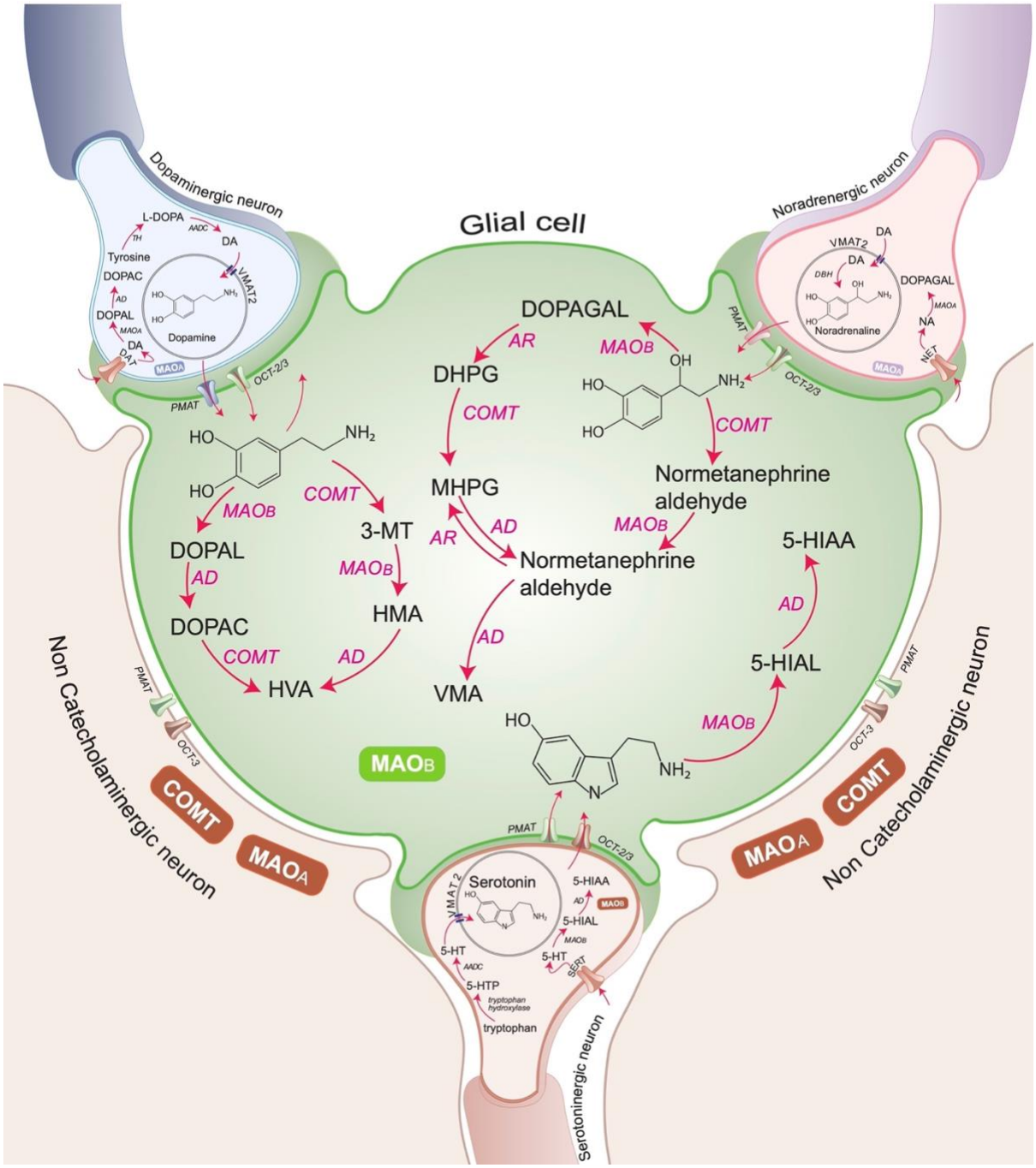

Figure 7 - Schematic of the catabolism and metabolism of monoamines.

Metabolic pathways of monoaminergic neurons. This drawing represents terminals of dopamine (DA) (top left), noradrenaline (NA) (top right) and serotonin (5-HT) neurons surrounding a glial cell and non-catecholaminergic neurons. L-amino acid decarboxylase (AADC); L-3,4-dihydroxyphenylalanine (L-DOPA) tyrosine hydroxylase (TH); Monoamine oxidase A and B (MAOA, MAOB); 3,4-Dihydroxyphenylacetaldehyde (DOPAL); Aldehyde dehydrogenase and reductase (AD, AR); 3,4-Dihydroxyphenylacetic acid (DOPAC); Catechol-o-methyltransferase (COMT), 3-Methoxytyramine (3-MT); homovanillic acid (HVA); dopamine beta-hydroxylase (DBH). 3,4-methoxyphenylacetaldehyde, (DOPEGAL); 3,4-

dihydroxyphenylglycol (DHPG); 3-Methoxy-4-hydroxyphenylglycol (MHPG); vanillylmandelic acid (VMA); 5-hydroxytryptophan (5-HTP); 5-hydroxyindole acetaldehyde (5-HIAL); 5-hydroxyindoleacetic acid (5-HIAA); vesicular monoamine transporter 2 (VMAT2); DA transporter (DAT); 5-HT transporter (SERT); NA transporter (NET); plasma membrane monoamine transporter (PMAT); organic cation transporter 2 and 3 (OCT-2, OCT-3). (VMAT2); DA transporter (DAT); 5-HT transporter (SERT); NA transporter (NET); plasma membrane monoamine transporter (PMAT); organic cation transporter 2 and 3 (OCT-2, OCT-3). (Butler et al., *in preparation a*).

Psychedelics' action on neuromodulation

Monoamines

The effect of psychedelics on monoaminergic systems is discussed thoroughly in *Publication Art I:* Classical psychedelics' action on brain monoaminergic systems (Butler, Ricci, et al., 2024). We will briefly mention some points to understand what the difficulties are to appreciate the impact of psychedelics on monoamines in the brain.

There are several ways to study monoaminergic systems. Techniques favouring temporal resolution include electrophysiology and intracerebral microdialysis. Whereas post-mortem tissue quantity measures offer a static measure of monoaminergic parameters. Monoamine tissue contents were, and are, sometimes used to extrapolate the electrical activity of these neurons or their neurotransmitter release. Nevertheless, the link between the electrical activity, neurotransmitter release, and the tissue measurements remains enigmatic. This is likely because monoaminergic neurons are subject to intricate regulatory mechanisms, both at their cell bodies and their terminals, involving homologous (self-regulating) and heterologous (regulated by other systems) controls. Such complex regulation contextualizes the anatomy of these large monoaminergic neurons.

The study of psychedelics on monoaminergic neurons is not clear, even for simple answers like the electrical activity of 5-HT, DA, and NA neurons. The initial belief that psychedelics inhibit the activity of 5-HT neurons is not reported for all psychedelics, including psilocybin and mescaline (see part on electrophysiology). It could be linked to the ability, or not, of the psychedelic to stimulate 5-HT_{1A}R like LSD or 5-MeO-DMT. However, DOI is also able to inhibit the activity of 5-HT DRN neurons, while increasing the release of 5-HT in the cortex. This effect was not blocked by ketanserin or ritanserin in one study (Garratt et al., 1991) and blocked by MDL-100,907 administered

at the excessive dose of 0.5 mg/kg in another study (Martin-Ruiz et al., 2001). Less is known for NA neurons as it is more challenging to monitor NA extracellular levels, and to record the electrical activity of LC neurons. Regarding DA, numerous studies have contributed to the influence of 5-HTR in the control of DA neuron activity. Figure 8 summarises the established modulation of 5-HT_{1A}R, 5-HT_{2A}R, 5-HT_{2B}R, and 5-HT_{2C}Rs on monoaminergic systems.

These receptors, often targets of 5-HT psychedelics, can exert opposite effects, for instance the action of 5-HT_{2A} and 5-HT_{2C}Rs on DA in the PFC can exert an excitatory and inhibitory response respectively. The second major consideration is that effects are region-dependent, cortical effects sometimes opposite to the subcortical effects via long loops and local regulation. Third, each receptor acts preferentially on different regulatory mechanisms. In the case of DA neurons, the 5-HT_{2C}R primarily acts on the electrical activity, while for 5-HT_{2A}R primarily acts on synthesis. The last point to be made here is that these effects are often state dependant, the consequences of 5-HT_{2A}R antagonism alone on DA is not apparent at rest, although supressed amphetamine-induced increased Dab (Alex & Pehek, 2007; De Deurwaerdère et al., 2021; De Deurwaerdère & Di Giovanni, 2017).

Acknowledging that most of these data have been obtained with selective compounds, often antagonists under diverse conditions, it is difficult to predict what the effect would be of a psychedelic with variable affinity (and perhaps biased-agonism) on each of these four receptor subtypes. DOI (5-HT_2R agonist) can behave as a 5-HT_{2A}R agonist in the presence of amphetamine to boost amphetamine-induced DA release (Ichikawa & Meltzer, 1995), or as a 5-HT_{2C}R agonist in the presence of morphine, to suppress morphine-induced DA release (Willins & Meltzer, 1998).

Reviewing this literature, there is no characteristic pharmacological response of monoamine systems to the diverse group of classical serotonergic psychedelics. Since the data are often limited to specific brain regions, there might be some brain regions in which the responses is clearer; however it cannot be addressed with current in vivo approaches.

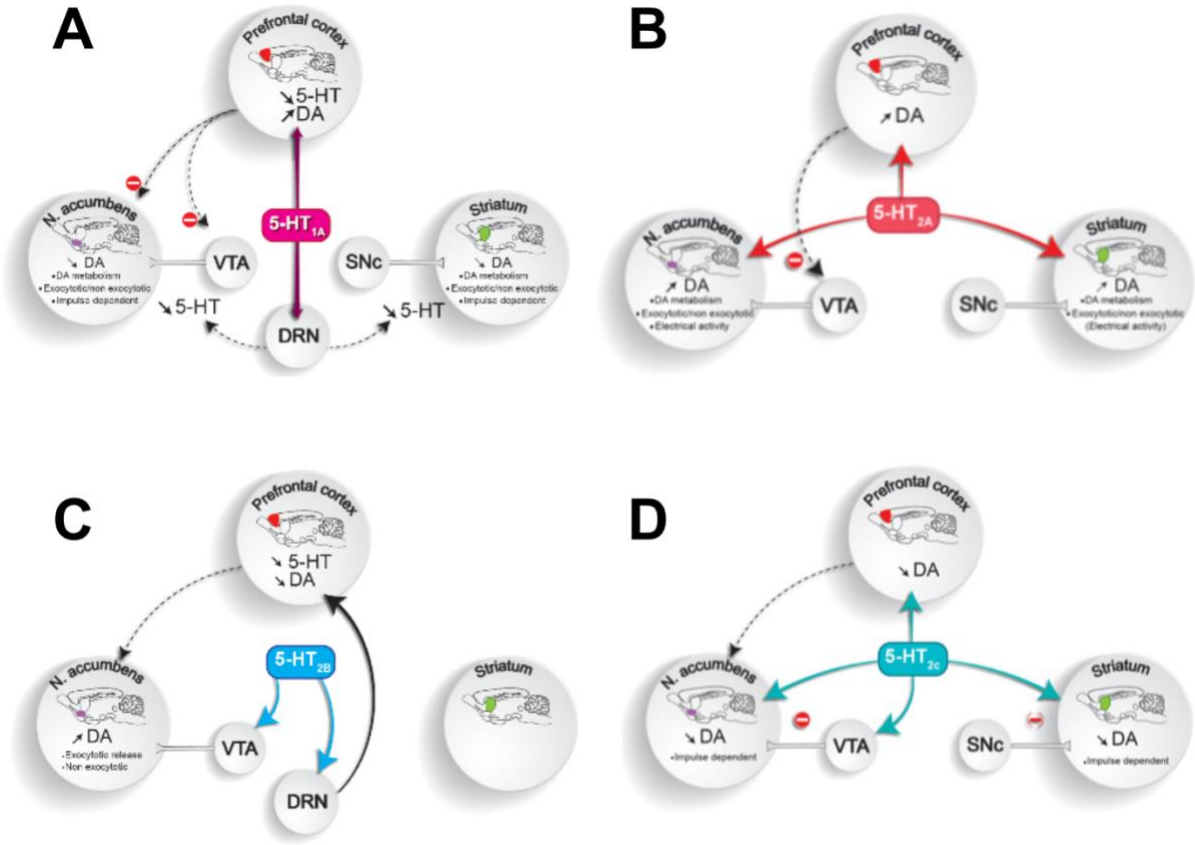

Figure 8 - Summary of 5-HTR actions on monoamine release.

Schematic of the actions of $5HT_{1A}R$, $5HT_{2A}R$, $5HT_{2B}R$ and $5HT_{2C}R$ on cortical, accumbal and striatal 5-HT, DA and NA release as inferred by microdialysis and electrophysiology studies in rodents, figure adapted from (De Deurwaerdère et al., 2021).

Executive neurotransmitters

Executive neurotransmitters, namely glutamate and GABA are also clearly implicated in the psychedelic mechanism of action. Indirect evidence includes the location of 5-HT_{2A}R on glutamatergic and GABAergic neurons (*see Brain localisation*), glutamatergic PNs and GABAergic INs in the cortex activated by psychedelics (*see Electrophysiology*), and the involvement of mGluR in psychedelic behaviour, electrophysiological, and neurochemical responses.

It has been recurrently shown that psychedelics enhance cortical glutamate (GLU). *In vivo* microdialysis in the rat somatosensory cortex showed increase GLU following both systemic and local DOI administration (Scruggs et al., 2003).The local action of DOI in the somatosensory cortex was blocked by 5-HT_{2A}R antagonist MDL-100,907. Neurotransmitters GABA and GLY were not increased by DOI locally or systemically. In the PFC both local and systemic LSD increased extracellular GLU, the latter also blocked by MDL-100,907 (Muschamp et al., 2004). DOM and 25I-MBOMe also increased PFC extracellular GLU, 25I-MBOMe shown to be 5-HT_{2A}R-dependent (Herian et al., 2019; Muschamp et al., 2004). Wojtaz et al. found increased PFC extracellular GLU and GABA following systemic administration of psilocybin (Wojtas et al., 2022, 2024). Beyond a clear role of cortical GLU, likely 5-HT_{2A}R-dependent, the data surrounding the effect of psychedelics on amino acid neurotransmitters is variable and somewhat sparce subcortically.

Psychedelics desynchronise brain connectivity and alter oscillations, suggesting an alteration in network activity (*see Neuroimaging to "Connectivity"*) – an effect that is likely accompanied by altered excitatory and inhibitory neurotransmission. Monoaminergic systems by their diffuse and widespread action could also play a major role in modulating executive neurotransmission. Additionally, to direct action of psychedelic on executive systems of neurotransmission, such as 5-HT_{2A}R on glutaminergic and GABAergic neurons, psychedelic action on brain wide monoaminergic systems would modulate excitatory and inhibitory tone.

Lastly, we should consider the metabolism of GLU and GABA, briefly summarised in Figure 9. Several of these metabolites, most notably aspartate, also act as neurotransmitters, however they are rarely considered when assessing psychedelic

action. Beyond microdialysis neurochemical quantification can also be done in postmortem tissue, which encapsulates not only extracellular levels (negligible) but also vesicular, cytoplasmic, and circulating amino acids.

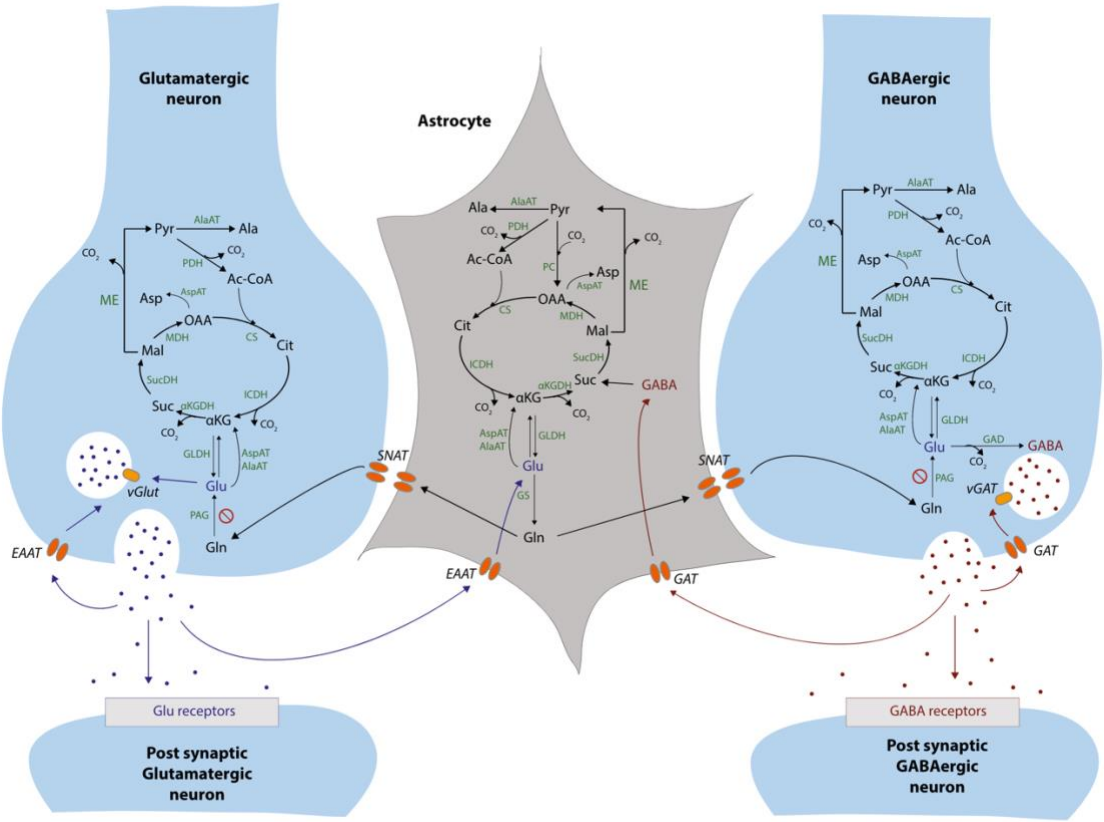

Figure 9 - Scheme of excitatory glutamate and inhibitory GABA metabolism in the brain.

Key enzymes include phosphate-activated glutaminase (PAG), glutamine synthetase (GS), glutamate dehydrogenase (GLDH), alanine aminotransferase (AlaAT), aspartate aminotransferase (AspAT), glutamic acid decarboxylase (GAD), gamma-aminobutyric acid aminotransferase (GABA-T), a-ketoglutarate dehydrogenase (aKGDH), succinate dehydrogenase (SucDH), malate dehydrogenase (MDH), malic enzyme (ME), pyruvate dehydrogenase (PDH), pyruvate carboxylase (PC), citrate synthase (CS), and isocitrate dehydrogenase (ICDH). Metabolites include glutamine (Gln), glutamate (Glu), gamma-aminobutyric acid (GABA), a-ketoglutarate (aKG), succinate (Suc), malate (Mal), oxaloacetate (OAA), citrate (Cit), aspartate (Asp), pyruvate (Pyr), alanine (Ala), and acetyl coenzyme A (Ac-CoA). The tricarboxylic acid (TCA) cycle plays a central role in coordinating these metabolic pathways. (Kosten et al., 2019)

Neurochemicals to neuroconectome

Correlative organisation of neurotransmission

Classically neurochemistry has consisted of quantitative methods calibrated against external standards such as high-pressure liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), quantitative autoradiography, or the use of radioactive tracers (in conjunction with PET, although this presents other challenges). These methods favour precision, scalability, and capacity for statistical validation compared to semi-quantitative methods such as fluorescence imaging, c-Fos expression, BOLD-fMRI, or EEG (Leopold et al., 2019).

A primary challenge in neurochemistry is temporo-spatial resolution, invasive techniques, such as microdialysis, move toward improved temporal resolution at the expense of spatial resolution. The insertion of a probe damages local environments limiting their insertion into multiple regions. On the other side of the spectrum we can prioritise spatial resolution working postmortem, i.e. at a single timepoint across multiple regions limited by dissection ability and measurement sensitivity.

Whatever the method data will inevitably have variability. From where this variability comes is of particular interest for the development of a correlative analysis. We cannot tell if such variability is methodological: systematic errors, equipment variability and human error, although great caution is taken to avoid this, or physiological variability. Methodological variability, or ‘noise’, could result in the loss of physiologically meaningful correlations and the gain of meaningless noise correlations, the difference between the two being near-impossible to distinguish after the fact. Shelving the views of reductive science for but a moment we may recognise that the brain is a complex system with emergent properties for which a nonlinear approach may be considered. For instance, reductionist science would suggest that variability would diminish the more precise and reduced the experiment from organism to sub-cellular compartment. However, variability between increasingly reduced disciplines had no difference in variability, favouring a scientific viewpoint of complexity where variability is intrinsic to the component parts of the system (Sapolsky & Balt, 1996).

The analysis of this thesis was done on quantitative data, which notably even in 1996, was less frequent in more reductive disciplines (Figure 10). Today, correlation of non-quantitative data is common practice within neuroscience, particularly for methods like fMRI, EEG, and MEG, where connectivity patterns, activation networks, or signal coherence are used to infer functional relationships between brain regions. We posit that such an analysis would be particularly meaningful when done with quantitative data, leveraging the variability of a chaotic system to understand something beyond the addition of its constituent parts.

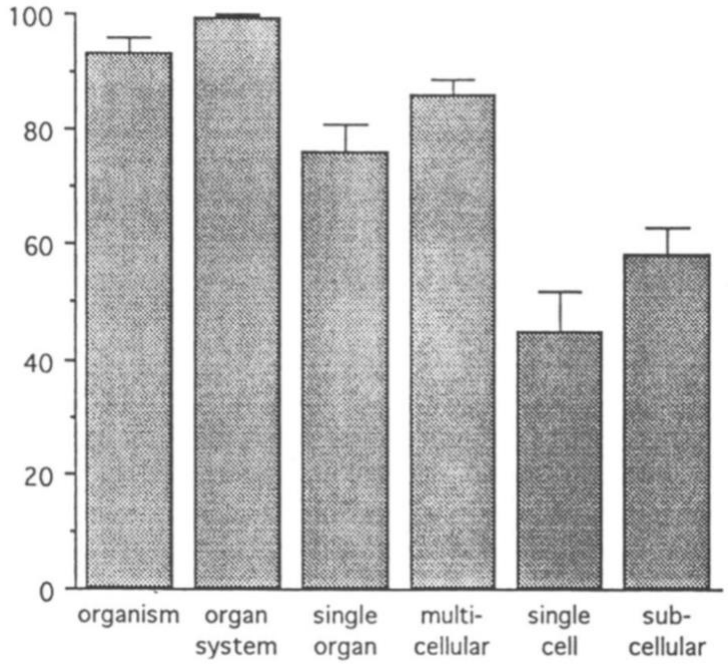

Figure 10 - Percentage of quantitative data as a function of reductive levels of techniques.

(Sapolsky & Balt, 1996)

The monoaminergic systems exhibit biological responses through their interaction across the brain (Fitoussi et al., 2013; Hensler et al., 2013). Knowledge of the biological functions and complex interaction between these systems is rather limited. Qualitative analysis is designed to address changes in the pattern of correlations within and between neurotransmitters systems, expanding upon the current regional specificity studied and adding a dimension of relativity between regions. Correlation between postmortem neurochemical tissue content is a technique that has been established in the lab, taking great care to minimise methodological variability (Dellu-Hagedorn et al., 2017). The meaning of these correlations is not firmly established.

Several reasons could be evoked to account for the lack of a consistent correlative patterns in controls, including the species, the age, the handling or training in distinct behavioural experiments, the number of animals/per group, the chromatographic analysis, and the size and location of the punch (Chagraoui et al., 2019; Dellu-Hagedorn et al., 2017; Puginier et al., 2019). It thus requires careful experimental design, such as analysis by region following brain region dissection to avoid unidirectional noise induced by differing HPLC conditions.

A correlative approach can provide additional insights into the organization of neurotransmitter systems, offering more than just differences in mean ± standard deviation (SD). Correlation between monoamine tissue content in various regions has been shown to be altered between genetic lines, pharmacological treatments, and conditions (Bharatiya et al., 2020; Marty et al., 2023; Midzyanovskaya et al., 2020; Mlost et al., 2024). For instance, the total number of correlations between DA tissue content has been shown to be reduced after 5-HT_{2C}R agonists (Figure 11). These subtle changes (number, and pattern or regional-organisation of correlations) occurred at the lowest dose of 5-HT_{2C}R agonists injection. At this low dose, both 5-HT_{2C}R agonists induced behavioural effects without altering DA extracellular levels in the striatum and the NAc as measured by intracerebral microdialysis (De Deurwaerdère, Ramos, et al., 2020; Lagière et al., 2018). This parameter of correlation confers an additional aspect on monoaminergic transmissions, beyond quantitative modulation, and might be extremely sensitive way in which to address changes in monoaminergic transmission.

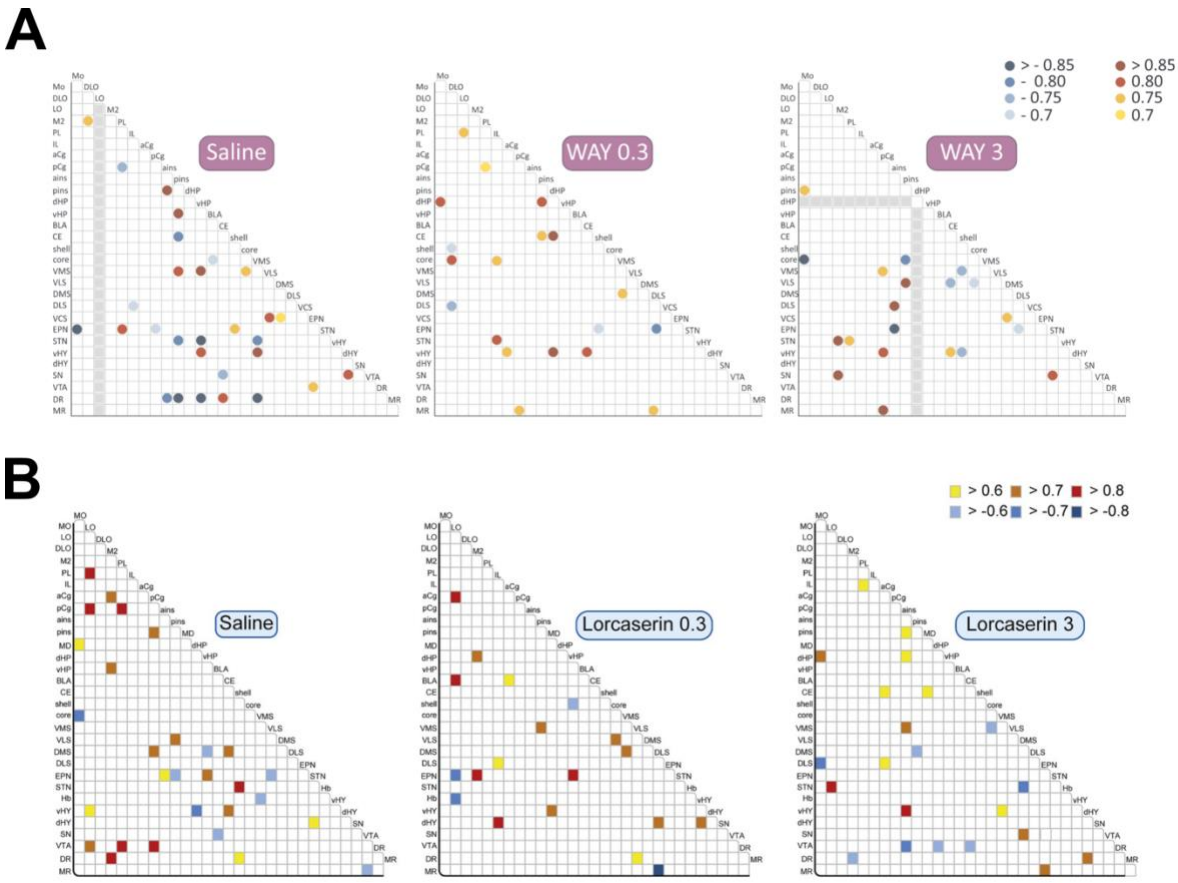

Figure 11 - Neurochemical connectivity in resting conditions modified 5HT$_{2C}$R agonists.

Total number of correlations between DA tissue content is reduced after 5-HT$_{2C}$R agonists, **A** WAY-163,909 (Chagraoui et al., 2019) and **B** Lorcaserin (De Deurwaerdère, Ramos, et al., 2020). *Brain region abbreviations are as follows: aCg, anterior cingulate cortex; ains, anterior insular cortex; BLA, basolateral amygdala; CE, central amygdala; dHP, dorsal hippocampus; dHY, dorsal hypothalamus; DLS, dorsolateral striatum; DMS, dorsomedial striatum; DLO, dorsolateral orbitofrontal cortex; MD, mediodorsal nucleus of the thalamus; DR, dorsal raphe nucleus; EPN, entopeduncular nucleus; Hb, habenula; IL, infralimbic cortex; LO, lateral orbitofrontal cortex; M2, motor cortex; MO/Mo, medial orbitofrontal cortex; MR, median raphe nucleus; NAc, nucleus accumbens; core, core of the nucleus accumbens; shell, shell of the nucleus accumbens; pCg, posterior cingulate cortex; pins, posterior insular cortex; pIns, posterior insular cortex; PL, prelimbic cortex; SN, substantia nigra; SNc, nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; vHP, ventral hippocampus; vHY, ventral hypothalamus; VLS, ventrolateral striatum; VMS, ventromedial striatum; VTA, ventral tegmental area.*

Nonetheless, to assert that these modifications are a consequence of the pharmacological treatment we must overcome inter-individual differences between treatment-groups as a repeated measure design is not possible postmortem. Note that in these two experiments, conducted in the same laboratory in male Sprague Dawley rats, the saline-cohort had few correlations that were different between experiments.

This difference in vehicle-treated animals is quite likely due to inter-individual differences. Animals in both experiments were taken from their home cage 45 min after IP injection for sacrifice. In this environment, each animal would have a unique internal state despite the consistent environment. Some engaged in feeding, others in grooming or resting, each state potentially impacting the neurochemical state of the animal immediately before sacrifice and consequent neurochemical quantification. Considering this measure of neurochemical connectivity as an emergent property of organism-environment interactions, we may be able to orchestrate the environment to induce specific brain states. This could mobilize monoaminergic systems, engaging attention, motor control, and arousal, and help us determine whether what we are measuring represents noise or an emergent system of neurochemical networks.

Aim of the Thesis

The aim of the study was to address the hypothesis that psychedelics alter the connectivity of neurotransmitter systems across multiple brain regions and territories via the 5-HT_{2A}R. Included in this were requirements that led to specific developments and deliveries throughout the duration of the thesis.

Pharmacology

We selected the preferential 5-HT_{2A}R agonist TCB-2 at three doses based on previous review by Di Giovanni and De Deurwaerdère (Di Giovanni & De Deurwaerdère, 2018).

0.3 mg/kg: minimal dose that is ineffective on head twitches
3 mg/kg: most effective dose in numerous studies
10 mg/kg: high dose to complete dose response, classically used in other studies

The 5-HT_{2A}R antagonist MDL-100,907 (volinanserin) has been chosen to block 5-HT_{2A}R (Sorensen et al., 1993). It has been reported to block the head twitches induced by psychedelics at the dose of 0.04 mg/kg and 0.1 mg/kg (Halberstadt et al., 2013; Moliner et al., 2023). We increased the dose to 0.2 mg/kg to extend its blockade until 1 hour and 15 minutes post-intrapleural administration, at which point the animals were sacrificed. Doses of 0.5 mg/kg or 1 mg/kg were not considered, as at these levels, MDL-100,907 loses its selectivity for the 5-HT_{2A}R, towards the 5-HT_{2C}R, and D_2R.

Behaviour

Anticipating months or years of neurochemical analyses, it was important to determine if the HTR induced by TCB-2 was consistent with the numerous data in the literature. This conditioned the placement of the mice in transparent cages to measure the HTR at regular time intervals until 50 minutes post TCB-2 or vehicle administration. The floor was a clear Perspex.

The forced exploratory behaviour was important to conform with the idea of Adams and Geyer (1986) that psychedelics would be better studied during the exploratory behaviour, one of the few unconditioned behaviours distinctive to psychedelics (Adams & Geyer, 1986; Halberstadt & Geyer, 2018). It was also relevant for

neurochemical connectivity to determine whether a change of environment could modify the correlative maps, studied to date only in resting conditions. With minimal behavioural experience and no access to a BPM, we fabricated a flooring made up of 4 distinct colours/textures to replace the Perspex floor of the isolation chamber. From 50 minutes post TCB-2 or vehicle the Perspex flooring was changed for the novel textured flooring and the resulting forced exploration was filmed from above.

Neurochemical approach and Dissection

The electrochemical detection of monoamines was established in the lab. For this project, we were also interested in the tissue quantity of amino acid neurotransmitters including GABA and glutamate, as the 5-HT_{2A}R are expressed by these neurons cortically. Thus, we built HPLC systems capable of monitoring amino acids in tissue extracts. The method was based on the protocol proposed by Dozanti and Yamamoto and was ameliorated to additionally measure glycine (Donzanti & Yamamoto, 1988). Monoamines and amino acids were then measured in parallel for each sample.

The brain regions were chosen according to their functions and belong to cognitive, motor, sensory, mood, and vegetative systems. The number of brain regions was sufficiently elevated to perform pertinent correlative analysis to approximate neurochemical connectivity.

Analysis and code development

Building a code for handling neurochemical/behavioural data

The previous analysis pipeline for neurochemical data in the lab was not satisfactory for an analysis of this scale. It required manual handling, particularly error prone for such a large dataset, across several software's eventually culminating in the construction of a correlogram reporting significant correlations in PowerPoint.

The code in python was developed in parallel with the experimental part of the thesis. Initially we verified the results from the code were similar in terms of descriptive statistics with the classical analysis pipeline used in the lab. Following this, data and figures were all handled by the code. The implementation of the code additionally

allowed for better data handling facilitating the development of novel methods of correlative analysis most notable graph theory, the application of which is discussed at length in *Art VI: Neurochemistry and postmortem data: toward the study of neurotransmitter connectivity*.

Publications

Art I - Classical psychedelics' action on brain monoaminergic systems

Questions and purpose of the review

Psychedelics exert brain wide effects exemplified by neuroimaging studies. Psychedelic action on monoaminergic systems have been demonstrated in localized brain regions or pairs of regions. However, monoaminergic systems act as interconnected neuromodulatory systems with local and global regulation projecting from midbrain nuclei throughout the brain. This review summarized the current literature on the effects of serotonergic psychedelics on 5-HT, DA and NA systems. It integrates electrophysiological studies on the activity monoaminergic neurons in midbrain nuclei with region-specific neurochemical analyses, as measured by microdialysis or post-mortem neurochemical quantification, to determine whether there is a characteristic action of classical psychedelics on monoaminergic systems.

International Journal of Biochemistry and Cell Biology 176 (2024) 106669

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

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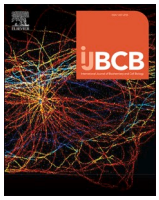

Classical psychedelics' action on brain monoaminergic systems

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ARTICLE INFO

Keywords
Serotonin
Dopamine
Noradrenaline
Psychedelics
Microdialysis
Single-cell extracellular recordings
Serotonin receptors

ABSTRACT

The study of the mechanism of action of classical psychedelics has gained significant interest due to their clinical potential in the treatment of several psychiatric conditions, including major depressive and anxiety disorders. These drugs bind 5-hydroxytryptamine receptors (5-HTR) including 5-HT_{1A}R, 5-HT_{2A}R, 5-HT_{2B}R, and/or 5-HT_{2C}R, as well as other targets. 5-HTRs regulate the activity of ascending monoaminergic neurons, a mechanism primarily involved in the action of classical antidepressant drugs, antipsychotics, and drugs of abuse. Sparse neurochemical data have been produced on the control of monoaminergic neuron activity in response to classical psychedelics. Here we review the available data in order to determine whether classical psychedelics have specific neurochemical effects on serotonergic, dopaminergic, and noradrenergic neurons. The data show that these drugs have disparate effects on each monoaminergic system, demonstrating a complex response with state-dependent and region-specific effects. For instance, several psychedelics inhibit the firing of serotonergic neurons, although this is not necessarily associated with a decrease in serotonin release in all regions. Noradrenergic neuron spontaneous activity also appears to be inhibited by psychedelics, also not necessarily associated with a decrease in noradrenaline release in all regions. Psychedelics influence on dopaminergic systems is also complex as the above-mentioned 5-HTRs may have opposing effects on dopaminergic neuron activity, in a state-dependent manner. There is an apparent lack of clear neuronal signature induced by psychedelics on monoaminergic neuron activity despite specific recurrent mechanisms. This review provides a current summary of the action of psychedelics on monoamine neuromodulators serotonin, dopamine and noradrenaline, compiling reoccurring and contradictory findings demonstrating that a monoamine signature of psychedelics, if applicable, would be state- and region-dependant.

1. Introduction

Classical psychedelics refer to psychoactive compounds with converging affinity for a subset of serotonergic receptors in addition to other possible targets. The mechanisms leading to the altered sensory experience and 'mind manifesting' effects remain elusive and are subject to debate. These drugs are still characterized based on their chemical class (e.g. ergolines, tryptamines, indolamines, phenylalkylamines), as were antipsychotic drugs some decades ago, before their mechanism of action was better understood. The interest in these compounds extends beyond their psychedelic effects as there is growing evidence of their antidepressant, anxiolytic, and anti-addictive properties, to cite a few (Bogenschutz et al., 2022; Carhart-Harris et al., 2021; Goodwin et al., 2022; Holze et al., 2024, 2023; Raison et al., 2023; Seybert et al., 2023).

Abbreviations: 5-HTR, 5-hydroxytryptamine receptors; DA, dopamine; 5-HT, serotonin, NA, noradrenaline; LSD, lysergic acid diethylamide; 25CN-NBOH, N-2-Hydroxybenzyl-2,5-Dimethoxy-4-Cyanophenylethylamine; M-CPP, meta-chlorophenylpiperazine; DRN, dorsal raphe nucleus; 5-MeO-DMT, 5-methoxy-dimethyl-tryptamine; PFC, medial prefrontal cortex; DMT, dimethyltryptamine; DOI, 2,5-dimethoxy-4-iodoamphetamine; MDL100, 907, volinanserin; $GABA_A$R, γ-amino-butyric acid A receptor; DOM, 2,5-dimethoxy-4-methylamphetamine; 25I-NBOMe, 2–4-iodo-2,5-dimethoxyphenyl-N-2-methoxybenzylethanamine; 5-HIAA, 5-hydroxyindoleacetic acid; SN, substantia nigra; VTA, ventral tegmental area; NAc, nucleus accumbens; DOB, 2,5-dimethoxy-4-bromoamphetamine; LC, locus coeruleus.

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https://doi.org/10.1016/j.biocel.2024.106669
Received 7 August 2024; Received in revised form 17 September 2024; Accepted 23 September 2024
Available online 25 September 2024
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However, the acute and long-term effects of classical psychedelics are complex. In this review, we summarize data mostly reporting on the acute effects of these compounds on central monoaminergic systems to determine whether their impact has a specific signature on neuromodulatory transmission.

Monoaminergic systems, encompassing neuronal populations synthesizing dopamine (DA), serotonin (5-HT), or noradrenaline (NA), are divergent and highly complex systems of neurotransmission. From cell bodies located in the brainstem, they innervate vast territories in the brain and spinal cord via large axonal domains harbouring numerous *en-passant* synapses. These systems are central to the mechanisms of action of antidepressants, anxiolytics, antipsychotics, and antiparkinsonian drugs, and are considered to contribute to numerous other brain afflictions (Di Giovanni et al., 2016). Knowing that psychedelic drugs bind monoaminergic receptors (Table 1), and display efficacy in multiple psychiatric diseases, it is conceivable that monoaminergic transmission participates in the mechanism of action of classical psychedelics.

The involvement of monoaminergic systems in the mechanism of action of lysergic acid diethylamide (LSD) started with evidence that LSD was a ligand of 5-HT receptors (5-HTR), which had just been described in the brain (Woolley and Shaw, 1954). Most classical psychedelics bind a subset of 5-HTR, the 5-HT_{2A}R subtype being recurrently proposed for the underlying effects of these agents. The preferential 5-HT_{2A}R agonist N-(2-Hydroxybenzyl)-2,5-Dimethoxy-4-Cyanophenylethylamine (25CN-NBOH, Hansen et al., 2014) is a psychedelic compound in humans (Nichols, 2016). However, other drugs also binding and stimulating 5-HT_{2A}R do not have clear psychedelic properties such as lisuride, meta-chlorophenylpiperazine (mCPP), quipazine or lorcaserin (Halberstadt and Geyer, 2018). These distinct abilities of 5-HT_{2A}R agonists to promote psychedelic effects could be explained through their specific influence on different intracellular signalling pathways, most of these compounds displaying biased agonist properties (Cao et al., 2022; González-Maeso et al., 2007). Additionally, this diversity within 5-HT_{2A}R agonists may be related to their differing pharmacological profiles, each with a unique combination of targets, some perhaps able to mask 'psychedelic' effects (Halberstadt and Geyer, 2018; Saraf et al., 2021). Irrespective of the inconsistent psychedelic effects triggered by 5-HT_{2A}R agonists, the 5-HT_{2A}R remains a likely substrate for these effects. 5-HT_{2A}R antagonist ketanserin, blocks the acute experiential effect in humans and head twitch response (a proxy for the hallucinogenic effects) in rodents (Becker et al., 2023; Vollenweider et al., 1998; Halberstadt, 2015; Murnane, 2018). However, other 5-HTR targets (5-HT_{2B}R, 5-HT_{2C}R, 5-HT_{1A}R and possibly 5-HT_5R and 5-HT_6R) are also known to modulate the effects of these drugs (Canal et al., 2010). Table 1 summarizes affinities of 5-HT_{2A}R agonists which are classical psychedelics and non-psychedelic drugs studied through electrophysiological and neurochemical exploration of the monoaminergic systems, as well as affinities of commonly used 5-HT_{2A}R antagonists.

2. Psychedelics and serotonergic neurons

Given their affinity for 5-HTRs, the serotonergic system has been considered the primary neurotransmitter system targeted by classical psychedelics, leading to the serotonin hypothesis of the action of hallucinogenic drugs dating back to the 1950's (Woolley and Shaw, 1954). The serotonin hypothesis posits that the 'mental disturbances' caused by LSD could be attributed to a reduction of serotonin tone in the brain. LSD has been shown to inhibit the electrical activity of serotonergic neurons for decades (Aghajanian et al., 1968), an effect occurring at the μg/kg range of doses (De Gregorio et al., 2016; Trulson and Jacobs, 1979). It has also been shown to reduce both the synthesis of 5-HT and its release (Pettibone and Pflueger, 1984). This inhibitory response is classically attributed to the activation of 5-HT_{1A} autoreceptors expressed by 5-HT neurons in the dorsal raphe nucleus (DRN). 5-methoxy-dimethyltryptamine (5-MeO-DMT) has a high affinity for the 5-HT_{1A}R and clear psychedelic effects (Table 1). 5-MeO-DMT has been reported to inhibit the activity of 5-HT neurons after peripheral injection or iontophoretic application in the DRN (Heidenreich and Rebec, 1989; Rogawski and Aghajanian, 1981; Trulson et al., 1981), and to reduce 5-HT release in the medial prefrontal cortex (PFC) after systemic injection or intra-cortical application (Riga et al., 2016). Similarly, dimethyltryptamine (DMT) and psilocin, the active metabolite of psilocybin, both reduce the activity of 5-HT neurons, presumably by presynaptic action (Trulson et al., 1981). However, psilocin did not reduce 5-HT release, rather increased 5-HT extracellular levels in the PFC (Sakashita et al., 2015). Other psychedelics like 2,5-dimethoxy-4-iodoamphetamine (DOI) also inhibit the electrical activity of 5-HT neurons and extracellular 5-HT levels in the PFC (Garratt et al., 1991). In its initial characterization, the inhibitory effect of DOI (100 μg/kg i.v.) was not blocked by the non-selective 5-HT_{2A}R antagonists ketanserin and ritanserin (Garratt et al., 1991). Ten years later, Martín-Ruiz et al., (2001) found that the inhibitory effect of 1 mg/kg DOI was blocked by a high dose (0.5 mg/kg) of the preferential 5-HT_{2A}R antagonist MDL100, 907 (volinanserin) and by the γ-aminobutyric acid A receptor ($GABA_A$R) antagonist picrotoxin (Martín-Ruiz et al., 2001).

In attempt to localise this effect, Garratt *et al.* administered DOI locally in DRN, demonstrating both reduced spontaneous DRN firing and PFC extracellular 5-HT (Garratt et al., 1991). However, *ex vivo* bath application of DOI on isolated DRN neurons did not reduce Ca^{2+} currents as LSD and 5-HT did via the 5-HT_{1A}R (Penington, 1996). Therefore, the inhibitory effect of 1 mg/kg DOI originating from the DRN could be mediated by an indirect action involving 5-HT_{2A}R expressed by GABAergic interneurons (Martín-Ruiz et al., 2001).

Regarding the PFC-DRN circuit, intra-PFC injection of DOI induced a local increase in 5-HT extracellular concentration in the PFC, accompanied by increased firing in a subset of DRN 5-HT neurons. This cortical effect of DOI was antagonised by 5-HT_{1A}R agonists and 5-HT_{2A}R antagonist (Martín-Ruiz et al., 2001; Amargós-Bosch et al., 2004; Frånberg et al., 2012). This finding suggests indirect modulation of DRN 5-HT neurons via cortical 5-$HT_{1A/2A}$R. 5-HT_{2A}R are often co-localised with 5-HT_{1A}R in the PFC. Layer 5 pyramidal DRN-projecting neurons display excitatory (5-HT_{2A}R) and inhibitory (5-HT_{1A}R) effects following DRN stimulation, projecting back to both 5-HT and GABAergic DRN neurons (Amargós-Bosch et al., 2004). This would facilitate a selective inhibitory or excitatory effect on DRN 5-HT neurons originating from the PFC.

Meanwhile, a higher systemic dose of DOI (2.5 mg/kg) did not alter the extracellular levels of 5-HT in the PFC (Gobert and Millan, 1999). This lack of effect could be speculatively related to the combination of the opposing mechanisms described above. Other phenethylamines like 2,5-dimethoxy-4-methylamphetamine (DOM) and mescaline produced mixed responses on the electrical activity of DRN 5-HT neurons (Trulson et al., 1981).

It is not known if newer benzyl-phenethylamines like 2-(4-iodo-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine (25I-NBOMe) alter the electrical activity of 5-HT neurons. It has been reported that 25I-NBOMe did not reduce (Miliano et al., 2019), but potentially increased 5-HT extracellular levels in the PFC, nucleus accumbens, and striatum as measured by intracerebral microdialysis (Herian et al., 2019). The increase in 5-HT extracellular levels induced by 25I-NBOMe could be partly consequent to a slight blockade of 5-HT reuptake sites (Zwartsen et al., 2017; see below for the dopaminergic section). Additionally, Herian et al., 2019 reported an increase in frontal cortical tissue level of both 5-hydroxyindoleacetic acid (5-HIAA; the primary metabolite of 5-HT) and 5-HT after the administration of 0.3–10 mg/kg 25I-NBOMe. The increase was proportionally higher for 5-HT compared to 5-HIAA, implying a decrease of the ratio 5-HIAA/5-HT, suggesting a reduction in 5-HT turnover. This could potentially be due to reduced electrical activity of 5-HT neurons, thereby accumulating unreleased tissue 5-HT.

Psychedelics exert pronounced effects on the serotonergic system via the activation of 5-HTRs, although they are not quite generalizable to a

Table 1
Affinities of various 5-HT ligands and hallucinogenic drugs. Ki affinities (nM) of relevant 5-HT ligands and psychedelics at serotonergic receptors 5-HT_{1A}R, 5-HT_{2A}R, 5-HT_{2B}R, 5-HT_{2C}R and receptors with <300 nM affinity. Values are from human tissue unless indicated ^{r} for rat o$^{r\ m}$ for mouse. Ki extracted from the PDSP Ki database (https://pdsp.unc.edu/databases/pdsp.php), averages are indicated (av), number of studies reported (n=), else specific reference provided. SERT: serotonin transporter; 5-HT_{1-7}: 5-HT receptor subtypes; α_{1-2}: Alpha adrenergic receptor subtypes; β_{1-2}: Beta adrenergic receptor subtypes; D2-D5: DA receptor subtypes; H1–2: histaminergic receptor subtypes; TrkB: tropomyosin receptor kinase B; σ: Sigma receptors.

	5-HT Receptor subtype				Other sites <300 nM	References
	1 A	2 A	2B	2 C		
5-HT_{2A}R agonists						
5-HT	3.32 (av)	9.84 (av)	8.71 (av)	9.09 (av)	5-HT_{1B} (av 4.3), 5-HT_{1D} (3.4), 5-HT_{1E} (av 7.5), 5-HT_{1F} (10), 5-HT_3 (269.2)^{m}, 5-HT_4 (125.9), 5-HT_{5A} (126), 5-HT_{5B} (7.8)^{r}, 5-HT_6 (av 87.3), 5-HT_7 (av 8.1), SERT (22)^{r}	From PDSP Ki Database (n=13)
TCB−2		0.73				(McLean et al., 2006)
LSD	2.29 (av)	0.73 (av)	0.98	2.91	5-HT_{1B} (3.9)^{r}, 5-HT_{1D} (14), 5-HT_{1E} (93)^{r}, 5-HT_{5A} (9)^{r}, 5-HT_{5B} (av. 3.2)^{r}, 5-HT_6 (2.3), 5-HT_7 (av. 104.5)^{r}, D1 (av 291.5), D2 (20), D3 (96), α_{2A} (12), β_1 (140)^{r}, TrkB (3.38)^{m}	From PDSP Ki Database (n=10) (Moliner et al., 2023)
DOI	2355^{r}	0.68 (av)	20.03 (av)	2.38 (av)		From PDSP Ki Database (n= 4)
DOM	>10,000 (av)	3.18 (av)	nd	25.7		From PDSP Ki Database (n= 2)
DOB	6326	0.89 (av)	35.28 (av)	6.31		From PDSP Ki Database (n=3)
Psilocybin	>10,000	13^{r}	98.7	>10,000^{r}	5-HT_{1E} (194.8)	From PDSP Ki Database (n= 1); PDSP certified Data;
Psilocin	49	25^{r}	nd	10^{r}	TrkB (6.73)^{m}	From PDSP Ki Database (n= 1) (Moliner et al., 2023)
Mescaline	4600	150^{r}	nd	>10,000		From PDSP Ki Database (n= 1)
Lisuride	0.32 (av)	1.4	1.07	6.31	5-HT_{1B} (18.6); 5-HT_{1D} (1), 5-HT_{5A} (3.1), 5-HT_6 (7.3), 5-HT_7 (6.8); α_{1A} (5.5), α_{1B} (16.6), α_{1D} (3), α_{2A} (0.1), α_{2B} (0.1); α_{2D} (0.8)^{r}, β_1 (67.6), β_2 (8), D1 (64.6), D2 (1.4), D3 (av 1), D4 (4.57), D5 (3.6), H2 (114.3)	From PDSP Ki Database (n= 8)
m-CPP	43.9	54.48(av)	30.28 (av)	13.04 (av)	5-HT_{1B} (av 251. 9), 5-HT_3 (50)^{r}, 5-HT_7 (162.5), α_{2B} (105.8), α^{2C} (123.5), SERT (av 265.1)	From PDSP Ki Database (n= 8); PDSP certified Data
Quipazine	1697	128.82	66.91 (av)	53.70	5-HT_3 (1.23)	From PDSP Ki Database (n= 4)
Ro60–0175	6 680	29.14 (av)	4.29 (av)	7.30		From PDSP Ki Database (n= 4)
Lorcaserin	700	112	174	15		(Thomsen *et al.*, 2008)
5-MeO-DMT	1.9	14^{r}	3884	538	5-HT_{1B} (74), 5-HT_{1D} (6.3), 5-HT_{5A} (276.6), 5-HT_6 (35.5), 5-HT_7 (3.9), D1 (79.5)	From PDSP Ki Database (n= 2)
DMT	450	131.50 (av)	107.6	166	5-HT_{1D} (93), 5-HT_6 (av 277.7), 5-HT_7 (87.5), α_{2B} (257.7), α_{2C} (258.6), D1 (271.1)	From PDSP Ki Database (n= 3)
25I-NBOMe	1800	0.2	nd	4.6	H1 (90)	(Rickli *et al.*, 2015)
5-HT_{2A}R antagonists						
MDL100, 907	>10 000 (av)	0.64 (av)	1 023.29	30.20	α_{2B} (270), H1 (66.7), σ (87)^{r}	From PDSP Ki Database (n= 4); PDSP certified Data
Ketanserin	5373.22 (av)	4.77 (av)	911.74 (av)	146.33 (av)	5-HT_{1D} (111), α_{2B} (199), H1 (1.79)	From PDSP Ki Database (n= 13)

characteristic psychedelic mechanism. A reduction of the activity of DRN 5-HT neurons is observed with several psychedelics (LSD, 5-MeO-DMT, DMT, psilocin, Garratt et al., 1991; Haigler and Aghajanian, 1973; Trulson et al., 1981). This reduction occurs in a subpopulation of neurons in the case of DOI and mescaline (Haigler and Aghajanian, 1973; Martín-Ruiz et al., 2001). Two mechanisms are conceivable for this reduction: first, 5-HTRs expressed by 5-HT neurons (5-HT_{1A}R, 5-HT_{1B}R, 5-HT_{1D}R, 5-HT_{2B}R, 5-HT_{6}R), and second, DRN 5-HTRs on GABAergic neurons (5-HT_{2A}R, 5-HT_{2B}R, 5-HT_{2C}R) which bind psychedelics to varying extents (Table 1, Beyeler et al., 2021; Ju et al., 2020, Cathala et al., 2019). Therefore, it is plausible that psychedelics induce opposite effects on 5-HT activity and extracellular levels at 5-HT terminal regions, such as PFC by acting on DRN and extra-DRN 5-HTRs (Fig. 1A).

Despite the involvement of 5-HTRs, the lack of a generalizable serotonergic signature of psychedelics on DRN neuron activity and serotonin release does not support the idea that the reduction of 5-HT tone plays a role in the alteration of consciousness. Furthermore, lisuride, known for its non-psychedelic effects despite similar affinities (Table 1), powerfully inhibits DRN neuronal activity (Rogawski and Aghajanian, 1979). The action of psychedelics on the activity of 5-HT neurons is likely not at the origin of their psychedelic effects. Early work in cats showed a dissociation between DRN firing and behaviour, with a decreased firing observed following 5-MeO-DMT, psilocin and LSD, not DOM or mescaline (Trulson et al., 1981). However, we cannot discard that acute psychedelic administration could have long term consequences on the activity of 5-HT neurons (Kidd et al., 1991) and participate in the therapeutic benefits of psychedelics.

3. Psychedelics and dopaminergic neurons

The absence of net subcortical effects of psychedelics on DA release in conjunction with an increase in cortical DA tone could be one of the markers of their global effect. The systemic injection of DOI increased DA release in the cortex (Gobert and Millan, 1999; Ichikawa et al., 2001). However, it did not alter the electrical activity of substantia nigra (SN) or ventral tegmental area (VTA) DA neurons, *in vivo* or *in vitro* (Di Matteo et al., 2000; Shi et al., 1995), or DA release in the striatum or nucleus accumbens (Ichikawa and Meltzer, 1995). Low concentrations of LSD (5–20 μg/kg i.v.) do not alter the activity of VTA DA neurons, however higher doses (50–120 μg/kg i.v.) decrease VTA DA firing activity, leading to a decrease in DA release attributed to several mechanisms including the direct stimulation of DA receptors (De Gregorio et al., 2016). Conversely, 25I-NBOMe has been shown to enhance DA release in the cortex, nucleus accumbens (NAc) and striatum (Herian et al., 2021, 2019), although others have failed to see such robust increase (Miliano et al., 2019). Psilocybin enhanced DA release in the NAc at 10, but not 1 and 5 mg/kg, an effect associated with a small reduction of PFC DA release (Sakashita et al., 2015).

LSD, 5-MeO-DMT, and DMT have moderate (<300 nM) affinity for DA receptors (predominantly D1; Table 1) which may contribute to their ability to modulate the DA system. However, other psychedelics do not. The combined participation of 5-HT_{1A}Rs, 5-$HT_{2A,B,C}$Rs in classical psychedelics' mechanism of action limits the prediction of their effects on DA neuron function (Fig. 1B). Agonism of 5-HT_{2A}R receptors could facilitate DA release in the striatum and NAc. However, this effect is state-dependent, meaning it depends on the current state of dopaminergic transmission (De Deurwaerdère, Di Giovanni, 2017; Ichikawa and Meltzer, 1995; Lucas et al., 2000; Nash, 1990; Nash and Brodkin, 1991; Palfreyman et al., 1993; Porras et al., 2002; Schmidt et al., 1992; Sorensen et al., 1993). Thus, the state of DA transmission, the action of DA post-synaptically and/or the electrical activity of DA neurons, unmasks 5-HT_{2A}R regulation on DA systems. For instance, while 5-HT_{2A}Rs play a permissive role in the activation of the DA system, this control is absent in resting conditions in subcortical regions. When administered prior to amphetamine or D_2 antagonist haloperidol (preferentially low doses for the striatum), 5-HT_{2A}R antagonists attenuate the induced DA release, as measured by microdialysis in the striatum or NAc (Porras et al., 2002; Lucas et al., 2000; Liégeois et al., 2002). Part of 5-HT_{2A}R-dependent increase of striatal DA occurs via DA synthesis (Schmidt et al., 1992), such as that induced by amphetamine, ecstasy, or low doses of some antipsychotics (De Deurwaerdère and Di Giovanni, 2017). However, the 5-HT_{2A}R control on DA release may be less restricted in the NAc, compared to the striatum or PFC, given that 5HT_{2A}R antagonism modifies 1 mg/kg haloperidol induced DA release in the NAc, but not in the PFC or striatum (De Deurwaerdère and Di Giovanni, 2017; Lucas et al., 2000; Liégeois et al., 2002).

It is noteworthy that systemic administration of 10 mg/kg psilocybin and psilocin increase NAc DA outflow (Wojtas et al., 2023; Sakashita et al., 2015), but the unique role of 5-HT_{2A}Rs in this effect is questionable at this dose (Table 1). Moreover, a state-dependent inhibitory response following nigral injection of 5-MeO-DMT and non-selective 5-HT_{2C}R agonist m-CPP was reported on striatal L-DOPA accumulation (Spampinato et al., 1985), suggesting state-dependent action on DA synthesis that could correspond to a 5-HT_{2C}R-dependent effect, another target of several psychedelics (Table 1).

5-HT_{2C}Rs are best described as having continuous, predominantly inhibitory, control over DA electrical activity and release (Di Giovanni et al., 1999). Tonic inhibition can be demonstrated by 5-HT_{2C}R antagonist administration, SB 242084, increasing cortical, NAc, and striatal DA extracellular levels (Pozzi et al., 2002; Gobert et al., 2000; De Deurwaerdère et al., 2004). This control is more complicated as the blockade of endogenous constitutive 5-HT_{2C}R activity with the 5-$HT_{2B/2C}$R inverse agonist SB 206553 increases NAc and striatal DA outflow *in vivo* (De Deurwaerdère et al., 2004) and can produce the opposite output on NAc DA release by acting in the PFC under specific conditions (see below, Leggio et al., 2009).

Like the 5-HT_{2A}R, the 5-HT_{2C}R impacts DA systems in a phasic manner that is also state-dependent. 5-HT_{2C}R antagonist and inverse agonist, SB 242084 and SB 206553, potentiate cocaine- and morphine-induced NAc and striatal extracellular DA outflow (Porras et al., 2002; Navailles et al., 2004). However, the phasic inhibitory control of 5-HT_{2C}R agonists occurs preferentially on impulse-stimulated DA release: it attenuates morphine and stress induced DA outflow (Willins and Meltzer, 1998; Pozzi et al., 2002), while it does not modulate cocaine-induced (impulse-independent) DA outflow (Navailles et al., 2004).

Therefore, one may predict 5-HT_{2C}R-dependent psychedelic action, agonism at 5-HT_{2C}Rs, would inhibit the electrical activity of DA neurons and DA release in the striatum and NAc, preferentially via the VTA-NAc pathway in resting states (Di Giovanni et al., 2000). Systemic injection of DOI did not alter basal NAc DA release, but suppressed morphine-induced DA release (Willins and Meltzer, 1998). Despite agonism of 5-HT_{2C}R, DOI's inability to inhibit basal DA in the NAc could be attributed to the concomitant stimulation of 5-HT_{2A}R, although in a resting state seems unlikely given the phasic (state-dependent) nature of 5-HT_{2A}Rs on DA systems. Alternatively, excitatory effects exerted by 5-HT_{2C}R agonists on DA neuron activity have also been described. DOI delivered in the striatum, housing terminals of DA neurons, increased striatal DA release in a 5-HT_{2C}R-dependent manner (Lucas and Spampinato, 2000). 5-HT_{2C}R inverse agonist, SB 206553, attenuates morphine-, haloperidol- and GPR-12909-induced NAc DA release when delivered into the PFC (Leggio et al., 2009). These data illustrate the multiple sites involving 5-HT_{2C}Rs in the control of DA neuron functions: the excitatory action of striatal 5-HT_{2C}Rs, the facilitatory constitutive activity of PFC 5-HT_{2C}Rs on subcortical DA, and the "classic" inhibitory that was first described (Prisco et al., 1994).

Again, 25CI-NBOMe is unique as it enhances DA release in the PFC of rats and mice (Herian et al., 2019; Miliano et al., 2019). This effect is preferentially observed in female mice, while DA release in the NAc is increased in both male and female mice (Miliano et al., 2019). Although we do not have clear arguments for these differences between male and female, both pharmacodynamic and pharmacokinetic explanations

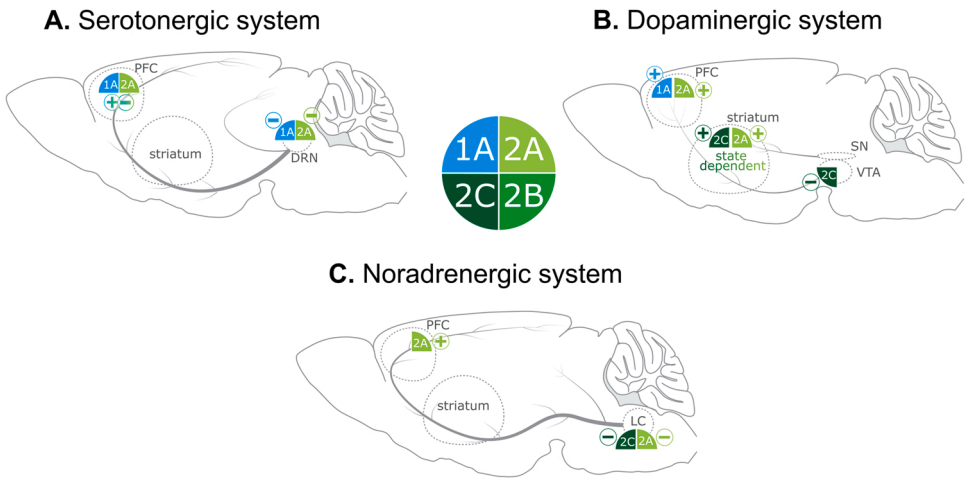

Fig. 1. 5-HTR subtypes involved in psychedelic monoaminergic modulation. A. Several psychedelics inhibit the electrical activity of DRN 5-HT neurons, likely via 5-HT_{1A} autoreceptors, and potentially 5-HT_{2C}R located on GABAergic interneurons. Cortical 5-HT_{1A} and 5-HT_{2A}R have been proposed to excite and inhibit subpopulations of DRN 5-HT neurons modulating 5-HT tone, accompanied by an increase in PFC 5-HT. B. Dopaminergic neurons in the VTA are under inhibitory control exerted by 5-HT_{2C}R (not necessarily locally). Conversely, local 5-HT_{2A}R and/or 5-HT_{2C}R receptors in the striatum/nucleus accumbens exert a state-dependent faciliatory effect on DA release. Cortical 5-HT_{1A}, 5-HT_{2A}, also have a general excitatory effect on cortical DA. C. Inhibition of LC firing observed by psychedelics (DOI, DOM) dependent on GABAergic interneurons and 5-HT_{2A} and/or 5-HT_{2C}Rs (outside the LC). Enhanced extracellular NA in the PFC induced by DOI is likely 5-HT_{2A}, possibly reflecting local excitatory actions. The 5-HT_{2B}R, a target of several psychedelics, was recently identified as an important regulator of the activity of dopaminergic and serotonergic neurons, yet more research is required with specific agonists. DRN, dorsal raphe nucleus; LC, locus coeruleus; PFC, medial prefrontal cortex; SN, substantia nigra; VTA, ventral tegmental area.

should be kept in mind. In the PFC, part of its effect is due to the stimulation of 5-HT_{2C}Rs (Herian et al., 2020, 2019). It is noteworthy, however, that 25I-NBOMe displays micromolar affinity for monoamine transporters (Zwartsen et al., 2017), as other compounds of this chemical class (Eshleman et al., 2018), and a minimal blockade of monoamine transporters could have magnifying effects in microdialysis experiments. The observation that a dose of 3 mg/kg of 25I-NBOMe in rats strongly involves cortical 5-HT_{1A}R on cortical DA release (Herian et al., 2020) while displaying modest affinity for 5-HT_{1A}R (500 nM) suggest that it could potentially impair DA reuptake in subcortical regions.

The 5-HT_{1A}R has been established to influence the activity of DA neurons, often studied in the context of atypical antipsychotic drugs. A broader influence of 5-HT_{1A}R agonists compared to other subtypes could be related to their ability to decrease the activity of 5-HT neurons via the stimulation of 5-HT_{1A} autoreceptors, thereby decreasing central 5-HT tone and mimicking antagonism at other subtypes. Therefore, 5-HT_{1A}R agonists mimic the action of 5-HT_{2A}R antagonists on activated DA neurons, speculatively 5-HT_3R and 5-HT_4R antagonists also, but not 5-HT_{2C}R antagonists (De Deurwaerdère and Di Giovanni, 2017).

In resting conditions, 5-HT_{1A}R agonists increase firing of VTA DA neurons (Arborelius et al., 1993; Lejeune and Millan, 1998), although not consistently increasing DA release in the NAc or striatum (De Deurwaerdère and Di Giovanni, 2017). 5-HT_{1A}R agonists have been reported to enhance PFC DA release (Díaz-Mataix et al., 2005; Sakaue et al., 2000). Consistent with a more pronounced increase in firing and localisation of 5-HT_{1A}R in the parabrachial pigmented nucleus of the VTA, associated with mesocortical DA projections (Arborelius et al., 1993; Doherty and Pickel, 2001). Despite the presence of VTA 5-HT_{1A}R, microiontophoretic application of the agonist 8-OH-DPAT into the VTA did not cause any change in the basal activity (Prisco et al., 1994) and the excitatory effect of 5-HT_{1A}R agonist BAYx3702 on VTA DA neurons was completely prevented by fronto-cortical transection (Díaz-Mataix et al., 2005). PFC 5-HT_{1A}R are expressed in pyramidal and GABAergic neurons. PFC GABAergic neurons have been implicated in the increased VTA firing observed with 5-HT_{1A}R agonists, their inhibition causing disinhibition of VTA projecting pyramidal neurons, thereby regulating VTA DA neurons (Llado-Pelfort et al., 2012).

An increasing body of evidence suggests that 5-HT_{2B}Rs also play a role in the control of monoaminergic neuron activity (Doly et al., 2017, 2009). However, a lack of selective 5-HT_{2B}R agonists and corresponding studies, makes prediction of 5-HT_{2B}R agonism in the psychedelic mechanism of action difficult. That said, 5-HT_{2B}R antagonists inhibit subcortical DA release (NAc) via a mechanism involving an increase in cortical DA release, mediated by 5-HT in the DRN (Auclair et al., 2010; Cathala et al., 2019; Devroye et al., 2018). This mechanism would be implicated in the neurotransmitter modulation induced by numerous pharmacological compounds, including antipsychotics (Devroye et al., 2018).

Currently, the literature suggests that psychedelics may be accompanied by an increase in cortical DA release (Gobert and Millan, 1999; Ichikawa et al., 2001), which could inhibit subcortical DA, explaining the lack of subcortical DA effects generally found (Di Matteo et al., 2000; Shi et al., 1995; Ichikawa and Meltzer, 1995; De Gregorio et al., 2016). However, there are exceptions to this in the literature. For instance, 25I-NBOMe shows subcortical DA increase as reported by Herian et al. (2021), (2019), and a high dose of psilocybin (10 mg/kg) also increases DA release in the NAc (Sakashita et al., 2015).

Psychedelics being devoid of action on DA release in the reward system, subcortically, is in line with their poor propensity to promote addiction (Nichols, 2016). Furthermore, their attenuation of morphine-induced subcortical DA (impulse-stimulated), via 5-HT_{2C}R agonism, may contribute to their anti-addictive properties (Willins and Meltzer, 1998).

Given that psychedelics bind with varying affinities to the four 5-HT receptors most involved in the control of DA function, each having state-dependent or opposing effects, it is beyond our current understanding to predict what effect each compound may induce on brain wide DA, let alone deduce a clear 'psychedelic' signature. This, however, may elucidate the contradictory findings that can be found in the literature, as variable experimental conditions could favour some controls over others given the state dependent and opposing action of target receptors (dose specific). This hypothesis would also explain the variability of behavioural responses to psychedelics, such as seen for locomotor activity (Halberstadt and Geyer, 2018).

4. Psychedelics and noradrenergic neurons

The action of psychedelics on NA neurons has been less investigated compared to serotonergic and dopaminergic neurons. Nonetheless, Halberstadt (2015) reviewed the tremendous inhibitory impact of psychedelics (LSD, DOI, DOM, 2,5-dimethoxy-4-bromoamphetamine (DOB), mescaline) on the spontaneous electrical activity of NA neurons, accompanied by an increased response to peripheral stimuli (Aghajanian, 1980; Chiang and Aston-Jones, 1993; Rasmussen and Aghajanian, 1986). This could have profound effects on sensory integration, alertness, and awareness. The initial post-mortem studies on whole brain reported that LSD and mescaline decreased NA tissue content (Leonard and Tonge, 1969; Pieri et al., 1978). This effect was also found in the hypothalamus following 2 mg/kg LSD (Sugrue, 1969). However, these results should be interpreted cautiously due to the doses used and the lack of region specificity.

It has been shown that 5-HT_{2C}Rs could modulate NA in the brain. Postmortem data using preferential 5-HT_{2C}R agonists WAY-163,909 and lorcaserin respectively did not alter and slightly increased NA tissue content in a subset of regions (Chagraoui et al., 2019; Di Giovanni et al., 2020). However, in the case of cortical NA it has been proposed that 5-HT_{2C}Rs assert a tonic inhibitory control (Millan et al., 1998). This is supported by increased extracellular NA following 5-HT_{2C}R antagonist SB 242084 and inverse agonist SB 206553 (Millan et al., 1998; Gobert and Millan, 1999). Furthermore, SB 206553 at 0.63 mg/kg shows a modest increase in cortical extracellular NA and 5-HT_{2C}R agonist Ro60–0175 at 1.25 mg/kg reduces extracellular cortical NA (Gobert and Millan, 1999; Gobert et al., 2000). Moreover, cumulative doses of Ro60–0175 reduce locus coeruleus (LC) neurons' firing, while SB 206553 increases their firing (Gobert and Millan, 1999). These data implicate the 5-HT_{2C}R in a psychedelic action, reducing central NA tone.

Consistent with the electrophysiological data, a microdialysis study reported a decrease in NA extracellular levels in the hippocampus following systemic injections of 0.5 mg/kg DOI and 1 mg/kg DOM (Done and Sharp, 1992). This effect likely involved 5-HT_{2A}R as ritanserin and spiperone, antagonists at the 5-HR_{2A}R, but not sulpiride, a D_2 antagonist, block the effect induced by DOI. However, this is independent from local actions of DOI in the hippocampus or the LC (Done and Sharp, 1992).

DOI-induced decrease in LC spontaneous activity is blocked by $GABA_A$R antagonists bicuculline and picrotoxin, suggesting indirect GABAergic modulation of the LC (Chiang and Aston-Jones, 1993). Indeed this 5-HT_2R mediated inhibition of LC NA neurons requires afferent GABAergic projections from the prepositus hypoglossal nucleus. However, this effect may not be due to local 5-HT_2R within the prepositus hypoglossal nucleus, as neither the agonist quipazine nor the antagonist ketanserin altered LC neurons firing when locally infused (Chiang and Aston-Jones, 1993; Gorea et al., 1991). Note here that distinguishing this as a 5-HT_{2A}R or 5-HT_{2C}R effect is difficult given the doses used and shared affinity across psychedelics.

Counter intuitive to the reduced LC spontaneous firing seen with DOI, systemic injection of 2.5 mg/kg DOI enhanced NA extracellular levels in the PFC. This effect was blocked by 0.04 mg/kg MDL100,907, but not 0.63 mg/kg SB 206553, implicating a mechanism at the terminal level indirectly controlled by 5-HT_{2A}R (Gobert and Millan, 1999). However, a dose of 0.27 mg/kg DOI did not alter the extracellular levels of NA in the PFC, but blocked haloperidol and clozapine induced NA increase (Westerink et al., 2001). It is unclear how DOI acts in this specific context because of the multiple targets bound by these antipsychotic drugs, particularly clozapine which blocks all 5-HT_2R subtypes.

In conclusion, the NA system is likely modulated by psychedelics in a region-dependent manner. The reduction in spontaneous firing in the LC and increased response to external stimuli may be characteristic of psychedelics, although the data is currently too limited to assert this claim. Fig. 1C illustrates that 5-HT_2R action likely plays a role in the modulation of the NA system, including action in the PFC and LC, acknowledging 5-HT_2Rs acting on the LC may not be localised there. The LC is a point of convergence of sensory information, described as a 'novelty detector' attributing salience to incoming stimuli, and modulating signal-to-noise ratios (Nichols, 2016). This indeed seems a likely site of psychedelic action given the profound alterations of sensory perception they reliably induce. In line with this speculative hypothesis, lisuride, devoid of such sensory alterations, increased spontaneous firing in the LC (Rogawski and Aghajanian, 1979).

5. Non-neuronal 5-HTR expressing glia

Neurons are not the only cell type in the brain that express 5-HTRs to which psychedelics may bind. Microglia, astrocytes, and oligodendrocytes also contain 5-HTRs, and we cannot exclude their participation in the psychedelic mechanism of action. Microglia, suggested to regulate neurogenesis and synaptic plasticity, express 5-HT_{2B}R, (Turkin et al., 2021). Astrocytes express the 5-HT_{1A}R, 5-HT_{2A}R, 5-HT_5R, 5-HT_7R, as well as serotonin reuptake transporter and noradrenaline reuptake transporter to some extent (Zhou et al., 2019). Oligodendrocytes, responsible for myelination of neurons, express 5-HT_{1A}R and 5-HT_{2A}R (Fan et al., 2015). Acknowledging that psychedelics promote neuroplasticity, their influence on extracellular matrix structures, such as perineuronal nets, is an emerging field that also converges with monoaminergic systems (Ly et al., 2018). Further research into the psychedelic mechanism in these non-neuronal populations is required.

6. Conclusion

Psychedelics bind receptors involved in the control of 5-HT, DA, and NA monoaminergic neurons. Receptors that can have opposite, region-dependent and state-dependent effects on both release and activity of monoaminergic neurons (Fig. 1). In this review, we find no single signature of the effect of psychedelics on monoaminergic neuron function.

The current literature on psychedelics contains highly variable data, both neurochemical and otherwise. Variability within a single psychedelic may be attributed to variable contexts —favouring some state-dependent pathways over others, variable dose/administration—higher doses acting on a broader range of receptors, and variable species—acknowledging known differences in monoaminergic pathways and receptor actions between rats, mice, cats, and humans (Ellenbroek and Youn, 2016). Additionally, serotonergic psychedelics have varying affinities for each of the 5-HTRs discussed, as well as additional targets, which could have confounding effects (Table 1). Each of these factors presents significant challenges in understanding the mechanism of psychedelics, the interest in which grows in light of their numerous clinical and preclinical effects (antidepressant, anxiolytic, psychotomimetic). The doses used in preclinical animal studies, as presented here, generally correspond to high doses in humans, consistent with the clinical studies establishing psychedelic efficacy. Direct measurement of monoaminergic systems in human studies is not currently possible due to the invasive nature of such measurements, making translation to humans challenging. Positron emission tomography to examine the binding potential of D_2-dopamine receptors following the administration of psilocybin, revealed altered binding potential in the caudate and putamen (Vollenweider, 1999). The authors attributed this effect to an increase in DA release induced by psilocybin.

Given the complexity of monoaminergic modulation, it is conceivable that psychedelics induce a subtle neurochemical effect resulting in profound phenomenological alterations. We have described the actions of its target receptors as state-dependent, as induced by pharmacology. It would serve future experimenters to consider state-dependency as induced by behaviour/environment/context, avoiding the perceptual vacuum of anaesthetics under which several psychedelic studies have been conducted. For instance, acknowledging that these compounds

alter perception, observing their effects in a challenged or active perceptual experience may facilitate identifying the subtle neurochemical modifications at play in altering it. A pertinent example is exploratory or investigatory behaviour, during which "animals are motivated to explore their surroundings in order to reduce perceptual uncertainty about the environment" (Halberstadt and Geyer, 2018; Berlyne, 1966). This idea is not unique and was proposed by Adams and Geyer as a model for hallucinogens. Authors used behavioural pattern monitoring on unconditioned behaviour of both voluntary and forced exploration following treatment with LSD in rats (Adams and Geyer, 1986). Beyond the ability of this paradigm to distinguish compounds behaviourally we may contrast activated neuromodulation with psychedelic neuromodulation. Such experiments consider the effect of external environment, as considered by clinicians 'set and setting' in the context of psychedelics, and its impact on neurochemical environment and therefore psychedelic mechanism.

CRediT authorship contribution statement

Jasmine Jade Butler: Writing – review & editing, Writing – original draft. **Chloé Aman:** Writing – review & editing, Data curation. **Daria Ricci:** Writing – review & editing. **Philippe De Deurwaerdère:** Writing – review & editing, Writing – original draft. **Anna Beyeler:** Writing – review & editing.

Declaration of Competing Interest

All the authors declare that they have no established conflicting financial interests or personal relationships that may have influenced the research presented in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

We acknowledge the support of the Région Nouvelle-Aquitaine, the 'Avenir program' of the French Institute of Health (INSERM), the 'Agence Nationale pour la Recherche' (ANR), the 'Cercle de la Fondation Schlumberger pour l'Education et la Recherche' (Cercle FSER), the 'Fondation pour la Recherche Médicale' (Equipe FRM) and the 'Fondation Bettencourt-Schueller' (impulscience 2023) to AB. We are grateful for the support of the Grand Programme de Recherche (GPR work package Adapsy) of the University of Bordeaux to PDD and AB.

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Summary Art I

Psychedelics have complex, state- and region-dependent effects on monoaminergic systems, influencing serotonin, dopamine, and noradrenaline in varied ways. While they often inhibit serotonergic and noradrenergic neuron activity, this doesn't consistently reduce neurotransmitter release across all regions. Their effects on dopaminergic systems are similarly nuanced, with opposing mechanisms depending on the state. Overall, psychedelics lack a clear monoamine signature but exhibit recurrent, context-specific mechanisms. A more relational approach to the interaction of monoaminergic systems between regions may provide new insights into the organisation or ‘connectivity’ perhaps parallelling the altered connectivity described in neuroimaging studies.

Art II - Deleting Snord115 genes in mice remodels monoaminergic systems activity in the brain toward cortico-subcortical imbalances

Questions and purpose of the study

The aim of this study was to compare monoaminergic systems between two genetic backgrounds of male mice, one with deletion of the *Snord115* gene, a small nucleolar RNA thought to be involved in the post transcriptional editing of the 5-HT_{2C}R. Investigation of monoaminergic systems was assessed via post-mortem neurochemical quantification of monoamines and metabolites in 12 distinct brain regions. The analysis of this data was developed from a classical quantitative comparison between tissue quantity or ratios indicative of turnover, towards a correlative approach identifying neurochemical connectivity between distinct regions.

OXFORD

Human Molecular Genetics, 2023, Vol. 32, 2, 244–261
https://doi.org/10.1093/hmg/ddac139
Advance access publication date 11 August 2022
Original Article

Deleting *Snord115* genes in mice remodels monoaminergic systems activity in the brain toward cortico-subcortical imbalances

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Abstract

The neuronal-specific SNORD115 has gathered interest because its deficiency may contribute to the pathophysiology of Prader-Willi syndrome (PWS), possibly by altering post-transcriptional regulation of the gene encoding the serotonin (HTR2C) receptor. Yet, Snord115-KO mice do not resume the main symptoms of PWS, and only subtle-altered A-to-I RNA editing of Htr2c mRNAs was uncovered. Because HTR2C signaling fine-tunes the activity of monoaminergic neurons, we addressed the hypothesis that lack of Snord115 alters monoaminergic systems. We first showed that *Snord115* was expressed in both monoaminergic and non-monoaminergic cells of the ventral tegmental area (VTA) and the dorsal raphe nucleus (DRN) harboring cell bodies of dopaminergic and serotonergic neurons, respectively. Measuring the tissue level of monoamines and metabolites, we found very few differences except that the content of homovanillic acid—a metabolite of dopamine—was decreased in the orbitofrontal and prefrontal cortex of Snord115-KO mice. The latter effects were, however, associated with a few changes in monoamine tissue content connectivity across the 12 sampled brain regions. Using *in vivo* single-cell extracellular recordings, we reported that the firing rate of VTA dopaminergic neurons and DRN serotonergic neurons was significantly increased in Snord115-KO mice. These neural circuit dysfunctions were not, however, associated with apparent defects in binge eating, conditioned place preference to cocaine, cocaine-induced hyperlocomotion or compulsive behavior. Altogether, our multiscale study shows that the absence of Snord115 impacts central monoaminergic circuits to an extent that does not elicit gross behavioral abnormalities.

Introduction

Box C/D small nucleolar RNAs (SNORD) belong to a well-known family of antisense small noncoding RNAs that direct sequence-specific 2'-O-methylations onto ribosomal RNAs, spliceosomal U-snRNAs or transfer RNAs (1). However, the precise mode of action of some of them has not yet been formally demonstrated. Among these, the neuronal-specific SNORD115 has received particular attention due to its apparent role in the post-transcriptional regulation of the gene encoding the 2C subtype of serotonin receptor (HTR2C) involved in the regulation of food intake, energy balance, motor neuron activity, anxiety, mood and cognition (2–4). The most parsimonious model posits that SNORD115, through base-pairing interaction, regulates alternative splicing and/or site-specific A-to-I RNA editing of HTR2C pre-mRNA which, in turn, leads to the synthesis of HTR2C receptor isoforms with diminished signaling activity (5–8).

SNORD115 is also remarkable in that its tandemly repeated genes are only expressed from the paternal chromosome (Fig. 1). Of relevance, *SNORD115* gene copies are embedded within a large imprinted gene cluster at human Chr.15q11q13 where deficiencies for paternally expressed alleles lead to the Prader–Willi Syndrome (PWS). This rare neurodevelopmental disorder is characterized by a complex set of metabolic, hormonal and neurological abnormalities, notably obsessive behavior related to food and eating which, in the absence of strict control, leads to morbid obesity (9–11). Although paternally inherited *SNORD115* alleles are deleted in the vast majority of PWS patients, loss of SNORD115 *per se* appears insufficient to provoke PWS (12). However, this does not rule out that disrupted HTR2C-dependent regulation, as predicted by loss of SNORD115, underlies some aspects of PWS etiology, notably increased food intake (4,5,7,13,14). Surprisingly, our most recent work shows that Snord115 loss-of-function in mice is not

Received: April 1, 2022. **Revised:** May 25, 2022. **Accepted:** June 9, 2022
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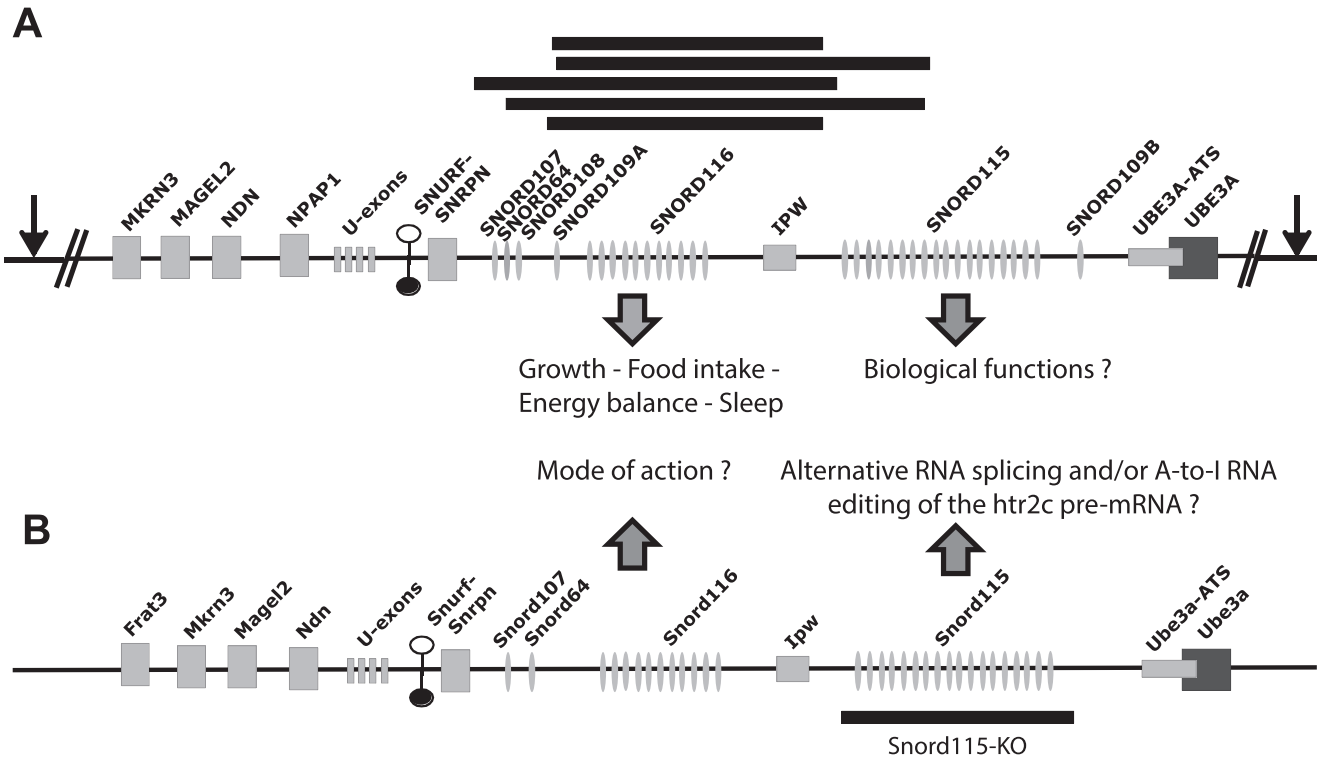

Figure 1. The brain-specific Snord115 is generated from tandemly repeated, paternally expressed non-coding DNA arrays. (**A**) Schematic representation of the imprinted gene cluster at human Chr.15q11q13. Tandemly repeated *SNORD115* genes are part of a large imprinted chromosomal region containing a dozen paternally expressed gene loci, including SNORD116 whose repeated gene organization recalls that of *SNORD115*, several conserved protein-coding genes (*MKRN3*, *MAGEL2*, *NDN*, *NPAP1*, *SNURF-SNRPN*) and as yet poorly characterized long non-coding RNA genes (anti-*UBE3A*, *IPW*, exon-U containing transcript). In most cases of PWS (60–75%), a de *novo* deletion of paternal origin removes all the paternal alleles (vertical BLACK arrows). Other common PWS cases involve inheritance of two maternal chromosomes 15 or rare genetic/epigenetic alterations (not shown). Of relevance in the context of our study, rare shorter deletions of paternal origin (horizontal BLACK bars) that mostly overlap *SNORD116* genes have also been identified in a few PWS patients (89–94). Defects in SNORD-mediated functions might therefore account for important disease outcomes. (**B**) Schematic representation of the imprinted gene cluster at human Chr.7C. Mouse genetics show that a targeted deletion of *Snord116* gene leads to growth retardation as well as alterations in feeding behavior, sleep architecture and energy homeostasis (for comprehensive reviews: (5,95,96)). While the mode of action of SNORD116 is unknown (direct RNA targets?), SNORD115 contains a perfect 18 nt-long conserved, D box-dependent antisense element to exon VI of the brain-specific serotonin (HTR2C) receptor mRNA. SNORD115 base-pairing is thought to influence alternative splicing and/or A-to-I RNA editing of HTR2C pre-mRNA. The horizontal black bar indicates the ∼300 kb-long CRISPR-Cas9-mediated deletion removing the entire *Snord115* gene array (15). Note that *Snord115* are intron-encoded and released after splicing of their non-coding host-gene transcripts (not shown). In contrast to their host-gene transcripts whose functions, if any, remain unknown, *Snord115* and *Snord116* are highly conserved among placental mammals (eutherian), indicating that they represent functional RNA entities under selective pressure. (A and B) The paternally and maternally expressed genes are depicted by light grey and dark grey symbols, respectively. SNORDs are symbolized by ovals while vertical and horizontal rectangles represent protein and non-protein coding genes, respectively. The imprinting center element (ICE) is indicated by filled and open lollipops (methylated and unmethylated DNA, respectively). The drawing is not to scale.

accompanied by any apparent abnormalities in HTR2C-mediated behaviors such as emotionality, homeostatic feeding or energy balance. In addition, and contrary to our expectations, post-transcriptional regulation of Htr2c pre-mRNA remained globally unaffected, with only a few subtle regional-specific changes in A-to-I RNA editing (15). These rather modest changes were in line with a previous study that provided the first in vivo evidence that ectopically expressed Snord115 could be involved in fine-tuning A-to-I RNA editing of Htr2c pre-mRNAs in mouse choroid plexus (16). The lack of robust phenotypic alterations suggests that adaptive mechanisms might have been triggered in such constitutive, germline-derived Snord115 knockout (KO) mouse model.

Monoaminergic systems are the prototypical systems involved in the adaptation of complex organisms (17–20). In mammals, these systems include the dopamine (DA) neurons from the substantia nigra (SN) and the ventral tegmental area (VTA), the serotonin (5-HT) neurons from the midbrain raphe nuclei and the noradrenaline (NA) neurons from the locus coeruleus. These monoaminergic neurons innervate all neurobiological networks at diverse densities and shape the activity of these networks via their multiple anatomical and functional cross-talks (21). Of particular interest, the activation of HTR2C receptor signaling in cortical and subcortical areas regulates the activity of monoaminergic neurons in a phasic and tonic manner (2,22–24). We then reasoned that disrupting *Snord115* genes could subtly alter the balance of monoaminergic features across the brain in line with the few apparent modifications it induces on post-transcriptional regulation of Htr2c pre-mRNA (15).

The main purpose of the present study was to probe the abovementioned hypothesis. This was achieved by combining mouse genetics, comprehensive measurements of monoamines levels (DA, NA, 5-HT and

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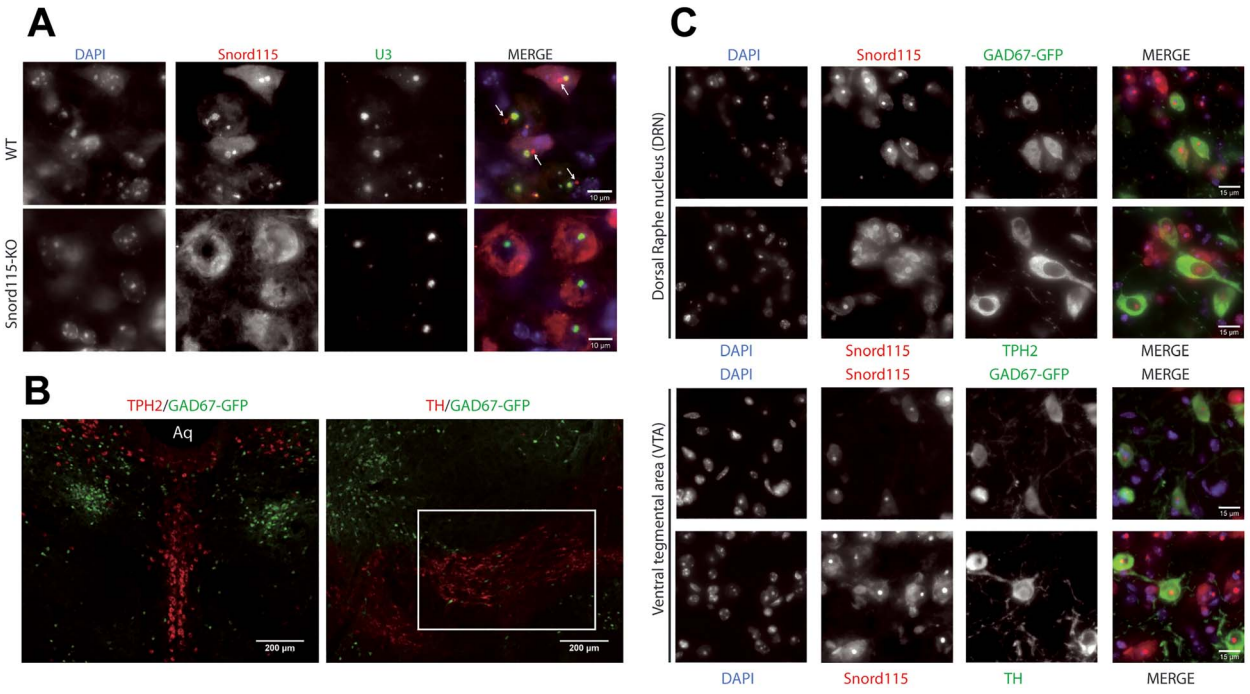

Figure 2. *Snord115* is expressed in GABAergic, dopamine (DA) and serotonin (5-HT) neurons. (**A**) Representative images from RNA FISH performed in lateral hippocampus of WT and Snord115-KO mice using Cy3-labeled Snord115 (red) and Cy5-labeled U3 (green, nucleolar marker) oligo-probes. White arrows indicate nucleoplasmic *Snord115* host gene transcripts detected at the transcription sites (25). Note that U3 probes also reveal additional punctuate signals, presumably Cajal bodies. (**B**) Representative images from IF co-immunostaining for GAD67-eGFP (green)/TPH2 (red) in the DRN (left) and GAD67-GFP (green)/TH (red) in the VTA (right). Aqueduct (Aq). VTA is boxed by a white rectangle. (**C**) Top panels: Representative images from IF staining for GAD67-eGFP and TPH2 followed by RNA FISH (Snord115) performed in the DRN. Bottom panels: Representative images from IF staining for GAD67-eGFP and TH followed by RNA FISH (Snord115) performed in the VTA. GAD67-eGFP staining was used as a marker for GABAergic interneurons, while TPH2 and TH staining identify DRN 5-HT neurons and VTA DA neurons, respectively. Nuclei were stained using DAPI.

their metabolites) in 12 distinct brain regions, *in vivo* extracellular electrophysiological recordings of VTA DA and DRN 5-HT neurons and behavioral analyses focusing on paradigms involving monoaminergic activities and/or HTR2C signaling. Altogether, we propose that lack of Snord115 leads to subtle remodeling of the monoaminergic circuits toward a lower dopaminergic tone in some frontal areas associated with higher electrical activity of VTA DA and DRN 5-HT neurons. These changes are, however, not conveyed in gross behavioral abnormalities.

Results

Expression of *Snord115* in dopaminergic and serotonergic neurons

We first sought to determine, via RNA fluorescent *in situ* hybridization (FISH), whether *Snord115* is expressed in monoaminergic neurons. We first validated the specificity of a mixture of Cy3-conjugated antisense oligonucleotide probes that recognize Snord115 as well as its host-gene transcript (hereafter named anti-Snord115 probes). In addition to cytoplasmic signals whose intensities vary greatly from one cell to another, anti-Snord115 probes generated strong, rounded nucleolar signals, as judged by their co-localization with those of the nucleolar U3 snoRNA marker (Fig. 2A, top panels). As expected, Snord115 nucleolar signals were no longer detected in the brain sections prepared from Snord115-KO mice, while cytoplasmic ones persist, indicating that the latter represent unspecific background signals (Fig. 2A, bottom panels). Finally, and consistent with previous observations made with primary neuronal cell cultures (25), anti-Snord115 probes also detected newly synthesized Snord115-host-gene transcripts at transcription sites (Fig. 2A, top). Snord115 distribution was then addressed by combining RNA FISH and immunofluorescence (IF) staining to Tyrosine hydroxylase (TH) and Tryptophan hydroxylase 2 (TPH2) used as specific markers for VTA DA and DRN 5-HT neurons, respectively. This was done using brain sections prepared from GAD67-GFP transgenic mice, thus allowing us to simultaneously detect GABAergic interneurons in the VTA (Fig. 2B-right) and DRN (Fig. 2B-left). As shown in Fig. 2C, top panels, IF staining of the DRN showed that most—if not all—GABAergic and 5-HT neurons displayed strong *Snord115* expression. In the VTA, the vast majority of GABAergic and DA neurons were also positive for Snord115 (Fig. 2C, bottom panels). Thus, Snord115 is strongly—but not exclusively—expressed in monoaminergic neurons.

Quantitative analysis of the effect of SNORD115 deletion on monoamines tissue content

The broad anatomo-functional dimension of monoaminergic systems can be addressed using post-mortem tissue content analysis of the monoamines and their metabolites across numerous brain regions (26,27). These tissue indexes represent the net effect of a combination of

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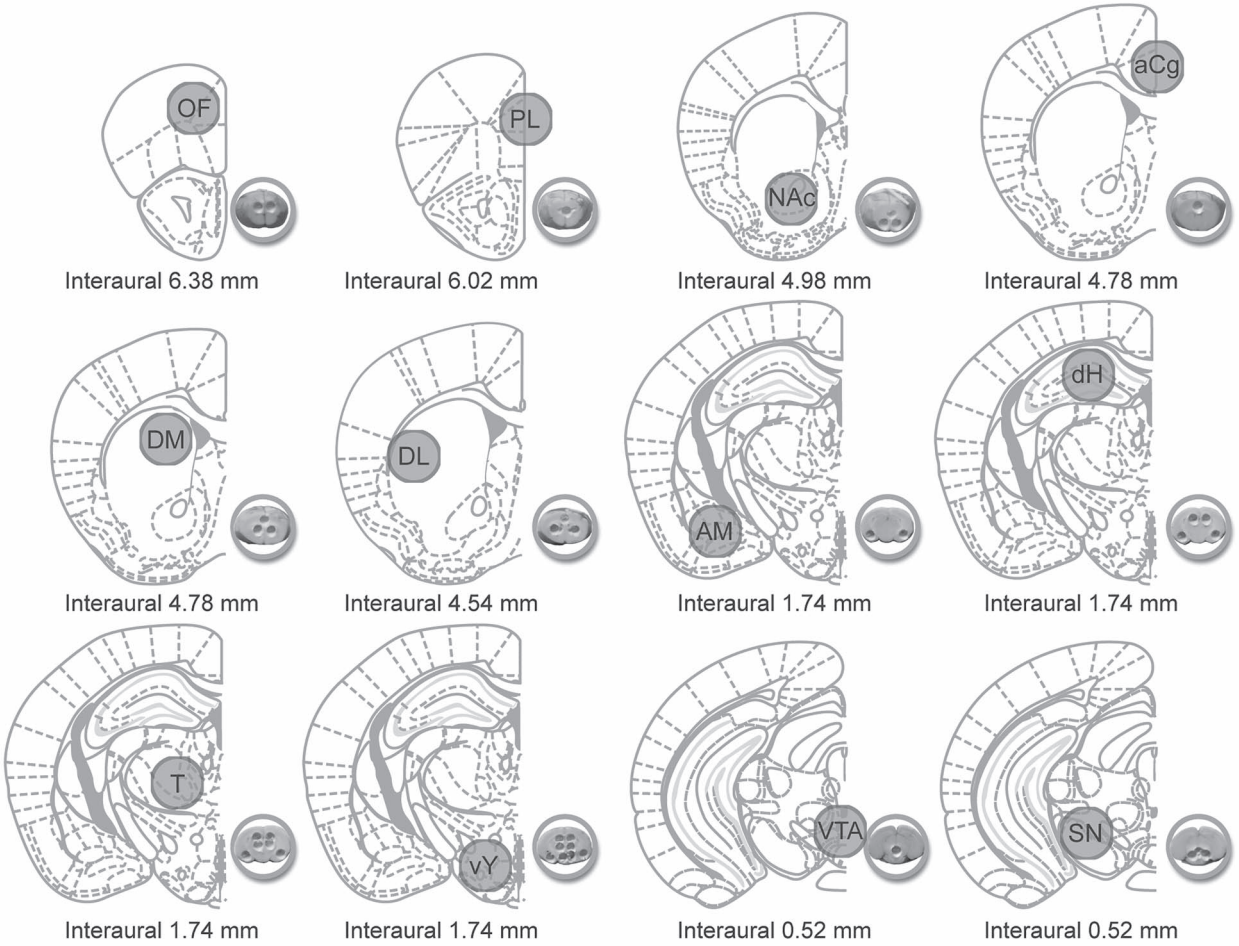

Figure 3. The approximate position of punched tissue from coronal sections of mouse brain. Adapted from (97). Cortical areas: orbitofrontal (OF, corresponding also to M2), prelimbic (PL) and anterior cingulate (aCg) cortices. Subcortical areas: dorsomedial (DM) and dorsolateral (DL) striatum, nucleus accumbens (NAc), dorsal hippocampus (dH), amygdala (AM), thalamus (T), ventral hypothalamus (vY), VTA and subthalamic nucleus (SN). Tissue was pooled from left and right cerebral hemispheres, with the exception of PL, aCg cortices and VTA using a frozen microtome (−25°C). Photomicrographs illustrate punched brain region (5-month-old males), atlas location indicated by interaural, multiple punches were taken at the same depth for structures aCg, DM at interaural 4.78 mm and AM, dH, T, vY at interaural 1.74. Mean tissue weight ± SEM was 1.79 mg ±0.037 ($n = 204$).

the numerous regulatory mechanisms that control the activity of terminals under resting conditions (28–30) which can be addressed via multiple correlative analyses between pairs of brain regions (27). This approach is sensitive enough to determine subtle differences in the neurochemical organization of monoaminergic systems previously shown to be sensitive to changes in HTR2C tone (31,32). The tissue content of monoamines and their metabolites have been measured in 12 brain regions dissected from Snord115-KO and wild-type (WT) littermates (Fig. 3). The results are reported in Tables 1 and 2. Aberrant criteria (see data analysis) excluded 3% of total data, similar for both single compounds and ratios (3.5 and 2.7%, respectively). Four percent of original data was lost due to experimental error, predominantly monoamine and metabolite data (7.5%) and to a lesser extent ratio (1%).

Consistent with previous studies in mice (33), tissue content of DA, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) was dramatically higher in the nucleus accumbens (NAc), dorsomedial (DM) and dorsolateral (DL) striatum of WT mice compared with other brain regions (Table 1). The indirect index of the DA turnover DOPAC/DA was congruently low, particularly in the striatum. A similar distribution of DA neurochemical markers in Snord115-KO mice was found with the noticeable exception of the HVA content in the orbitofrontal (OF, $P = 0.03$) and the prelimbic (PL, $P = 0.04$) cortices which was significantly lower in Snord115-KO mice (Table 1). The ratios HVA/DA ($P = 0.02$) and HVA/DOPAC ($P = 0.005$) were also reduced in the OF cortex, but not in the PL (data not shown). Despite the lack of marked difference in DOPAC and DA levels in the SN, the ratio DOPAC/DA was slightly higher in the SN of Snord115-KO mice ($P = 0.04$). No significance was found for DA itself. No other differences were observed in other brain regions (Table 1), and 3-methoxytyramine (3-MT), another metabolite of DA, was measured in the NAc and striatal regions only. Reaching ∼300 pg/mg of tissue depending on the striatal sub-regions in WT,

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Table 1. Tissue content of DA and its metabolites (pg/mg) and the ratio DOPAC/DA in various brain regions of WT and Snord115-KO mice

	DA		DOPAC		HVA		DOPAC/DA	
region	WT	SNORD	WT	SNORD	WT	SNORD	WT	SNORD
OF	10.8 ± 1.2	11.2 ± 0.96	26 ± 3	25 ± 3	69 ± 10	45 ± 3*	2.48 ± 0.2	2.36 ± 0.31
PL	41 ± 5	29 ± 4	17 ± 2	16 ± 3	116 ± 8	86 ± 11*	0.54 ± 0.08	0.55 ± 0.05
aCg	89 ± 12	80 ± 7	75 ± 9	67 ± 7	213 ± 23	201 ± 24	0.79 ± 0.09	0.84 ± 0.08
NAC	1591 ± 266	1544 ± 215	904 ± 136	1036 ± 109	601 ± 112	620 ± 59	0.6 ± 0.05	0.76 ± 0.11
DMS	4107 ± 356	4535 ± 1047	1561 ± 162	1496 ± 195	670 ± 74	723 ± 143	0.36 ± 0.03	0.39 ± 0.04
DLS	6064 ± 666	5028 ± 176	802 ± 92	790 ± 82	500 ± 60	448 ± 20	0.13 ± 0.01	0.15 ± 0.01
Th	6 ± 0.9	8 ± 1	20 ± 1	22 ± 2	72 ± 8	77 ± 6	3.85 ± 0.58	2.67 ± 0.22
AM	240 ± 54	287 ± 48.8	83 ± 19	121 ± 26	150 ± 22.6	215 ± 49	0.33 ± 0.05	0.46 ± 0.09
dH	48 ± 6	44 ± 4	13 ± 1.8	13 ± 2	39 ± 3.78	41.8 ± 5.3	0.3 ± 0.03	0.3 ± 0.02
VTA	215 ± 41	234 ± 38	191 ± 37	207 ± 27	205 ± 30.1	239 ± 18.8	0.88 ± 0.05	0.91 ± 0.06
SN	259 ± 54	223 ± 46	141 ± 15	136 ± 21	265 ± 36	252 ± 40.3	0.64 ± 0.04	0.76 ± 0.04*
vY	128 ± 19	106 ± 14	105 ± 8	101 ± 13.1	171 ± 18.8	136 ± 16.9	0.98 ± 0.07	0.99 ± 0.08

All values are expressed as mean ± SEM (pg/mg of tissue for individual compounds) except the ratio. Significance is indicated as follows: *$P < 0.05$ (Students *t*-test). ($n = 8$–10 except in the DL where $n = 5$ due to an experimental error).

Table 2. Tissue content of NA, 5-HT and its metabolite (pg/mg) and the ratio 5-HIAA/5-HT in various brain regions of WT and Snord115-KO mice

	NA		5-HT		5-HIAA		5-HIAA/5-HT	
region	WT	SNORD	WT	SNORD	WT	SNORD	WT	SNORD
OF	197 ± 12	174 ± 12	161 ± 15	155 ± 12	169 ± 18	140 ± 15	1.11 ± 0.13	0.96 ± 0.13
PL	340 ± 39	306 ± 24	158 ± 14	134 ± 16	108 ± 12	112 ± 14	0.7 ± 0.07	0.85 ± 0.07
aCg	586 ± 58	522 ± 55	78 ± 8	87 ± 10	147 ± 15	141 ± 14	2.34 ± 0.35	1.61 ± 0.17
NAC	130 ± 20	128 ± 15	299 ± 42	298 ± 26	210 ± 34	199 ± 11	0.71 ± 0.06	0.96 ± 0.05
DMS	50 ± 3	65 ± 11	193 ± 13	192 ± 41	120 ± 10	134 ± 26	0.65 ± 0.06	0.76 ± 0.04
DLS	25 ± 2	25 ± 3	156 ± 18	138 ± 6	114 ± 12	96 ± 4	0.75 ± 0.04	0.81 ± 0.05
Th	178 ± 11	199 ± 16	107 ± 10	127 ± 14	290 ± 13	304 ± 22	3.05 ± 0.25	2.38 ± 0.14*
AM	814 ± 182	451 ± 43	454 ± 38	422 ± 56	297 ± 27	322 ± 19	0.66 ± 0.04	0.74 ± 0.06
dH	239 ± 23	281 ± 23	334 ± 49	381 ± 23	554 ± 65	580 ± 56	1.85 ± 0.2	1.53 ± 0.14
VTA	409 ± 31	369 ± 22	450 ± 30	393 ± 29	529 ± 48	463 ± 34	1.3 ± 0.01	1.21 ± 0.09
SN	259 ± 20	217 ± 22	1068 ± 174	980 ± 128	632 ± 36	541 ± 28	0.7 ± 0.08	0.6 ± 0.04
vY	548 ± 56	646 ± 71	717 ± 61	506 ± 39*	624 ± 52	488 ± 45	0.89 ± 0.06	0.97 ± 0.05

All values are expressed as mean ± SEM (pg/mg of tissue for individual compounds) except the ratio. Significance is indicated as follows: *$P < 0.05$ (Students *t*-test). ($n = 8$–10 except in the DL where $n = 5$ due to an experimental error).

its concentrations were similar in Snord115-KO mice (data not shown). Table 2 reports the neurochemical data for Noradrenalin (NA), 5-HT, its metabolite 5-hydroxyindoleacetic acid (5-HIAA) and the ratio 5-HIAA/5-HT. At variance with DA, NA tissue content in WT mice was highest in the anterior cingulate cortex (aCg), the ventral hypothalamus (vY) and VTA and lowest in the striatal areas. A similar distribution of NA in Snord115-KO mice was found. Consistently, the levels of NA metabolites vanillylmandelic acid (VMA, in pg/mg: 728 ± 78WT and 1019 ± 298 Snord115-KO) and 3-methoxy-4-hydroxyphenylethylenglycol (MHPG, 447 ± 235 WT and 171 ± 24 SNORD115-KO), measured only in the aCg, were similar in both groups (data not shown). The content of 5-HT and its metabolite 5-HIAA were highest in the amygdala (AM), SN, and VTA. A similar distribution was reported in Snord115-KO mice with two exceptions. The 5-HIAA/5-HT ratio in the T ($P = 0.04$) and the 5-HT content in the vY ($P = 0.01$) were both significantly reduced in Snord115-KO mice (Table 2). The monoaminergic precursors L-tyrosine (L-TYR) and L-tryptophan (L-TRP) were detected in three brain regions including the aCg, PL and AM. Their concentrations (over 4200 pg/mg for L-TYR and 1900 pg/mg for L-TRP) were also similar in Snord115-KO mice (data not shown). With the notable exception of HVA in the OF and PL and the content of 5-HT in vY, we conclude that overall monoamine levels remain largely unchanged in all Snord115-KO brain areas examined.

Qualitative analysis of the effect of *Snord115* deletion on monoamines tissue content

Correlative analyses of monoamine content were expected to unmask, between two conditions, qualitative changes of the organization of monoamines in a single brain region or between brain regions. The approach is well designed for monoamines which innervate the brain from a limited number of brain regions but with high level of reciprocal interactions between regions (27,34). Fig. 4A depicts the relationships between the metabolites and the neurotransmitters acknowledging that the catabolism of monoamines can occur at both

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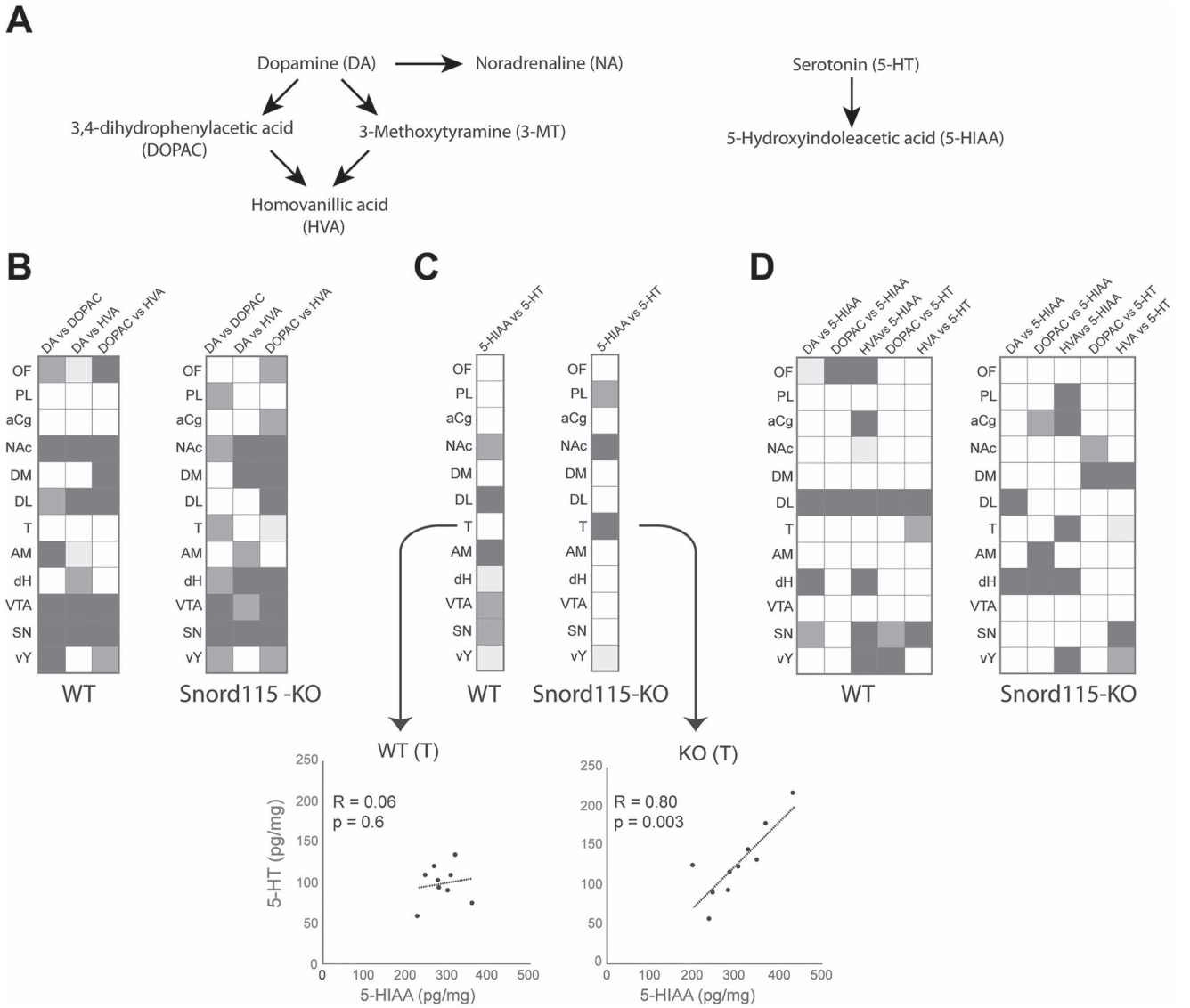

Figure 4. Pearson product correlations between relevant neurotransmitter metabolite pairs within a single brain region of Snord115-deleted and WT mice. (**A**) Relationships between monoamine neurotransmitters and their related degradation products. (**B**) Correlations within the dopaminergic system, (**C**) within the serotonergic system and (**D**) between the serotonergic and dopaminergic systems. Only significant correlations are shown, with increasing intensity of box filling corresponding to $P < 0.05$, $P < 0.01$ and $P < 0.001$. Only positive correlations were found whatever the parameters. C (bottom): 5-HT (*y*-axis) and 5-HIAA (*x*-axis) pg/mg tissue content correlation in the thalamus (T) of WT or Snord115-KO mice. Pearson correlation coefficient (R) and two tailed *P*-values with a CI95% (*P*) are displayed.

presynaptic and postsynaptic compartments. While the correlative analyses could be extensive (26), we have limited them. We examined inter-substrate correlations within a single brain region, as indicated in the matrix for the relationships in DA parameters (Fig. 4B), 5-HT parameters (Fig. 4C) as well as the cross-relationship between DA and 5-HT parameters (Fig. 4D).

As previously reported (26), a correlative link between the substrate and the product in WT mice was consistently found in 7 of 12 brain regions for DA and DOPAC, DA and HVA, and DOPAC and HVA (Fig. 4B). Notably five regions (OF, NAc, SN, DL and VTA) significantly correlated between all three substrate product pairs. The number of correlations for the DA system between substrate and product increased in Snord115-KO mice, particularly for the relationship between DOPAC and HVA (ten correlations). While the correlations in the NAc, the SN and the VTA were kept similar, there was a loss of the substrate/product relationship in the OF and the DL. Conversely, the link between substrates/products was reinforced in the dH and the DM (Fig. 4B). As shown in Fig. 4C, 5-HT and 5-HIAA contents in WT mice correlated significantly in seven regions which were exclusively observed in subcortical areas. In Snord115-KO mice, only the NAc and the vY maintained correlations between substrates and all other subcortical 5-HT correlations were lost. Of note, Snord115-KO mice gained two novel correlations, notably in the T as illustrated by the correlation of Pearson shown in Fig. 4C, bottom. Opposite trends in correlations for the direct and indirect markers of the DA and 5-HT metabolisms led us to study the possible correlations between DA and 5-HT

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transmitters and metabolites within a brain region in Snord115-KO versus WT mice (Fig. 4D), acknowledging that the catabolic pathways for DA and 5-HT share some cells and enzymes (35,36). We found some correlations between DA and 5-HT parameters (for instance DA versus 5-HIAA or HVA versus 5-HIAA). These cross-system correlations were slightly less numerous compared to the number of correlations for the substrate versus its product except for HVA versus 5-HIAA in WT mice. The number of these correlations was diminished in Snord115-KO mice, congruent with opposite trends in the DA and 5-HT systems. Intriguingly, the correlations in OF, SN and DL reported in WT mice were almost lost in Snord115-KO mice whereas single correlations were gained in the DM striatum, PL and aCg cortices, T, AM and dH.

The second analysis addresses the correlations of one monoamine, metabolite or the ratio between brain regions to determine whether the *Snord115* deletion impacts the organization of monoaminergic systems across the brain by changing the correlation maps. As summarized in correlograms presented in Fig. 5, brain regions in Snord115-KO mice showed an increase in subcortical correlations for DA content as compared to WT mice (from 2 to 6), with an equivalent number of positive and negative correlations. Similarly, the number of correlations for DOPAC was increased in Snord15-KO mice (from 4 to 7), particularly in cortical regions OF (gained three positive correlations: DL, AM and SN) and aCg (gained one positive correlation: dH) (data not shown). Inversely, HVA lost correlations in Snord115-KO mice (WT 6, SNORD 4); all cortical correlations were lost (data not shown). The number of correlations was constant for DOPAC/DA between WT and knockout mice, but the pattern was totally different (Fig. 5). Conversely, an overall loss in 5-HT correlations was reported in Snord115-KO mice (2 relative to 4), and the remaining positive correlations were distinct from WT. The inter-regional correlations (8 relative to 6) of the metabolite 5-HIAA were increased in Snord115-KO mice, both in cortical (particularly the aCg) and subcortical (dH and AM) regions (data not shown). The correlations of the 5-HIAA/5-HT ratio increased in Snord115-KO mice (7 relative to 5), negative cortical correlations (in the PL and aCg cortices) and positive striatal (DM and DL) correlations were gained (Fig. 5). Altogether, our quantitative and correlative biochemical analyses unveil subtle remodeling of monoaminergic systems, notably the decrease in HVA level in prefrontal areas, and a trend to establish additional correlations between brain regions for DA and NA.

Increased basal firing rate of dopaminergic and serotonin neurons in Snord115-KO mice

The question then arises as to whether the abovementioned qualitative reorganization of monoaminergic networks across brain regions is accompanied by changes in neuronal functional activities. Using anesthetized mice, we then investigated this possibility by performing *in vivo* single-cell extracellular recordings of DRN 5-HT and VTA DA neurons at the level of their cell bodies. Such *in vivo* electrophysiological studies are particularly well adapted since they interrogate neuronal activities in biological settings where long-range connectivity across brain regions is preserved, notably functional interactions between 5-HT and DA systems (37,38). As reported in Figs 6A–C, we first recorded DRN 5-HT neurons which could be distinguished by their spike trains typical properties (i.e. slow and regular firing rate; (39)). Remarkably, Snord115-KO mice exhibited significant higher spontaneous firing rates of DRN 5-HT neurons (+55%), as compared to WT littermates (Fig. 6A). The specificity of these alterations was further confirmed by showing that electrophysiological proprieties of DRN 5-HT neurons in Snord115-KO mice bearing a maternally inherited deletion (i.e. *Snord115* is normally expressed) were similar to those observed in WT littermates (+5%; Fig. 6B). When basal firing of VTA DA neurons was monitored, according to their bursting activity and their triphasic action potentials (40), we found that Snord115-KO mice having a paternally, but not maternally transmitted, deletion displayed significant increased activities (+40%), as compared to WT littermates (Figs 6D–F). Based on these *in vivo* recordings, we conclude that genetic ablation of *Snord115* genes impacts—directly or indirectly—the excitabilities of DRN and VTA neuronal circuitries.

Lack of Snord115 does not influence reward behaviors

To fully appreciate the physiological consequences that might result from changes in the reorganization of monoaminergic networks and/or altered neuronal activities, a series of behavioral tests were performed. We first measured the reward response of cocaine by employing conditioned place preference (CPP) that relies on DA and 5-HT transmissions including HTR2C signaling (41–43). The conditioning sessions consists of 4 consecutive days (two daily sessions at 4 h intervals) during which i.p. injections of the rewarding drug (cocaine at 5 mg/kg or 20 mg/kg) or vehicle (0.9% saline solution) were repeatedly associated with two different chambers (Fig. 7A, top). On the fifth day, time spent in each chamber was recorded over a 20-min period and a CPP is observed if the time spent in the cocaine-paired chamber is longer than that of in the vehicle-paired chamber. Results are presented in Fig. 7A, bottom. During habituation (Day 0), mice explored equally the two chambers. During the test session (Day 5), and as expected, both WT and Snord115-KO mice spent significantly more time in the cocaine-paired compartment at either 5 or 20 mg/kg. The CPP response of Snord115-KO mice was, however, in the normal range compared to WT littermates. Increased locomotor activity in Snord115-KO mice after cocaine treatments, as measured during conditioning, did not differ from the one observed in WT littermates, whether at 5 or 20 mg/kg (Fig. 7B). Thus, we consider

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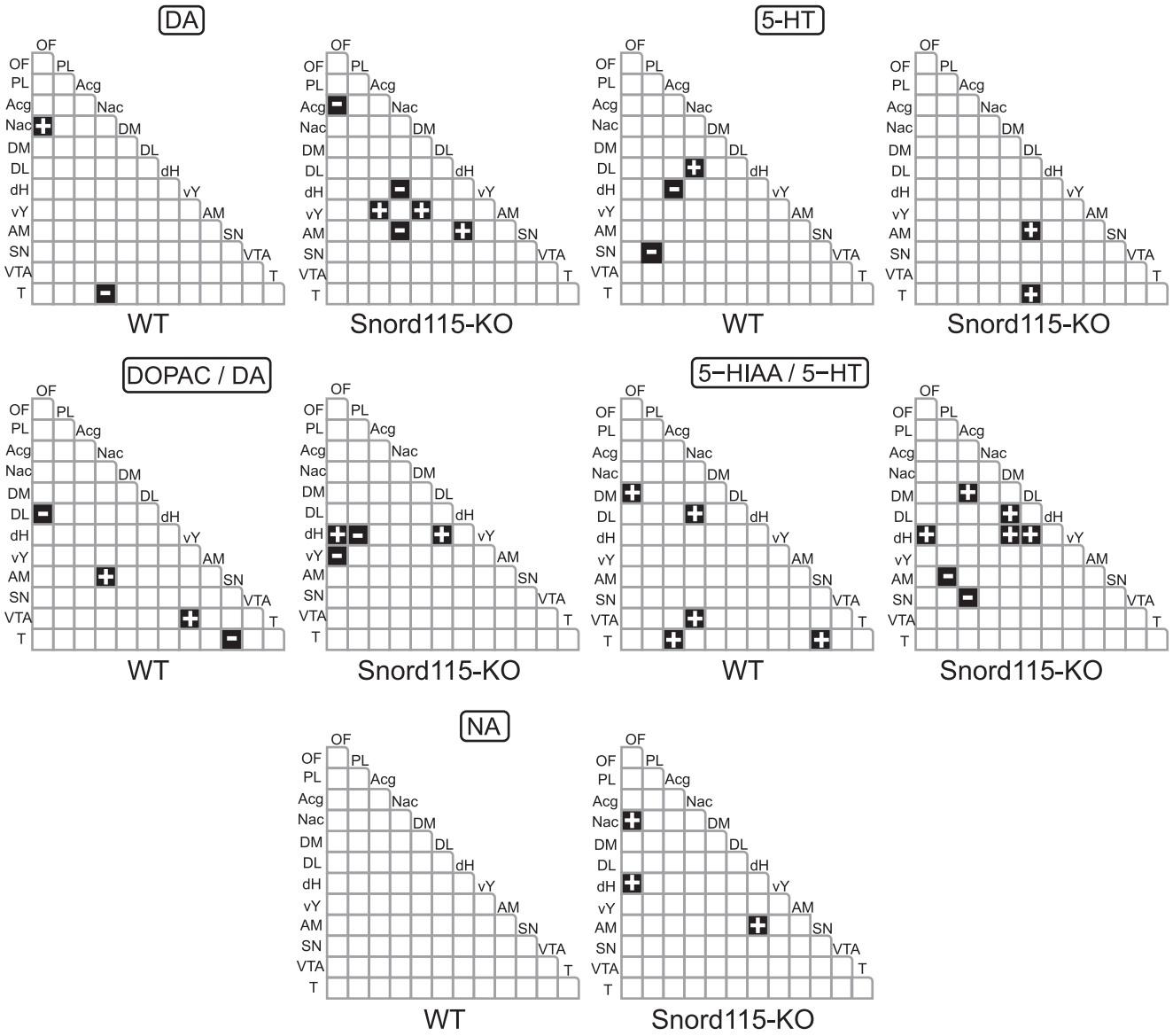

Figure 5. Correlograms of DA and the ratio DOPAC/DA, 5-HT and the ratio 5-HIAA/5-HT, and NA across the sampled brain regions. Only significant correlations ($P < 0.05$) are shown with + and − inside box indicating positive and negative correlations, respectively.

that Snord115-KO mice behave normally after repeated exposure to cocaine.

Lack of Snord115 does not impact on binge eating

We then examined binge-like eating for which a role of HTR2C on VTA DA neurons was recently established (44). Two cohorts of Snord115-KO mice and WT littermates were subjected to two types of diets, as depicted in Fig. 7C, top. Cohort 1 had free access to regular chow (CD) or high-fat diet (HFD) pellets during the entire experiment ('continuous mice') while cohort 2 had access to CD and HFD pellets for only 24 h (on Monday) and for the rest of the week, they were given chow diet ('intermittent mice'). The amount of HFD versus CD ingested was then monitored once a week (each Monday) during a short period of time (2.5 h), and this procedure was repeated for 4 consecutive weeks (4 binge cycles). As exemplified for cycle n1, mice from both cohorts showed a net preference for palatable HFD as compared with CD (Fig. 7C-middle). Moreover, and in agreement with prior studies (44), HFD intake was markedly increased in 'intermittent' as compared with 'continuous' mice (an increase by a ∼3–4 factor). Since excessive caloric intake persisted for at least 4 cycles without any sign of habituation (Fig. 7C, bottom), we concluded that such intermittent HFD administration elicited robust binge consumption. Under these experimental settings, the feeding behavior of Snord115-KO mice was, however, in the normal range as compared with WT littermates (Fig. 7C, bottom). Thus, the absence of Snord115 has no apparent impact on compulsive eating or on attraction to palatable foods.

Lack of Snord115 does not alter compulsive behavior

Altered compulsive behaviors were previously noticed in mice lacking the htr2c receptor (45) and drugs

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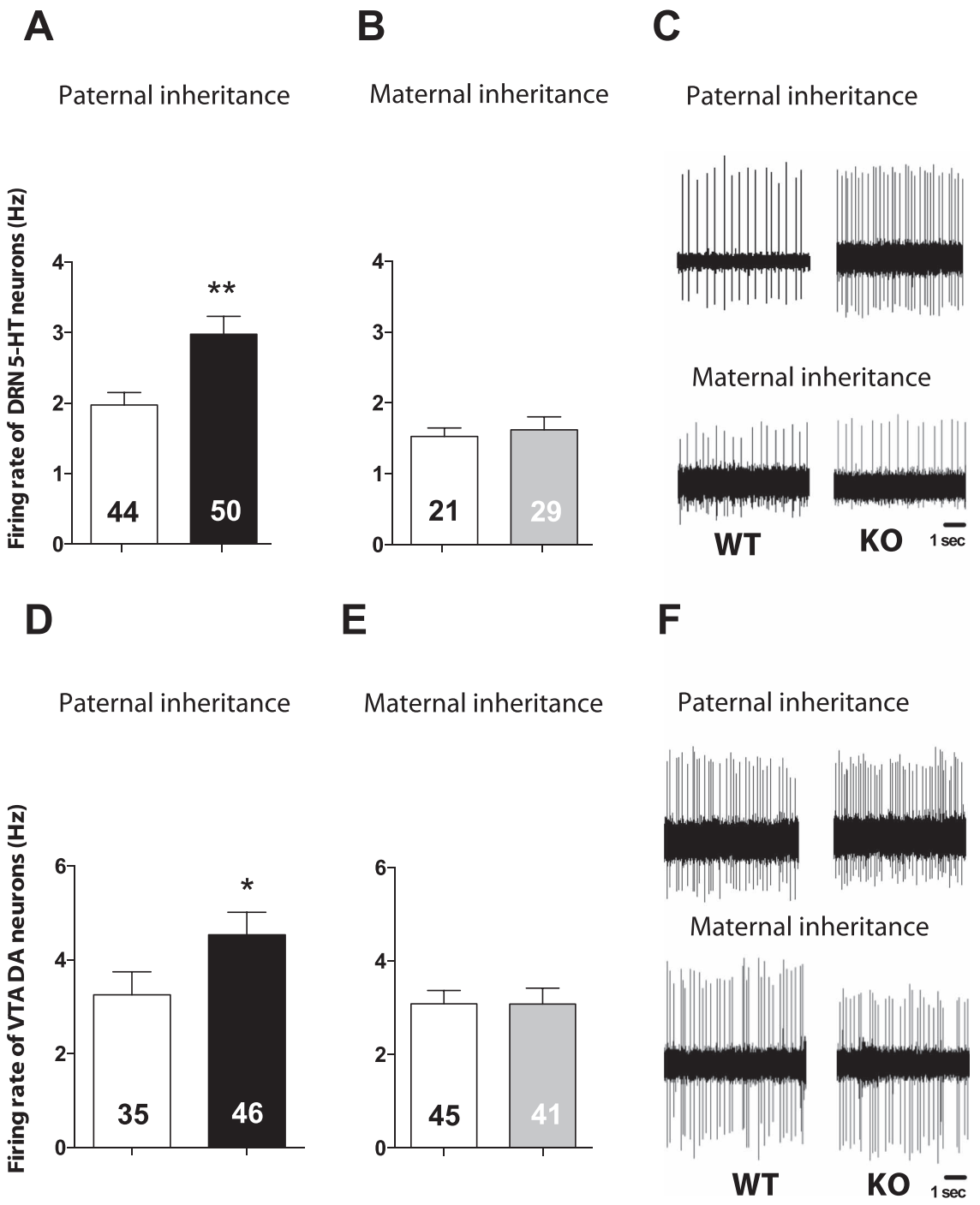

Figure 6. Snord115-deficient mice exhibit altered excitabilities of DRN and VTA neuronal circuitries. Neuronal firing (frequency, Hz) of 5-HT and DA neurons recorded in the DRN (**A** and **B**) and the VTA (**D** and **E**) of WT littermates (white) or genetically modified mice with a paternally inherited (black) or maternally inherited (grey) deletions. (**C–F**) Examples of typical recordings obtained in each group are shown. Note that *in vivo* extracellular unit recording assesses neuronal discharge (whether a neuron fires an action potential or not), but it does not provide any reliable information regarding the amplitude of the recorded action potential. Indeed, this latter largely depends on various parameters, among others the distance between the electrode tip and recording soma, which are technically impossible to control during *in vivo* extracellular recordings. Results are shown as mean ± SEM and the number of neurons recorded for each genotype is indicated within histograms. *$P < 0.05$; **$P < 0.01$ (two-tailed Student's *t*-test). Recordings of DRN 5-HT neurons were performed with 3–6 months old female mice (WTpat ($n = 6$), KOpat ($n = 7$), WTmat ($n = 2$), KOmat ($n = 3$)), while recording of VTA DA neurons was performed with 3–6 months old male mice (WTpat ($n = 8$), KOpat ($n = 8$), WTmat ($n = 5$), KOmat ($n = 7$)).

targeting serotoninergic or dopaminergic systems affect the behavior of rodents when examined by the Marble-burying test commonly employed to screen obsessive–compulsive-like phenotypes (46). This prompted us to examine the repetitive behavior of Snord115-KO mice using the Marble-burying test that assesses the naturally occurring burying behavior of rodents when they are in the presence of objects such as marbles, i.e. the greater the number of buried marbles, the more likely it is that the animal is committed in compulsive behavior.

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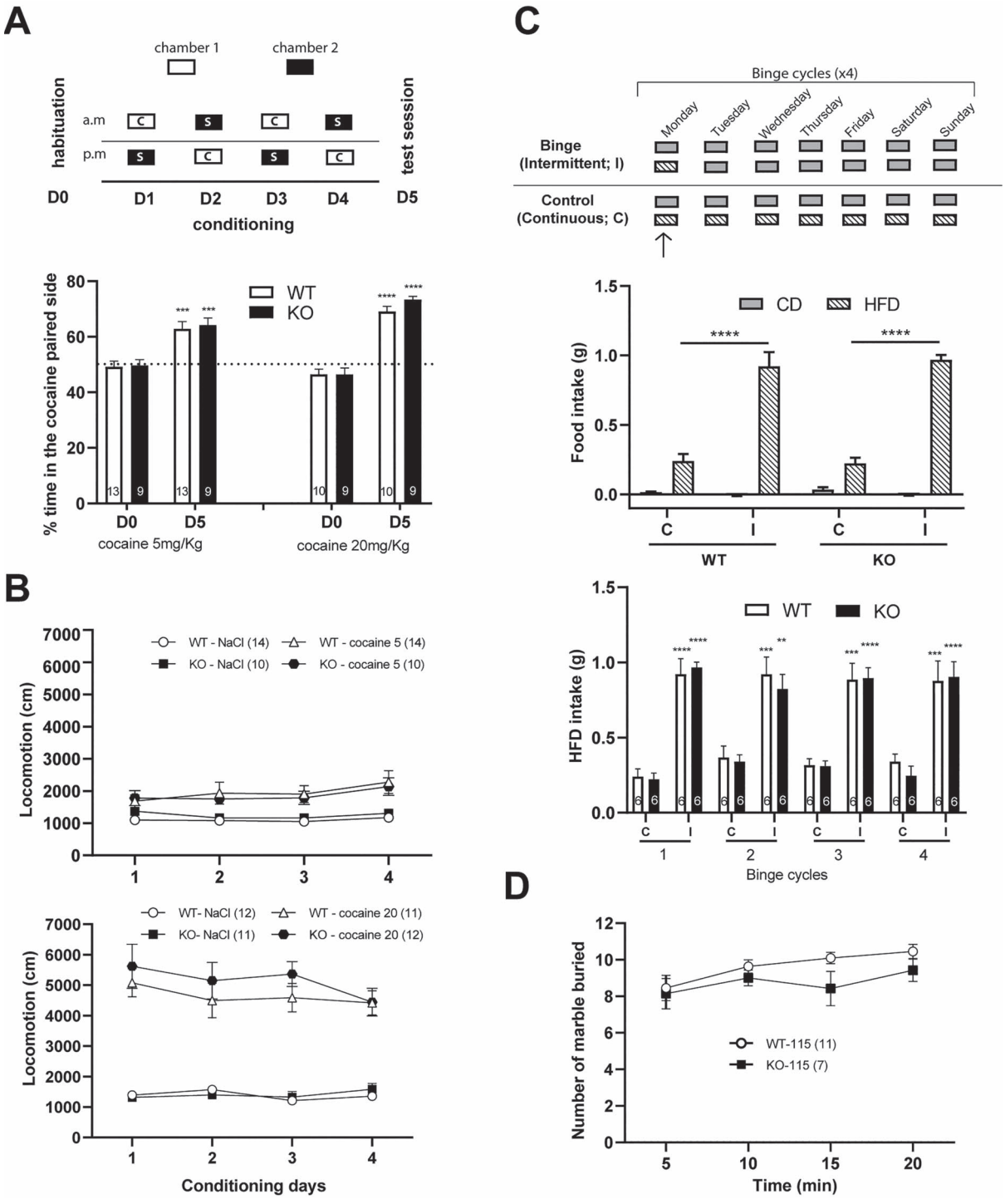

Figure 7. Reward-related and repetitive-like phenotypes remain unaffected in male Snord115-KO mice. (**A**) CPP for cocaine. Top: Experimental procedure for acquisition of CPP to cocaine. Half of the mice were conditioned with the reciprocal pair association, namely saline solution (S) and cocaine (C) administration (20 or 5 mg/kg) were associated with chambers 1 and 2, respectively (not shown). Bottom: Motivation for obtaining cocaine remains unchanged in 3–4-months-old Snord115-deficient (black), as compared with WT mice (white). Dashed line represents the 50% chance value. (**B**) Cocaine-induced hyperlocomotion. Exposure to cocaine at low (top) or high (bottom) concentration does not affect spontaneous locomotion of 3–4-months-old Snord115-deficient (black) as compared with WT mice (white). Due to video tracking issues, two mice had to be removed from the analysis: one cocaine-treated Snord115-KO (20 mg/kg) and one vehicle-treated WT mice. (**C**) Binge-like eating phenotype. Top: Experimental procedure for assessing binge-like eating phenotype. Two groups of 4–5-months-old Snord115-KO and WT mice were exposed to two types of diet plans. 'Intermittent (I) mice' were exposed to both chow (gray) and HFD (hatched) pellets from Monday (11:00 am) to Tuesday (11:00 am) and they were then exposed to only chow pellets for the rest of the week. This procedure was repeated for 4 consecutive weeks defining four binge cycles. As controls, 'Continuous (C) mice' were exposed to both chow and HFD pellets for the entire study. HFD and chow intake was measured each Monday for 2.5 h (as denoted by a short arrow). Middle: HFD and CD intake of Snord115-deficient and WT mice during the binge cycle 1 after exposure to continuous (C) or intermittent (I) diets. Bottom: HFD intake of Snord115-KO (black) and WT (white) mice after exposure to continuous (C) or intermittent (I) diets. Four consecutive binge cycles are shown. (**D**) Marble-burying behavior. The number of marbles buried by 6-months-old Snord115-KO (black) and WT (white) mice over a 20-min period. (A–D) Results are shown as mean ± SEM and the numbers of mice analyzed are indicated within histograms or into brackets. ***$P < 0.001$ and ****$P < 0.0001$.

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As shown in Fig. 7D, no significant difference in the numbers of marbles buried was found between the two genotypes over a 20 min period. Thus, digging repetitive-like behaviors remain unaffected in Snord115-KO mice.

Discussion

Monoaminergic systems broadly act in the brain and play a role in the regulation of adaptive behaviors. Whether the deletion of the *Snord115* genes could alter monoaminergic neuron function and ultimately related behaviors was the goal of the present study. The results show that *Snord115* genes are widely expressed in monoaminergic nuclei of the mesencephalon including DA and 5-HT neurons. Its constitutive targeted deletion leads to subtle modifications of monoamine neurochemistry, likely reflecting a lower DA activity in frontal areas associated with an increased activity of mesencephalic VTA DA and DRN 5-HT neurons. Despite this apparent remodeling of monoaminergic systems, behaviors of male Snord115-KO mice examined so far were seemingly normal.

Toward a low dopaminergic, cortical tone in Snord115-deleted mice

We report a neurochemical database of tissue monoamines and metabolites that highlights the heterogeneity of monoamines quantities across brain regions in ranges that are compatible with previous data in rodents (27,33). This comprehensive analysis reveals that deleting *Snord115* genes subtly alters the monoamine quantities in sampled brain areas. Yet, a decrease in 5-HT content was reported in the vY, but it was not associated with a decrease in 5-HIAA content. This latter finding rather suggests a change in the biochemical activity of 5-HT terminals, also reported at the level of the thalamus with the 5-HIAA/5-HT ratio.

Interestingly, we also noticed decreased HVA content in the OF and the PL cortices. Tissue content of HVA in the cortex or other brain regions poorly innervated by DA fibers could cautiously be used as an indirect index of DA transmission (47). Indeed, at variance with areas densely innervated by DA fibers like the striatum, the clearance of DA in the cortex/hippocampus involves the noradrenaline transporter (NET) (48–50) and the COMT located outside DA terminals (51–53). The decrease in tissue HVA in the cortex of Snord115-deficient mice is strongly suggestive of a lower DA tone at rest in the cortex of Snord115-deleted mice.

The neurochemical database offers the possibility to address qualitative changes of monoamines and metabolism using multiple correlative analyses across the brain (27,34). The number of correlations between DA or 5-HT and the respective metabolites in single brain regions were in line with previous studies in WT mice (26,33), but this number was increased and decreased for the DA and 5-HT systems in Snord115-deleted mice, respectively. This result suggests a modest functional reorganization of the central DA and 5-HT systems in an opposite manner. Although the relationship between a neurotransmitter and its metabolites in a single brain region is intuitive, the correlations are not always significant which might be due to the complexity of monoamines metabolism (54). DA metabolism indices correlated with 5-HT metabolism markers in some regions, especially in the striatum, suggesting that DA and 5-HT metabolic pathways are somehow intertwined. It has been reported that an increase in striatal 5-HT concentration occurred in the terminals of DA neurons in MAOA-deficient mice (55). It is also interesting that the number of correlations between DA and its products DOPAC and HVA increased in Snord115-deficient mice while the correlations between 5-HT and its metabolite 5-HIAA decreased in subcortical areas, supporting the idea of the interference of both monoaminergic metabolic pathways with each other in a single brain region. In structures that are poorly innervated with DA projections such as Th, dHP and the cortices, the correlations between DA and its metabolites found are few to none in WT mice while knockout mice expressed correlations notably in dHP and Th. These regions also have high tissue contents of NA compared with DA (Table 2) suggesting the influence of NA metabolism on DA marker concentrations (56–60).

Toward the activation of monoaminergic neurons in Snord115-deleted mice

A decrease in cortical DA tone in Snord115-KO mice could have important consequences on subcortical monoaminergic functions, as it is acknowledged that DA tone in the cortex inhibits subcortical DA and 5-HT neuronal activities (61–63). In line with this possibility, the electrical activity of VTA DA and DRN 5-HT neurons was increased in Snord115-deleted mice. The apparent dissociation between the electrophysiological data and the post-mortem biochemical results is not so surprising as the electrical activity has a weak incidence on the *ex vivo* tissue content of monoamines at terminal regions unless strong phasic stimulation is applied on monoaminergic cells (28,30,64).

Whether some of the reported changes in neuronal activities are directly due to impaired HTR2C signaling is an attractive hypothesis. Although the loss of *Snord115* genes had apparently only marginal effects on post-transcriptional regulation of Htr2c pre-mRNA, including the striatum, the DRN or the prefrontal cortex (15), it is tempting to surmise that reduced activity of HTR2C may account for some of the neurochemical results and increased firing rates of VTA DA and DRN 5-HT neurons. Indeed, opposite to what we observed in Snord115-deleted mice, the stimulation of HTR2C receptors increased HVA content in the prefrontal cortex (65) and reduced the correlations of the tissue content for DA across brain regions (31,32,65). In regard to the electrophysiological data, numerous pharmacological studies reported that activation of HRT2C, presumably those expressed on GABAergic interneurons, inhibits the

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firing rates of VTA DA and DRN 5-HT neurons (24,66–74). Such an effect is often accompanied by a decrease in *in vivo* extracellular levels of neurotransmitters at terminals, but it is not always the case (22,32,75,76). Conversely, the administration of HTR2C antagonists may enhance DA neuron electrical activity and DA release at terminal regions of DA neurons (22,70,75,77), whereas data regarding their effect on 5-HT neurons is less evident, often reporting no effect (75). More sophisticated studies are now required to formally demonstrate whether changes in excitability of VTA and DRN neurons are causally linked to defective htr2c signaling in GABAergic interneurons. Given the broad expression profile of *Snord115*, they might also arise from cell-autonomous defects in DA and 5-HT neurons, or even result from defects in other still unidentified Snord115-expressing cells. Because the expression profile of *Snord115* overwhelms the classical distribution of Htr2c mRNA, its deletion might also alter additional unsuspected processes beyond its predicted regulatory roles in modulating htr2c response. Finally, and in light of the functional interactions between the VTA and DRN, the increased firing of 5-HT neurons in Snord115-KO mice could be a consequence of the high firing rate of DA neurons. It is well documented that D2 receptors are expressed in the DRN and that their activation depolarizes 5-HT neurons (78,79).

Interpreting minimal phenotypes of SNORD115-deficient mice

Our study spotlights the hypothesis that lack of Snord115 remodels slightly monoaminergic function in the brain, yet it does not highlight a specific functional domain in which Snord115-KO mice could be strongly defective: the cocaine-induced reward response and locomotor hyperactivity, binge-like eating and repetitive digging behaviors remained unaffected. Given that these behaviors are regulated by 5-HT, sometimes in an opposite manner compared to DA (80), net changes in behavioral responses are likely to be meager because the 5-HT neurons also display higher activity. Despite higher rates of neuronal discharges, adaptive mechanisms in Snord115-KO mice (e.g. reuptake, degradation of neurotransmitters and changes of receptors) may also attenuate physiological consequences at the organism level. Thus, defects in behavioral responses of Snord115-KO mice could only be apparent under specific circumstances, perhaps depending on cortico-subcortical crosstalk. It is also worth mentioning that dopaminergic and serotoninergic dysfunctions have already been described in other PWS mouse models lacking *Magel2* or Ndn genes (81–84). Thus, *Snord115* genes might have more detrimental defects when the other adjacent, paternally expressed, protein-coding genes are also defective, which is the case in the vast majority of PWS patients, and in the PWS-IC mouse for which impairments in HTR2C-mediated behaviors were attributed to lack of Snord115 (13,14,85). We believe that identifying such environmental contexts or sensitized genetic backgrounds represents one of the most exciting, yet challenging goals, to further the biology of SNORD115 including its putative contribution in PWS.

Materials and Methods

Mice housing and breeding

Animal procedures were approved by the University of Toulouse and CNRS Institutional Animal Care Committee (DAP2016061716367988 and DAP 2020073111298508). The animal housing facility met CNRS standards. Mice were housed in a temperature-controlled room with a 12 h light–dark cycler and access to water and food ad libitum, either regular chow diet or HFD diet (ssniff® DIO—60 kJ% fat (Lard)). Since *Snord115* is expressed only from the paternal chromosome (15), Snord115-KO mice and their WT littermate controls were generated by crossing heterozygous males with WT females (the normally active allele is deleted). For electrophysiology experiments, a batch of control mice were added by performing reciprocal crossing (heterozygous females × WT males) which generate genetically modified mice but whose *Snord115* expression is maintained (the normally silent allele is deleted). Behavioral analyses were conducted with males of 3–6 months of age (Snord115-KO versus WT littermates) we backcrossed to C57BL/6 J genetic background (F10-F15). Upon euthanasia, tissues were harvested, weighted and immediately snap frozen in liquid nitrogen and stored at −80°C.

Tissue collection of brain regions

Dissected brain of 5-month-old males (F11) were snap frozen in liquid nitrogen and stored at −80°C. Isolation of brain areas was performed in a frozen microtome; bilateral punches of discrete regions were selected using a magnifying glass, punched with an 800 μm stainless steel cannula and pooled in pre-weighed, 0.6 mL Eppendorf tubes (Fig. 3). The bilateral punches were taken from 12 distinct brain regions including the orbitofrontal cortex (OF), motor cortex (M2), the prelimbic cortex (PL), the anterior cingulate cortex (aCg), the dorsal part of hippocampus (dHP), the core of NAc core, two quadrants of the striatum—dorsomedial (DM) striatum (DMS), dorsolateral striatum (DLS), the thalamus (Th), the vY, the SN and the VTA. A camera was used to capture pictures of punches of all brain regions.

Tissue processing and neurochemical analysis

The day of the biochemical analysis, Eppendorf tubes containing the brain tissue of one region were retrieved from deep freezer and placed on ice. Tubes were brought next to the same scales used to weigh the empty tubes, wiped to remove ice or water and weighed (usually between 1 and 3 mg of tissue and did not differ between Snord115-KO and WT mice between all brain regions) (26). Tissues were homogenized in 100 μL of 0.1 N HClO4, sonicated and centrifuged at 13 000 rpm for 30 min at 4°C. Aliquots (10–20 μL) of the supernatants were injected

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into the high-pressure liquid chromatography (HPLC) system coupled with electrochemical detection (HPLC-ECD) without dilution in the mobile phase.

Chromatographic analysis

Tissue concentrations of monoamines and their metabolites were measured using two sensitive HPLC-ECD systems: one was a classical setup with a single cell for electrochemical detection, the second comprised two cells placed in series (see below). With the two systems, we were able to measure NA, DA, 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), DOPAC, HVA and 3-methoxytyramine (3-MT) whenever their concentration was above the limit of detection. With the second system corresponding to an upgrade of the chromatographic conditions, we were able to add the detection of both tryptophan (L-TRP) and tyrosine (L-TYR), as well as vanylmandelic acid (VMA) and 3-Methoxy-4-hydroxyphenylglycol (MHPG), two metabolites of NA. We also tested other compounds such as 5-hydroxytryptophan, adrenaline, L-DOPA or 3-O-methyldopa. These compounds could be separated from the other ones, but their endogenous levels were too low to be studied. Most brain regions were analyzed using the classical system with the exception of the aCg, OF and PL cortices. HPLC injections were performed blind of the experimental group to ensure no bias.

Samples were kept on ice after the centrifugation (series of 8 or 10 samples at maximum) and the supernatants were injected using a manual injector (Rheodyne 7725i, C.I.L.-Cluzeau, Sainte-Foy-La-Grande, France) into Equisil ODS (C18) HPLC column (150 × 4.6 mm, 5 μm; C.I.L.-Cluzeau) preceded by a Brownlee–Newgard precolumn (RP-8, 15 × 3.2 mm, 7 μm; C.I.L.-Cluzeau). The mobile phase, delivered at 1–1.4 mL/min flow rate, depending on the HPLC system (HPLC pump LC20-AD, Shimadzu, France) was as follows (in mM): 60 NaH2PO4, 0.1 disodium EDTA, and 2 octane sulfonic acid plus 7% methanol, approximately adjusted to pH 4 with orthophosphoric acid and filtered through a 0.22 mm Millipore filter. The temperature of the column was maintained at 40°C.

Detection of monoamines and metabolites was performed with a coulometric cell (cell 5011, ESA, Paris, France) coupled to a programmable detector (Coulochem II, ESA) (86). Each coulometric cell has two electrodes. In the classical HPLC-ECD system, the potentials of the electrodes were set at +350 and −270 mV. In the second system where two coulometric cells were in series (thus, four electrodes), potentials were set at +150, +300, +450 and + 600 mV (an example of this kind of setup is described in a recent publication; (54)). Output signals are transited via an interface (ULYSS, Toulouse) to a classic computer equipped with the software AZURE (Toulouse, France). The calibration curves for each compound on each system were performed once peaks corresponding to the different compounds of interest were clearly separated (final adjustments with sodium octyl sulfonate and phosphoric acid). Calibration curves were calculated using three concentrations and considered acceptable when the r^2 of the straight line reached 0.99. The electrochemical detector used allows for selecting the appropriate gain for the recorded current and for changing the gain during the chromatogram. A 5–10 nA/V gain was needed when the quantity of compounds in a given sample was low and correspondingly produced a small current upon oxidation (small height of the peak). It was used for measuring DA parameters in brain regions like the cortices, hippocampus and thalamus. A 1 μA/V gain was appropriate when the quantities of compounds were very large, as is commonly the situation for DA in the striatum (86). Thus, the programmed changes of gain during the course of a chromatogram allowed for selecting an appropriate gain for each compound and each brain region. Under these conditions, the sensitivity for NA, DA, 5-HT, DOPAC, HVA and 5-HIAA varied slightly between the systems used, but it approximately corresponded with 5, 2, 15, 15, 40 and 10 pg/20 μL, respectively, with a signal/noise ratio of 3:1. The limit of detection of 3-MT, VMA, MHPG, L-Tyr and L-Trp was poor (not calculated), but we could manage their analysis due to their high concentrations in some tissues. Standard solutions containing all the compounds of interest at 1 ng/10 μL were systematically injected each day before and after a series of samples.

In vivo electrophysiological recordings

Male and female mice were anesthetized with chloral hydrate (400 mg/kg, i.p.) and then placed in a stereotaxic apparatus. Single-unit recordings of serotonergic neurons located in the dorsal raphe nucleus (DRN) were performed using a glass micropipette (Stoelting, Wood Dale, IL, USA) prepared from a gravitational puller (Narishige, London, UK) and filled with a 2 M NaCl solution to reach an impedance ranging between 2.5 and 5 MΩ. Recording micropipettes were dipped into the DRN of female mice (between 3 and 6 months of age, F2-F5) using the following coordinates: 0.2–0.5 mm posterior to the interaural line on the midline and lowered at 3.5 mm from the brain surface. Serotonergic neurons were characterized using two main criteria: a regular firing rate (0.5–2.5 Hz) and a long-duration positive action potential. Several tracks were performed to measure the average discharge frequency of DRN 5-HT neurons and the number of neurons recorded per track. Regarding the recording of dopaminergic neurons, micropipettes were dipped into the VTA of male mice (between 3 and 6 months of age, F11) using the following coordinates: −3 to −3.3 mm posterior, ±0.3 to 0.6 mm lateral and lowered at 4.1–4.6 mm from the brain surface. Dopaminergic neurons were identified according to the criteria previously described (Grace and Bunney, 1983). Briefly, DA neurons were identified from a typical triphasic action potential with a marked negative deflection; a characteristic long duration (2.5 ms) often with an inflection or 'notch' on the rising phase; and a slow spontaneous firing rate (2–10 Hz) with an irregular single

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spiking pattern and slow bursting activity (characterized by spike-amplitude decrement). The onset of a burst was defined as two spikes occurring <80 ms apart, with the termination of the spike being defined as two spikes occurring >160 ms apart.

RNA *in situ* hybridization and immunofluorescence staining on mouse brain tissues

Adult GAD67-eGFP (G42) transgenic mice (87) were transcardially perfused with 4% PFA solution and dissected brains were post-fixed for 48 h in 4% paraformaldehyde before being stored at 4°C in PBS 1X/30% sucrose/0.1% sodium azide. The fixed brain was coronally sectioned (Cryostat microtome; 20 μm) and sections were stored at −20°C in a cryoprotectant solution until use. Slices were first rinsed in PBS and then blocked for 15 min with 0.25% Triton, H_2O_2 10%, MetOH 10% in PBS. After three to four washes in PBS, slices were blocked in Normal Donkey serum (NDS 5%) in PBS for 60 min before overnight incubation at room temperature with rabbit anti-TH antibodies (AB152, 1/1000) or rabbit anti-TPH2 antibodies (Invitrogen PA1–778, 1/1000) or goat anti-GFP antibodies (Rockland, 1/2500). After two washes in PBST for 20 min, slices were incubated for 90 min at room temperature with Donkey anti-rabbit conjugated to Alexa Fluor®647 (Invitrogen A31573, Massachusettes, USA; 1/250) or Donkey anti-goat conjugated to Alexa Fluor® 488 (Lifetechonologies A11055, California, USA; 1/250). Slices were washed three times with PBS for 5 min before being subjected to pre-hybridization for 60 min at 37°C in PBS containing 15% formamide, 2XSSC, 10% dextran sulfate, 150 μg/mL yeast tRNA. Overnight hybridization at 37°C were carried out in 100 μL of hybridization buffer supplemented with a mixture of Cy3-labeled oligonucleotide probes (300 ng each): Snord115: Cy3-TTGAGCATGAATTTTATGTCATCACCTTTCTTCATGACAT T-Cy3; Intron_ 115 (1): Cy3-ATCACCTTTGTTCATGACATT GGGTTGTCATCATTGACCCAGTCCTAGA CAGAATT-Cy3; intron115 (2):Cy3-CTCCTGTGACCCCTCGCCCTGGTTGGG TCTCAGCG TAATCCTATTGAGCATG-Cy3; G1exon: Cy3-CTGTTATCTGTGTTTCTCTGCCTT CCAAG GTGGCATGTC-Cy3; G2exon: Cy3-TTGGTCACATGGAACTGGAGAGGGATT GCTGG GACGAAATGTCTTC-Cy3. The nucleolar (U3) marker was visualized with a mixture of two Cy5-labeled oligonucleotide probes (U3 (1): Cy5-AA CTTCTCTAG-TAACGAACTATA GAAATGATCCCTGAAAGTATAG; U3 (2): Cy5-CACCCTCCCAAAGGAGGGAAGAA CGATCATCAATG-GCAGGAGGCAG). Samples were then washed at room temperature in 15% formamide, 2XSSC (20 min, twice), 1XSSC (10 min) and mounted in Moviol DAPI (0.1 mg/mL). Images are obtained using a Retiga R6 CCD camera (TELEDYNE Qimaging, Canada) adapted to a wide-field microscope (DM6000 Leica, Wetzlar, Germany) with 5X (PL Fluostar 0.15 NA) and 40X (PL APO 0.85 NA) objectives, SEMROCK filters and a source (LED3 Leica, Wetzlar, Germany). Maximum intensity images from z-stacks were acquired with Metamorph software (Molecular Devices, California, USA) and images were merged using ImageJ.

Cocaine-induced CPP

The device consists of two chambers (each 16 × 15 × 25cm) which differ in the color and geometric shapes of the walls as well as the texture of the floor: Chamber 1 (floor circle pattern, wall with black and white strips) and Chamber 2 (floor square pattern, gray wall). During the familiarization period (Day 0, morning), male Snord115-KO and WT mice (3–4 months of age; F10-F11) have free access to the two chambers for 10 min via a removable door. During the habituation period or pre-test (Day 0, afternoon), mice are placed back in the device for 20 min and thanks to the video tracking system (Ethovision software (Noldus, USA)), the time spent in each chamber is recorded. This verifies that mice do not show a strong preference for one of the two chambers. During the conditioning period (D1-D4), each mouse was intraperitoneally injected (twice a day) by cocaine (SIGMA, C5776; 5 or 20 mg/kg) and saline (control) solution (0.9% NaCl) and then immediately placed in one chamber for 20 min. Through video tracking, the distance (cm) traveled by the injected mice is recorded. To avoid any bias, for each genotype, half mice were given cocaine in the morning and the other half in the afternoon. This conditioning procedure was done in chamber 1 and chamber 2 (Fig. 7A). During habituation (Day 0), mice are supposed to explore equally the two chambers. However, we noticed that 1 WT and 1 KO mouse for the group of cocaine (5 mg/kg) and 2 WT and 3 KO mice for the group cocaine (20 mg/kg) had a basal preference greater than 65% for one of the two compartments. In order not to introduce bias, these mice were removed from the analyses. During the test (D5), mice are introduced into the device with free access to the two chambers for 20 min. The time spent in each chamber is measured and the reinforcing effects of cocaine are inferred from an increase in the time spent in the cocaine-paired chamber.

Binge-like eating

Binge-like eating was mostly assessed as described in (44). After 1-week period of familiarization during which mice have been individualized in new cages (Lxhxl: 332 × 150 × 130 mm) with access to regular chow diet (SAFE A04 3339 kcal/g). Mice were then randomly assigned to either 'intermittent (binge)' or 'continuous chow/HFD (control)' cohorts. Male Snord115-KO and WT littermates (4–5 months of age, F14-F15) from 'Intermittent cohort' were submitted for 48 h (Monday–Tuesday) to both HFD (ssniff® DIO—60 kJ% fat (Lard); 5.15 kcal/g) and chow pellets (habituation). 'Continuous mice' were exposed to both chow and HFD for the entire study. 'Intermittent mice' were exposed to both chow and HFD on Monday at 11:am and starting from Tuesday at 11: am they had only access to chow diet for the rest of the week. Binge-like eating behavior of 'not intermittent' mice was assessed each Monday

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between 11:am and 1:30 pm. Food intake of 'Continuous mice' was determined at the same time as described for intermittent mice. This procedure was repeated during four consecutive weeks ($n = 4$ binge cycles).

Marble burying

This test related to the fact that rodents display burying behavior—also referred to as 'defensive burying'—and this digging behavior is thought to reflect highly repetitive perseverative phenotypes (88). Twelve glass marbles are placed on sawdust in the home-cage (4 rows of 3 marbles) and the number of marbles buried at 2/3 is measured every 5 min. Male Snord115-KO and WT mice (6 months of age; F15) were tested for 20 min.

Statistical data analysis

The tissue levels of monoamines (NA, DA and 5-HT) and respective metabolites were expressed in pg/mg of tissue weight. The indirect index of the turnover corresponding to the ratio between the metabolite and its parent neurotransmitter (DOPAC/DA and 5-HIAA/5-HT) was also calculated. For each brain region, the data were presented as the mean ± SEM of values. Aberrant data were discarded on the basis of the value outside the range of the average mean ± two standard deviations (27). Comparisons of two groups were analyzed using a two-tailed Student's unpaired *t*-test. Correlations were performed using Bravais-Pearson's *r* correlation test for the content of each monoamine, metabolite and the ratio ($n = 12$/group before outliers). The presented values for correlations were uncorrected for the *P*-values. Correlation was considered significant at the 5% level. For electrophysiology, action potentials (spikes) of DA and 5-HT neurons were detected using the spike sorting algorithm, with version 6.02 of Spike2 software (Cambridge Electronic Design, Cambridge, UK). Two-tailed Student's *t*-test was used to compare firing rates in Snord115-KO bearing a paternally (or maternally) inherited deletion versus their wild-type littermates. For CPP, the percentage of time spent in the cocaine-paired compartment was analyzed using a one-sample *t*-test (comparison to the 50% chance value). The effects of cocaine between the two genotypes were analyzed by a two-way ANOVA with genotype and time as factors. The effects of treatment on locomotion were analyzed by a three-way ANOVA with treatment, genotype and time as factors. For binge-like eating, a three-way ANOVA with genotype, diet and cohort as factors, followed by post-hoc Bonferroni was used to compare CD versus HFD food intake. Comparison between Continuous and Intermittent cohorts, WT and Snord115-KO mice at the same cycle was analyzed by a three-way ANOVA with genotype, cohort and cycle as factors, followed by post hoc Bonferroni. For the marble burying test, a two-way ANOVA was used with time and genotype as factors. Graphpad Prism (version 8) statistical software was used for behavioral data analysis. Data are presented as mean ± SEM and statistically significant differences are indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

Acknowledgements

We thank Lionel Mouledous and Lionel Dahan (CRCA-CBI, Toulouse), as well as Guillaume Canal, for critical readings of the manuscript. We are grateful to Fabrice Ango from the Institute for Neurosciences of Montpellier (INM) for the gift of adult brain samples dissected from Gad67-GFP mice (G42 line). The authors greatly acknowledge the Mouse Behavioral Core (MBC- CBI -ANEXPLO, Toulouse, France) for its expertise and assistance in setting up behavioral apparatus and procedures.

Conflict of Interest statement. The authors declare that they have no conflict of interest.

Funding

'Foundation pour la Recherche Médicale' (FRM; DEQ20160334936); Agence Nationale de la Recherche (ANR-18-CE12-0008-01); Foundation for Prader-Willi Research (FPWR); CBI intramural funding research grant (awarded to J.C. and B.G.).

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Summary Art II

Despite a lack of robust phenotypic alterations, and marginal effects on posttranscriptional regulation of 5-HT_{2C}R pre-mRNA, *Snord115* knockout animals' monoaminergic systems were distinct. Dopamine metabolite quantity was reduced in the orbitofrontal and prefrontal cortex of *Snord115* knockout mice. Additionally, the firing rate of VTA dopaminergic and DRN serotonergic neurons was increased in *Snord115* knockout mice. Both findings suggest a modification of monoaminergic systems insufficient to produce a distinct phenotype in *SNord115* knockout mice. The investigation of correlation between neurotransmitter quantity, either of metabolite and neurotransmitter within each region (Figure 4), or within neurotransmitters (or turnover ratios) between regions (Figure 5), was distinct for 5-HT, DA and NA systems. A caveat to this correlative approach was the lack of correlation density, out of 72 possible correlations no more than 6 (8.3%) were found to be significant for any condition or neurotransmitter. Prior to sacrifice these animals were in their home cage, given the nature of postmortem neurochemistry we are capturing the state of monoaminergic systems in this condition, at rest or unstimulated. We postulate that this may not be the optimal condition to assess monoaminergic differences in regional connectivity and that a stimulus to mobilise monoaminergic systems may serve to better asses altered regional neurochemical connectivity.

Art III (*submitted*) - $5HT_{2A}$ Receptors Structure Monoaminergic Connectivity in the male Mouse Brain

Questions and purpose of the study

Aiming to move toward a brain wide understanding of neurochemical systems, particularly in the context of psychedelics, postmortem quantification of monoamines and metabolites across 28 brain regions was performed. Prior to sacrifice a paradigm of forced exploration was invoked to mobilise neurotransmitter systems, to better revel alterations inducted by psychedelic TCB-2. This work additionally assesses the role of the 5-HT_{2A}R in the action of TCB-2 using 5-HT_{2A}R antagonist MDL-100,907. This paper fostered the application of graph theory to correlative neurochemical analysis, constructing networks reflecting the connectivity between brain regions both within and between monoaminergic systems.

Progress in Neuropsychopharmacology & Biological Psychiatry

5-HT2A Receptors Structure Monoaminergic Connectivity in the male Mouse Brain

--Manuscript Draft--

Manuscript Number:	
Article Type:	VSI: Psychedelic Research
Keywords:	psychedelic; correlations; MDL-100,907; neurochemistry; exploratory behaviour
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Abstract:	The mechanism of action of serotonergic psychedelics is being increasingly explored worldwide due to their clinical benefits in various psychiatric conditions. Beyond the stimulation of serotonin 2A (5-HT2A) receptors, psychedelics may desynchronize activity between brain regions, but the involvement of corresponding neurotransmission systems has been largely overlooked. Given that monoaminergic systems connect all brain regions and play a role in exploratory behaviour, we hypothesized that psychedelics disrupt the coherence of monoamines across brain regions during forced exploratory behaviour in mice. Using post-mortem tissue quantification of serotonin (5-HT), dopamine (DA), noradrenaline (NA) and their metabolites in 28 distinct brain regions, we report a dense and highly organized pattern of correlations within and between monoamines in vehicle-treated mice. This organisation was disrupted by both the psychedelic 5-HT2A receptor agonist TCB-2 (0.3, 3 and 10 mg/kg) and the antagonist MDL-100,907 (0.2 mg/kg), both decreasing correlations. The combination MDL-100,907 and TCB-2 partially restored some correlations. Quantitatively, TCB-2 dose-dependently decreased 5-HT turnover (metabolite/5-HT) across all brain regions and the ratio 3-methoxytyramine/DA in the striatum. TCB-2 also enhanced markers of the DA and NA systems in certain brain regions, notably the anterior cingulate cortex. MDL-100,907, which had minimal impact on monoamine levels by itself, reduced TCB-2 (3 mg/kg)-induced head twitches and monoamine effects in the anterior cingulate cortex but did not affect the TCB-2-induced decrease in 5-HT turnover across the brain. These data suggest that the functional connectivity of monoaminergic systems during exploration is highly sensitive to modulation by 5-HT2A receptor agonist and antagonist.
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Prof Louis Gendron
EiC Progress in Neuropsychopharmacology and Biological Psychiatry
University of Sherbrooke
Canada

Friday December 6th, 2024

Object : Submission of a research article to PNBP

Dear Professor Gendron,

We wish to publish a manuscript entitled "*5-HT$_{2A}$ receptors structure Monoaminergic Connectivity in the Male Mouse Brain*", in the special issue covering psychedelics in Progress in Neuropsychopharmacology and Biological Psychiatry. We hereby submit our work authored by Jasmine Jade Butler, Margherita Virgili, Giuseppe Di Giovanni, Abdeslam Chagraoui, Anna Beyeler, Philippe De Deurwaerdère.

We feel that the article is important because it offers another light on the ability of psychedelics to desynchronise the activity between brain regions. The desynchronization of brain regions reported in humans does not inform at all on neurotransmitters systems. Among neurotransmitters systems, monoaminergic neurons with serotonin (5-HT), dopamine (DA), and noradrenaline (NA) play a role in connecting different neurobiological networks. Our study investigates the mechanisms underlying the effects of serotonergic psychedelics on brain function in mice, with a focus on the interplay between 5-HT$_{2A}$ receptor (5-HT$_{2A}$R) activity and monoaminergic systems during exploratory behaviour.

This original study addresses the identification of a coherent organisation of monoamine systems during exploratory behaviour and its disruption by action on the 5-HT_{2A}R (not only the psychedelic, but also the antagonist). This study works to bridge the gap between brain wide monoaminergic networks and their modulation through the 5-HT_{2A}R, targeted by several clinically relevant compounds including antidepressants, atypical-antipsychotics, and psychedelics.

In terms of peer-reviewing if the paper passes the editorial expertise, our work belong both to neurochemistry of neurotransmitter systems in multiple brain regions, and to biological networks connectivity in brain. It is not developed in the current literature. So, we proposed some reviewers who could make the job to ameliorate the initial submission.

We look forward to hearing from you.

Sincerely,
Philippe De Deurwaerdère

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5-HT$_{2A}$ Receptors Structure Monoaminergic Connectivity in the male Mouse Brain

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Acknowledgments

We thank the support of the University of Bordeaux and the Centre de la Recherche Nationale et Scientifique and the Région Nouvelle-Aquitaine. We acknowledge the support of the 'Avenir program' of the French Institute of Health (INSERM), the 'Agence Nationale pour la Recherche' (ANR), the 'Cercle de la Fondation Schlumberger pour l'Education et la Recherche' (Cercle FSER), the 'Fondation pour la Recherche Médicale' (Equipe FRM) and the 'Fondation Bettencourt-Schueller' (impulscience 2023) to AB. We are grateful for the support of the Grand Programme de Recherche (GPR work package Adapsy) of the University of Bordeaux to PDD and AB. JJB was fellow recipient of PhD grant from the Ministère de la Recherche et de l'Enseignement Superieur, and received support from the EUR graduate School of Bordeaux Neurocampus.

Keywords

psychedelic, correlations, MDL-100,907, neurochemistry, exploratory behaviour

Abstract:

The mechanism of action of serotonergic psychedelics is being increasingly explored worldwide due to their clinical benefits in various psychiatric conditions. Beyond the stimulation of serotonin 2A (5-HT_{2A}) receptors, psychedelics may desynchronize activity between brain regions, but the involvement of corresponding neurotransmission systems has been largely overlooked. Given that monoaminergic systems connect all brain regions and play a role in exploratory behaviour, we hypothesized that psychedelics disrupt the coherence of monoamines across brain regions during forced exploratory behaviour in mice. Using post-mortem tissue quantification of serotonin (5-HT), dopamine (DA), noradrenaline (NA) and their metabolites in 28 distinct brain regions, we report a dense and highly organized pattern of correlations within and between monoamines in vehicle-treated mice. This organisation was disrupted by both the psychedelic 5-HT_{2A} receptor agonist TCB-2 (0.3, 3 and 10 mg/kg) and the antagonist MDL-100,907 (0.2 mg/kg), both decreasing correlations. The combination MDL-100,907 and TCB-2 partially restored some correlations. Quantitatively, TCB-2 dose-dependently decreased 5-HT turnover (metabolite/5-HT) across all brain regions and the ratio 3-methoxytyramine/DA in the striatum. TCB-2 also enhanced markers of the DA and NA systems in certain brain regions, notably the anterior cingulate cortex. MDL-100,907, which had minimal impact on monoamine levels by itself, reduced TCB-2 (3 mg/kg)-induced head twitches and monoamine effects in the anterior cingulate cortex but did not affect the TCB-2-induced decrease in 5-HT turnover across the brain. These data suggest that the functional connectivity of monoaminergic systems during exploration is highly sensitive to modulation by 5-HT_{2A} receptor agonist and antagonist.

Abbreviations:

OF (orbitofrontal cortex), PL (prelimbic cortex), aCC (anterior cingulate cortex), *IC (insular cortex)**, M1 (primary motor cortex), SJ (somatosensory cortex jaw), S1L/R (left/right primary somatosensory cortex), *S1L6L/R (left/right primary somatosensory cortex layer 6)*,* AC (auditory cortex), V1 (primary visual cortex), A (amygdala), dH/vH (dorsal/ventral hippocampus), NAc (nucleus accumbens), DM/DL (dorsomedial/dorsolateral striatum), VM/VL (ventromedial/ventrolateral striatum), MD (medial dorsal nucleus), VPL/R (left/right ventral posteromedial nucleus), DLG (lateral geniculate nucleus), Y (lateral hypothalamus), SC (superior colliculus), SN (substantia nigra), VTA (ventral tegmental area), DR (dorsal raphe nucleus), MR (median raphe nucleus), CB (cerebellum), TCB-2 (4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide), MDL-100,907 ((R)-(+)-α-(2,3-Dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperinemethanol, volinanserin), L-DOPA (L-3,4-dihydroxyphenylalanine), NA (noradrenaline), DOPAC (3,4-Dihydroxyphenylacetic acid), 5-HTP (5-hydroxytryptophan), DA (dopamine), 5-HIAA (5-hydroxyindoleacetic acid), HVA (homovanillic acid), 5-HT (5-hydroxytryptamine, serotonin), ** indicates regions that were excluded due to striatal contamination.*

1. Introduction

Classical serotonergic psychedelics profoundly alter cognition and perception (Nichols, 2004). The mind action of psychedelics is primarily attributed to activation of the serotonin 2A receptor (5-HT_{2A}R) in humans (Becker *et al.,* 2023; Vollenweider, 1998; Madsen *et al.,* 2019), approximated in mice with the head twitch response (HTR) (Halberstadt and Geyer, 2013, 2018; Nichols, 2016; Halberstadt *et al.*, 2020). A better understanding of their mechanisms is required in view of their clinical efficacy for depression, anxiety, and substance abuse disorders (Bogenschutz *et al.,* 2022; Carhart-Harris *et al.,* 2021, 2018; Goodwin *et al.,* 2022; Holze *et al.,* 2024; Palhano-Fontes *et al.,* 2019; Reckweg *et al.,* 2023). However, psychedelic action beyond 5-HT_{2A} receptor activation remains unknown.

Human and rodent studies report brain-wide alterations induced by psychedelics, often inferred trough functional extrapolation of gross indices of brain activity (Carhart-Harris *et al.,* 2012; Timmermanna *et al.,* 2022; Kwan *et al.,* 2022), and plasticity (Grieco *et al.,* 2022; Ly *et al.,* 2018). It has been recently proposed that psilocybin and LSD desynchronize functional connectivity between brain regions, within resting-state networks and oscillatory rhythms (Siegel *et al.*, 2024; Müller *et al.,* 2018; Carhart-Harris *et al.,* 2016; Muthukumaraswamy *et al.*, 2013). While the neurochemical systems involved in such desynchronization remain unknown, they likely include monoaminergic neurons (Butler *et al.*,2024), as serotonin (5-HT), dopamine (DA), and noradrenaline (NA) neurons diffusely and unevenly innervate the brain (Di Giovanni *et al.*, 2016). Psychedelics have been shown to alter the electrical activity of monoaminergic neurons (Di Gregorio *et al.*, 2016; Garratt *et al.*, 1991) and modulate the release of monoamines at terminal fields (Herian *et al.*, 2021; Wojtas *et al.,* 2022; Garratt *et al.*, 1991; Martin-Ruiz *et al.*, 2001). While these data remain unclear (Butler *et al.*, 2024), important insights into the actions of psychedelics may be gained by studying the function of monoaminergic systems through post-mortem tissue analysis of monoamine content across various brain regions, to inform network analyses (Fitoussi *et al*., 2013; Marty *et al.*, 2023; De Deurwaerdère *et al.*, 2020).

A ‘forced’ exploration protocol is a paradigm that has been used to study psychedelic compounds in rats and mice, in conjunction with antagonists implicating receptor involvement in specific behaviours (Chen *et al.,* 2023; Rodriguiz *et al.,* 2021). Psychedelics can alter exploratory behaviour, generally toward less organized paths with more frequent irregular direction changes (Odland *et al.,* 2022; Adams and Geyer, 1985, 1986). Exploration and novelty likely mobilise monoaminergic systems, particularly DA (Lisman and Grace, 2005; Düzel *et al.,* 2010) and NA neurons (Vankov *et al.*, 1995; Schomaker and Meeter, 2014). Acknowledging the importance of the environment and brain state on the psychedelic effect (Halberstadt and Geyer, 2018), a forced exploration protocol would induce a specific ‘exploratory’ brain state mobilising monoaminergic systems that could be altered by psychedelics.

To address monoaminergic modifications induced by 5-HT_{2A} receptor activation in mice, we used (4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide (TCB-2; 0.3, 3, 10 mg/kg), an agonist with sub-nanomolar affinity (Di Giovanni and De Deurwaerdere, 2018; Fox *et al.,* 2010; Takaba *et al.,* 2024), either alone or in the presence of the 5-HT_{2A} receptor antagonist (R)-(+)-α-(2,3-Dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperinemethanol (MDL-100,907; 0.2 mg/kg; Halberstadt *et al.,* 2015). The efficacy of compounds was verified using the established head twitch response (HTR). Forced exploratory behaviour was addressed 50 minutes after TCB-2 or vehicle administration, for 10 min, in which the Perspex floor was substituted for a textured floor prior to sacrifice (Figure 1A). Monoamine and metabolite content were quantified (Figure 1C) in 28 brain regions, including lateralisation for the ventral posteromedial nucleus and somatosensory cortex (Figure 1B), to report changes in tissue quantity and correlation across the mouse brain.

2. Materials and Methods

2.1. Animals

All experimental procedures in mice were carried out in accordance with the European Communities Council Directive (86/609/EEC + 2010/63/UE) and the Institutional Animal Care Committee of the University of Bordeaux. All efforts were made to minimize animal suffering and reduce the number of animals used.

9–11-week-old male C57BL6/J mice weighing 21.8-27.4g were used. Animals were kept in the animal facility (University of Bordeaux, France), with access to standard chow and water ad libitum, in a constant temperature (21 ± 2°C) and humidity (60%) under a 12h light/dark cycle.

2.2. Pharmacological treatment and experimental design

TCB-2 (4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide and MDL-100907 *((R)-(+)-α-(2,3-Dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperinemethanol)* were diluted the morning of experiments as free base in NaCl 0.9% and

Tween 0.3% in NaCl 0.9%, respectively, and stored on ice. MDL-100907 (0.2 mg/kg)/vehicle was injected i.p. (5 ml/kg), 15 minutes prior to TCB-2/vehicle i.p. (7.5 ml/kg). For each experimental group animals were pseudo-randomised (at least each treatment per day in a random order) and treatments were administered in a blinded fashion between 11 am and 2 pm (maximum 8 mice/day). The doses of TCB-2 were chosen to span from a behaviourally low active dose (0.3 mg/kg), active dose (3 mg/kg), and high dose (10 mg/kg) (Di Giovanni and De Deurwaerdère, 2018). The involvement of 5-HT_{2A} receptor was addressed with the 5-HT_{2A} receptor antagonist MDL-100,907 only on the effects triggered by the dose of 3 mg/kg TCB-2 (Di Giovanni and De Deurwaerdère, 2018). The solvent of MDL-100,907 was administered in controls, and in mice receiving 0.3 mg/kg or 10 mg/kg. The dose of MDL-100,907 was chosen with respect to a previous article reporting its ability to block head twitch (HT) at 0.1 mg/kg although in a limited amount of time (Halberstadt *et al., 2015*). Thus, we used 0.2 mg/kg to maximise 5-HT_{2A} receptor blockade at 1h15minutes, until sacrifice.

Following MDL-100,907 or vehicle administration mice were placed in an isolated chamber (33x33x25cm) for behavioural observation and injected with TCB-2 or vehicle 15 minutes later. Head twitch responses (HTR) were observed for 1 minute at 20-, 30-, 40- and 50-minutes post TCB-2 or vehicle. Following the last HTR measurement the Perspex floor of the observation chamber was switched for a novel 'textured' floor composed by 4 distinct textures: red felt, brown hessian, white sponge and orange microfiber chenille. Mice were exposed to this floor for the 10 minutes prior to sacrifice.

2.3. Tissue sampling and conditioning

Mice were sacrificed, cervical dislocation, 60 minutes after agonist/vehicle injection (see pharmacological treatment). The brain was immediately removed, placed in isopentane (-35 ± 5^{o}C) and stored in a freezer at -80^{o}C. Brains were then sliced in a cryostat at -24^{o}C, and bilateral 'punches' were taken encompassing various structures using a 800µm steel

cannula except for the median and dorsal raphe nuclei (one central punch encompassing the left and right parts). Punches were pooled between hemispheres apart from the primary somatosensory cortex (S1L, S1R) and ventral posteromedial nucleus (VPL, VPR). The selected regions belonged to cognitive, motor, limbic, sensory and vegetative systems. Punches were deposited in pre-weighed tubes and returned to -80°C storage. Samples were reweighed on the day of biochemical analysis to determine the weight of the tissue (in mg) and 100µl of perchloric acid ($HClO_4$ 0.1N, 4°C) added. By region, tissue weight did not vary between treatments (see table S3). Samples were then sonicated for approximately 7 seconds and centrifuged at 13,000rpm for 30 minutes at 4°C. 10-20µl of supernatant was injected directly into a high-pressure liquid chromatography (HPLC) system.

2.4. HPLC and electrochemical detection conditions

Tissue concentration of monoamines was measured by HPLC coupled to electrochemical detection. The mobile phase was composed of methanol (7%), NaH_2PO_4 (70mM), triethylamine (100µl/l), EDTA (0.1mM), sodium octyl sulphate (100mg/l) diluted in deionised water (pH 4.2, adjusted with orthophosphoric acid) (Marty *et al.,* 2023). It was filtered (0.22µm) prior to installation. The mobile phase was passed through the column (Equisil ODS C18, 150 x 4.6mm, particle size 5µm 120Å, C.I.L. Cluzeau, Sainte-Foy-La-Grande, France) at a flow rate of 1.2ml/min using an HPLC pump (LC-20AD, Schimadzu, France) to reach a coulometric detection cell (Cell 5014, ESA, Paris, France) with electrodes at +350mV (oxidation) and -270mV (reduction). Samples were injected using a manual injection valve (Rheodyne, model 7725i, C.I.L. Cluzeau) equipped with a 20µl loop. Coulometric cell was connected to detector (Coulochem II, ESA, Paris, France) linked to a computer with software for chromatogram acquisition (Azur 5.0, Alphasmos, France).

Once good chromatographic separation was achieved (no overlapping peaks), systems were calibrated using standard solutions of 0.25-1ng/10ul, adapted according to brain area.

Gain was set using a timeline on the detector ranging from 5nA to 1µA. Daily injection of standards preceded sample quantification to verify the chromatographic conditions. Sensitivity limit ranged from 0.7 pg/20µL (DA) to 11 pg/20µL (HVA) with a signal to noise ratio 3:1.

2.5 Statistical analysis

2.5.1 Normality and Outlier Testing

Tissue quantities of monoamines and related compounds were reported as the mean content +/-SD in ng/mg for each group and each brain region. Outliers were excluded using Grubbs' test, $p<0.05$. Data was considered parametric, as 94% of compound-region-treatment groups (1287 out of 1365 unique combinations) were normally distributed (Shapiro-Wilk, $p < 0.05$).

2.5.2 Quantitative Analysis

For both HTR and tissue levels analysis, statistical analysis was split to address the dose response and the interaction of TCB-2 and MDL-100907. For the dose response (vehicles group, 0.3, 3, 10 mg/kg TCB-2), we used a one-way ANOVA. In case of significance, it was followed by the post hoc Tukey's test. A two-way ANOVA was used to address the interaction between TCB-2 (3 mg/kg) and MDL-100,907. In case of significance, it was followed by the post hoc Tukey's test.

2.5.3 Correlation Analysis

Correlative analysis of monoamine tissue quantity in the investigated regions was done using the Pearson correlation coefficient, for either individual neurotransmitters or pairs of neurotransmitters. This analysis was conducted separately for each experimental group with significance set at $p<0.05$. Significant correlations comprise a correlation matrix that may be displayed as a correlogram or used to construct a network, where each brain region is a node and correlations the weighted edges between.

Working with networks allows for further quantification of patterns of correlation, starting with the density of the network, which represents the percentage of correlations (edges) relative to all possible connections. It also enables the identification of highly connected brain regions (hubs) based on node degree, the number of edges of a given node. Finally, degree distribution—specifically the standard deviation of node degrees—can provide insight into network organization. Networks with a higher standard deviation of node degree are more heterogeneous in connectivity, with both hubs (highly connected regions) and isolated or less connected regions. In contrast, lower standard deviations reflect more uniform connectivity, where a structure organized by regions is less apparent.

2.5.4 Data and Code availability

Quantitative data for each compound-region-treatment combination was compiled manually in excel and input into a custom Python code for neurochemical multivariate analysis currently being developed, code available upon request.

3. Results

3.1. Behaviour

TCB-2 increased HTR relative to vehicle-treated mice, with the largest increase seen after 3 mg/kg TCB-2 compared to 0.3 and 10 mg/kg doses. The effect peaked at 20 min post injection [t20: one-way ANOVA $F(3, 31)=44.36$, $p<0.01$] and progressively decreased until 50 min post injection [t50: $F(3, 31) = 4.70$, $p<0.01$; Figure 1A)]. Pretreatment with MDL-100,907 blocked the effect of 3 mg/kg TCB-2 at t20, t30 and t40 [two-way ANOVA $F(1, 36) = 28.26$, $p < 0.001$, for raw data and ANOVA's table S1 and S2)]. No HTR were observed following administration of MDL-100,907 alone.

3.2. Quantitative distribution of monoamines in selected brain regions

The tissue quantity of monoamines and their metabolites in vehicle-treated animals exposed to the textured floor is presented in Table 1 for each brain region. As expected (Fitoussi *et al.*, 2013; De Deurwaerdère *et al.*, 2020), tissue DA and its metabolites had a greater heterogeneity across brain regions, with the striatum exhibiting the highest concentrations. Elevated DA levels in the inner parts of the somatosensory (left and right), as well as the insular cortex were due to small fraction of striatal/accumbal tissue contamination (Fig 1B), leading to their exclusion from the study. In contrast, NA and 5-HT tissue contents were more homogenously distributed among the brain areas (Table 1).

In vehicle-treated mice, significant correlations of tissue contents were observed between some regions but not others (Figure 1D). Figure 1E illustrates the organisation of regions by structure, conserved in all correlograms, with the primary metabolite of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA) for vehicles-treated mice. Alternatively, the correlation matrix can be represented as a network using graph theory, where each region is a node, and significant correlations are represented as edges (Figure 1E). A network is a mathematical object consisting of system of elements, nodes, that are connected by edges (see 3.4. Correlative analysis).

3.3. Correlative analysis of monoamine tissue content within a monoamine system following TCB-2 and MDL-100,907 administration

Maintaining the axis organisation by region subclasses as shown in Figure 1E, Figure 2 illustrates the correlations within 5-HT, DA, and NA systems. Vehicle-treated animals exhibit distinct, predominantly positive correlation patterns between regional tissue concentrations of 5-HT, DA, and NA. Notably, the total number of correlations was reduced following administration of both TCB-2 and MDL-100,907 for each monoamine (table S4).

Table 1 – Monoamine tissue quantity in vehicle treated animals (mean +/- SD in ng/mg, n=6-11).

Region	5-HT	5-HIAA	DA	DOPAC	HVA	3MT	NA
OF	0.137 ± 0.071	0.148 ± 0.068	0.015 ± 0.007	0.037 ± 0.012	0.093 ± 0.046		0.240 ± 0.075
PL	0.097 ± 0.047	0.106 ± 0.046	0.019 ± 0.008	0.032 ± 0.011	0.082 ± 0.033		0.111 ± 0.069
aCC	0.052 ± 0.021	0.140 ± 0.059	0.016 ± 0.005	0.023 ± 0.010	0.056 ± 0.021		0.228 ± 0.055
M1	0.165 ± 0.075	0.034 ± 0.016	0.013 ± 0.004	0.006 ± 0.005	0.046 ± 0.018		0.171 ± 0.055
SJ	0.116 ± 0.076	0.136 ± 0.091	0.004 ± 0.004	0.047 ± 0.023	0.125 ± 0.063		0.084 ± 0.052
S1L	0.066 ± 0.027	0.095 ± 0.030	0.005 ± 0.003	0.006 ± 0.001	0.034 ± 0.009		0.205 ± 0.038
S1R	0.092 ± 0.051	0.129 ± 0.080	0.004 ± 0.002	0.006 ± 0.005	0.047 ± 0.031		0.272 ± 0.081
AC	0.070 ± 0.030	0.088 ± 0.044	0.009 ± 0.002	0.025 ± 0.022	0.033 ± 0.016		0.153 ± 0.051
V1	0.055 ± 0.035	0.056 ± 0.027	0.008 ± 0.004	0.002 ± 0.002	0.003 ± 0.003		0.187 ± 0.076
A	0.242 ± 0.097	0.317 ± 0.101	0.189 ± 0.108	0.163 ± 0.127	0.121 ± 0.055	0.015 ± 0.005	0.298 ± 0.069
dH	0.136 ± 0.053	0.241 ± 0.083	0.032 ± 0.034	0.012 ± 0.014	0.026 ± 0.012		0.131 ± 0.035
vH	0.236 ± 0.060	0.447 ± 0.085	0.019 ± 0.013	0.029 ± 0.015	0.025 ± 0.006		0.232 ± 0.042
NAc	0.129 ± 0.059	0.145 ± 0.063	1.717 ± 0.487	0.801 ± 0.278	0.505 ± 0.168	0.084 ± 0.028	0.094 ± 0.016
VM	0.266 ± 0.139	0.216 ± 0.118	3.342 ± 1.604	1.049 ± 0.480	0.782 ± 0.425	0.155 ± 0.076	0.220 ± 0.121
DM	0.175 ± 0.093	0.186 ± 0.094	3.977 ± 1.402	1.022 ± 0.300	0.660 ± 0.245	0.212 ± 0.067	0.139 ± 0.056
VL	0.259 ± 0.121	0.226 ± 0.102	7.328 ± 1.055	1.379 ± 0.448	1.396 ± 0.402	0.318 ± 0.059	0.179 ± 0.038
DL	0.165 ± 0.090	0.198 ± 0.104	4.787 ± 1.371	1.160 ± 0.413	1.000 ± 0.374	0.244 ± 0.073	0.084 ± 0.033
MD	0.150 ± 0.063	0.203 ± 0.100	0.055 ± 0.040	0.054 ± 0.028	0.054 ± 0.021		0.552 ± 0.224
VPL	0.111 ± 0.058	0.287 ± 0.130	0.006 ± 0.004	0.022 ± 0.014	0.047 ± 0.021		0.074 ± 0.043
VPR	0.069 ± 0.060	0.275 ± 0.153	0.007 ± 0.005	0.017 ± 0.011	0.048 ± 0.022		0.156 ± 0.053
DLG	0.134 ± 0.080	0.349 ± 0.253	0.005 ± 0.002	0.007 ± 0.005	0.026 ± 0.015		0.182 ± 0.090
HY	0.168 ± 0.124	0.231 ± 0.131	0.034 ± 0.030	0.019 ± 0.008	0.062 ± 0.028		0.302 ± 0.151
SC	0.183 ± 0.089	0.268 ± 0.046	0.042 ± 0.021	0.027 ± 0.012	0.017 ± 0.012		0.159 ± 0.057
SN	0.438 ± 0.151	0.259 ± 0.072	0.108 ± 0.058	0.078 ± 0.031	0.134 ± 0.035	0.008 ± 0.005	0.153 ± 0.031
VTA	0.228 ± 0.140	0.268 ± 0.180	0.283 ± 0.171	0.199 ± 0.096	0.143 ± 0.067	0.014 ± 0.005	0.238 ± 0.114
DR	0.675 ± 0.367	0.471 ± 0.260	0.052 ± 0.031	0.042 ± 0.016	0.066 ± 0.026		0.611 ± 0.227
MR	0.285 ± 0.170	0.316 ± 0.139	0.016 ± 0.012	0.029 ± 0.015	0.068 ± 0.030		0.254 ± 0.100
CB	0.059 ± 0.040	0.084 ± 0.040	0.035 ± 0.020	0.027 ± 0.011	0.025 ± 0.012		0.285 ± 0.101

3.2.1 Correlations of 5-HT tissue levels across brain regions

Vehicle-treated animals exhibited an unprecedented number of positive correlations for the 5-HT tissue content across brain regions (47.6%, 180 total). Highly correlated regions included striatal areas (22:VM; 18:NAc; 17:V; 13:DM; 11:DL), cortical regions (19:OF, aCC; 18:S1R; 17:M1), the medial dorsal nucleus (20:MD), and mesencephalic areas (16:SC; 14:VTA, MR; 7:DR). Notably, the ventral hippocampus and the substantia nigra showed no correlations in vehicle-treated animals. TCB-2 administration resulted in a dose-dependent reduction of 5-HT correlations, decreasing to 19.1% (72 total) at 0.3 mg/kg, 15.6% (59 total) at 3 mg/kg and 11.3% (47 total) at 10 mg/kg. This dose-dependent loss of correlations was particularly evident in the anterior cingulate cortex (aCC), which exhibited 19 correlations in vehicle-treated animals, compared to 10, 4 and 1 following 0.3, 3, and 10 mg/kg of TCB-2, respectively. While some striatal and fronto-cortical positive correlations remained at 0.3 (11:DL, OF, V1; 10:DM, VL, PL, aCC) or 3 (8:DL; 7:VL, DM OF; 6:PL) mg/kg TCB-2, negative correlations (-3:DR; -5:S1R) emerged at 3, which were absent at 10 mg/kg.

MDL-100,907 also reduced the number of the 5-HT tissue content correlations to 13% (45 total) compared to vehicle-treated animals. Cortical correlations were notably diminished, especially those involving sensory cortices (5:AC; 3:V1; 2:S1L; 0:S1R), in contrast to the orbitofrontal cortex which retained 9 correlations (9:OF). In MDL-100,907-treated animals, 5-HT tissue content in the aCC exhibited exclusively negative correlations (-3:aCC).

The correlation pattern observed with the combination MDL-100,907 and TCB-2 differed markedly from that of MDL-100,907 alone. It more closely resembles that of vehicle-treated animals with no negative correlations but with a reduced total correlation number (25.5%, 82 total). Notably, correlations involving mesencephalic regions were sparse (4:SC; 3:DR;

0:MR), but included the substantia nigra (4:SN). No correlations were observed involving the auditory cortex and aCC, or the nucleus accumbens.

3.2.2 DA tissue level correlations across the brain

In vehicle-treated animals, the number of correlations of the DA tissue content was elevated (17.2%, 65 total) compared to what has been shown in previous studies (Gros *et al.*, 2021; Fitoussi *et al.*, 2013), with a notable concentration in the prefrontal regions, striatum, and mesencephalon. The highest correlations were observed in the prelimbic cortex and aCC (11:PL; 10:aCC), as well as striatal regions (12:VM; 9:DM; 8:DL; 5:NAc; 3:VL). Surprisingly, the DA content of the substantia nigra showed only negative correlations with other regions (-9:SN).

TCB-2 dose-dependently reduced the total number of DA content correlations to 10% (38 total), 6.6% (25) and 5% (19) at 0.3, 3 and 10 mg/kg, respectively. The loss of correlations in striatal areas (9:DM; 3:VL; 2:VM, NAc; 1:DL), and the aCC (4:aCC) observed at 0.3 mg/kg TCB-2, were less pronounced that those obtained following administration of 3 (-3:VL; 2:DL; 1:PL, -1:aCC; 0:VM, DM, NAc) or 10 mg/kg (2:DL; 1:aCC, PL, VM, NAc; 0:DM, VL).

MDL-100,907 alone reduced the correlation density of DA tissue contents (6.6%, 25 total), particularly in striatal regions (3:NAc, VM; 2:VL; 1;DM, DL), prelimbic cortex and aCC (2:PL; -1:aCC), and mesencephalic regions (see Figure S1). In contrast, motor and sensory cortices showed an increase in correlations (2:M1, S1L 4; SJ; 1:S1R; Figure S1). When MDL-100,907 was combined with TCB-2, the correlation profile of DA tissue content was distinct from either treatment alone, showing a higher number of correlations (8.2%, 31 total). Few mesencephalic or striatal correlations were present (4:VM; 2:DM, DL; 0:VL, NAc), while diencephalic (5:DLG, VPR; 4:HY; 1:VPL), and cortical (8:PL; 7:S1L; 5:M1; 2:OF, SJ, S1R; 1:aCC) regions showed increased correlations.

3.2.3 NA tissue level correlations across the brain

NA tissue level correlations reached 22.2% (84 total) in vehicles-treated animals and were highly organized, primarily involving cortical regions (15:OF; 13:SJ, V1; 11:AC; 10:aCC; 9:PL; 8:M1), ventromedial striatum (13:VM), diencephalon (11:DLG; 5:VPR; 3:VPL), and mesencephalon (10:VTA; 8:MR; 6:DR; 3:SC).

In contrast to DA and 5-HT, the greatest loss of correlations relative to vehicle-treated animals was seen at 0.3 mg/kg TCB-2 (5.8%, 22 total), compared to 3 mg/kg (7.9%, 30 total) and 10 mg/kg (7.1%, 27 total) TCB-2. Negative correlations involving cortical regions were observed without a clear organisation.

MDL-100,907 alone reduced the number of NA tissue content correlations to 6.8% (26 total, 11 negative correlations). The few correlations observed were mostly within cortical (5:OF, PL) and diencephalic (4:DLG; 3:VPL, HY) regions. However, the number of correlations increased in the combination MDL-100,907/TCB-2, which displayed a unique pattern of correlations compared to either compound alone. Diencephalic regions correlated within themselves (10:DLG; 6:VPL; 4:VPR) and with several cortical regions (8:PL; 7:M1; 6:SJ; 4:OF, S1R).

3.4. Correlative analysis between monoaminergic systems across the brain

3.4.1 Correlative analysis of 5-HT and DA tissue

In vehicle-treated mice, vertically organized bands of correlations emerged, where 5-HT tissue content across the brain correlated with DA in specific regions. This pattern was most evident in fronto-cortical (30:PL; 27:aCC), striatal (33:VM; 30:DL; 22:DM; 19:VL; 15:NAc) mesencephalic (26:SC; 23:VTA; 15:MR; -13:SN; 12:DR), cerebellum (18:CB), and some thalamic regions. Hemispheric differences were noted in both the ventroposterior thalamus and somatosensory cortex (31:VPR; 22:S1L; 7:S1R, VPL).

TCB-2 dose-dependently reduced the number of correlations from 31.1% in vehicle-treated animals (235 total) to 15.3% (116 total), 10.2% (77 total), and 7.3% (55 total) in 0.3, 3, and 10 mg/kg TCB-2, respectively. At higher doses of TCB-2 (3 and 10 mg/kg), correlations were less organised, with a notable increase in negative correlations.

MDL-100,907 alone reduced the density of correlations to 9.3% (70 total), with the highest number of negative correlations (16 total). Most correlations were observed in the dorsal geniculate nucleus and frontal cortices (11:OF; 10:PL, SJ, DLG). Administration of the combination MDL-100,907/TCB-2 induced profiles that were dissimilar to those obtained with TCB-2 or MDL-100,907 alone, with a higher number of correlations (15.2%, 115 total), and a unique pattern. DA tissue content in cortical regions (across a broader range than in vehicle-treated animals), correlated with fronto-cortical, striatal, and diencephalic 5-HT (20:PL; 16:M1; 15:S1L; 9:SJ; 7:S1R; 5:aCC; 4:OF). Correlations with 5-HT and DA in the mesencephalon and cerebellum were sparse.

3.4.2 Correlative analysis of NA and DA tissue content

The correlations between DA and NA tissue levels (20.9%, 158 total) in vehicle-treated animals were well-organized. DA tissue content in the prelimbic cortex and aCC (22:PL; 20:aCC), striatum (24:VM; 14:DL; 13:DM; 3:VL; 2:NAc), and mesencephalon (15:SC, VTA, DR; 12:MR; -10:SN) correlated with NA tissue content. NA in the dorsal raphe correlated with DA across a wider subset of regions.

TCB-2 dose-dependently reduced the number of correlations between NA-DA tissue levels to 9% (68 total), 7.7% (58 total), and 7% (53 total) at 0.3, 3, and 10 mg/kg, respectively. The highest number of negative correlations occurred with 3 mg/kg TCB-2 (18 total), with most correlations being scattered, including 6 negative correlations with NA in the primary somatosensory cortex (-4:S1R; -2:S1L).

MDL-100,907 also reduced the number of correlations between NA and DA tissue content across the brain (8.2%, 62 total). These correlations were diffuse, with 17 negative correlations. In contrast, the combination MDL-100,907/TCB-2, revealed more distinct horizontal bands. DA tissue content across the brain correlated with cortical (16:PL; 15:M1; 9:SJ; 6:S1L, S1R; 3:OF), ventral hippocampal (13:vH), diencephalic (17:DLG; 12:VPR; 6:VPL; 4:HY), and cerebellar NA tissue content (8:CB).

3.4.3 Correlative analysis of 5-HT and NA tissue content

The number of significant correlations was highest between 5-HT and NA tissue content (38%, 286 total), and the pattern was highly organised. Vertical bands of positive correlations showed that 5-HT across the brain correlated with NA in the cortex (35:OF; 32:aCC; 29:AC; 28:SJ, V1; 26:PL; 25:M1), ventromedial striatum (40:VM), diencephalon (28:DLG; 23:VPL; 18:VPR), mesencephalon (25:MR; 17:DR; 18:SC; 1:SN), and cerebellum (17:CB).

TCB-2 reduced the total number of correlations at 0.3 mg/kg (11.8%, 89 total), 3 (15.1%, 114 total), and 10 mg/kg (9.7%, 73 total), while increasing the total number of negative

correlations to 8, 34, and 15, at each dose respectively. Cortical correlations involving NA were specific at 0.3 mg/kg (18:OF; 16:PL), wider at 3 mg/kg (19:M1; 16:OF; 15:PL, V1; 14:S1L; 7:S1R) with negative correlations, and lacked clear organization at 10 mg/kg. Notably, the correlations of NA and 5-HT in a single brain region (diagonal of the panels) were numerous in all situations.

MDL-100,907 reduced the number of correlations (17.3%, 92 total, 20 negative) compared to vehicle-treated animals, with a unique in distribution. Striatal 5-HT correlations were scattered compared to vehicles, while mesencephalic correlations were reduced to a lesser extent than with TCB-2. Positive correlations with fronto-cortical NA partially remained (14:OF; 12:PL), and 9 negative correlations with NA in somatosensory, auditory and visual cortices appeared.

The combination MDL-100,907 and TCB-2 resulted in correlations that vaguely resembled the profile obtained in vehicle-treated animals, except the low number of mesencephalic correlations.

3.4.4. Organisation as measured by degree distribution between treatments

Throughout this paper we have referred to the organisation of correlations. In attempt to illustrate the principle of an organised vs disorganised correlation network we calculated the standard deviation (SD) of the node degree, *i.e.* the distribution of correlations by region, referred to as degree distribution (Figure 4A). This measure is indicative of regions having variable degrees of connectivity, implying a regional organization of correlative patterns. The highest SD for each monoamine network, alone or in combination, was in vehicle-treated mice (Figure 4B). The SD is dose-dependently reduced by TCB-2 and by MDL-100,907. Across all networks the degree distribution is partially restored to that of organised vehicle-treated and MDL-100,907/TCB-2-treated animals.

3.5. Quantitative analysis of the effect of TCB-2 on monoamines and related compounds

Brain-wide 5-HT tissue content was significantly increased by TCB-2 in 24/28 regions at 10 mg/kg, and 8/28 at 3 mg/kg TCB-2. The ratio of 5-HT's primary metabolite 5-HIAA over 5-HT, taken as an indirect index of turnover, was reduced in all regions after 3 and 10 mg/kg TCB-2 (Figure 5A), except for the amygdala at 3 mg/kg. The magnitude of the decrease differed between regions, with the lowest decrease found in the dorsolateral striatum and the highest in some cortical and thalamic regions. Of note, 0.3 mg/kg TCB-2 increased the ratio 5-HIAA/5-HT in the cerebellum.

TCB-2 induced subtle and region-dependent effects on the dopaminergic system (Figure 5B). Increased DA was seen at 3 and 10, but not 0.3 mg/kg, in the nucleus accumbens, aCC and the primary somatosensory cortex. At 10 mg/kg only, DA levels were increased in the amygdala, lateral geniculate nucleus, lateral hypothalamus, superior colliculus, and dorsal raphe (Figure 5B). The ratios of DA metabolites were dose-dependently modified by TCB-2 in a subset of regions (Figure 5B). Specifically, 3-MT/DA was dose-dependently decreased by TCB-2 in the nucleus accumbens and all striatal quadrants, reaching significance for 3 and 10 mg/kg. In the dorsomedial striatum 3, not 10 mg/kg, TCB-2 slightly enhanced the ratio DOPAC/DA. Additionally, 0.3 mg/kg TCB-2 increased DOPAC/DA and HVA/DA in the cerebellum, and HVA/DA in the dorsal hippocampus and superior colliculus. Of note, HVA in the aCC was increased at 3 and 10 mg/kg TCB-2, and in the ventral hippocampus and hypothalamus at 10 mg/kg TCB-2. Other DA metabolites were modified in a subset of regions at various doses, including at 0.3 mg/kg (see supplementary table S5).

NA tissue content was modified in a dose- and region-dependent manner (Figure 5C). TCB-2 at 0.3 mg/kg increased NA in the lateral hypothalamus and ventral tegmental area. A

significant increase in NA content was observed in the aCC and the ventral hippocampus after 3 and 10 mg/kg. Moreover, at 10 mg/kg, TCB-2 enhanced NA content in nucleus accumbens, left ventroposterior thalamus, substantia nigra, hypothalamus, and superior colliculus.

3.5.1 Effect of MDL-100,907 on the changes of monoamine parameters induced by 3 mg/kg TCB-2

Brain-wide modifications of the serotonergic system mostly persist after pretreatment with 5-HT_{2A} receptor antagonist MDL-100,907. The two-way ANOVA indicated an interaction between TCB-2 and MDL-100,907 on tissue 5-HT in the ventral hippocampus, aCC and auditory cortex. An interaction between TCB-2 and MDL-100,907 was observed in four regions for the ratio 5-HIAA/5-HT. In the dorsomedial striatum, a partial blockade of TCB-2 by MDL-100,907 was observed. In the ventral hippocampus, right primary somatosensory cortex and ventral tegmental area, the interactions can be explained by an increase of 5-HIAA/5-HT by MDL-100,907 that was no longer present when TCB-2 was administered (Figure 6A).

The excitatory effect of TCB-2 on DA tissue content was blocked by MDL-100,907 in the aCC, but not in the nucleus accumbens or the primary somatosensory cortex. A TCB-2 and MDL-100,907 interaction was observed in the median raphe nucleus, resulting from a non-significant increase in DA by MDL-100,907 [two-way ANOVA, $F(1, 33) = 5.883$, $p = 0.021$, Tukey's test] and combination treatment reducing DA. The reduction of the ratio 3-MT/DA by TCB-2 was significantly blocked by MDL-100,907 in striatal regions but not in the nucleus accumbens, [two-way ANOVA, $F(1,38)=2.64$, $p = 0.113$]. An interaction in the dorsomedial striatum occurred for the ratio DOPAC/DA, likely corresponding to the increase in DOPAC/DA induced by MDL-100,907 alone.

The increase in NA tissue content induced by TCB-2 was blocked by MDL-100,907 in the aCC but not in the ventral hippocampus [two-way ANOVA, $F(1,39)=2.09$, $p = 0.156$].

4. Discussion

This study aimed to elucidate the effect of the psychedelic compound TCB-2, via 5-HT_{2A} receptors, on monoamine levels across multiple brain regions during a protocol of forced investigatory behaviour. The primary finding is that forced investigatory behaviour was associated with high number of correlations of 5-HT tissue content and specific, unique patterns for DA and NA. These patterns were disrupted by both TCB-2 and MDL-100,907. TCB-2, but not MDL-100,907, induced heterogeneous changes in monoamine tissue levels via both 5-HT_{2A} receptor and non-5-HT_{2A}R-mediated mechanisms. These data establish a strong link between 5-HT_{2A} receptor and the coherence of monoamines function across the brain during exploratory behaviour.

The forced investigatory behaviour protocol was inspired by Halberstadt and Geyer (2018) and Adams and Geyer (1985) to investigate the mechanism of action of psychedelics during exploratory behaviour. Surprisingly, the exposure of vehicle-treated mice to the textured floor corresponded with high number of correlations of 5-HT tissue content between pairs of brain regions (48%), and to a lesser extent NA (22%). These values are remarkably high compared to previous studies in naive or vehicle-injected rodents in which the density of correlations corresponded to 5-10% of the total comparisons (Fitoussi *et al.*, 2013; Puginier *et al.*, 2019; De Deurwaerdère *et al.*, 2020; Chagraoui *et al.*, 2019; Gros *et al.*, 2021; Marty *et al.,* 2023). The second peculiarity was the specific distribution of correlations for the DA and NA systems, which were distinct across cortical, striatal, and thalamic areas. When examining the relationships between pairs of monoamines throughout the brain, this regional organisation became more pronounced, suggesting that the distribution of 5-HT correlations was shaped by the catecholamines' pattern. The characterisation of organisation was

initially observed by eye in correlograms as horizontal, or vertical, bands where the regional quantity of a compound correlated extensively throughout the brain with either itself or another monoamine. We then worked to quantify this organisation through the degree distribution of the network and found that the standard deviation of the number of correlations in each brain region corresponded to the loss in organisation observed (Figure 4). Additionally, we observed lateralized correlations in a subset of regions. DA contents of the left somatosensory cortex and the right ventral posteromedial nucleus of the thalamus correlated with 5-HT tissue content across several regions to a greater extent than their opposing hemispheres. To our knowledge, it is the first visualisation of the distinct involvement of monoaminergic systems in response to a novel environment across multiple brain regions and neurobiological networks.

We hypothesize that these specific patterns are imposed by the change of flooring 10 min before sacrifice, likely related to forced exploration. DA neurons of the ventral tegmental area and substantia nigra increase their activity in a context of novelty and reward (Lisman and Grace, 2005; Düzel *et al.,* 2010; Waelti *et al.,* 2001). NA neurons of the locus coeruleus show heightened activity during novel exploration (Vankov *et al.,* 1995; Schomaker and Meeter, 2014). The observed pattern of correlations, structured by DA and NA when paired, aligns with the involvement of mesencephalic DA systems in the exploratory behaviour (Le Moal and Simon, 1991), and NA systems in processing novelty, stress, arousal, and sensory stimuli (Sarah, 2009; Foote *et al.,* 1980). Interestingly, unlike the ventral tegmental vehicle-correlations, DA content in the substantia nigra had exclusively negative correlations with DA, NA, and 5-HT in other regions. These opposite correlations suggest that meso-cortico-limbic and nigrostriatal related pathways are differently regulated in this exploration paradigm. The extensive number of correlations for 5-HT is compatible with its involvement in virtually all brain functions (Jacobs and Azmitia, 1992; Soubrié, 1986; Baque-Cazenave

et al., 2020), potentially reflecting its heightened reactivity in a variety of conditions (Gros *et al.*, 2021; Bharatiya *et al.*, 2019).

Before subjecting mice to the novel environment, we verified that TCB-2 increased the HTR at 3 and 10, but not 0.3 mg/kg (Di Giovanni and De Deurwaerdère, 2018) in a 5-HT_{2A} receptor antagonist (MDL-100,907)-sensitive manner (Halberstadt *et al.,* 2015; González-Maeso *et al.,* 2007). TCB-2 dramatically reduced the number of correlations for all monoamines, which was dose-dependent for 5-HT and DA systems. Furthermore, the specific organisation of correlations reported in vehicle-treated animals was absent at 3 and 10 mg/kg TCB-2 for all monoamines, whether analysed alone or by monoamine pairs. Negative correlations were most prevalent with TCB-2 at 3 and 10 mg/kg, with no clear organisation.

The quantitative effects of TCB-2 on DA systems may underline the disruption of correlations, as DA activity seems to play a role in organizing correlations in vehicle-treated animals. TCB-2 increased DA tissue content while decreasing the ratio 3MT/DA in the nucleus accumbens and all striatal quadrants. Since the production of 3-MT depends on catechol-O-methyl transferase, which is not expressed by catecholaminergic cells in rodents (Schendzielorz and Oinas, 2013), a decreased 3-MT/DA ratio suggests that striatal DA tone is decreased after 3 and 10 mg/kg TCB-2 administration. These effects were blocked by MDL-100,907, implicating 5-HT_{2A}Rs in mediating TCB-2's effects on DA. Previous research has demonstrated that 5-HT_{2A}Rs modulate striatal and accumbal DA neuron activity, exerting excitatory and state-dependent influences on extracellular DA levels (De Deurwaerdère and Di Giovanni, 2017; De Deurwaerdère *et al.*, 2021). While a reduction of correlations has been observed with a 5-HT_{2C}R agonists and a decrease of DA neuron activity in resting states (Di Matteo *et al.*, 2000; De Deurwaerdère *et al.*, 2020; Chagraoui *et*

al., 2019), there is no experimental evidence to suggest that TCB-2 triggers 5-HT_{2C}R-dependent responses.

One of the most peculiar findings of this study is the disruption of correlation profiles by the 5-HT_{2A} receptor antagonist MDL-100,907, comparable to TCB-2. While TCB-2 significantly altered monoamine tissue contents, MDL-100,907 had sparse and weak quantitative effects. Although our experimental conditions do not correspond to a resting state, a minimal impact of MDL-100,907 on monoamine tissue levels aligns with numerous electrophysiological and *in vivo* neurochemical studies reporting a lack of effect of preferential 5-HT_{2A} receptor antagonists in resting conditions (Schmidt *et al.,* 1992; Gobert and Millan, 1999; De Deurwaerdère and Spampinato, 1999, Di Matteo *et al.*, 2000). This suggests that blockade of the 5-HT_{2A} receptor tone alters the synchronisation of monoaminergic systems during exploratory behaviour without significantly altering monoamine tissue content. These data are intriguing because MDL-100,907, which has demonstrated antipsychotic-like effects in animals (Kehne *et al.,* 1996; Schmidt *et al.,* 1995), has not shown similar efficacy in clinical studies (Kapur and Mamo, 2003). Our results suggest that its mechanism of action may involve desynchronization of monoaminergic networks during exploratory demand, a potential explanation for its distinct *in vivo* pharmacological profile.

The correlation profiles obtained after TCB-2 (at any dose) and MDL-100907 are unique. While it is tempting to speculate that these distinct profiles correspond to inner states associated with each condition, an understanding of the consequences of remaining correlations is insufficient. Correlative analysis relies on a cohort of animals for each treatment, and inter-individual differences (Dellu-Hagedorn *et al.*, 2018) may shape these distinct correlation profiles. Despite this limitation, our findings indicate that the textured floor represents a sufficiently strong stimulus to elicit specific correlation patterns, regardless of

the cohort composition. Notably, the pattern of correlations observed at 0.3 mg/kg TCB-2, while marked by a reduction in total correlations, remained somewhat organized. Furthermore, the correlation profile in the MDL-100,907/TCB-2-treated group bore similarities to those of the vehicle-treated group for all monoamines, including a notable reduction of negative correlations. Conversely, a lack of clear organization of correlations, or monoaminergic connectivity in mice treated with higher doses of TCB-2 (3 and 10 mg/kg) or MDL-100,907 could be attributed to their dissociative properties. These findings point to the complexity of monoaminergic interactions and their modulation by 5-HT_{2A}R-targeting compounds, suggesting that the dissociative and exploratory states induced by these pharmacological and environmental manipulations profoundly influence brain-wide monoaminergic connectivity.

TCB-2's action in the presence of 5-HT_{2A} receptor antagonist MDL-100,907 gave a distinct profile of correlation compared to MDL-100,907 alone, supporting the hypothesis that TCB-2 acts on targets beyond 5-HT_{2A} receptor (Di Giovanni and De Deurwaerdère, 2018). In line with this evidence, TCB-2 increased 5-HT tissue content in several brain regions and decreased the ratio 5-HIAA/5-HT across all brain regions. Apart from the partial blockade of 5-HT effects in a few brain regions, including the aCC, most 5-HT effects of TCB-2 were not reduced by MDL-100,907. The psychedelic 5-HT_2 receptor agonist 2,5-Dimethoxy-4-iodoamphetamine (DOI) reduces the activity of 5-HT DRN neurons and 5-HT release, but the role of 5-HT_{2A} receptor in this mechanism remains a point of contention (Marin-Ruiz et al., 2001; Garratt *et al.,* 1991). Although inhibition of 5-HT neuron activity has been reported for various psychedelics, a reduction in serotonergic tone is unlikely to underlie their psychedelic effects (Pieri *et al.,* 1978; Trulson *et al.,* 1981; Butler *et al.,* 2024). Our findings corroborate previous reports that TCB-2 modulates 5-HT tone (Guilhemsang *et al.*, 2023), showing TCB-2's action was disturbed following 4-para-chloro-phenylalanine (PCPA), and further reveal that some of its effects are independent of 5-HT_{2A} receptors.

The aCC emerges as a particularly interesting region in our study due to its significant neurochemical changes under the influence of psychedelic TCB-2. TCB-2 increased the tissue levels of all three monoamines in the aCC, while MDL-100,907 reduced or blocked these effects, implicating the 5-HT_{2A} receptor in psychedelic monoaminergic modulation in the aCC. This finding aligns with prior human studies (Carhart-Harris *et al.,* 2012; Castelhano *et al.,* 2021), highlighting the aCC as a pivotal region in the mechanism underlying psychedelic action. The aCC's inclusion in the default mode network, which psychedelics often downregulate, is frequently linked to ego dissolution and altered self-awareness (Carhart-Harris *et al.,* 2016). Additionally, its role in the salience network, underlines its importance in attributing significance to stimuli (Seeley *et al.,* 2007). Human studies propose the aCC as a unique integrative hub between cognitive and emotional processes, with altered activity in this region associated with changes in self-awareness, sociability, and mood disorders such as depression (Allman *et al.,* 2001; Devinsky *et al.,* 1995). As part of the limbic system, the aCC is well-positioned to mediate the cognitive and emotional alterations induced by psychedelics. Supporting this notion, in rats, a local administration of LSD into the aCC substitutes for systemic administration of LSD in a dose-dependent manner, a process mediated by 5-HT_{2A} receptors, but not by 5-HT_{2C} receptors or 5-HT_{1A} receptors (Gresch *et al.,* 2007). This would be in line with the importance of cortical 5-HT_{2A} receptors, sufficient to elicit HTR (González-Maeso *et al.,* 2007). Our data contribute to this growing body of evidence, demonstrating the aCC's involvement in 5-HT_{2A} receptor dependent psychedelic effects on monoamine systems.

We bring up some evidence that 5-HT_{2A} receptor ligands can disrupt brain lateralized mechanisms. Addressed solely in the left and right somatosensory cortex and ventral posteromedial thalamus, the monoaminergic connectivity was distinct between left and right hemispheres during exploration in vehicle-treated animals. For 5-HT-DA correlations, the

left primary somatosensory cortex displayed a high level of correlations (S1L), relative to few correlations with the left ventral posteromedial thalamus (VPL). The inverse was true for the right hemisphere. The connectivity profile between hemispheres for these two structures was disrupted with TCB-2 and MDL-100,907 with some quantitative effects on both 5-HT and DA. One interesting change was the negative correlations between NA and 5-HT in mice receiving TCB-2 (3 mg/kg), and to a lower extent MDL-100,907 or the combination. Lateralized psychedelic effects are compatible with recent data (Jeanblanc *et al.,* 2024). Although not directly addressed in human studies reported fMRI and EEG data appear to have lateral differences that are not explicitly discussed by authors (Siegel *et al.,* 2023; Timmerman *et al.,* 2022; Muthukumaraswamy *et al.,* 2013; Carhart-Harris *et al.,* 2016). At this stage we cannot exclude that altered brain lateralization processes by psychedelics (or 5-HT_{2A} receptor antagonism) play a major role in their effects. This, and the sex distinction which has not been covered in our study, would need deeper analyses.

In conclusion, our findings highlight that a specific organisation within and between monoaminergic system is mobilised by exploration and novelty and this intricate organisation is disrupted by both agonists and antagonists of the 5-HT_{2A}R. The observed loss of inter-regional neurotransmitter connectivity and organisation following psychedelic TCB-2 may mirror the reported altered functional connectivity in human studies following psychedelics (Siegel *et al.,* 2024; Müller *et al.,* 2018; Carhart-Harris *et al.,* 2016). Monoaminergic systems, therefore, may play a role in the dissociation within networks that has been associated with the psychedelic experience. Although it is premature to assert that the therapeutic or mind-manifesting effects of psychedelics could be related to widespread re-organization of monoaminergic systems operating within numerous neurobiological networks, our data suggest that such re-organisation could contribute to increased neuroplasticity observed following psychedelics (Ly *et al.,* 2018). Our data, add depth to the

anatomo-functional understanding of psychedelic mechanisms of action, proposing that the effects are not confined to isolated brain regions but involve a dynamic interplay across several neurofunctional systems. This expanded view underscores the complexity of psychedelic-induced alterations and their potential links to therapeutic outcomes.

Figure 1 – Experimental timeline and design reporting head twitch response, brain region dissection, chromatographic analysis, and the correlogram structure.
A) Experimental timeline reporting the quantification of head twitch response (HTR) count/minute 20-, 30-, 40- and 50-min post TCB-2, vehicle, and/or MDL-100,907. MDL-100,907 was administered 15 min prior TCB-2 (3 mg/kg). The vehicle of MDL-100,907 was administered in all groups. *p<0.05 indicates significance relative to vehicle (Tukey's test, one-way ANOVA or two-way ANOVA when combining TCB-2 with MDL-100,907). Notably, no HTR was recorded in the presence of MDL-100,907.
B) Dissection of brain regions on horizontal slices using a 'punch' technique; examples of removed tissue overlaid at relevant bregma (Paxinos and Franklin, 2004). Tissue weights ranged from 1-4 mg, with no significant difference between treatments (Table S3).
Brain regions include: orbitofrontal cortex (OF), prelimbic cortex (PL), anterior cingulate cortex (aCC), insular cortex (IC)*, primary motor cortex (M1), somatosensory cortex jaw (SJ), left/right primary somatosensory cortex(S1L/R), left/right primary somatosensory cortex layer 6 (S1L6L/R)*, auditory cortex (AC), primary visual cortex (V1), amygdala (A), dorsal/ventral hippocampus (dH/vH), nucleus accumbens (NAc), dorsomedial/dorsolateral striatum (DM/DL), ventromedial/ventrolateral striatum (VM/VL), medial dorsal nucleus (MD), left/right ventral posteromedial nucleus (VPL/R), lateral geniculate nucleus (DLG), lateral hypothalamus (HY), superior colliculus (SC), substantia nigra (SN), ventral tegmental area (VTA), dorsal raphe nucleus (DR), median raphe nucleus (MR), cerebellum (CB). Regions marked with * indicates regions that were excluded due to striatal contamination.
C) Chromatogram of 0.1 ng/μl standard of measured compounds, listed in order of elution: L-3,4-dihydroxyphenylalanine (L-DOPA), noradrenaline (NA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptophan (5-HTP), dopamine (DA), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), serotonin (5-hydroxytryptamine, 5-HT).
D) Pearson correlations between 5-HIAA tissue quantity for a significant (MD and aCC) and not significant (DLG and VPR) pair of brain regions.
E) Regions colour coded by structure and ordered in correlogram of significant Pearson R correlations (red for positive and blue for negative) between tissue 5-HIAA across all regions, alternat network representation shown above. Horizontal brain image adapted from the Mouse Brain Library, Atlas 232, https://www.mbl.org/atlas232/atlas232_frame.html.

Figure 2 – Correlograms within a monoaminergic system under various conditions: **A** 5-HT, **B** DA, and **C** NA. Conditions include vehicle (V), TCB-2 at doses of 0.3, 3, and 10 mg/kg, 0.2 mg/kg MDL-100,907 (MDL), and 3 mg/kg TCB-2 with MDL-100,907 pretreatment (MDL+3). Significant Pearson's R correlations are depicted in red for positive and blue for negative (scale at top right), with $p < 0.05$. An example positive correlation for 5-HT and a negative correlation for DA are highlighted within the marked square in the matrix. Brain

regions are arranged as in Figure 1E, ordered by cortical, telencephalic, diencephalic, mesencephalic, and cerebellar areas. See figure S1 for network depiction of regions subsections and table S4 for basic network parameters.

Figure 3 – Correlograms between a monoamine tissue content for **A** 5-HT-DA, **B** NA-DA, and **C** 5-HT-NA under various conditions: vehicle (V), TCB-2 at doses of 0.3, 3, and 10 mg/kg, 0.2 mg/kg MDL-100,907 (MDL), and 3 mg/kg TCB-2 with MDL-100,907 pretreatment (MDL+3). Significant Pearson's R correlations are indicated in red for positive and blue for negative (scale at top right), with $p < 0.05$. Brain regions are organized as in Figure 1E; from left to right and top to bottom by cortical, telencephalic, diencephalic, mesencephalic, and cerebellar areas. See table S4 for basic network parameters.

Figure 4 – Network degree distribution, proposed proxy for regional organisation. **A** Networks for NA vehicle (left) and 3mg/kg TCB-2 (right) correlations and histogram of network degree distribution, standard deviation(SD) indicated. **B** Degree distributions of 5-HT, NA, DA, 5-HT-DA, 5-HT-NA, NA-DA networks for conditions: vehicle (V), TCB-2 at doses of 0.3, 3, and 10 mg/kg, 0.2 mg/kg MDL-100,907 (MDL), and 3 mg/kg TCB-2 with MDL-100,907 pretreatment (MDL+3).

Figure 5 – TCB-2 dose response 0.3, 3, and 10 mg/kg on mean tissue quantity, ng/mg ± STD. **A** 5-HT and ratio 5-HIAA/5-HT for all brain regions. **B** DA and its metabolite ratios **C** NA in regions with a significant effect. *$p < 0.05$, Tukey's test. For all regions see table S5 for statistical report.

Figure 6 – 5-HT_{2A}R-dependent modulation of monoamines by TCB-2 and MDL-100,907, showing mean tissue quantities (ng/mg ± SD) **A** 5-HT and its ratio for all regions.
B DA and its ratios and **C** NA in regions in where MDL-100,907 and TCB-2 significantly interacted (two-way ANOVA) are reported. Post-hoc significance is reported as *$p<0.05$ for significant differences of TCB-2 or MDL-100,907 alone relative to vehicles or # $p<0.05$ relative to MDL-100,907 (Tukey's test). For all regions see table S6.

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Graphical abstract

5-HT correlations during exploration

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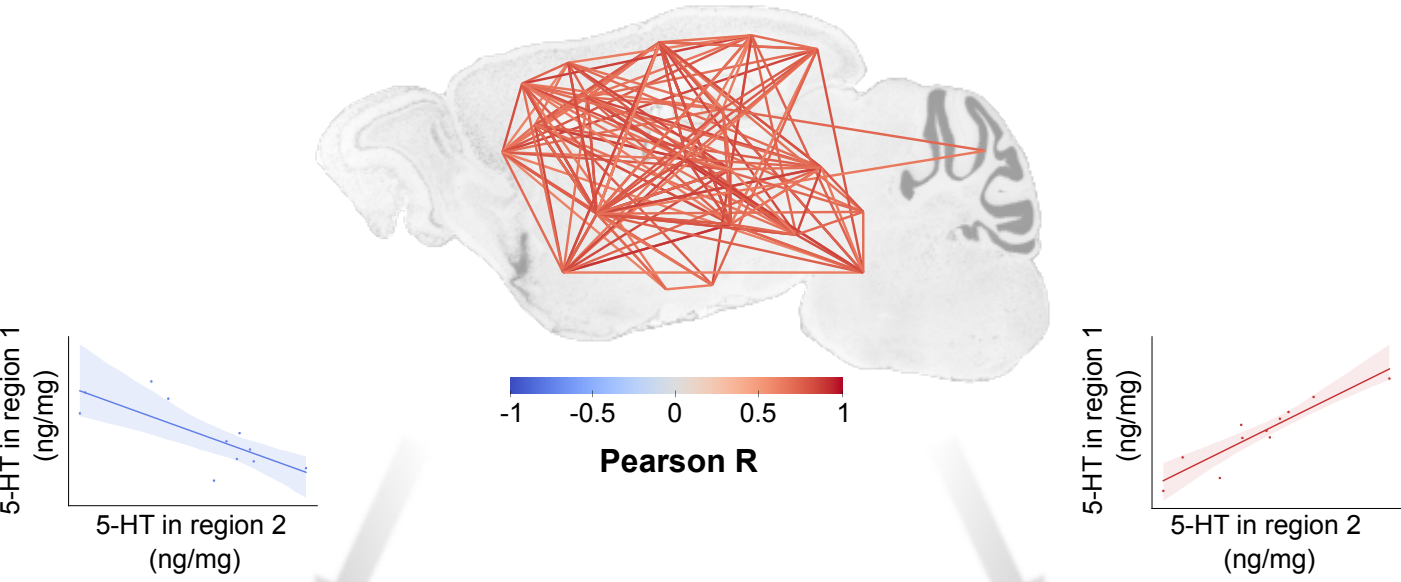

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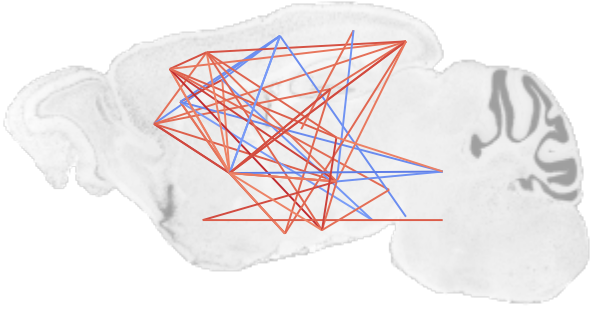

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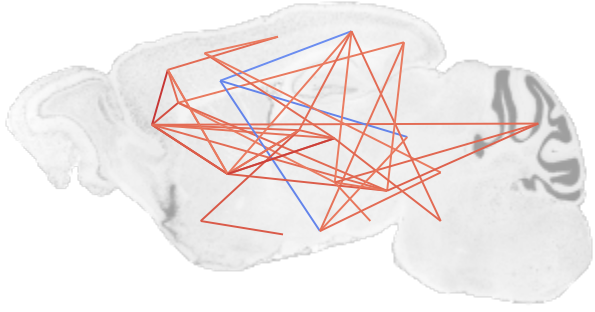

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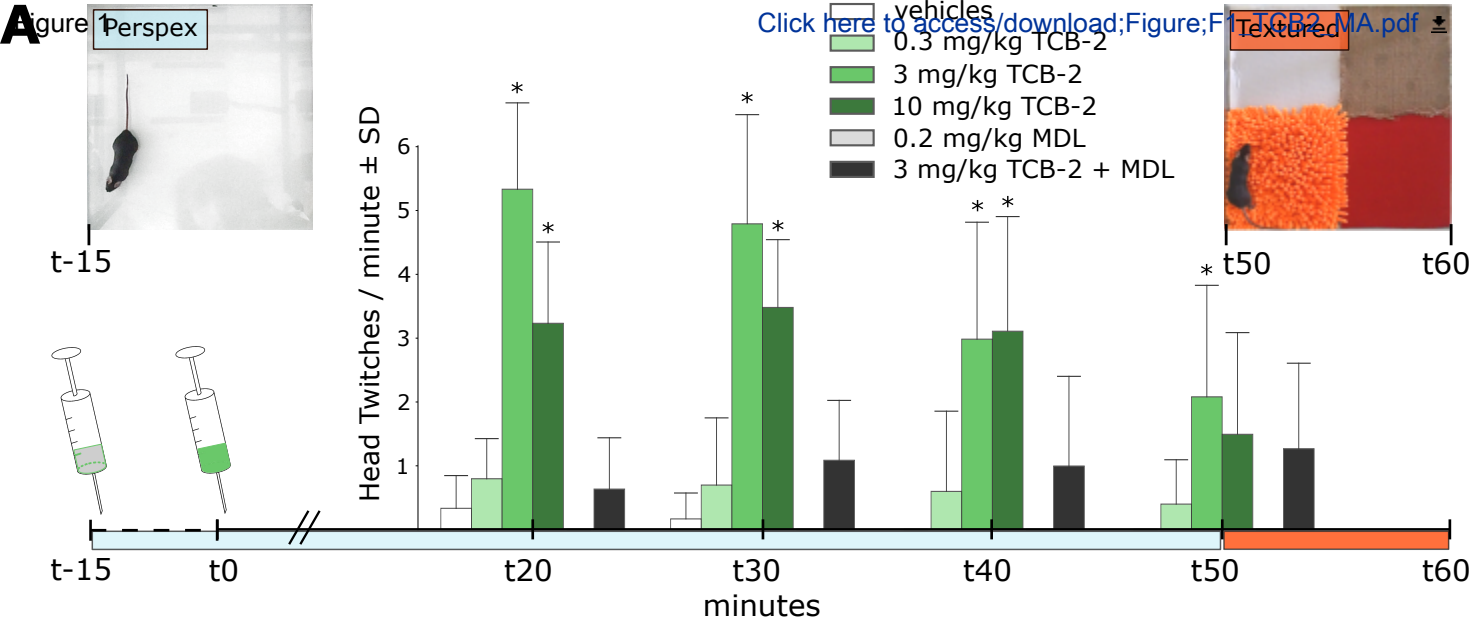

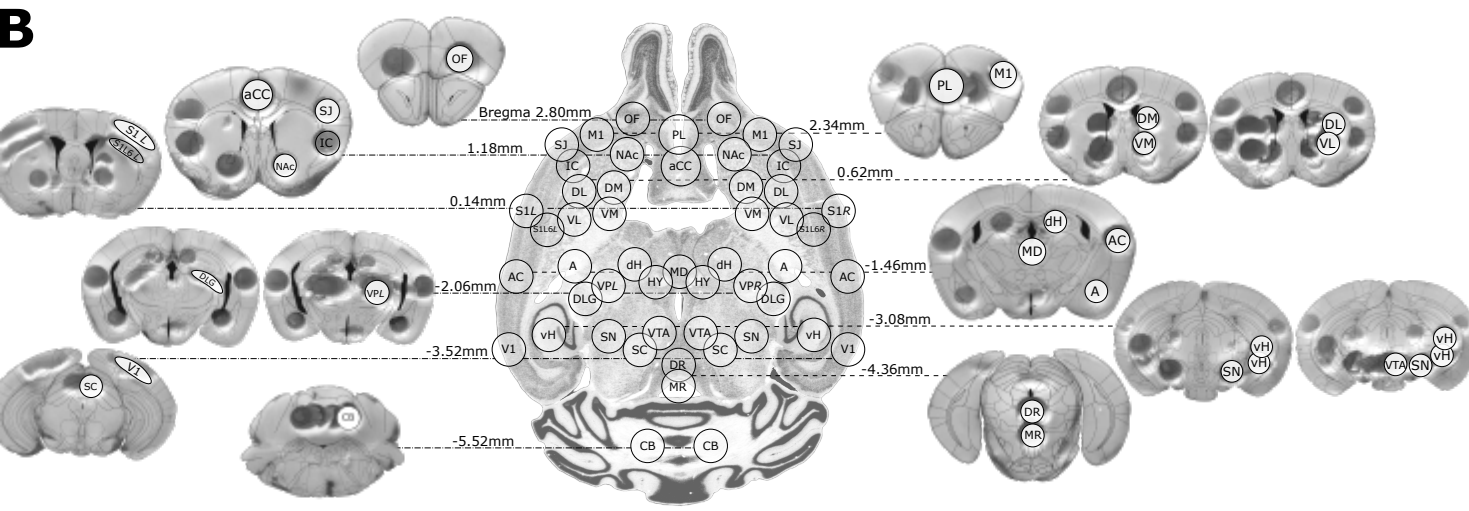

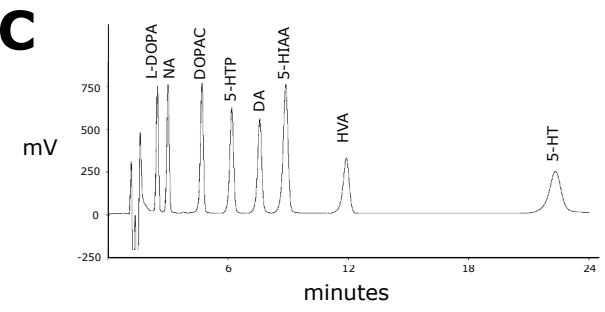

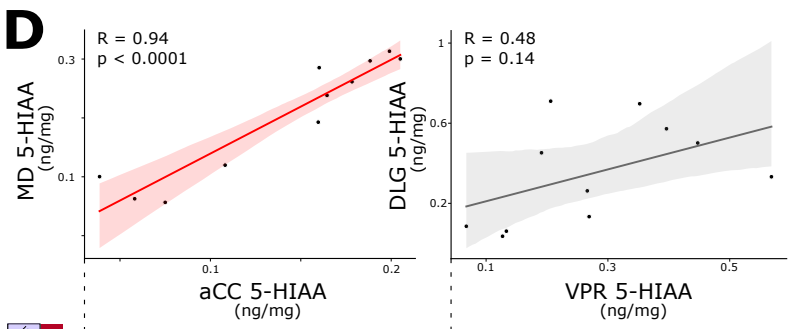

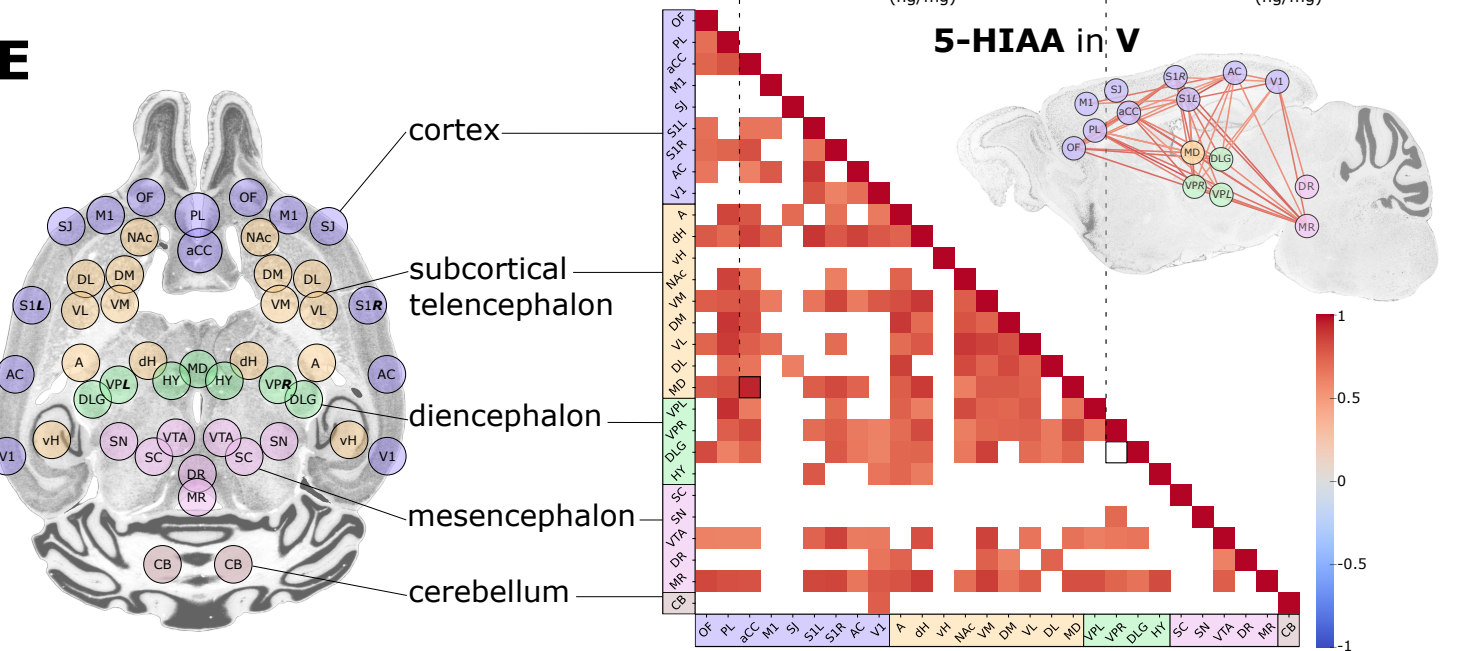

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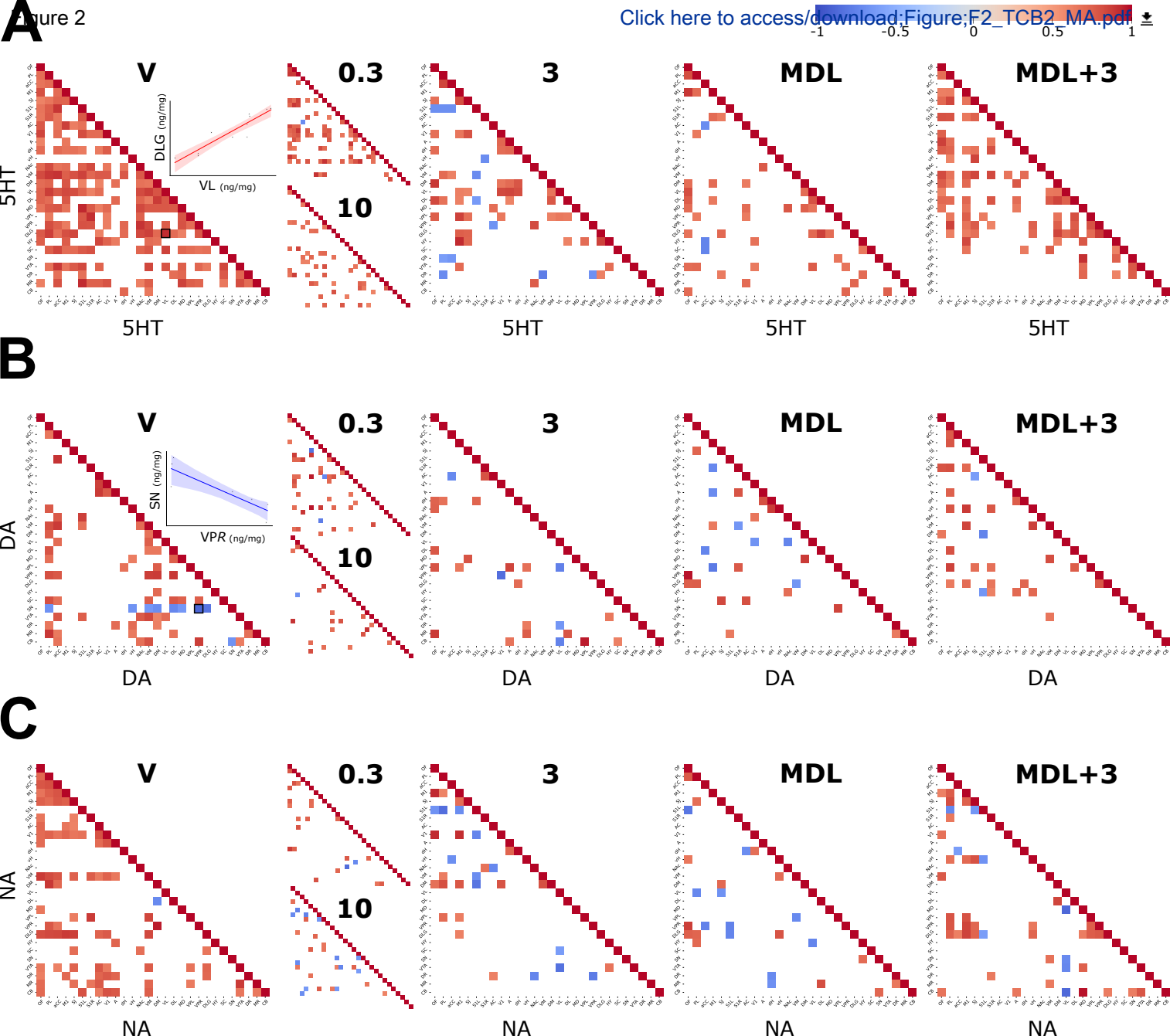

Figure 3

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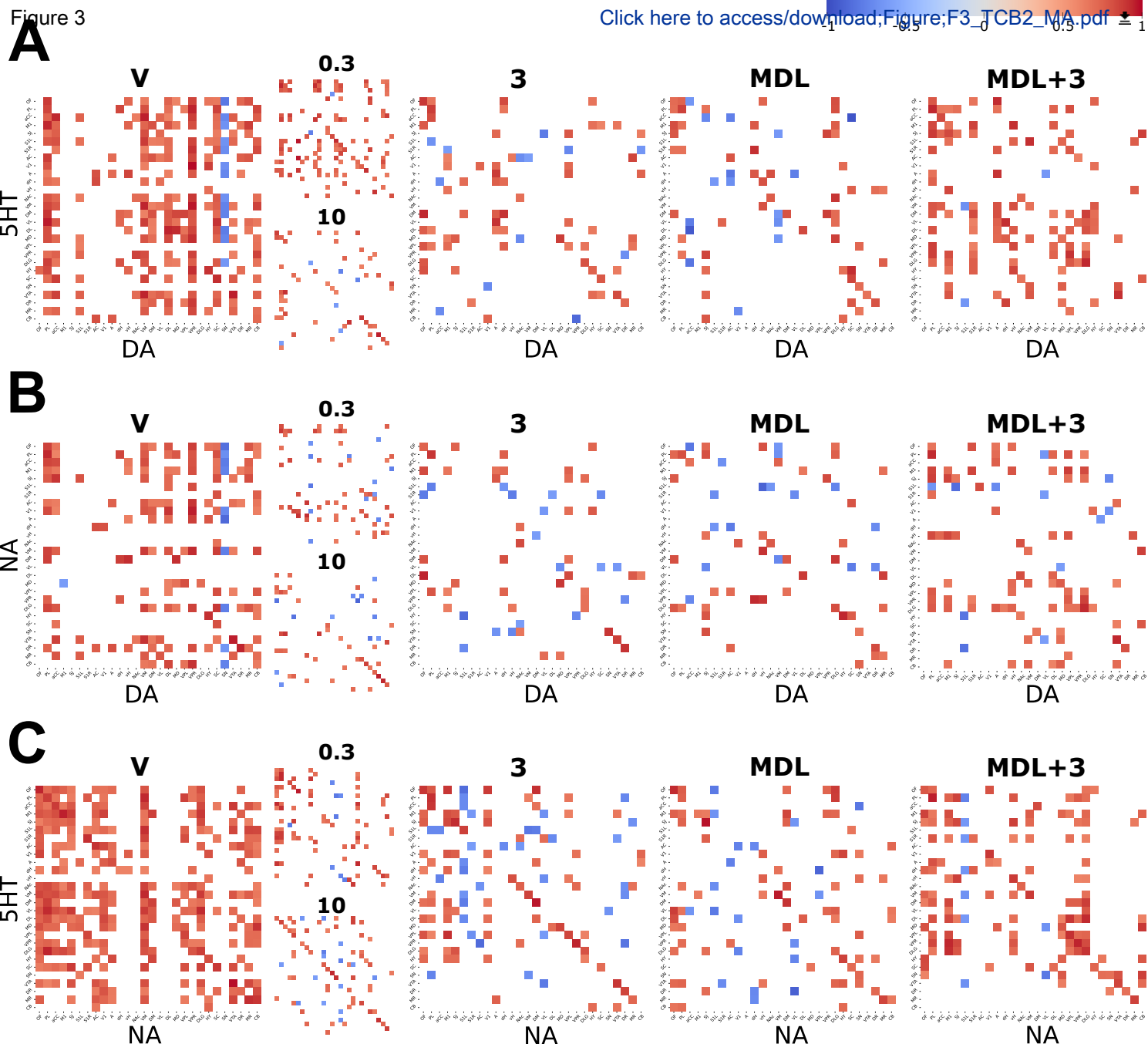

A
Figure 4
Click here to access/download;Figure;F4_TCB2_MA_.p
SD = 4.69
SD = 2.05
Node count (region count)
Node Degree (correlation count)
dH, vH, VL, SN
NAc, DL, MD, HY
S1L
VPR, SC
DM
S1R, VPL
DR
A
M1, MR, CB
PL
aCC, VTA
AC, DLG
SJ, V1, VM
OF
DL, MD, SN, MR, CB
aCC, SJ, S1R, A, dH, vH, VPR, DLG, HY, SC
AC, VL, VPL, VTA
NAc, VM, DR
OF, V1
PL
S1L, DM
M1
B
Degree distribution (SD)
5-HT
NA
DA
5-HT-DA
5-HT-NA
NA-DA
Vehicles
0.3 TCB-2
3 TCB-2
10 TCB-2
MDL
MDL/TCB-2

Click here to access/download;Figure;F5_TCB2_MA.pdf

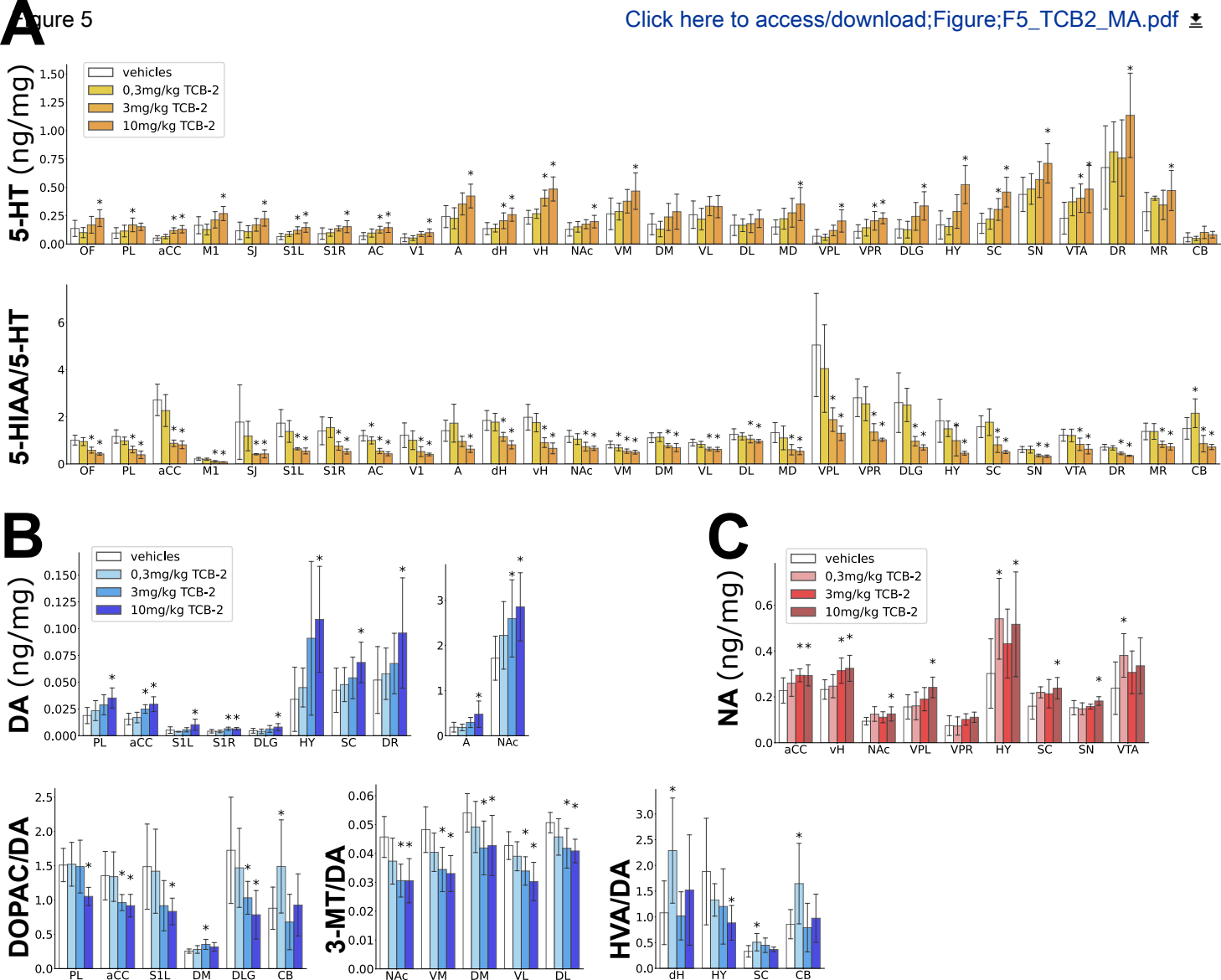

Figure 6

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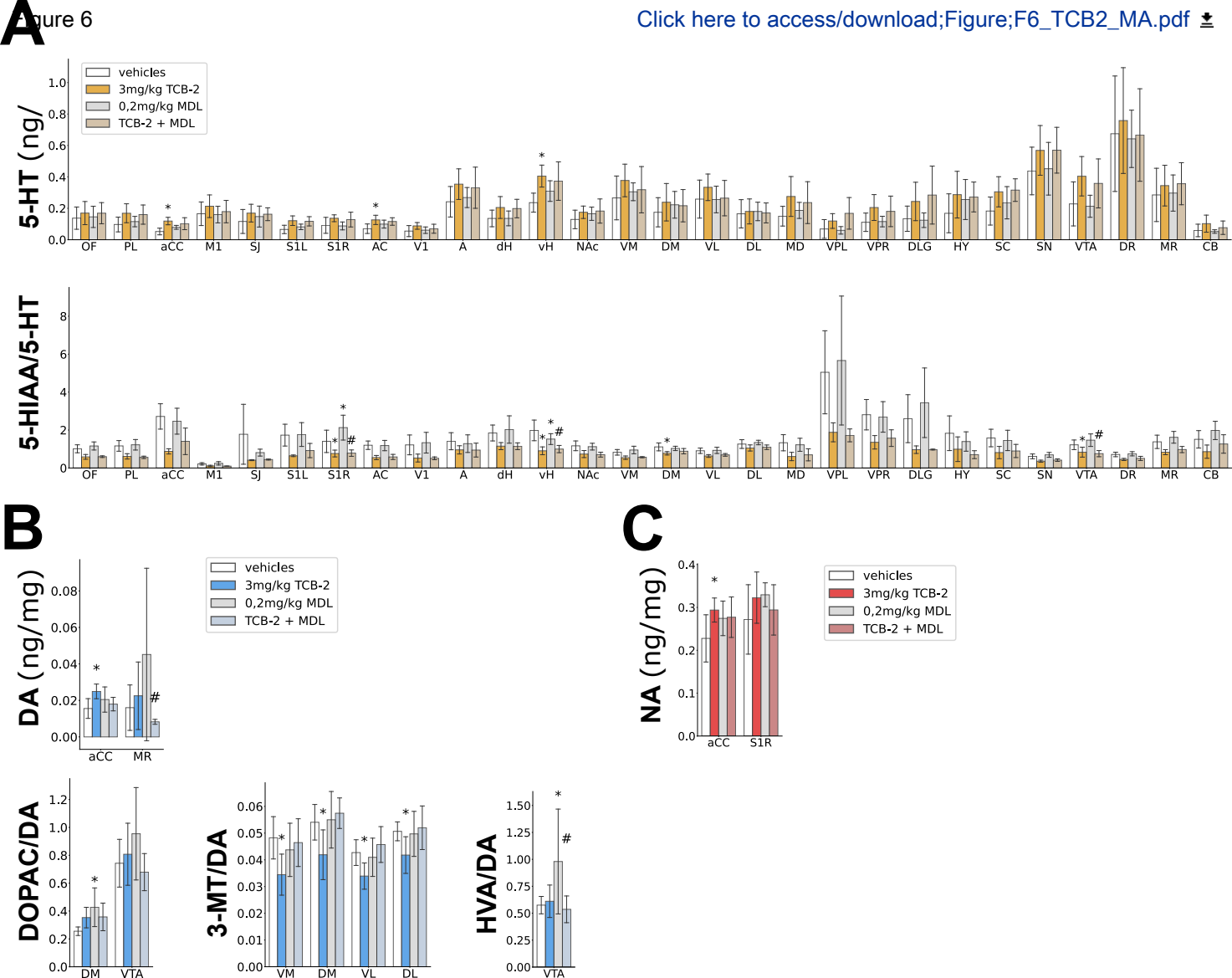

Declaration of Interest Statement

All the authors declare that they have no established conflicting financial interests or personal relationships that may have influenced the research presented in this paper.

Ethical Statement

All procedures with animals were conducted in accordance with the local ethics committee of the University of Bordeaux and the European Committee Council Directive (approval number 2023121921231115). All efforts were made to minimize animal suffering and reduce the number of animals used.

5-HT_{2A} Receptors Structure Monoaminergic Connectivity in the male Mouse Brain

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Summary Art III

Following the novel forced exploratory paradigm vehicle-treated animals had a correlation density unprecedented in the lab, the highest density was observed for 5-HT tissue quantity between regions at 47.6%. The high density of correlations in vehicle-treated animals additionally revealed an organisation within and between monoaminergic systems, providing insight into the interaction between regions and monoaminergic systems when mobilised for exploratory behaviour. Psychedelics TCB-2, dose dependently disrupts these dense and highly organized correlations. An effect that is accompanied by a dose-dependent reduction in serotonin turnover across brain regions and altered dopamine and noradrenaline markers, especially in the anterior cingulate cortex. Curiously antagonist MDL-100,907 also abolished organization vehicle-correlations, while the combination TCB-2/MDL-100,907 partially restored this organization. MDL-100,907, with minimal effects alone, reduced TCB-2-induced head twitches and monoamine changes but did not reverse decreased serotonin turnover, implicating TCB-2 targets beyond the 5-HT_{2A}R. Furthermore, this suggests monoaminergic functional connectivity during exploration is highly sensitive to 5-HT_{2A}R modulation.

Art IV *(in preparation a)* – 5-HT_{2A} Receptor Activation and Blockade Disrupt Amino Acid Connectivity in the Male Mouse Brain: Possible Role of Monoaminergic

Questions and purpose of the study

This study extends the determination of the impact of the 5-HT_{2A}R agonist TCB-2 and the 5-HT_{2A}R antagonist MDL-100,907 on neurotransmitter systems in the brain. The hypothesis is that 5-HT_{2A}R ligands would impact amino acid neurotransmitter systems (glutamate, aspartate, GABA, glycine, and taurine) at different locations in the mouse brain following a protocol of forced exploratory behaviour. Tissue concentrations of amino acid neurotransmitters were measured using HPLC coupled to electrochemical detection. Analysis of the data included monoamines (previous article) to determine possible nature of the interaction of monoamines and amino acid neurotransmitters, and behavioural parameters measured during the last 10 minutes of the experiment to study the effect of 5-HT_{2A}R ligands on exploration. Combining all measured transmitters in different parts of the brain, and behaviours, this study addresses the distributed function of various neurotransmitters in exploration and its sensitivity to 5-HT_{2A}R ligands including psychedelic TCB-2.

5-HT_{2A} Receptor Activation and Blockade Disrupt Amino Acid Connectivity in the Male Mouse Brain: Possible Role of Monoaminergic Neurotransmitters

Jasmine Jade Butler[1,2], Margherita Virgili[1,2], Giuseppe Di Giovanni[3], Joeri Bordes[1,6], Leo Sont[1,6], Abdeslam Chagraoui[4,5], Anna Beyeler[1,6], Philippe De Deurwaerdère[1,2*]

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Acknowledgments

We thank the support of the University of Bordeaux, the Centre de la Recherche Nationale et Scientifique and the Région Nouvelle-Aquitaine. We acknowledge the support of the 'Avenir program' of the French Institute of Health (INSERM), the 'Agence Nationale pour la Recherche' (ANR), the 'Cercle de la Fondation Schlumberger pour l'Education et la Recherche' (Cercle FSER), the 'Fondation pour la Recherche Médicale' (Equipe FRM) and the 'Fondation Bettencourt-Schueller' (impulscience 2023) to AB. We are grateful for the support of the Grand Programme de Recherche (GPR work package Adapsy) of the University of Bordeaux to PDD and AB. JJB was a recipient of PhD grant from the Ministère de la Recherche et de l'Enseignement Superieur, and received additional support from the EUR graduate School of Bordeaux Neurocampus.

Abstract

Drugs targeting the serotonin 2A receptor subtype (5-HT_{2A}R), such as serotonergic psychedelics or antipsychotic drugs, are presumed to act on executive neurotransmitter systems, although comprehensive brain-wide neurochemical data remains scarce. Here, we report the effect of the 5-HT_{2A}R agonist TCB-2 and antagonist MDL-100,907 on amino acid tissue concentrations of glutamate, GABA, aspartate, taurine, and glycine in 28 brain regions following a forced exploratory paradigm in mice. In vehicle-treated mice, amino acid tissue content correlations displayed a high density and clear regional organisation, particularly in relation to dopamine and noradrenaline. While TCB-2 and MDL-100,907 minimally affected absolute amino acid levels, they strongly reduced the number of correlations within and between regions for all amino acids alone or combined with monoamines. MDL-100,907, without effect by itself, failed to antagonize most TCB-2 altered behaviours. Correlative analyses of behaviour with regional amino acid and monoamine tissue content revealed distinct patterns in vehicle-treated mice, altered in mice treated with TCB-2 or MDL-100,907. These findings suggest that following novel exploration the neurochemical connectivity of amino acid neurotransmitters is regionally organized in relation to catecholamines, dopamine and noradrenaline, and that this organization is sensitive to 5-HT_{2A}R ligands. This desynchronization of neurotransmitters may offer a new perspective on the altered functional connectivity following psychedelic 5-HT_{2A}R agonists.

Keywords

Psychedelic, correlations, MDL-100,907, neurochemistry, exploratory behaviour

Abbreviations

OF (orbitofrontal cortex), PL (prelimbic cortex), aCC (anterior cingulate cortex), M1 (primary motor cortex), SJ (somatosensory cortex jaw), S1L/R (left/right primary somatosensory cortex), AC (auditory cortex), V1 (primary visual cortex), A (amygdala), dH/vH (dorsal/ventral hippocampus), NAc (nucleus accumbens), DM/DL (dorsomedial/dorsolateral striatum), VM/VL (ventromedial/ventrolateral striatum), MD (medial dorsal nucleus), VPL/R (left/right ventral posteromedial nucleus), DLG (lateral geniculate nucleus), Y (lateral hypothalamus), SC (superior colliculus), SN (substantia nigra), VTA (ventral tegmental area), DR (dorsal raphe nucleus), MR (median raphe nucleus), CB (cerebellum), TCB-2 (4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide), MDL-100,907 ((*R)-(+)-α-(2,3-Dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperinemethanol*, volinanserin), GLU (glutamate), ASP (aspartate), GLY (glycine), GABA (γ-aminobutyric acid), TAU (taurine), 5-HT (5-hydroxytryptamine, serotonin), DA (dopamine), NA (noradrenaline).

1. Introduction

The serotonin 2A receptor subtype (5-HT_{2A}R) has garnered significant interest with the resurgence of clinical and preclinical research on serotonergic psychedelics, which share a converging agonist action on the 5-HT_{2A}Rs (Kwan et al., 2022; Nichols, 2016). Psychedelics exhibit antidepressant, anxiolytic, and anti-addictive properties, that may be distinct from their hallucinogenic/psychotomimetic effects (Becker et al., 2023; Madsen et al., 2019; Vollenweider et al., 1998). However, the role of 5-HT_{2A}R in mediating these therapeutic benefits remains a topic of ongoing debate (Moliner et al., 2023; Sekssaoui et al., 2024). This renewed interest in the 5-HT_{2A}R also builds up upon existing evidence suggesting that antagonism at the 5-HT_{2A}R, a component of the pharmacological profile of atypical antipsychotics, may confer benefits in treating schizophrenia (Kapur & Remington, 1996; Meltzer et al., 2003). Despite its clinical relevance, the role of 5-HT_{2A}R in regulating brain neurotransmitter systems remains elusive.

Immunohistochemical, *in situ* hybridization, and autoradiographic studies have shown that 5-HT_{2A}Rs are widely expressed in the mammalian cortex, and more limited expression in specific subcortical regions (Nichols, 2016). In the cortex, 5-HT_{2A}Rs are expressed by both GABAergic and glutamatergic neurons in rodents and primates (De Almeida & Mengod, 2007; Jakab & Goldman-Rakic, 2000; Weber & Andrade, 2010). Electrophysiological and biochemical evidence supports both direct and indirect actions of 5-HT_{2A}R-targeting agonists on amino acid neurotransmission, most notably through elevated cortical glutamate (GLU) levels (Aghajanian & Marek, 1999; Scruggs et al., 2003; Wojtas et al., 2024). The modulation of the excitation/inhibition balance by 5-HT_{2A}Rs may play a crucial role in shaping connectivity between brain regions and regulating oscillatory rhythms in the brain. Psychedelics have been shown to alter brain connectivity (Carhart-Harris et al., 2012; Kwan et al., 2022; Timmermann et al., 2023) and desynchronize functional connectivity between brain regions within resting-state networks and oscillatory rhythms (Carhart-Harris et al., 2016; Celada et al., 2008; Müller et al., 2018; Muthukumaraswamy et al., 2013; Siegel et al., 2024). These connectivity changes

may be mediated by altered excitatory and inhibitory amino acid neurotransmission in the brain.

The neurochemical quantification of neurotransmitter content is not feasible in humans; however, in animals, it is possible to measure neurotransmitter levels across multiple brain regions post-mortem. Previous research from our lab indicates that monoamine neurotransmitter connectivity is relatively low in resting conditions (Fitoussi et al., 2013; Marty et al., 2023). In contrast, following forced exploration, the correlation density among monoamines is much higher and exhibits organized patterns across the brain (Butler *et al.*, *submitted*). Novel exploration serves as an unconditioned behavioural assay for studying the effects of psychedelics (Halberstadt & Geyer, 2018). Based on these observations, we hypothesized that 5-HT_{2A}R stimulation could influence the tissue levels and connectivity of excitatory and inhibitory neurotransmitter systems, accompanying psychedelic-induced altered exploration.

To investigate this hypothesis, male C57Bl6/J were placed in a Plexiglas chamber for 60 minutes following administration of a 5-HT_{2A}R agonist. During the final 10 minutes, the Plexiglas floor was replaced with a novel floor featuring four distinct textures and colours to encourage exploration. The tissue content of the amino acids GLU, aspartate (ASP), γ-aminobutyric acid (GABA), glycine (GLY), and taurine (TAU) were quantified in 28 brain regions of mice treated with either vehicle, the 5-HT_{2A}R agonist TCB-2 (0.3, 3, or 10 mg/kg), the 5-HT_{2A}R antagonist MDL-100,907 (0.2 mg/kg), or a combination of MDL-100,907 (0.2 mg/kg) and TCB-2 (3 mg/kg). The amino acid data used in this work was obtained from the same samples from mice previously studied on monoamines (Butler et al., submitted).

2. Methods

2.1. Animals

All experimental procedures in mice were carried out in accordance with the European Communities Council Directive (86/609/EEC + 2010/63/UE) and the Institutional Animal Care Committee of the University of Bordeaux. Every effort was made to minimize animal suffering and reduce the number of animals used. 9-11-week-old male C57BL6/J mice weighing 21.8-27.4 g, were used in this study. Animals were kept in the animal facility (University of Bordeaux, France), with access to standard chow and water ad libitum, in a constant temperature (21 ± 2 ^{o}C) and humidity (60%) under a 12 h light/dark cycle.

This data corresponds to a larger neurochemical dataset, the action of TCB-2 and MDL-100,907 has been previously described for monoaminergic systems (Butler *et al*., *submitted*). For details on the dissection method and quantifications of monoamines post-mortem see our previous publications (Chagraoui et al., 2019; De Deurwaerdère et al., 2020; Butler *et al*., *submitted*).

2.2. Experimental design and pharmacological treatment

TCB-2 (4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl) methylamine hydrobromide and MDL-100,907 *((R)-(+)-α-(2,3-Dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperinemethanol)* were freshly diluted the day of experiments as free base in NaCl 0.9% and Tween 0.3% in NaCl 0.9%, respectively, and stored on ice. MDL-100,907 (0.2 mg/kg) or vehicle was administered intraperitoneally (i.p.) at 5 ml/kg, 15 min prior to TCB-2 (3 mg/kg) or vehicle i.p. at 7.5 ml/kg. Animals were pseudo-randomized for each experimental group (at least one treatment per day in a random order) and

treatments were administered in a blinded fashion between 11 am and 2 pm (maximum 8 mice per day). The doses of TCB-2 were selected to represent a behaviourally low active dose (0.3 mg/kg), an active dose (3 mg/kg), and a high dose (10 mg/kg) (Di Giovanni & De Deurwaerdère, 2018). The solvent of MDL-100,907 was administered in controls, and in mice receiving 0.3 mg/kg or 10 mg/kg. The 0.2 mg/kg dose of MDL-100,907 was chosen with respect to a previous article reporting its ability to block head twitch response (HTR) at 0.1 mg/kg although in a limited amount of time (Halberstadt, 2015). Thus, 0.2 mg/kg was selected to maximize 5-HT_{2A}R blockade for 1h 15 minutes, until sacrifice.

After MDL-100,907 or vehicle administration mice were placed in an isolated chamber (33 x 33 x 25 cm) for behavioural observation and then injected with TCB-2 or vehicle 15 min later (Figure 1A). Head twitch responses (HTR) were observed for 1 min at 20, 30, 40 and 50 min post TCB-2 or vehicle administration. Following the last HTR, the Perspex floor was replaced with a novel 'textured' floor composed of 4 distinct textures: red felt, brown hessian, white sponge and orange microfiber chenille. Mice were allowed to explore this new floor for the 10 minutes prior to sacrifice and their exploration videorecorded (8 minutes) from above.

2.3. Post-mortem Neurochemical quantification

Tissue concentrations of monoamines and amino acids were measured using distinct HPLC systems coupled to electrochemical detection, each optimized for the specific conditions required by the analytes. The procedures and the quantities of monoamines and their metabolites in each sampled brain region were the subject of a submitted article (Butler *et al*., *submitted*).

Amino acid mobile phase consisted of methanol (28%), $NaH_2PO_4 \cdot 2H_2O$ (70 mM), $Na_2HPO_4 \cdot 2H_2O$ (50 mM), EDTA (0.1 mM), and was diluted in deionised water (pH 6.1, adjusted with orthophosphoric acid). Prior to installation, mobile phases were filtered (0.22 µm). Pre-column derivatization with o-pthalaldehyde (OPA)/β-mercaptoethanol (BME) was used to enhance amino acid signal. The stock derivative reagent, composed of methanol (10%), OPA (2.7 mg/ml), BME (0.5 µl/ml), sodium-tetraborate (0.09 M) in deionised water, was made 24 h prior to use and kept for no more than 3 days. On the day of injection the stock derivative reagent was diluted 1:10 in sodium-tetraborate (0.1 M) and 50 µl added to 25 µl of standard or sample supernatant 2 minutes prior to injection (Donzanti & Yamamoto, 1988). Samples were injected using a manual injection valve (Rheodyne, model 7725i, C.I.L. Cluzeau, Sainte-Foy-La-Grande, France) with a 20 µl loop. The amino acid mobile phase was passed through the column (Force C18, 100 x 4.6 mm, particle size 3 µm, Restek, France) at a flow rate of 1 ml/min using an HPLC pump (System Gold 118 Solvent Module, Beckman Coulter, France). Post separation mobile phase was passed through a coulometric detection cell (Cell 5011, ESA, Paris, France) with electrodes at -400 mV (reduction) and +600 mV (oxidation). Coulometric cells were connected to detectors (Coulochem II, ESA, Paris, France) which were interfaced with a computer with software for chromatogram acquisition (Azur 5.0, France).

Systems were calibrated flowing the separation of compounds using orthophosphoric acid. Amino acid standards (≤3.3 ng/10 µl; Figure 1C) were used to create a three-point calibration curve for each compound, adjusted according to the specific brain area. Gain was configured using a timeline method on the detector, ranging from 200

nA to 1 µA. Daily injections of standards were performed prior to sample quantification to verify the time of elution and calibration.

2.4. Behavioural analysis

In addition to HTR measured at 20, 30, 40 and 50 min post TCB-2 or vehicle administration, exploratory behaviour was assessed from 50 to 58 min. Exploration was videorecorded from above after switching the observation chamber flooring, from a Perspex to textured (Figure 1A). Video data were analysed using a trained network in DeepLabCut 2.3.6 to identify 14 points on the mouse: nose, ears, shoulders and hips, 3 spine points and 4 tail points (Figure 4C) (Lauer et al., 2022). Path tracing was calculated using the mouse's centre point, normalized to real-world dimensions, for calculating distance travelled. Behaviours such as huddling, sniffing and wall-climbing were extracted using DeepOF v2.0 package in Python (Bordes et al., 2023; Miranda et al., 2023). Huddling was identified through machine learning-based annotation, wall climbing was defined as the nose reaching beyond wall by >10 mm, and sniffing was defined by a nose-to-object distance of <10 mm, nose speed >50 mm/s and locomotion speed <50 mm/s.

2.5. Statistical analysis

2.5.1 Normality and Outlier Testing

The neurochemical dataset contains tissue content of each measured compound, amino acid, monoamine or metabolite, in each brain region sampled. The behavioural dataset contains behaviours as % of total time (8 min) or as the total time in seconds for each behaviour. Outliers were identified and removed using the Grubbs test, with a maximum of 2 iterations, from both the neurochemical and behavioural dataset. All

behavioural data was normally distributed (Shapiro-Wilk, $p<0.05$). For the neurochemical dataset, 95.5 % (1254 /1314 compound region combinations) of the data for GLU, GABA, GLY, ASP, TAU, 5-HT, DA and NA in each region were normally distributed (Shapiro-Wilk, $p<0.05$). Therefore, both datasets were treated as parametric.

2.5.2 Quantitative Analysis

Quantitative statistical analysis was divided into two approaches to address the dose-response effects and the interaction TCB-2 and MDL-100,907. For the dose response-analysis, involving the groups treated with vehicles, 0.3, 3, 10 mg/kg TCB-2, a one-way ANOVA was performed. To examine the interaction between TCB-2 (3 mg/kg) and MDL-100,907, involving the groups treated with vehicles, 3 mg/kg TCB-2, 0.2 mg/kg MDL-100,907 and the combination TCB-2/MDL-100,907, a two-way ANOVA was used. When significance was detected, a post hoc Tukey's test was performed.

2.5.3 Correlation Analysis

Correlative analysis of tissue quantity was performed using the Pearson correlation coefficient to examine relationships between individual neurotransmitters, pairs of neurotransmitters or neurotransmitter and behaviour. This analysis was conducted independently for each experimental group, with significance set at $p<0.05$. Significant correlations were compiled into a matrix, which may be visualized as a correlogram.

2.5.4 Network Analysis

The resulting Pearson correlation matrices of neurotransmitters between brain regions were used to construct networks, where brain regions were represented as nodes and

significant correlations as edges. Basic network parameters, such as density, were compared across groups. Since all analysed networks shared an identical set of nodes, no additional normalization was applied.

2.5.5 Data and Code availability

Quantitative data for each compound-region-treatment combination was manually compiled in MS Excel and subsequently processed using a custom Python script designed for neurochemical multivariate analysis (code available upon request).

3. Results

3.1. Neurotransmitter quantity

Tissue quantities of amino acid neurotransmitters for vehicle-treated animals are reported in Table 1. GLU was generally found in the highest concentrations across the brain, followed by TAU, ASP, GABA then GLY. GLU levels were homogeneous across brain regions, TAU was most abundant in the striatum, cortex and cerebellum, ASP concentrations peaked in the striatum and somatosensory jaw cortex. GABA was highest in the ventral striatum, superior colliculus and substantia nigra, whereas GLY was most prominent in the superior colliculus.

Table 1 – Quantity of amino acid neurotransmitters GLU, ASP, GABA, GLY, and TAU across the mouse brain. See table S1 and S2 for statistical analysis between dose response and agonist antagonist treatments.

region	ASP	GABA	GLU	GLY	TAU
OF	166.8 ± 66.7	124.2 ± 53.1	1083.8 ± 433.8	59.0 ± 25.9	747.3 ± 324.0
PL	184.1 ± 65.6	96.7 ± 43.6	862.7 ± 349.6	57.9 ± 16.9	631.3 ± 275.2
aCC	186.6 ± 81.1	88.6 ± 39.3	782.3 ± 325.2	44.2 ± 17.2	519.6 ± 233.3
M1	182.7 ± 61.3	118.0 ± 34.3	872.8 ± 308.4	95.7 ± 19.7	680.5 ± 190.2
SJ	439.0 ± 161.9	74.2 ± 36.6	856.7 ± 322.5	49.5 ± 19.9	439.1 ± 173.2
S1L	199.1 ± 83.9	105.3 ± 37.4	637.3 ± 216.5	36.4 ± 11.2	559.6 ± 203.2
S1R	196.2 ± 117.0	115.7 ± 59.5	730.6 ± 354.3	38.4 ± 19.2	557.6 ± 292.9
AC	106.8 ± 44.2	74.0 ± 36.1	644.6 ± 268.5	42.8 ± 18.3	572.5 ± 237.3
V1	119.3 ± 63.8	99.0 ± 37.7	896.8 ± 457.2	70.4 ± 22.1	714.2 ± 268.2
A	96.4 ± 28.5	110.3 ± 32.9	563.7 ± 138.3	32.9 ± 6.8	355.2 ± 77.1
dH	152.2 ± 54.4	172.0 ± 52.8	745.8 ± 244.3	42.7 ± 12.5	643.7 ± 189.3
vH	325.1 ± 82.8	114.5 ± 33.4	853.9 ± 218.8	40.0 ± 10.0	475.1 ± 181.7
NAc	294.0 ± 81.5	87.8 ± 30.1	497.9 ± 147.0	30.5 ± 1.7	442.5 ± 156.6
VM	407.9 ± 204.8	179.8 ± 99.1	603.6 ± 293.6	41.4 ± 19.1	655.9 ± 297.7
DM	546.7 ± 217.4	96.7 ± 37.8	747.6 ± 262.5	52.4 ± 16.4	869.3 ± 317.8
VL	535.4 ± 178.9	215.8 ± 140.8	784.5 ± 214.2	54.5 ± 15.0	1072.2 ± 331.0
DL	517.2 ± 186.8	116.2 ± 43.2	771.9 ± 279.4	49.6 ± 15.3	952.6 ± 320.3
MD	123.1 ± 42.8	124.3 ± 49.4	626.3 ± 233.7	50.5 ± 17.3	336.9 ± 123.9
VPL	171.9 ± 90.4	74.4 ± 50.0	521.4 ± 271.5	31.2 ± 16.4	226.3 ± 133.7
VPR	193.8 ± 48.0	87.8 ± 48.4	506.0 ± 173.9	38.1 ± 11.1	240.4 ± 85.0
DLG	189.1 ± 115.2	92.6 ± 56.6	582.1 ± 338.7	30.3 ± 14.9	294.7 ± 171.5
SC	113.9 ± 38.5	205.9 ± 85.8	576.6 ± 268.5	93.3 ± 35.4	260.1 ± 100.7
SN	130.2 ± 37.4	298.1 ± 103.6	350.6 ± 126.5	29.4 ± 10.8	359.0 ± 134.0
VTA	111.0 ± 58.6	137.2 ± 67.0	339.6 ± 171.9	34.7 ± 15.9	181.6 ± 82.3
DR	110.0 ± 41.6	156.0 ± 59.1	408.3 ± 176.3	58.8 ± 15.1	199.5 ± 97.3
MR	59.2 ± 15.7	106.0 ± 55.0	256.1 ± 93.8	43.1 ± 14.7	140.5 ± 61.9
CB	157.8 ± 62.9	96.9 ± 41.5	751.6 ± 296.6	45.5 ± 14.4	489.5 ± 170.7

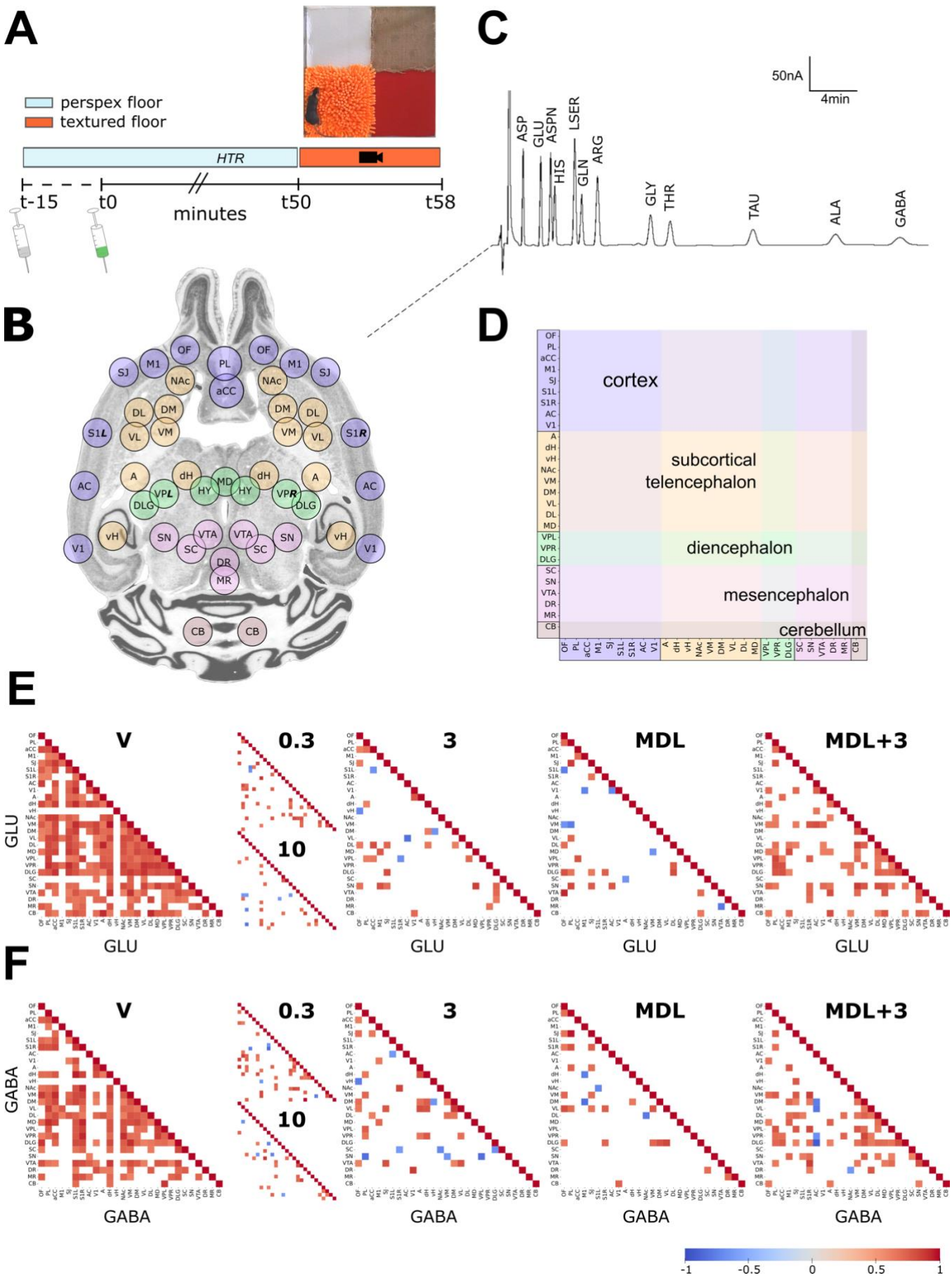
TCB-2 (0.3, 3 or 10 mg/kg) enhanced ASP levels in the dorsal (dH) and ventral (vH) hippocampus, the dorsolateral striatum (DL), the superior colliculus (SC) and the median raphe nucleus (MR), although GLU levels remain unchanged (one-way ANOVA, $p>0.05$; Figure S1). GLY levels were elevated in primary visual cortex (V1), SC, and vH while TAU levels were enhanced in SC, dorsolateral striatum (DL) and dH. GABA levels were enhanced only in the SC. Notably, in the SC, the 0.3 mg/kg dose of TCB-2 was the most effective across all transmitters, at variance from other brain regions. MDL-100,907 (0.2 mg/kg) interacted with TCB-2 (3 mg/kg) to affect ASP levels in anterior cingulate cortex (aCC), V1, vH, MR, and substantia nigra (SN), with MDL-100,907 alone increasing ASP levels in the SN (Figure S1). No interaction was

observed for GABA, and only one interaction was found for GLU and TAU in V1. However, the compounds interacted to alter GLY content in V1, vH, and SN (Supplementary Table S1 and S2, Figure S1).

3.2. Organized Correlation

3.2.1. Within neurotransmitter systems

Neurochemical connectivity is described by the correlation of neurotransmitter levels between brain regions. Significant ($p<0.05$) Pearson correlations of regional tissue quantity of neurotransmitters are reported in correlograms, with correlation colour indicating the Pearson r value. Figure 1B shows the brain regions sampled, while Figure 1D presents the conserved regional organization for all correlograms, grouped into the cortex, subcortical telencephalon, diencephalon, mesencephalon and cerebellum, ordered from antero-dorsal to posterior-rostral. As previously reported for monoamines, executive neurotransmitters such as GLU and GABA displayed strong interregional correlation in vehicle-treated animals (Figure 1E-F). Similar patterns were observed for ASP and TAU, although GLY had fewer correlations (Figure S2). Figure 2A shows the density of correlations for each neurotransmitter system network. In vehicle-treated animals, GLU, TAU, 5-HT, ASP and GABA had significant correlations between more than 40% of all possible region pairs. Catecholamines and GLY exhibited fewer correlations. The density of correlations within a neurotransmitter system decreased in response to MDL-100,907 alone and dose dependently with TCB-2. Despite this reduction, when MDL-100,907 was combined with 3 mg/kg of TCB-2, the correlation density was marginally restored and hierarchy resembled that of vehicle-treated animals. Additionally, the ratio of negative/positive significant correlations within a neurotransmitter system increased with TCB-2 (3 and 10 mg/kg) or MDL-100,907 alone, but not with their combined administration (Figure 2B).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1

Figure 1 – Correlation within neurotransmitters systems. A Experimental timeline showing the administration of MDL-100,907/vehicle at t-15, TCB-2/vehicle at t0, HTR measurement from t20 to t50 and novel flooring from t50 to t58. **B** Brain regions color-coded by structure, with a horizontal brain image adapted from the Mouse Brain Library, Atlas 232, https://www.mbl.org/atlas232/atlas232_frame.html. **C** Standard chromatogram displaying 3.3 ng/10µl of amino acids: Aspartate (ASP), Glutamate (GLU), Asparagine (ASPN), Histidine (HIS), L-serine (LSER), Glutamine (GLU), Arginine (ARG), Glycine (GLY), Threonine (THR), Taurine (TAU), Alanine (ALA), gamma-aminobutyric acid (GABA). **D** Organizational structure of brain regions used in all correlogram displays. Correlograms of **E** Glutamate (GLU) correlations and **F** GABA correlations between brain regions. Significant Pearson correlations indicated according to their Pearson R: red for positive and blue for negative, $p<0.05$.

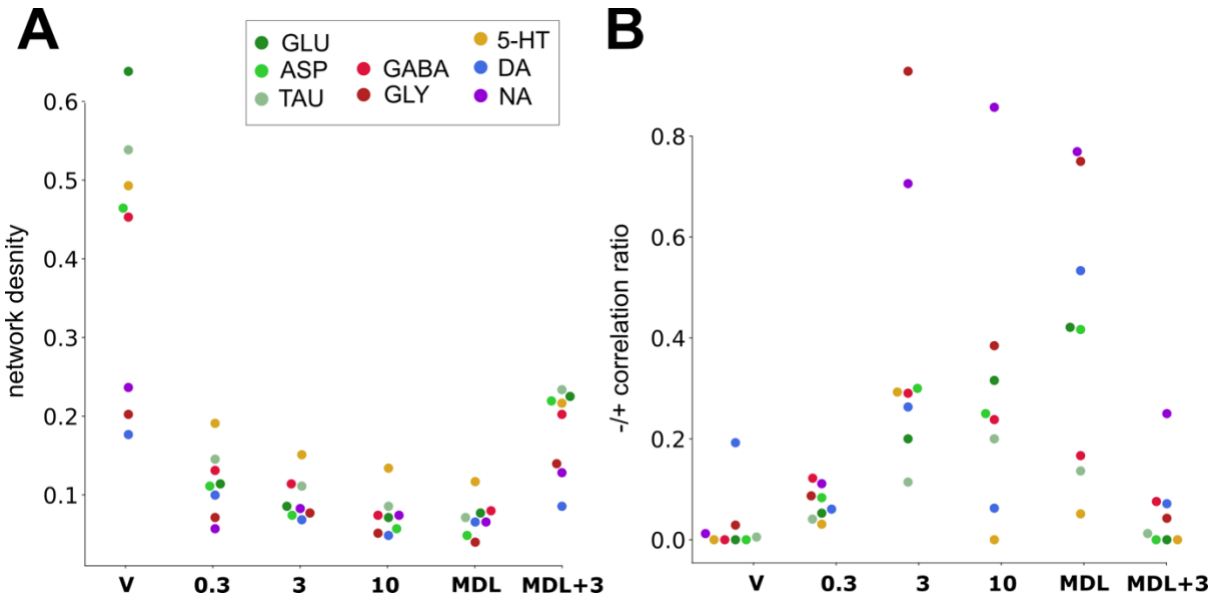

Figure 2 – Neurotransmitter connectivity as described by correlative networks is reduced by TCB-2 (0.3, 3, 10) and MDL-100,907. A Density of network correlations within neurotransmitter networks and **B** ratio of negative/positive correlations for vehicle- (V), 0.3, 3, 10 mg/kg TCB-2-, MDL-100,907- (MDL) and drugs co-administration animals (MDL+3). For detailed neurotransmitter correlograms, see Figure S2.

3.2.2. Between neurotransmitter systems

The relative connectivity between neurotransmitter systems was assessed using Pearson R correlations between regional tissue levels of neurotransmitter pairs. The resulting correlation matrices were used to construct networks of tissue correlations between brain regions. Figure 3 shows the organization of executive neurotransmitters, GLU and GABA, with catecholamines DA (Figure 2A, B) and NA (Figure 2C, D) in correlograms structured as in Figure 1D. Similar to correlations within

a neurotransmitter system, network density is highest in vehicle-treated animals (Figure 4A). Furthermore, a clear regional structure is apparent for neurotransmitter correlations with catecholamines, visible as vertical bands of correlation. This clear organization is not observed when correlating 5-HT with amino acid neurotransmitters (Figure S5).

DA in the prelimbic cortex (PL), aCC and to a lesser extent left somatosensory cortex (S1L) correlates with GLU, GABA, ASP, TAU and GLY across the brain (Figure 3A-B, S3). Subcortical DA in the striatum (VM, DM, VL, DL), mediodorsal and particularly right ventral posteromedial nucleus of the thalamus (MD, VPL, VPR), SC, SN, ventral tegmental area (VTA) and cerebellum (CB) also tend to correlate with amino acid neurotransmitters across the brain in vehicle-treated animals. Notably, DA in the SN had only negative correlations across the brain with all other neurotransmitters.

The regional organization imposed by NA in vehicle-treated animals included all cortical regions, with the least extensive correlations observed in the somatosensory cortex, although preferentially in the S1R, in contrast to DA. Subcortical NA tissue content in the amygdala (A), medial striatum (VM>DM), diencephalon (VPL, VPR, DLG), VTA, medial and dorsal raphe (MR, DR), CB and to a lesser extent the SC tended to correlate with amino acid neurotransmitters across the brain (Figure 3C-D).

TCB-2 at all doses and MDL-100,907 significantly reduced the number of correlations between neurotransmitter systems (Figure 4A). Both DA and NA regional organization of correlations is no longer evident in the presence of TCB-2 (3 and 10 mg/kg) or MDL-100,907 alone (Figure 3). However, the combination TCB-2/MDL-100,907 exhibited an increased density relative to either MDL-100,907 or 3 mg/kg TCB-2 alone and bore resemblance to the organization described for vehicle-treated animals.

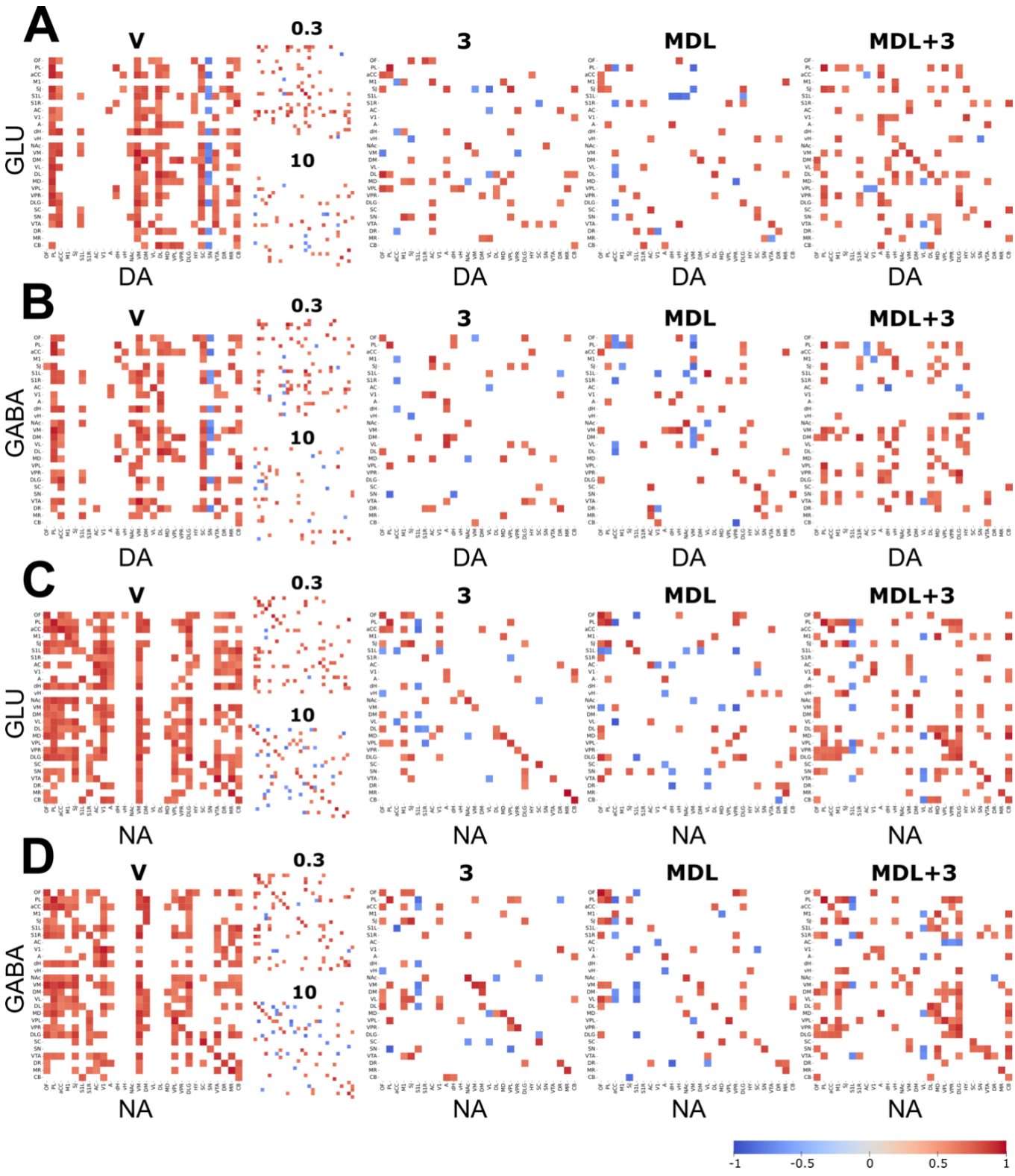

Figure 3 – Organization of correlations between amino acid neurotransmitters and catecholamines DA and NA. A-B DA correlations with Glutamate (GLU) and GABA, respectively. **C-D** NA correlations with Glutamate (GLU) and GABA, respectively.

Despite a general loss in neurotransmitter connectivity with both the agonist and the antagonist, an increase in the number of negative correlations was observed for 3 and 10 mg/kg TCB-2, as well as MDL-100,907 alone. This increase in negative correlations is reflected by the heightened ratio of negative/positive correlations (Figure 4B). Negative correlations were most prominent with catecholamines regardless of treatment, as indicated by purple/blue hues in Figure 4B. Additionally, catecholamine connectivity also tended to have the lowest network density (Figure 3A).

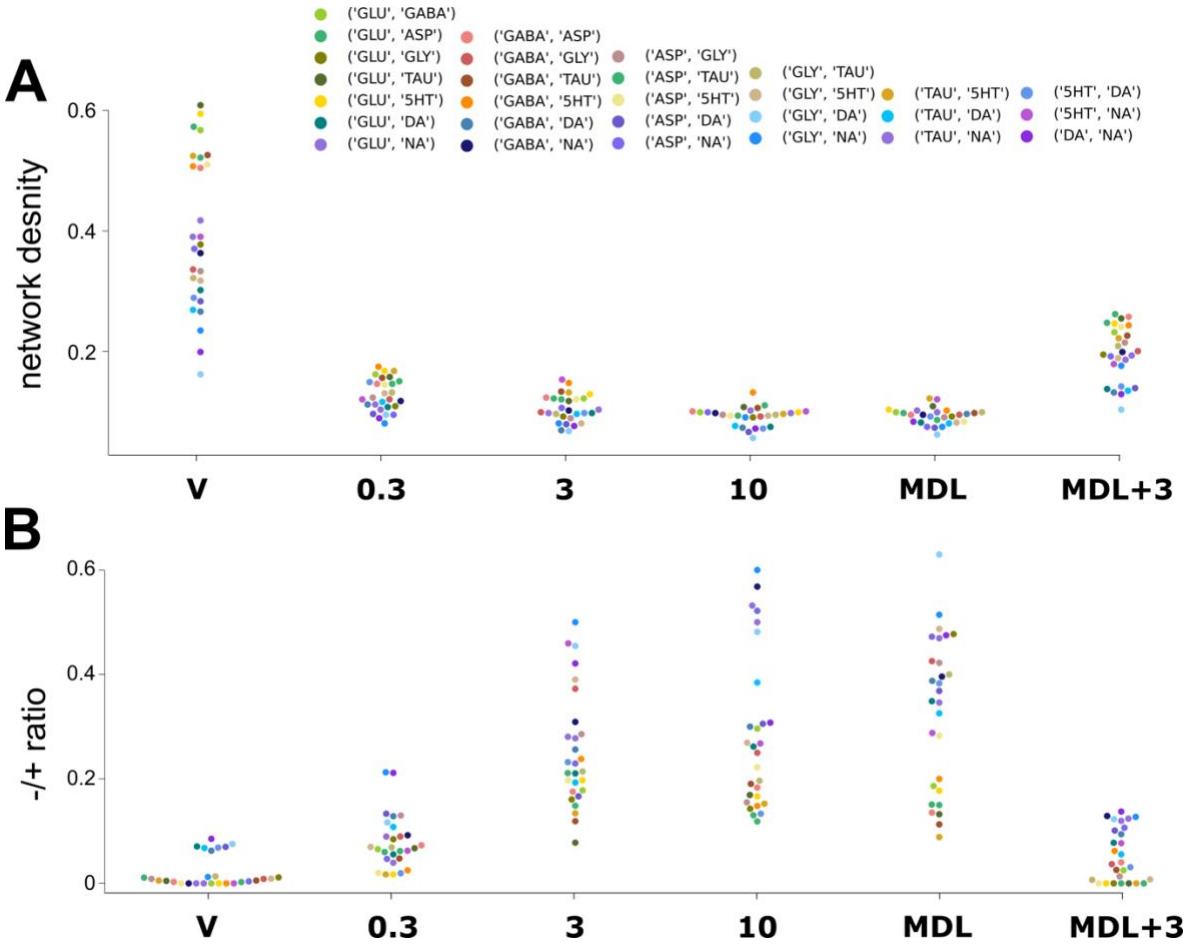

Figure 4 – Connectivity between neurotransmitters as described by correlative networks. A Density of network correlations between neurotransmitter pairs and **B** ratio of negative/positive significant correlations for vehicle- (V), 0.3, 3, 10 mg/kg TCB-2-, MDL-100,907- (MDL), and drugs co-administration (MDL+3). For complete correlograms of neurotransmitter pairs, see Figures S3, S4 and S5.

3.3. Behaviour

During forced exploratory behaviour from 50-58 min after TCB-2 or vehicle administration, just prior to sacrifice, behavioural differences between TCB-2- (particularly 3 and 10 mg/kg) and MDL-100,907-treated animals were observed. The TCB-2 dose-response data showed increased locomotion at 3 mg/kg relative to vehicle-treated animals (one-way ANOVA, $p<0.01$). Increased locomotion was not blocked by MDL-100,907 (two-way ANOVA, $p=0.67$). Sniffing and climbing behaviours were quantified during exploration, with vehicle-treated animals spending the most time on both activities. The dose-response with TCB-2 revealed that at 3 and 10 mg/kg TCB-2 reduced climbing and sniffing time (Figure 4A; Table S3). No interaction was found between TCB-2 3 mg/kg and MDL-100,907 for these behaviours (two-way ANOVA, $p>0.05$; Table S4). MDL-100,907 alone did not alter any behaviour measured, including huddling, which was also unaffected by TCB-2 at any dose.

Locomotion was also assessed by path-tracing of each animal during the exploratory period (Figure 5B). Vehicle-treated animals tended to move along the boundaries of each textured floor and the walls of the arena, with no place preference. This tracing of edges was less apparent in TCB-2 (3 and 10 mg/kg), and MDL-100,907-treated animals. Vehicle-treated animals spent approximately 25% of the time on each texture/quadrant (Figure 5C). TCB-2 at doses of 3 and 10 mg/kg induced a significant place preference, with the percentage of time spent on the white flooring (top left) reduced in favour of the orange flooring (bottom left). MDL-100,907 alone did not induce a significant preference. In the combination, MDL-100,907 did not block the TCB-2 induced increased time on orange floor, however, did attenuate the reduced time spent on the white floor (two-way ANOVA $p=0.226$ and $p=0.007$, respectively).

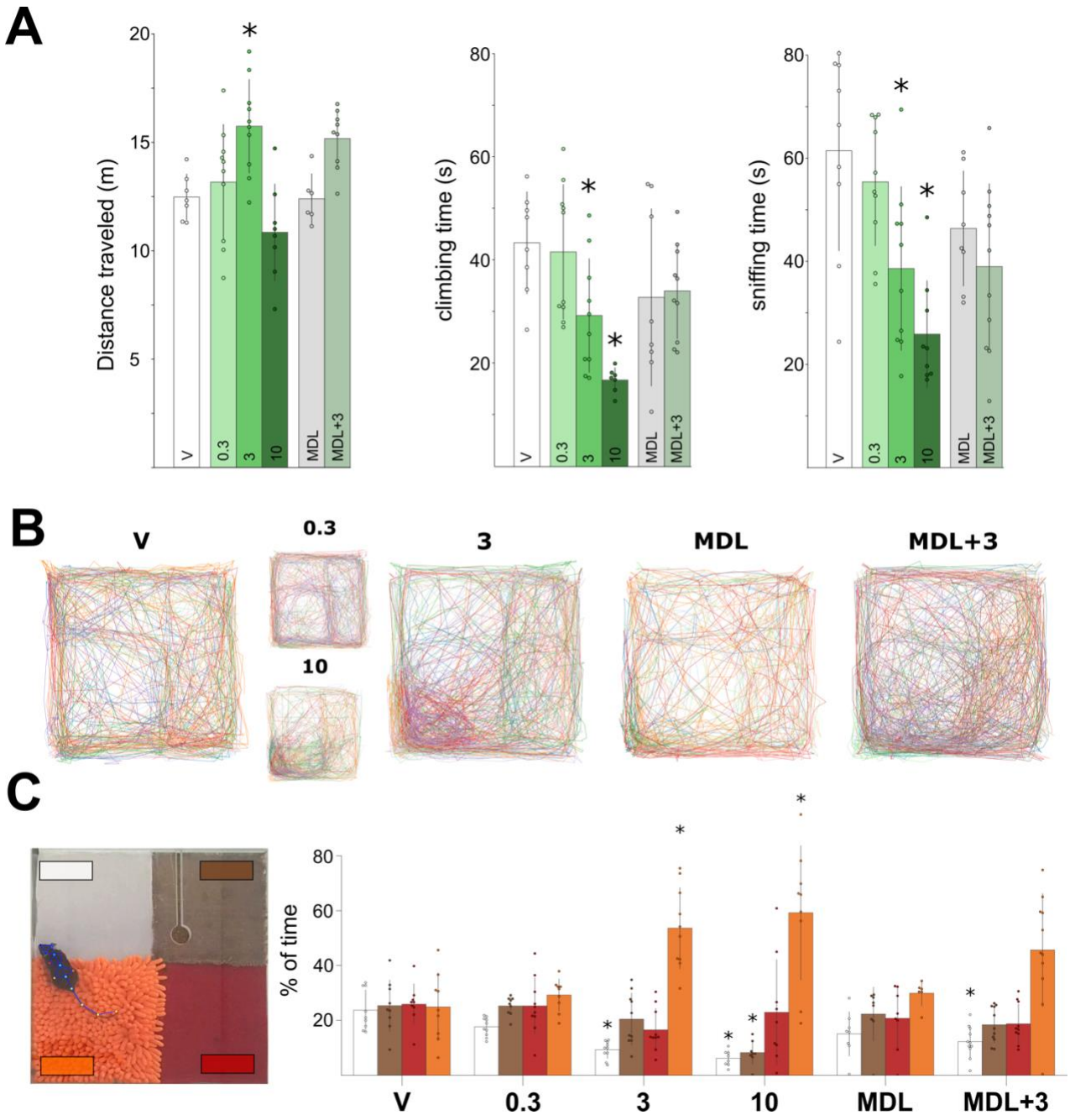

Figure 5 – Behavioural modifications induced by TCB-2 and MDL-100,907 (0.2 mg/kg), 50-58 minutes post TCB-2 or vehicle administration. A Distance travelled in meters, and time spent climbing and sniffing in seconds, reported as mean ± standard deviation. **B** Path traces of each animal during exploration for vehicle (V), TCB-2 (0.3, 3, 10), MDL-100,907 (MDL) or drugs co-administration (MDL+3) treated-mice. **C** Time spent (%) on each textured floor. Significance is reported Tukey's test * $p<0.05$, following a significant one-way ANOVA (V, 0.3, 3, 10; Table S3), or two-way ANOVA (V, 3, MDL, MDL+3: Table S4).

3.4. Correlations between behaviours and neurotransmitters by regions

Behavioural measures correlate selectively with neurotransmitters in distinct brain regions (Figure 6). In vehicle-treated mice, significant correlations recurrently occurred between the time spent on the orange floor (mostly positive) and the red floor (mostly negative) with each neurotransmitter system. The other correlations were sparser and specific to particular neurotransmitter-behaviour pairs, such as climbing and 5-HT (in S1R, AC, NAc, DM, SN). The number of correlations was similar after TCB-2 (3 mg/kg) administration, but the correlations with the orange floor, but not the red floor, were reduced. After MDL-100,907 treatment alone, few cortical correlations were observed, which were predominately negative. The lowest number of correlations was reported for the combination of MDL-100,907 and TCB-2 (MDL+3; Figure 3), with only isolated correlations with GLU, DA or NA in the cortex, and no correlations for DA or NA in the brainstem.

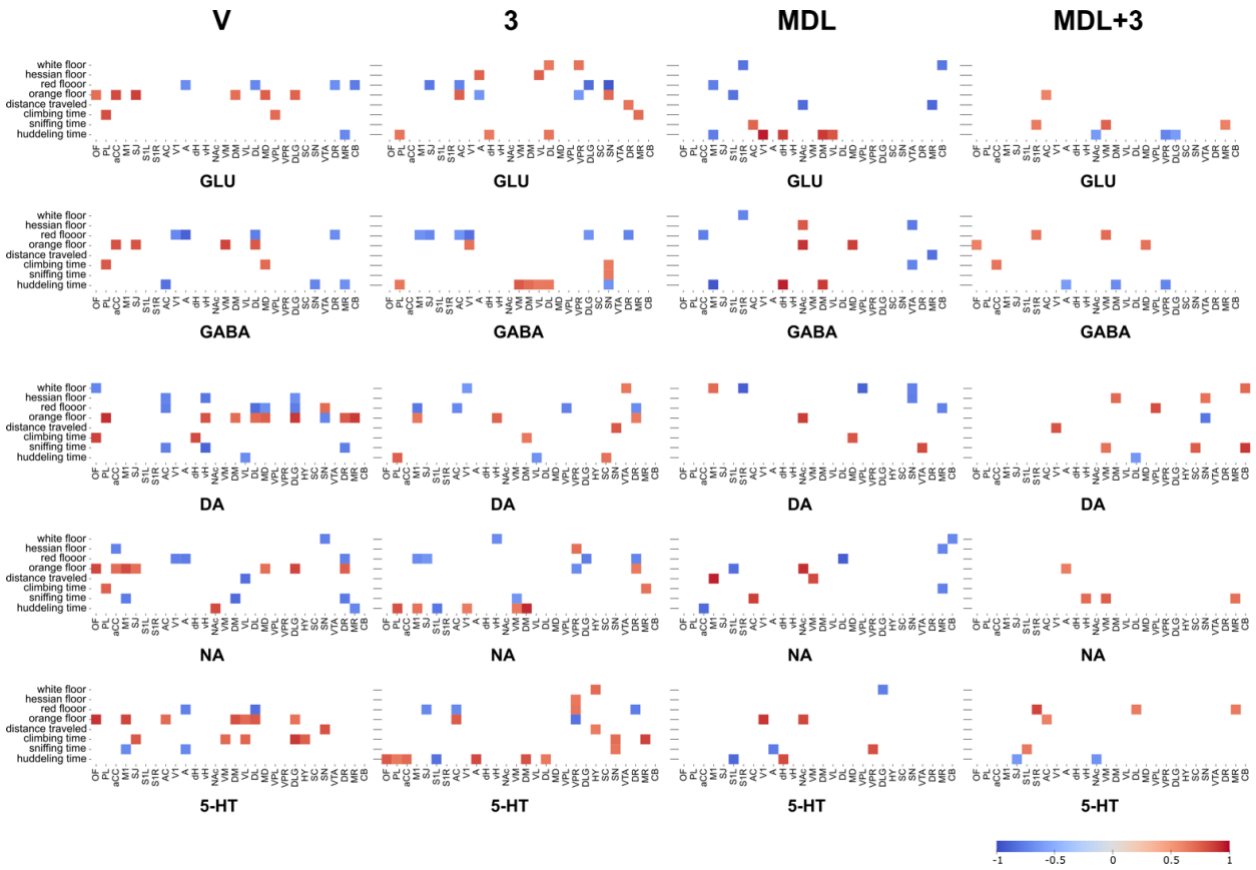

Figure 6 - Correlations between behavioural parameters and regional neurotransmitter quantity. Correlations between GLU, GABA, DA, NA, and 5-HT tissue content and behavioural measures—including percentage of time spent on white, hessian, red, and orange flooring, distance travelled, climbing, sniffing, and huddling—were assessed for vehicle (V), 3 mg/kg TCB-2 (3), 0.2 mg/kg MDL-100,907 (MDL), and drugs co-administration (MDL+3). For correlations observed with 0.3 and 10 mg/kg TCB-2, refer to Figure S8.

4. Discussion

We report here, for the first time, the tissue concentration of 5 major amino acid neurotransmitters in 28 regions of the mouse brain after a protocol of forced exploratory behaviour. The results show an impressive density of correlations for amino acids between brain regions in vehicle-treated mice, that was dramatically reduced by both the psychedelic TCB-2 and the 5-HT_{2A}R antagonist MDL-100,907. Unlike MDL-100,907, TCB-2 significantly altered exploration, locomotor activity, and other behaviours that were not blocked by MDL-100,907. Certain behavioural features, particularly time spent on specific component of the novel environment, correlated with regional concentrations of neurotransmitters including monoamines, and were altered by both 5-HT_{2A}R ligands. These results highlight distributed neurochemical networks engaged in exploratory behaviour that are disturbed by 5-HT_{2A}R ligands.

The reported amino acid tissue quantities align with previous post-mortem findings using HPLC (Kim *et al*., 2014; Donzanti and Yamamoto, 1988). Remarkably, the connectivity profile of amino acids was dense in vehicle-treated animals. The connectivity of amino acid neurotransmitters in resting conditions is presumably low, like for monoamines (Fitoussi *et al*., 2013; Marty *et al*., 2023), and the high level of connectivity is likely related to the forced exploration of novel environment. The density of network connectivity was highest for GLU between regions, and the lowest for GLY. This pattern also extends to correlations between compounds reaching the highest density of any network (60.8%) for GLU-TAU in vehicle-treated mice. Interestingly, TAU showed a striking resemblance to GLU, which is not so surprising in view of the data showing that GLU receptors promote TAU release (Gobert et al., 2011; Oja & Saransaari, 2000), while GLY was clearly under a different type of connectivity. The ventral hippocampus (vH), at variance with the dorsal (dH), rarely exhibited correlations. This is likely not a detection error as we found the same for monoamines, which were analysed with different HPLC systems. Thus, the lack of correlative links in the vH might be specific of the exploratory environment.

Correlating amino acids with DA or NA tissue content revealed a regionally structured pattern, corresponding to either catecholamine, in vehicle-treated animals. Tissue DA in the prelimbic and anterior cingulate cortices (PL, aCC) had a clear tendency to

correlate with other neurotransmitters across the brain, NA had this tendency for a boarder range of cortical regions, illustrating the differential influences of DA and NA in the cortex and more generally in the brain (Cerpa et al., 2021; Shinohara et al., 2020). Additionally, DA in the SN, having exclusively negative correlations even with its direct projections to the striatum, provides evidence that these correlative maps correspond to emerging properties of the system rather than the anatomical link between two regions. This functional catecholamine signature is particularly pertinent given the correlative pattern obtained with 5-HT which is without specific signature, closer to what we saw with amino acids (Figure S5).

Catecholamines appear to shape connectivity responses on other neurotransmitters, which corresponds to their responsiveness to novelty, stress, and arousal (Düzel et al., 2010; Lisman & Grace, 2005; Schomaker & Meeter, 2014; Vankov et al., 1995). This point is important because TCB-2 and MDL-100,907 reduced the density of correlations and increased the proportion of negative correlations, with no apparent regional organization. The loss of a clear organization or pattern of amino acids correlations suggests that TCB-2 (3 and 10 mg/kg) and MDL-100,907 disturb the structuration of the responses of neurochemical systems, possibly by altering catecholaminergic transmission. Even though the patterns obtained with TCB-2 and MDL-100,907 were different, the analysis through graph theory, which seemed appropriate for our quantitative data (Bullmore & Sporns, 2009) was impaired by the loss of density of correlations induced by 5-HT_{2A}R ligands. In our recent work (Butler *et al*., *submitted*), we hypothesized that regional organization of correlations between monoamines could be quantified by the variability of connections (correlations) associated with each node (region). Specifically, an organized system would exhibit greater variability in connections, with some regions showing high correlations (aCC) and others exhibiting little to no correlation (vH). A common metric to quantify this variability is the standard deviation of node degree. However, this metric, along with others such as clustering coefficient, is intrinsically influenced by graph density, confounding comparisons across networks with differing densities (Figure S6) (Van Wijk et al., 2010). To normalize densities, higher-density networks must have edges (significant correlations) removed to match the lowest-density network, this approach risks dismissing biologically significant correlations based on a ranking that may not be representative of their biological importance.

The quantitative data on amino acids did not reveal substantial effects of 5-HT_{2A}R ligands, particularly on GLU and GABA levels. TCB-2 increased slightly ASP levels in 5 brain regions including the ventral and dorsal hippocampus (vH, dH). TAU and GLY were also modified in 3 brain regions. TAU is an intriguing compound that can interact with the ionotropic and metabotropic GLY receptors, with GABA receptors depending on their composition, and possibly on NMDA receptors under specific circumstances (Chan et al., 2013; Laboute et al., 2023). Given that TAU concentrations are higher than GABA in some brain regions and GLY in all brain regions, TAU should not be excluded from the global inhibitory neurotransmitters in the brain (Albrecht & Schousboe, 2005; Gobert et al., 2011). Post-mortem data for amino acids (mM range) mask release and extracellular events (µM range) (Gobert et al., 2011), which have been reported following 5-HT_{2A}R stimulation, at least for GLU and GABA in the frontal cortex, the hippocampus, the striatum and the NAc (Aghajanian & Marek, 1999; Herian et al., 2020, 2021; Scruggs et al., 2003; Wojtas et al., 2024). Yet the distinct distributions of the sampled amino acids across tissues, and the correlative specificities, suggest that these values include, in part, neurotransmission.

Following the two highest doses of TCB-2, mice spent more time on the orange floor at the expense of the white floor, an effect that remained present (for the orange floor) in the combination TCB-2/MDL-100,907 treatment. TCB-2 at 3 mg/kg increased distance travelled. This was expected based on previous experiments (Halberstadt *et al*., 2013). However, the unexpected lack of effect at 10 mg/kg TCB-2 recalls that our experimental conditions do not permit clear comparison with systems designed for studying locomotor activity. The increased locomotion is unlikely to account for the altered time spent on different textures because avoidance of the white floor and preference for the orange, occurs with (TCB-2, 3 mg/kg), or without (TCB-2, 10 mg/kg) altered locomotor activity. TCB-2 at 3 and 10 mg/kg reduced time spent sniffing and climbing, with no change in time spent huddling. Not all the behaviour changes induced by TCB-2 (3 mg/kg) are related to 5-HT_{2A}R as they are still observed in the presence of MDL-100,907, or not significantly reduced (climbing). The lower time spent on the white floor induced by TCB-2 was slightly attenuated by MDL-100,907, like the trend toward increased time on the orange floor although it did not reach significance in this latter case.

These behavioural effects parallel the neurochemical findings, suggesting that TCB-2 triggers mechanisms other than the stimulation of 5-HT_{2A}R. If MDL-100,907 fully blocked TCB-2's effects, the correlative maps with amino acids alone or combined with monoamines would have been similar between the MDL-100,907 and combination TCB-2/MDL-100,907 group. This was clearly not the case. Not only was the density of correlations increasing upon the combination, with a more organized profile, but there was also smaller proportion of negative correlations compared to the patterns obtained with each separate treatment. This situation of partial recovery upon the combination of drugs corresponds to what we saw with monoamines. It is unclear what is behind this partial recovery, corresponding to a situation where the novel environment regains its impact on brain neurochemistry over the blockade of 5-HT_{2A}R. We cannot exclude that this in-between situation corresponds to a competition of TCB-2 and MDL-100,907 at 5-HT_{2A}Rs, leading to the situation approximating the results obtained with 0.3 mg/kg TCB-2: a reduced yet organized pattern of correlations, with few negative correlations. Another plausible explanation is that TCB-2 acts both on 5-HT_{2A}R and on yet unidentified sites as previously discussed (Di Giovanni & De Deurwaerdère, 2018). The dose of 0.2 mg/kg MDL-100,907 was chosen to maximize 5-HT_{2A}R blocked 1h 15 minutes after injection, a dose 2-5 times higher than previously sufficient to block 5-HT_{2A}R-dependent behavioural effects, but not too high (for instance 0.5 or 1 mg/kg) for which we suspect non-selective effects. Correspondingly, 5-HT_{2A}R antagonists clearly blocked the head twitches induced by TCB-2 as previously reported (Butler *et al*., *submitted*; Fox et al., 2010; Halberstadt & Geyer, 2013). Some of the quantitative DA and NA effects or amino acid (ASP) effects induced by TCB-2 were blocked by MDL-100,907 at this time point. Conversely, in addition to the behavioural data, we previously reported that TCB-2 reduced the ratio 5-HIAA/5-HT in all brain regions often insensitive to MDL-100,907 pretreatment (Butler *et al*., *submitted*). As some effects of TCB-2 were prevented by pretreatment of MDL-100,907, in this and our and previous works, it is more likely that there are several targets of TCB-2 either partly involving the 5-HT_{2A}R, or not at all.

The combination of multiple neurochemical parameters with behaviours allows for addressing correlative links between the two. The most intriguing finding within this exploration context is that the correlations may indicate that vehicle-treated mice can perceive distinctions among the various sections of their environment, even though

the time spent was the same on average for the four quadrants. Indeed, we found several, mostly positive correlations of all transmitters for the orange floor, and, to a lower extent, negative correlations for the red floor. The engagement of brain regions was not necessarily similar for neurotransmitters, suggesting distributed influence of neurotransmitter systems in the exploration of the environment. It is noteworthy that visual, tactile, and auditory sensory modalities are altered by the psychedelics 25I-NBOMe in a dose-dependent manner (Miliano et al., 2019). TCB-2 and MDL-100,907 can induce similar effects in the rodent continuous performance task (Rahbarnia et al., 2023), and reduced the correlations of neurotransmitters for the orange floor. However, only TCB-2 induced place preference for the orange floor associated with the avoidance of the white floor. These behavioural data suggested that TCB-2 reduced exploration of the novel environment.

The tissue levels of these amino acids do not concern only the content of vesicles of exocytosis as these compounds are circulating in the extracellular space and participating in metabolism (Yudkoff, 2017). The metabolic value of these parameters is also interesting in view of the numerous homeostatic processes including metabolic pathways and transporters aiming at maintaining their concentrations (Gobert et al., 2011; Yudkoff, 2017). The overall combination of quantitative and correlative approaches confirmed that the concentrations were kept similar upon 5-HT_{2A}R ligands injection, but with distinct relationships between the parameters (loss of correlations). As far as 5-HT_{2A}R agonists are concerned, numerous data provided evidence that 5-HT_{2A}R stimulation produced changes in body temperature (Fox et al., 2010), possibly acting on brown adipose tissue (Ootsuka & Blessing, 2006), modulated microvascular activity in the brain (Cohen et al., 1999), and strongly acted on arterial blood flow in a state-dependent manner (Buchborn et al., 2020). These data should be considered when interpreting human fMRI data addressing the effect of psychedelics on brain hemodynamics (Buchborn et al., 2020). The altered connectivity following 5-HT_{2A}R ligands (Carhart-Harris et al., 2016; Müller et al., 2018; Muthukumaraswamy et al., 2013; Siegel et al., 2024) could also include vascular and metabolic effects. The extent to which metabolic dimension is involved in the loss of correlations induced by 5-HT_{2A}R agents is currently addressed using multivariate analyses as recently proposed (Parrot, submitted).

In conclusion, our data show intriguing correlation patterns of amino acid neurotransmitter contents, across multiple brain regions, of mice submitted to a protocol of forced exploration. The connectivity of amino acid content was suppressed by the 5-HT_{2A}R agonist and antagonist TCB-2 and MDL-100,907, respectively, without strongly altering tissue quantities of these compounds. Some components of the exploration were correlating with tissue neurotransmitters from different brain regions, all suppressed by 5-HT_{2A}R agents. As this analysis develops, we may be able to make more definitive claims about the relationship between neurotransmitter systems during exploration and psychedelic action.

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7. Supplementary Files

Table S1 – Dose response one-way ANOVA results for TCB-2 for amino acid neurotransmitter tissue content.

	ASP	GLU	GLY	TAU	GABA
OF	F(3, 39) = 0.404, p = 0.75	F(3, 38) = 1.35, p = 0.27	F(3, 37) = 1.12, p = 0.36	F(3, 39) = 1.56, p = 0.22	F(3, 39) = 0.893, p = 0.45
PL	F(3, 39) = 1.29, p = 0.29	F(3, 39) = 0.104, p = 0.96	F(3, 38) = 0.931, p = 0.44	F(3, 39) = 0.48, p = 0.7	F(3, 39) = 0.647, p = 0.59
aCC	F(3, 39) = 1.85, p = 0.15	F(3, 38) = 0.763, p = 0.52	F(3, 38) = 1.14, p = 0.34	F(3, 39) = 1.97, p = 0.13	F(3, 38) = 0.429, p = 0.73
M1	F(3, 39) = 1.05, p = 0.38	F(3, 39) = 0.536, p = 0.66	F(3, 38) = 1.67, p = 0.19	F(3, 39) = 0.868, p = 0.47	F(3, 39) = 0.737, p = 0.54
SJ	F(3, 37) = 0.114, p = 0.95	F(3, 38) = 0.461, p = 0.71	F(3, 37) = 0.298, p = 0.83	F(3, 39) = 1.1, p = 0.36	F(3, 39) = 0.434, p = 0.73
S1L	F(3, 39) = 0.607, p = 0.61	F(3, 38) = 0.732, p = 0.54	F(3, 39) = 0.642, p = 0.59	F(3, 39) = 1.18, p = 0.33	F(3, 39) = 0.415, p = 0.74
S1R	F(3, 39) = 1.17, p = 0.33	F(3, 39) = 1.89, p = 0.15	F(3, 39) = 1.91, p = 0.14	F(3, 39) = 2.29, p = 0.093	F(3, 39) = 1.07, p = 0.37
AC	F(3, 39) = 0.89, p = 0.45	F(3, 39) = 0.826, p = 0.49	F(3, 38) = 0.827, p = 0.49	F(3, 39) = 0.527, p = 0.67	F(3, 39) = 0.93, p = 0.44
V1	F(3, 39) = 1.43, p = 0.25	F(3, 38) = 0.579, p = 0.63	F(3, 38) = 3, p = 0.043 *	F(3, 38) = 2.06, p = 0.12	F(3, 39) = 0.845, p = 0.48
A	F(3, 38) = 2.19, p = 0.11	F(3, 39) = 0.989, p = 0.41	F(3, 39) = 2.8, p = 0.053	F(3, 38) = 3.21, p = 0.034 *	F(3, 38) = 2.2, p = 0.1
dH	F(3, 39) = 4.27, p = 0.011 *	F(3, 39) = 1.68, p = 0.19	F(3, 38) = 2.19, p = 0.1	F(3, 39) = 3.34, p = 0.029 *	F(3, 39) = 2.1, p = 0.12
vH	F(3, 39) = 4.62, p = 0.0074 *	F(3, 39) = 2.54, p = 0.07	F(3, 37) = 3.11, p = 0.038 *	F(3, 39) = 2.44, p = 0.078	F(3, 39) = 2.04, p = 0.12
NAc	F(3, 36) = 2.43, p = 0.081	F(3, 38) = 1.3, p = 0.29	F(3, 37) = 2.19, p = 0.11	F(3, 38) = 2.49, p = 0.075	F(3, 37) = 0.235, p = 0.87
VM	F(3, 39) = 1.07, p = 0.37	F(3, 39) = 1.79, p = 0.16	F(3, 38) = 2.26, p = 0.097	F(3, 39) = 1.47, p = 0.24	F(3, 39) = 1.62, p = 0.2
DM	F(3, 39) = 2.62, p = 0.064	F(3, 39) = 0.731, p = 0.54	F(3, 39) = 1.67, p = 0.19	F(3, 39) = 1.66, p = 0.19	F(3, 39) = 1.83, p = 0.16
VL	F(3, 39) = 1.22, p = 0.32	F(3, 39) = 0.875, p = 0.46	F(3, 39) = 1.19, p = 0.33	F(3, 39) = 2.13, p = 0.11	F(3, 39) = 2.04, p = 0.12
DL	F(3, 38) = 3.04, p = 0.04 *	F(3, 36) = 2.37, p = 0.086	F(3, 36) = 1.08, p = 0.37	F(3, 36) = 3.32, p = 0.031 *	F(3, 38) = 1.03, p = 0.39
MD	F(3, 39) = 0.0197, p = 1	F(3, 39) = 0.283, p = 0.84	F(3, 39) = 0.952, p = 0.43	F(3, 39) = 0.435, p = 0.73	F(3, 39) = 0.663, p = 0.58
VPL	F(3, 38) = 2.07, p = 0.12	F(3, 38) = 1.61, p = 0.2	F(3, 38) = 1.53, p = 0.22	F(3, 38) = 2.2, p = 0.1	F(3, 38) = 1.83, p = 0.16
VPR	F(3, 38) = 0.426, p = 0.74	F(3, 39) = 0.575, p = 0.63	F(3, 39) = 0.605, p = 0.62	F(3, 39) = 0.816, p = 0.49	F(3, 39) = 0.644, p = 0.59
DLG	F(3, 39) = 1.11, p = 0.35	F(3, 39) = 0.779, p = 0.51	F(3, 38) = 0.444, p = 0.72	F(3, 39) = 0.994, p = 0.41	F(3, 39) = 0.432, p = 0.73
SC	F(3, 37) = 4.79, p = 0.0064 *	F(3, 36) = 1.83, p = 0.16	F(3, 37) = 4.62, p = 0.0076 *	F(3, 38) = 3.31, p = 0.03 *	F(3, 38) = 3.08, p = 0.039 *
SN	F(3, 39) = 1.53, p = 0.22	F(3, 37) = 1.5, p = 0.23	F(3, 39) = 1.33, p = 0.28	F(3, 38) = 1.33, p = 0.28	F(3, 38) = 0.818, p = 0.49
VTA	F(3, 38) = 1.61, p = 0.2	F(3, 38) = 0.811, p = 0.5	F(3, 38) = 1.44, p = 0.25	F(3, 38) = 1.89, p = 0.15	F(3, 38) = 1.05, p = 0.38
DR	F(3, 39) = 0.94, p = 0.43	F(3, 39) = 0.465, p = 0.71	F(3, 38) = 1.71, p = 0.18	F(3, 39) = 0.388, p = 0.76	F(3, 39) = 0.72, p = 0.55
MR	F(3, 35) = 3.59, p = 0.023 *	F(3, 37) = 2.01, p = 0.13	F(3, 35) = 1.39, p = 0.26	F(3, 37) = 0.908, p = 0.45	F(3, 38) = 1.03, p = 0.39
CB	F(3, 37) = 0.918, p = 0.44	F(3, 39) = 0.283, p = 0.84	F(3, 37) = 0.954, p = 0.42	F(3, 38) = 1.68, p = 0.19	F(3, 37) = 1.24, p = 0.31

Table S2 – Agonist Antagonist amino acid neurotransmitters					
	ASP	**GLU**	**GLY**	**TAU**	**GABA**
OF	F(1, 39) = 0.202, p = 0.655	F(1, 39) = 0.084, p = 0.774	F(1, 39) = 0.013, p = 0.91	F(1, 39) = 0.297, p = 0.589	F(1, 39) = 0.108, p = 0.744
PL	F(1, 38) = 1.096, p = 0.302	F(1, 38) = 0.098, p = 0.756	F(1, 38) = 0.84, p = 0.365	F(1, 38) = 0.154, p = 0.697	F(1, 38) = 0.113, p = 0.739
aCC	F(1, 39) = 4.251, p = 0.046 *	F(1, 39) = 3.675, p = 0.063	F(1, 39) = 2.019, p = 0.163	F(1, 39) = 3.868, p = 0.056	F(1, 39) = 1.243, p = 0.272
M1	F(1, 38) = 0.842, p = 0.365	F(1, 39) = 0.008, p = 0.931	F(1, 39) = 0.015, p = 0.902	F(1, 39) = 0.531, p = 0.471	F(1, 38) = 0.005, p = 0.944
SJ	F(1, 39) = 0.026, p = 0.872	F(1, 39) = 0.033, p = 0.857	F(1, 38) = 0.629, p = 0.433	F(1, 38) = 0.396, p = 0.533	F(1, 39) = 0.413, p = 0.524
S1L	F(1, 39) = 1.345, p = 0.253	F(1, 39) = 1.649, p = 0.207	F(1, 39) = 0.327, p = 0.571	F(1, 39) = 1.782, p = 0.19	F(1, 39) = 0.539, p = 0.467
S1R	F(1, 39) = 1.899, p = 0.176	F(1, 39) = 3.659, p = 0.063	F(1, 39) = 2.465, p = 0.125	F(1, 39) = 1.648, p = 0.207	F(1, 39) = 1.017, p = 0.319
AC	F(1, 39) = 1.115, p = 0.297	F(1, 39) = 0.894, p = 0.35	F(1, 37) = 2.532, p = 0.12	F(1, 39) = 0.517, p = 0.476	F(1, 37) = 0.623, p = 0.435
V1	F(1, 38) = 6.145, p = 0.018 *	F(1, 37) = 4.53, p = 0.04 *	F(1, 38) = 5.669, p = 0.022 *	F(1, 39) = 4.564, p = 0.039 *	F(1, 39) = 2.79, p = 0.103
A	F(1, 39) = 1.896, p = 0.176	F(1, 39) = 1.254, p = 0.27	F(1, 39) = 0.986, p = 0.327	F(1, 38) = 1.977, p = 0.168	F(1, 38) = 1.28, p = 0.265
dH	F(1, 39) = 2.414, p = 0.128	F(1, 39) = 0.619, p = 0.436	F(1, 39) = 0.346, p = 0.56	F(1, 39) = 1.739, p = 0.195	F(1, 38) = 1.355, p = 0.252
vH	F(1, 39) = 4.242, p = 0.046 *	F(1, 39) = 2.077, p = 0.157	F(1, 37) = 5.019, p = 0.031 *	F(1, 39) = 2.46, p = 0.125	F(1, 39) = 0.396, p = 0.533
NAc	F(1, 38) = 3.688, p = 0.062	F(1, 39) = 2.645, p = 0.112	F(1, 37) = 1.75, p = 0.194	F(1, 38) = 2.631, p = 0.113	F(1, 37) = 0.007, p = 0.934
VM	F(1, 38) = 1.713, p = 0.198	F(1, 38) = 1.239, p = 0.273	F(1, 37) = 1.211, p = 0.278	F(1, 38) = 1.232, p = 0.274	F(1, 38) = 1.048, p = 0.312
DM	F(1, 39) = 1.812, p = 0.186	F(1, 39) = 0.889, p = 0.352	F(1, 39) = 1.465, p = 0.233	F(1, 39) = 1.283, p = 0.264	F(1, 39) = 1.665, p = 0.205
VL	F(1, 39) = 0.107, p = 0.745	F(1, 39) = 0.121, p = 0.73	F(1, 38) = 0.927, p = 0.342	F(1, 39) = 0.329, p = 0.569	F(1, 39) = 0.089, p = 0.767
DL	F(1, 39) = 1.003, p = 0.323	F(1, 39) = 0.104, p = 0.749	F(1, 39) = 0.421, p = 0.52	F(1, 39) = 0.142, p = 0.708	F(1, 39) = 0.219, p = 0.643
MD	F(1, 39) = 0.006, p = 0.938	F(1, 39) = 0.001, p = 0.981	F(1, 39) = 0.749, p = 0.392	F(1, 39) = 0.137, p = 0.713	F(1, 39) = 0.345, p = 0.56
VPL	F(1, 37) = 0.164, p = 0.687	F(1, 36) = 0.17, p = 0.683	F(1, 37) = 0.227, p = 0.637	F(1, 36) = 0.001, p = 0.981	F(1, 37) = 0.494, p = 0.486
VPR	F(1, 38) = 0.052, p = 0.821	F(1, 39) = 0.897, p = 0.349	F(1, 37) = 0.751, p = 0.392	F(1, 39) = 0.248, p = 0.622	F(1, 39) = 0.005, p = 0.943
DLG	F(1, 39) = 0.475, p = 0.495	F(1, 39) = 0.016, p = 0.901	F(1, 39) = 0, p = 0.992	F(1, 39) = 0.129, p = 0.721	F(1, 39) = 0.159, p = 0.692
SC	F(1, 37) = 0.639, p = 0.429	F(1, 38) = 0.002, p = 0.961	F(1, 37) = 0.026, p = 0.873	F(1, 38) = 0.006, p = 0.941	F(1, 38) = 0.079, p = 0.781
SN	F(1, 39) = 4.41, p = 0.042 *	F(1, 37) = 1.763, p = 0.192	F(1, 39) = 7.261, p = 0.01 *	F(1, 38) = 2.513, p = 0.121	F(1, 39) = 0.579, p = 0.451
VTA	F(1, 38) = 1.742, p = 0.195	F(1, 37) = 0.331, p = 0.569	F(1, 38) = 1.321, p = 0.258	F(1, 38) = 1.149, p = 0.291	F(1, 39) = 1.702, p = 0.2
DR	F(1, 39) = 0.66, p = 0.421	F(1, 39) = 0.387, p = 0.537	F(1, 37) = 1.309, p = 0.26	F(1, 39) = 0.213, p = 0.647	F(1, 39) = 0.13, p = 0.721
MR	F(1, 36) = 7.146, p = 0.011 *	F(1, 37) = 3.551, p = 0.067	F(1, 35) = 2.987, p = 0.093	F(1, 38) = 2.237, p = 0.143	F(1, 38) = 1.6, p = 0.214
CB	F(1, 38) = 1.584, p = 0.216	F(1, 39) = 0.699, p = 0.408	F(1, 37) = 1.898, p = 0.177	F(1, 39) = 1.761, p = 0.192	F(1, 37) = 2.34, p = 0.135

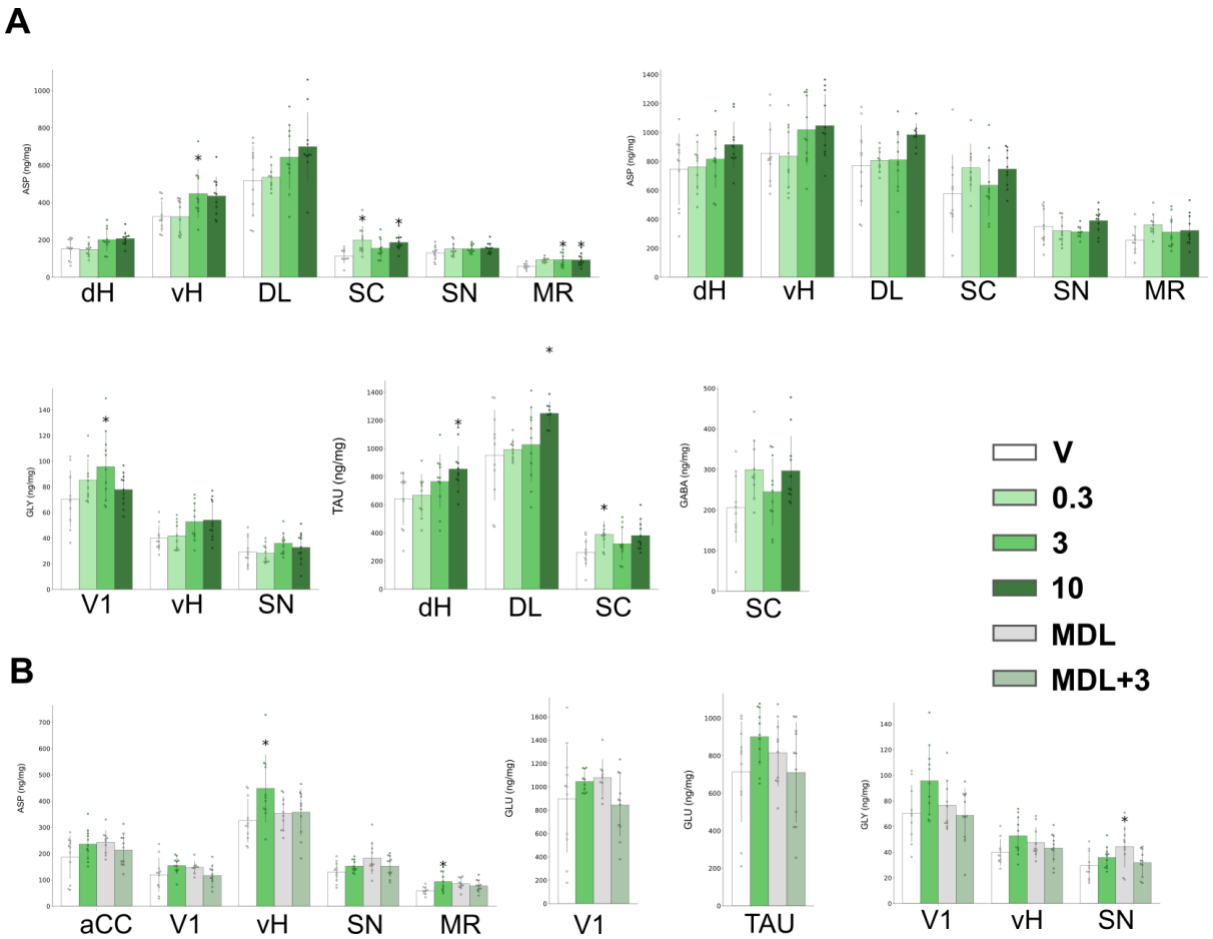

Figure S1- Significant tissue amino acid neurotransmitter tissue quantity for **A** dose response (one-way ANOVA, $p<0.01$; table S2**) B** agonist antagonist (two-way ANOVA, $p<0.01$). Histograms of mean ± SD of neurotransmitter quantity.

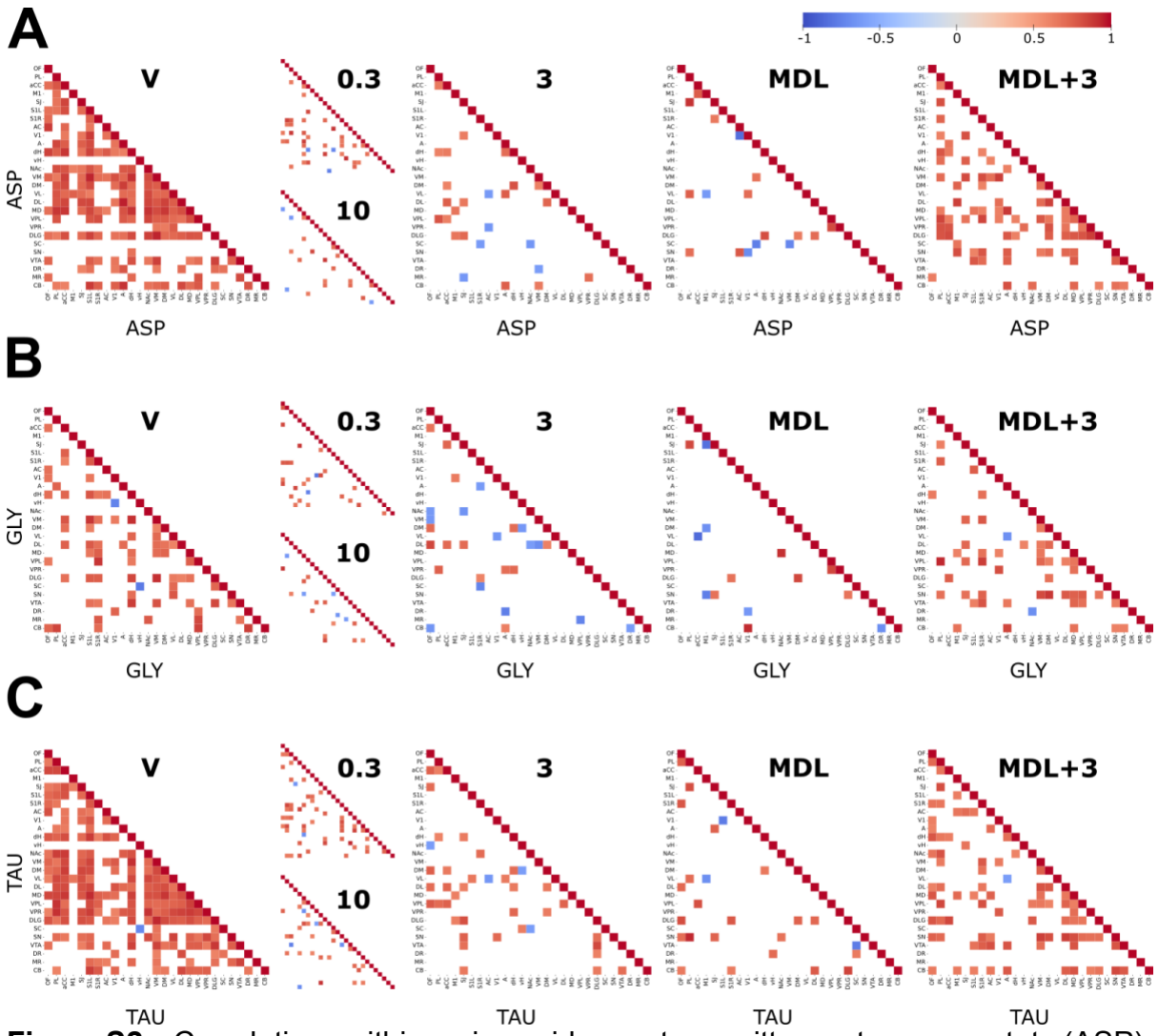

Figure S2 – Correlations within amino acid neurotransmitter systems aspartate (ASP), glycine (GLY), and taurine (TAU).

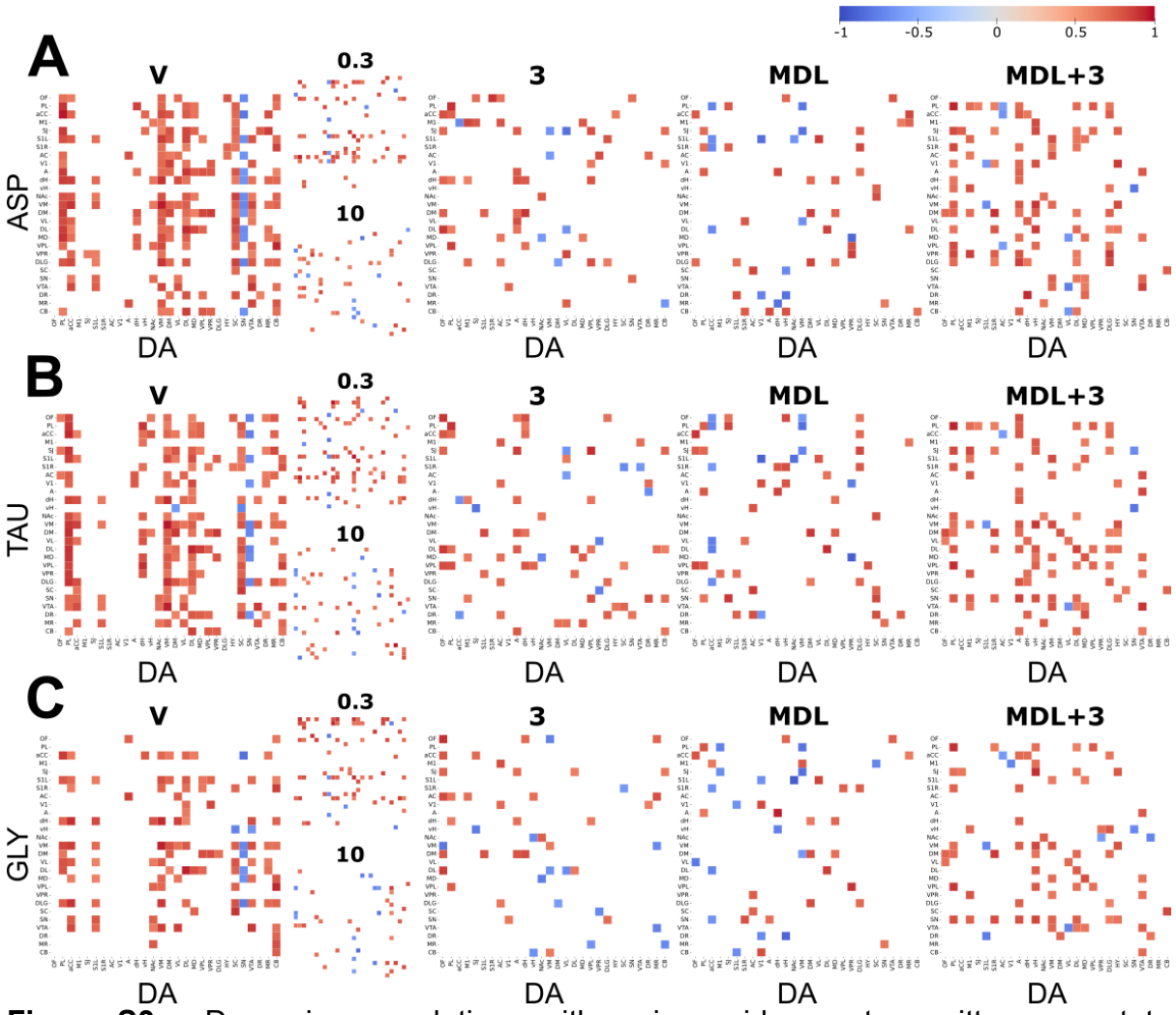

Figure S3 – Dopamine correlations with amino acideneurotransmitters aspartate (ASP), taurine (TAU) and glycine (GLY).

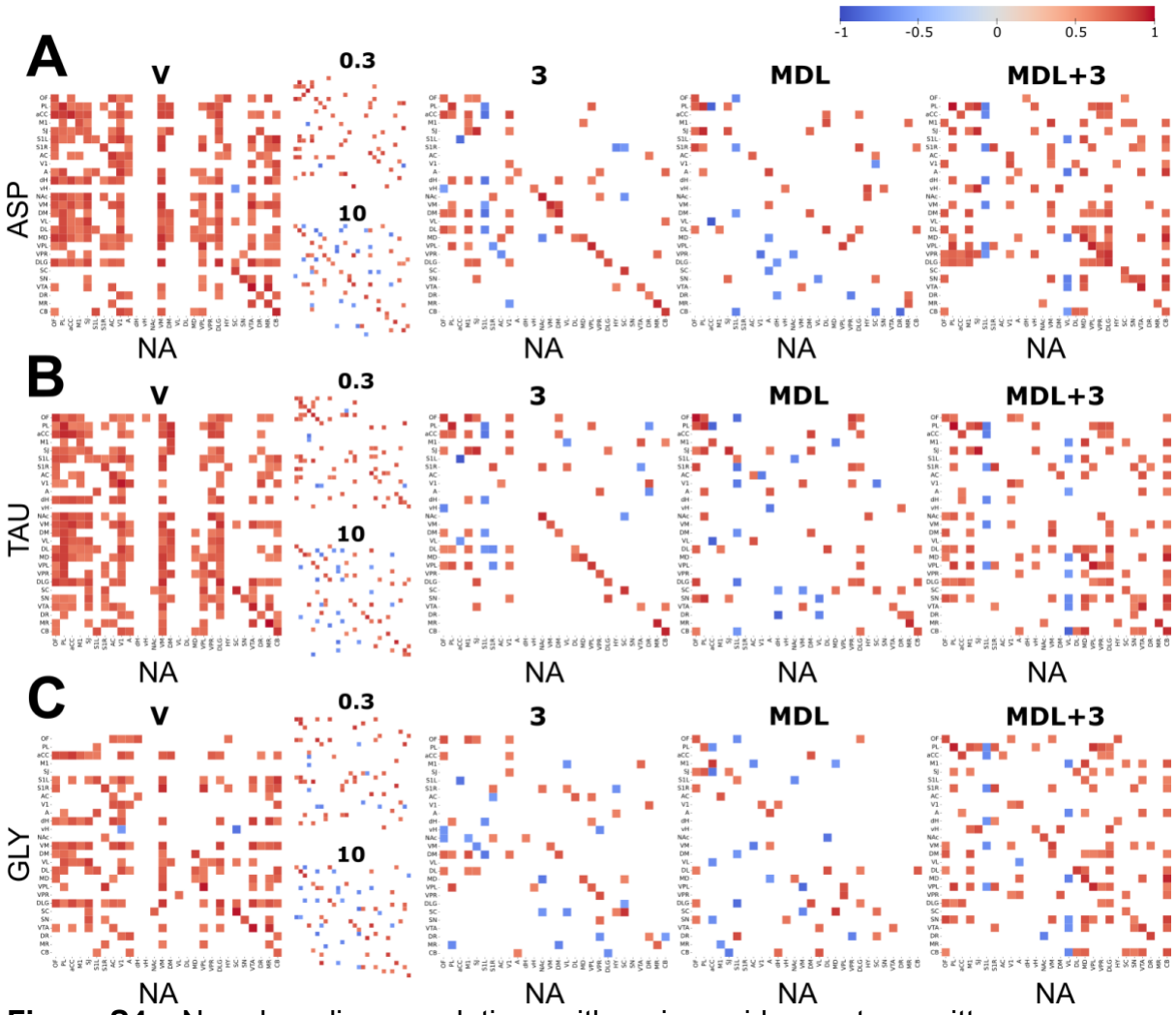

Figure S4 – Noradrenaline correlations with amino acid neurotransmitters.

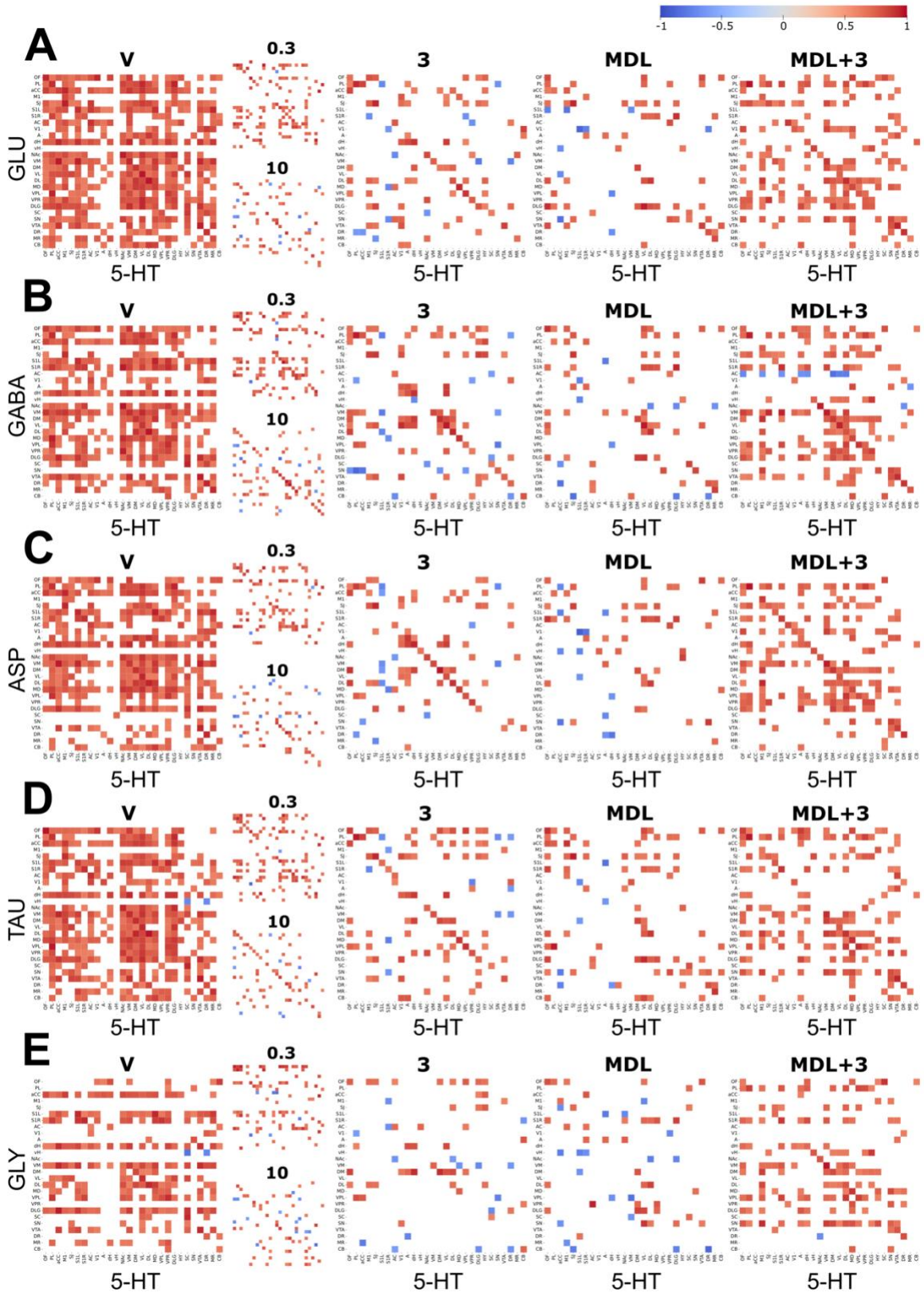

Figure S5 – Serotonin correlations with amino acid neurotransmitters glutamate (GLU), GABA, aspartate (ASP), taurine (TAU) and glycine (GLY).

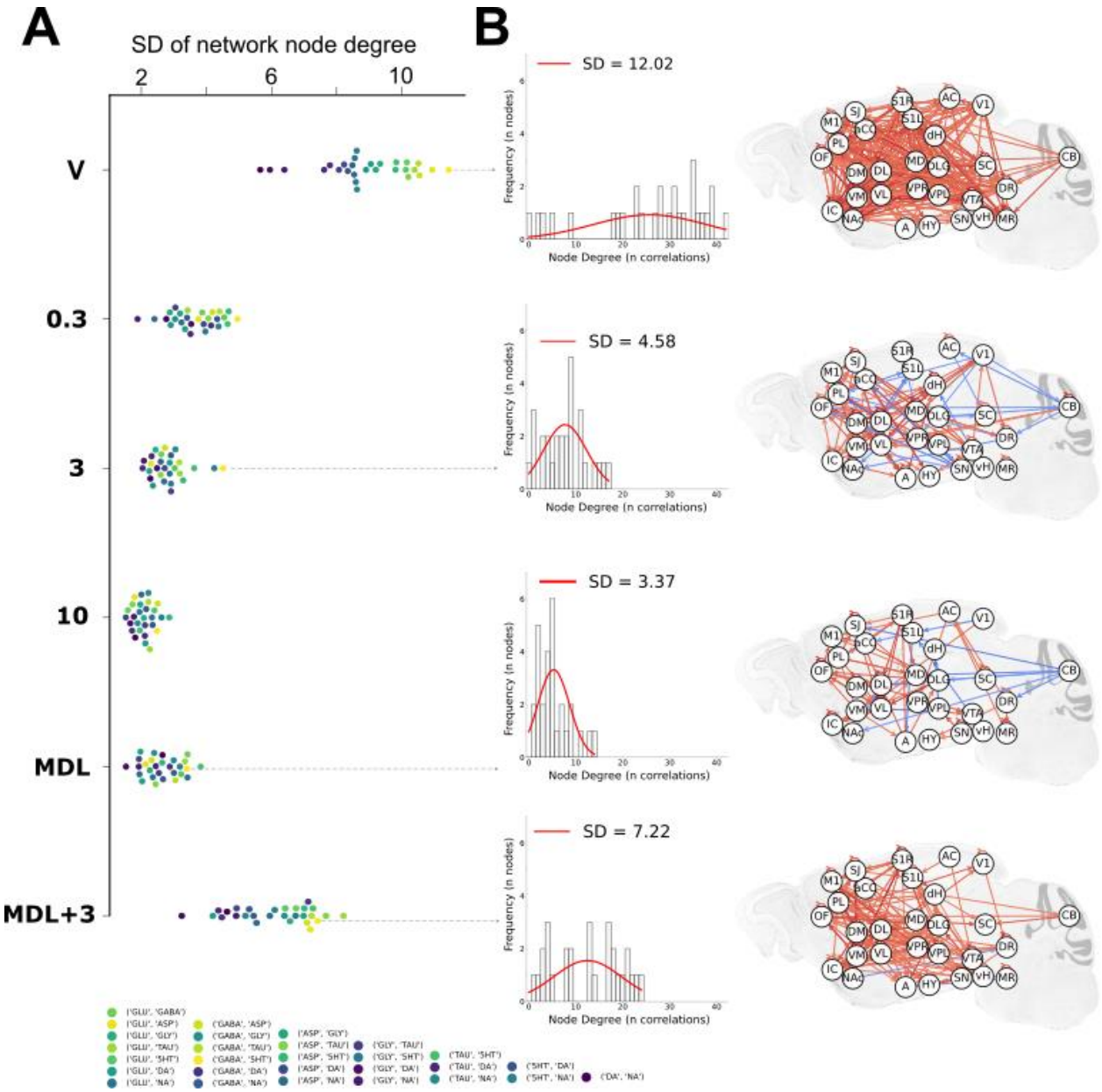

Figure S6 – SD of network node degree for neurotransmitter pairs. **A** Standard deviation (SD) of the node degree for each network of Pearson correlations between neurotransmitters pairs. **B** Network degree distribution histogram (left) and network (right) for the GLU-5-HT network in vehicle (V), 3mg/kg TCB-2 (3), 0.2 mg/kg MDL-100,907 (MDL) and combination (MDL+3) treated animals.

Table S3 – TCB-2 dose response one-way ANOVA results for exploration behavior.	
	one-way ANOVA
distance travelled	F(3, 31) = 7.93, p = 0.00046 *
climbing	F(3, 33) = 11.1, p = 3.3e-05 *
sniffing	F(3, 33) = 12.4, p = 1.4e-05 *
huddle	F(3, 34) = 1.39, p = 0.26
white floor	F(3, 33) = 27, p = 5.3e-09 *
hessian floor	F(3, 32) = 9.89, p = 9.1e-05 *
red floor	F(3, 34) = 1.25, p = 0.31
orange floor	F(3, 33) = 10.8, p = 4.2e-05 *

Table S4 – TCB-2 and MDL-100,907 two-way ANOVA (agonist antagonist) for exploration behavior.	
	two-way ANOVA
distance travelled	F(1, 28) = 0.187, p = 0.669
climbing	F(1, 32) = 3.631, p = 0.066
sniffing	F(1, 34) = 2.18, p = 0.149
huddle	F(1, 33) = 0.003, p = 0.955
white floor	F(1, 34) = 8.181, p = 0.007 *
hessian floor	F(1, 34) = 0.029, p = 0.866
red floor	F(1, 33) = 1.736, p = 0.197
orange floor	F(1, 32) = 1.525, p = 0.226

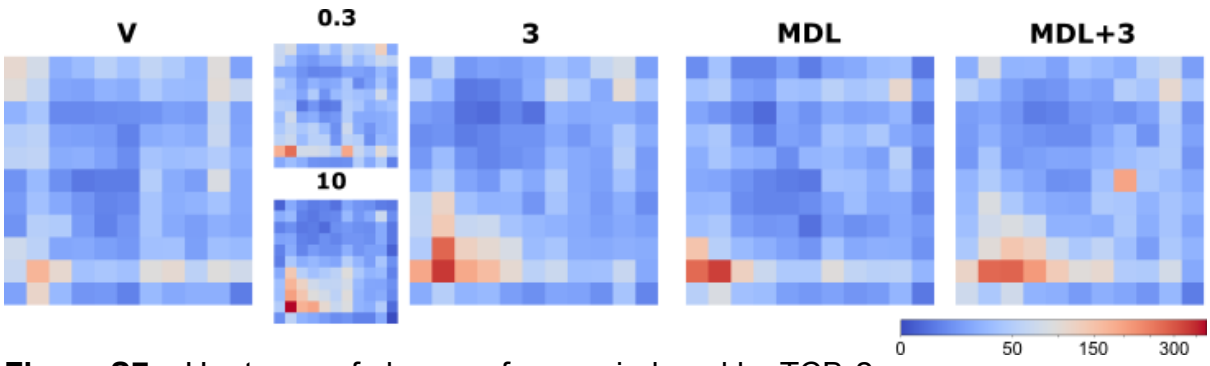

Figure S7 – Heat map of place preference induced by TCB-2.

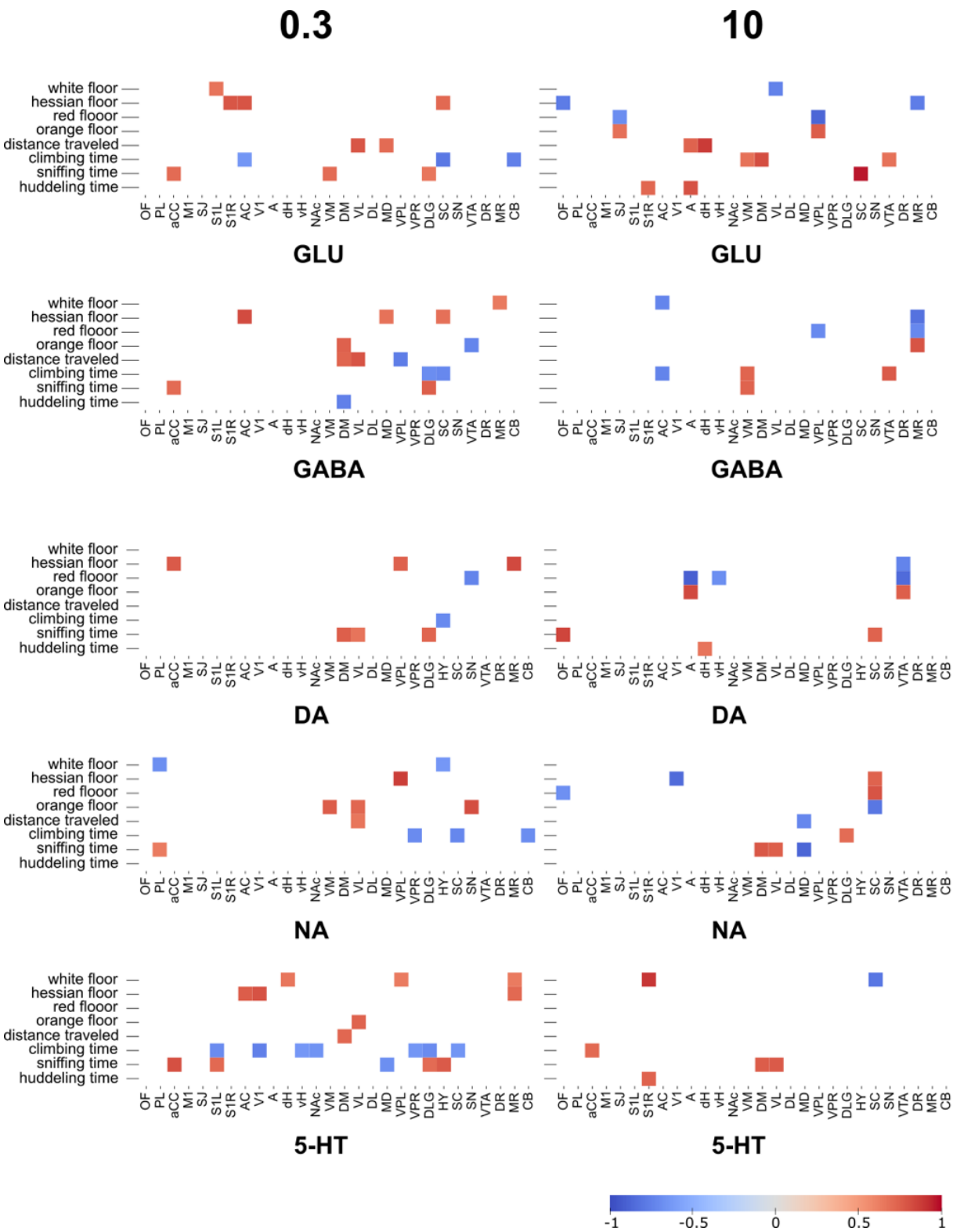

Figure S8 – Correlations between behavior and neurotransmitters GLU, GABA, DA, NA and 5-HT for 0.3 and 10mg/kg TCB-2.

Summary Art IV

This study reports the broad distribution of tissue levels of glutamate, aspartate, GABA, glycine, and taurine across the mouse brain. The density of positive tissue content correlations was tremendously high in vehicle-treated mice, and a clear regional-organisation of correlations was apparent when correlating tissue content of amino acids with that of DA and NA, but not 5-HT. This result extends the hypothesis of the first article that catecholamines play an important role in organizing brain neurochemical systems in response to novelty and/or exploration. TCB-2 (0.3, 3, and 10 mg/kg) barely altered the tissue levels of amino acid neurotransmitters but dose-dependently decreased the number of correlations for all of them. MDL-100,907 did not alter tissue content of amino acid neurotransmitters but also dramatically reduced the number of correlations as well as the organized pattern seen in vehicle animals exposed to the novel flooring. Both TCB-2 and MDL-100,907 increased the proportion of negative correlations. TCB-2 induced changes in some behaviours and notably it dose-dependently decreased sniffing and climbing but enhanced locomotor activity at 3 mg/kg only. The exploration of the floor was homogenous in vehicles (time spent on the different textures), but not after TCB-2 treatment. In fact, specific, mostly positive correlations of neurotransmitters with one texture of the floor in vehicle-treated mice disappeared in TCB-2-treated mice corresponding to an increase in time spent on that texture. The role of 5-HT_{2A}R in this effect was not clear-cut. By addressing tissue contents of neurotransmitters following exploration, this study offers the first analysis of distributed changes of neurotransmitters with respect to a specific environmental context. The study indicates that 5-HT_{2A}Rs have a profound impact on the connectivity of neurotransmitters in a forced exploratory behaviour. It also raises concerns about the meaning of tissue measurement of amino acids which likely overwhelms their status as neurotransmitters, towards metabolic markers.

Art V – Neurochemical Databases: Purpose and Expectations

Questions and purpose of the viewpoint

The purpose of this viewpoint was to highlight the necessity of neurochemical database comparable to that of the Allen Brain Atlas database for electrophysiology, but for neurochemistry. This idea progressively grew up with the progress on the neurochemical analysis code in the lab, which would facilitate the handling of datasets in this kind of database. Offering the additional possibility to combine past neurochemical experiments, not only from the lab, into metanalyses.

ACS Chemical Neuroscience

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Neurochemical Databases: Purpose and Expectations

Published as part of ACS Chemical Neuroscience special issue "Monitoring Molecules in Neuroscience 2024".

Jasmine Jade Butler,[⊥] Sandrine Parrot,[⊥] Chloé Aman, and Philippe De Deurwaerdère*

Cite This: https://doi.org/10.1021/acschemneuro.4c00812 Read Online

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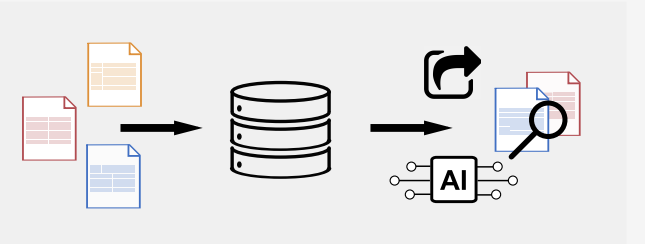
ABSTRACT: The exploration of increasingly specific brain structures and their relationships, in more nuanced ways, has facilitated the generation of databases for gene expression, connectivity, cell morphology, and electrophysiology. However, neurochemistry, the study of neurochemical environment and transmission, has not yet warranted a public database, despite the plethora of data published. From our viewpoint, a neurochemical database is overdue and would allow the field of neurochemistry to develop facilitating, standardization and reference values, reproducibility, resource efficiency, preservation and accessibility of raw data, hypothesis development and exploration, and metadata analysis. We propose to use acquired and published data from tissue content analysis to initialize this database.



KEYWORDS: *Neurochemistry, database, monoamines, standardization, tissue content, microdialysis, brain areas*

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The value of open-access databases in neuroscience is increasingly evident as the tools for large-scale data collection and analysis improve. These databases allow exploration of data sets beyond initial publications, allowing for cross-study meta-analyses and overcoming the reproducibility challenges associated with isolated studies. The Allen Brain Atlas, founded in 2003, provides a vast resource of gene expression data, neuronal connectivity, cell morphology, and electrophysiological properties in both rodent and human brains. More specific databases include NeuroMorpho.org for neuroanatomy and NeuroElectro.org for electrophysiology. However, a significant gap remains in the availability of standardized, quantitative neurochemical data in existing databases.

Previously the chemical measurement of neurotransmitters was not considered a high throughput method; however, the quantity of neurochemical data is growing due to the analysis of wider central nervous system territories and the possibility of measuring more constituents in postmortem studies or microdialysis samples. In work from 2017, we performed postmortem analysis of 6 monoamines and metabolites, and 2 ratios in response to monoamine oxidase inhibitors, across 3 brain regions in 51 Sprague Dawley rats.[1] Including the study of the ratios, 1200 parameters were then aliased manually through Excel and statistical softs. In a more recent study, we addressed the effect of a 5-HT_{2A} receptor agonist on the tissue content of 7 monoamines and metabolites and 13 amino acids, across 30 brain regions in 70 C57Bl/6J mice. This experiment generated a data set of 42 000 parameters, excluding metabolite-to-neurotransmitter ratios. These new data sets are, in fact, designed to perform multivariate analyses, currently underrepresented in neurochemistry,[2] with Python code currently under development. The *in vivo* monitoring of compounds is also developing. The advent of powerful analytical methods coupled to intracerebral microdialysis offers the possibility of measuring a variety of compounds in the extracellular space, in a dynamic, temporal manner.[3] We could argue that "the Gain is in the Analytical Chain" by analogy with "The Gains in Brain Are Mainly in the Stain" (Floyd Bloom, 1992),[4] but the consequence is major for neurochemists. From the *in vivo* study of a single neurotransmitter sampled in one brain region in response to various conditions, newer analyses report a neurochemical cloud encompassing different neurotransmitters, metabolic markers, and lipids that calls for new types of analyses, and questions.[5]

A proposed entity-relationship diagram with the flexibility to include additional non-neurochemical measures, such as behavior, is detailed in Figure 1. Central entities Projects, Subjects, and DataTypes store parameters pertaining to each level such as researcher, species, and units, respectively. Additional attributes such as behavioral phenotype, genotype,

Received: December 2, 2024
Accepted: December 19, 2024

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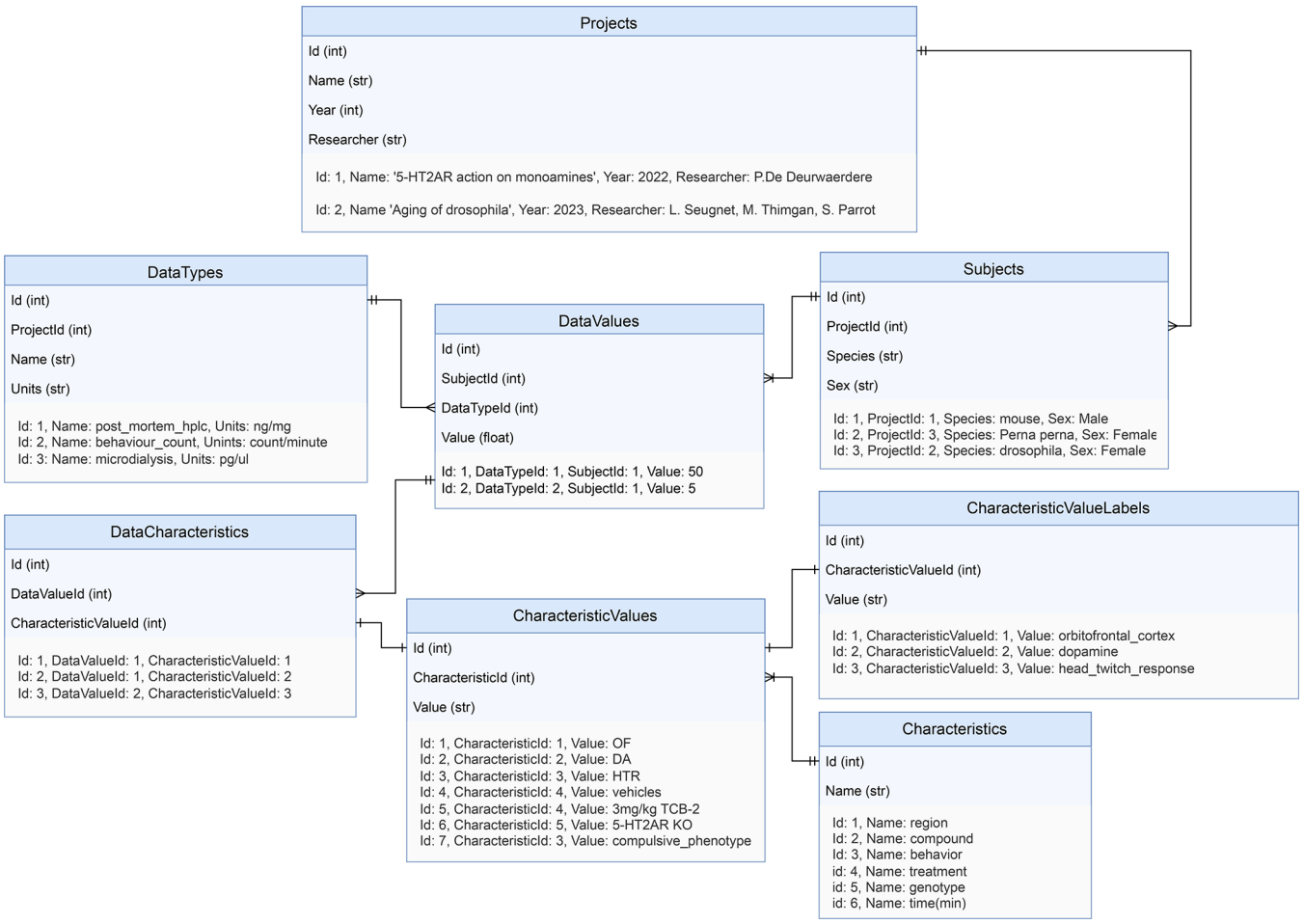

Figure 1. Schematic entity-relationship diagram for a neurochemical multimodal research relational database. Central entities include Projects, Subjects, and DataTypes, which categorizes data by type and units. The DataValues table links specific measurements to individual subjects and their corresponding data types. Flexible associations are achieved through DataCharacteristics and CharacteristicValues, which allow for the inclusion of metadata, such as regions (OF, orbitofrontal cortex), compounds (DA, dopamine), behavior (HTR, head twitch response), treatment (3 mg/kg TCB-2, (4-bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide), or time where applicable. This schema is designed to accommodate both neurochemical and behavioral data, with extensibility for future attributes. Data type is indicated after each attribute, string (str), integer (int), float (float).

age, physiological data, and treatment may be included as DataCharacteristics (Figure 1). This additional data inclusion, particularly behavior, would promote the development of functional neurochemistry and the search for dimensions governing, or sensitive to, neurochemicals.[2]

Such a database would serve as neurochemical atlas for different species, strains, and genetic models providing a reference for neurochemical values and moving toward standardization and reproducibility within neurochemistry. Additionally, it would favor the reduction of animal use, and resource efficiency. The inclusion of these additional parameters would allow data exploration and hypothesis development.

In line with developing analytical techniques, a neurochemical database would support the development of multivariate analysis in neurochemistry. The availability of raw data would permit correlative analyses of previously uninvestigated data[1] and interproject comparison. Ensuring the availability and accessibility of acquired and published data may eventually permit the implementation of artificial intelligence in the meta-analysis and modeling of neurochemical systems.

Concluding this viewpoint on a humorous tone, we conceived such a database to hold acquired postmortem neurochemical data, published and unpublished, from different experiments analyzed in Bordeaux and Lyon. While neurochemistry rarely permits poetry, our Excel files, some approaching 20 years old, showcase an impressive level of creativity in the abbreviations for brain regions and parameters. For instance, the prefrontal cortex can be abbreviated as PC, PFC, mPC, mPFC, PL, or PLC. If the researcher's native language is French, we may add to this letter puzzle CP, CPF, CPm, CPFm, or CPL. Beyond nonstandardized parameter abbreviations and differential structures of existing data (raw or processed) lies the nature of the experimental design, such as differential experimental groupings in behavioral phenotyping experiments. All this variability within only two research centers illustrates the need for an improved, standardized handling of neurochemical data.

Considerable time is required time to consolidate and standardize old files and develop the relation between entities that would best serve in a relational neurochemical database. Despite the considerable time required for the development and inclusion of acquired data sets, we believe that a

https://doi.org/10.1021/acschemneuro.4c00812
ACS Chem. Neurosci. XXXX, XXX, XXX−XXX

neurochemical database is overdue and will be of immense value to the scientific community. The neurochemical data of today are for future generations.

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Author Contributions

⊥J.J.B. and S.P.: equal contribution. J.J.B., S.P., and P.D.D. worked on the database design, development of ideas, and writing the manuscript. C.A. worked on writing the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by grants from the University of Bordeaux, the University Claude Bernard Lyon 1, and the French research organisms INSERM and CNRS. J.J.B. and C.A. were fellowship recipients from the Ministere de la Recherche et de l'Enseignement Superieur. The authors thank the GPR Brain of the University of Bordeaux and notably the subgroups Adapsy and Inhibition for their support. We thank Remi Ferreira and Roua Bouhouch for their participation in the first architecture of the neurochemical database and their work on old Excel files.

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Summary Art V

This viewpoint highlights the value of having a database for the increasingly large neurochemical datasets collected, often beyond the scope of a single study, and with the possibility of multiple methods of analysis. Beyond the availability of data, a database would move toward standard references for the field and unify a currently disparate neurochemical literature. This project is being constructed in parallel with the neurochemical analysis code. The integration of both the database and an open-source analysis package we hope will favour metanalysis between studies.

Art VI *(in preparation b)* –Neurochemistry and post-mortem neurotransmitters: toward the study of neurochemical connectivity.

Questions and purpose of the review

This review is designed to discuss the development of further methods of multivariate analysis within the field of neurochemistry. As discussed above neurochemical datasets are getting larger and consequently data handling is being improved. With these improvements comes the opportunity of novel kinds of analysis, such as neurochemical connectivity as assessed by tissue neurotransmitter correlation between regions and compounds. This paper discusses the relevance of tissue measures of neurotransmitters, particularly considering monoaminergic systems, and the sensitivity of this correlative analysis beyond that of quantitative modulation.

Neurochemistry and post-mortem neurotransmitters: toward the study of neurochemical connectivity.

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Keywords: monoamines, correlative analysis, variability, neurochemistry, brain, graph theory

Abstract

The purpose of this article is to address the technical requirements to perform complex analysis of neurochemical, postmortem, quantitative data on neurotransmitter systems. It covers the basic, technical aspects, addressing distinct possibilities for specific purposes, and deals with new types of neurochemical analysis beyond the pure quantitative aspects. While the usual lack of temporality and the blur meaning of neurotransmitter tissue content are weaknesses of this approach, postmortem measurement offers the possibility to quantitatively address the content of neurotransmitters in multiple part of the central nervous system with specific requirements. It thus expands the study of neurotransmitter systems beyond a bunch of brain regions and allows for describing possible inter-regional influence within a neurotransmitter system and between neurotransmitter systems across the CNS. Through this non-preceding discussion, this article will push forward the use of neurochemical data towards multiple dimensions of analysis, mostly based on monoaminergic systems and their connectivity.

Highlights

Neurochemical measurement of monoamines is a quantitative method

The variability of neurochemical data due to analytical devices, and protocols can be managed to conserve biological variability capturing inter-individual differences in a cohort

Correlative analysis of monoamine content across the CNS is sensitive and extends upon classical quantitative analyses

Correlative maps are sensitive to experimental conditions and inter-individual differences

Graph theory can be applied to neurochemical datasets to better describe neurochemical connectivity (between and within neurotransmitter systems)

1. Introduction

Monoaminergic systems, including dopamine (DA), serotonin (5-HT), noradrenaline (NA), play a role in virtually all cerebral functions. Their widespread innervation across the central nervous system (CNS), particularly for NA and 5-HT neurons, implies multiple actions in partnership with other neurotransmitter systems. Modification of their activity has been identified in most, if not all, neuropsychiatric and neurological diseases (Di Giovanni et al., 2016). The modifications are never that clear though such as in depression, anxiety, schizophrenia, or drug abuse. The benefit from monoaminergic-based treatments such as in depression, anxiety, schizophrenia, or Parkinson's disease is still used as an argument to indicate that the defects of monoaminergic systems are causally involved in those pathologies. It is a simplistic view of monoaminergic systems even though we can consider that the monoaminergic disturbances in other devastating diseases, including Alzheimer's disease, addiction, and epilepsy, did not lead yet to approved monoamine-based treatments (De Deurwaerdere and Di Giovanni, 2017; De Deurwaerdère and Di Giovanni, 2020; Di Giovanni et al., 2016; Simic et al., 2017; Svob Strac et al., 2016b, 2016a; Tomkins and Sellers, 2001). The reality is that we still do not exactly understand the function of monoaminergic systems.

There are different ways to study the activity of these monoaminergic systems including electrophysiology, anatomy, behaviour, and biochemistry. Post-mortem measurements of monoamines and their metabolites have been used for several years to approximate the function of monoaminergic neurons. These measures have been convenient to determine the functional status of monoaminergic systems in physiology, diverse conditions relative to environment and drugs, pathologies, mechanisms of action of drugs, and animal models of brain diseases. This kind of measurement can be undertaken in vertebrates and invertebrates, the function of monoaminergic systems (mostly DA and 5-HT) being quite conserved in the animal kingdom (Bacque-Cazenave et al., 2020; Cenci and Björklund, 2020; Jacobs and Azmitia, 1992; Smidt and Burbach, 2009). There are yet several differences of monoamine metabolisms across species and even strong regional differences in a given species but the general idea of their functioning remains the same: volume transmission, and a balance between neuron-like function and gland (Descarries and Mechawar, 2000). These

tissue parameters were and are sometimes used to extrapolate the findings towards the electrical activity of the neurons or the release of the neurotransmitter (Aby et al., 2022; Shannon et al., 1986). Nonetheless, the links between the electrical activity, the release of the neurotransmitter, and the tissue measurements are still enigmatic, and interpretation between the parameters is always speculative. Ultimately, the postmortem measurements were less used because they could only give a static measure of the monoaminergic parameters in contrast to electrophysiology, push-pull cannula, intracerebral microdialysis, and more recently biosensors. On the other hand, the above-mentioned *in vivo* approaches cannot offer a widespread picture of monoaminergic function in the CNS because they are invasive. Considering that in most animal models, researchers report specific, regional changes of monoamine tissue markers, in specific conditions, the regional balances for a monoamine and between monoamines appear an important feature of monoaminergic systems (Bharatiya et al., 2020; Mlost et al., 2024). Being quantitative and possibly invading several CNS regions, the post-mortem parameters are markers of choice to address kinds of connectivity within and between neurotransmitters systems across the CNS using correlative and multivariate analyses.

In this review, we explore the diverse conditions in which tissue monoamines and relative compounds are measured and focus on the meaning of these indexes. We take into an account the widespread innervation of monoaminergic systems, their morphology, and their regulations. From in-house data developed over 15 years, we extend the analysis to qualitative analyses of monoamines tissue parameters across the brain using multiple correlations within a monoaminergic system and between monoaminergic systems and/or other systems. We finally discuss the use of multivariate analysis of different types to approximate connectivity of neurotransmitter systems.

2. Gross anatomy of monoaminergic neurons

Pathways of monoaminergic systems project from the brainstem to different brain areas and the spinal cord, and they include dopaminergic neurons, located in the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNc), noradrenergic neurons, arising at least from locus coeruleus (LC), serotonergic

neurons, originating from median raphe nuclei (MRN) and dorsal raphe nuclei (DRN) (Aston-Jones, 2004; Bjorklund and Dunnett, 2007; Gerfen et al., 1987; Hale and Lowry, 2011; Steinbusch, 1984). The monoaminergic innervation of the CNS is more complex.

2.1. Dopaminergic system

DA neurons are a heterogeneous group of cells that are localized in the mesencephalic, diencephalic and olfactory bulb regions of the brain. However, most DA cells reside in the ventral part of the mesencephalon (Bjorklund and Dunnett, 2007). The major DA pathways are the nigrostriatal and the mesolimbic that originate from mesodiencephalic DA neurons which correspond to A8, A9 and A10 group of cells. The nigrostriatal pathway corresponds to DA neurons originating from the lateral SNpc (A9 cell group) that project into the striatum (caudate nucleus and putamen). These neurons are particularly studied because their degeneration has been involved in the development of Parkinson's disease (Cenci and Björklund, 2020; Grace and Bunney, 1985). The VTA DA cell bodies (A10 cell group) send DA projections to the nucleus accumbens, the ventromedial striatum, and extend towards structures closely associated with the limbic system (Deutch et al., 1987; Di Giovanni et al., 2009; Gerfen et al., 1987; Kalivas, 1993; White, 1996). The mesocortical DA pathways also originating from VTA innervate different cortices (Bannon and Roth, 1983; Deutch, 1993; Tassin et al., 1976).

The SN also comprises of the pars reticulata (SNpr) area which mainly contains γ-amino-butyric acid (GABA)-ergic neurons that constitutes major efferences of the basal ganglia. Nonetheless, the SNpr also contains some DA neurons projecting to the striatum (Bjorklund and Dunnett, 2007; Matsuda et al., 2009). There are also some less defined DA neurons in the A11 nucleus, responsible for the mere DA innervation of the spinal cord (Barraud et al., 2010; Koblinger et al., 2014). Other DA centres can also be found in the hypothalamus (A12 nucleus), playing a role in neuroendocrine processes via its projection in the pituitary gland (the tuberoinfundibular pathway) (Bjorklund and Dunnett, 2007). Some DA neurons have been reported in the DRN (Halberstadt and Balaban, 2008; Stratford and Wirtshafter, 1990).

2.2. Serotoninergic system

The 5-HT innervation of the CNS is widespread so that most CNS territories receive 5-HT projections at various degrees. The 5-HT cell bodies located in the medial and dorsal

raphe nucleus are mostly responsible this broad 5-HT mapping (Azmitia and Segal, 1978; Steinbusch, 1984). The DRN is the brain region housing the highest proportion of 5-HT, which is estimated to correspond to 30-56% of 5-HT neurons in the CNS depending on the species (Jacobs and Azmitia, 1992). 5-HT neurons may co-express and release other neurotransmitters such as GABA, glutamate and nitric oxide in addition to neuropeptides such as corticotropin-releasing factor, substance P or cholecystokinin (De Deurwaerdere and Di Giovanni, 2017; Jacobs and Azmitia, 1992; Ren et al., 2019). As far as glutamate is concerned, it has been well described that 5-HT neurons express the glutamate vesicular transporter Vglut3 (Amilhon et al., 2010). The 5-HT-containing cell bodies of the DRN send projections to DA cells located both in the VTA, SNpc, and their terminal fields including the NAc, mPFC, amygdala, and the striatum (De Deurwaerdere and Di Giovanni, 2017). They also innervate the midbrain regions housing the NA neurons including the locus coeruleus (Aston-Jones et al., 1991; Muzerelle et al., 2016; Steinbusch, 1984).

The 5-HT neurons in the DRN are heterogeneous, expressing more or less some proteins like vGlut3 (Commons, 2020; Okaty et al., 2020, 2015; Ren et al., 2019). These data are in line with previous anatomical studies showing the existence of at least six distinct pathways from the DRN (Hale and Lowry, 2011): dorsal part to the central and basolateral nuclei of amygdala, the dorsal hypothalamic area and the mPFC; ventral part to sensorimotor cortex and the caudate putamen; lateral part to the lateral hypothalamus or superior colliculus; rostral parts to the caudate putamen and the SN; caudal part to the amygdala, ventral hippocampus, and thalamic nuclei (Lowry et al., 2008; Steinbusch, 1984).

The MRN contains less 5-HT cell bodies, comprising approximately 5% of the neurons in the nucleus that project in hippocampus (Ren et al., 2019; Steinbusch, 1984, 1981). Although the MRN innervates several brain regions, the projections from this nucleus to at least the basal ganglia or the mPFC are presumably not releasing 5-HT (De Deurwaerdere and Di Giovanni, 2017; Jacobs and Azmitia, 1992). MRN 5-HT projections innervate specific regions such as the dorsal hippocampus or lateral septum even if it does not intervene alone. Other 5-HT cell bodies from the nucleus reticularis tegmenti pontis, the raphe pontis, and the raphe magnus can innervate the medial septum (Köhler et al., 1982). It could come also from the raphe obscurus (Vertes and Martin, 1988)

without being able to clearly determine the 5-HT nature of these neurons as suggested in the opossum (Martin et al., 1985).

2.3. Noradrenergic system

The adrenergic cell bodies are dispersed across 7 distinct nuclei (A1 to A7) in the brainstem and innervate in almost all regions of the brain. The NA system corresponds mainly to the locus coeruleus (LC, A6). A1, A5 and A7 clusters are responsible for some ascending and ventral NA projections to the septum, the bed nucleus of the stria terminalis or the hypothalamus (HY). The LC, in which 50% of neurons are adrenergic, is present in all mammalian species and is located dorsally in the pons. It is mainly responsible for the adrenergic innervation of the telencephalon and represents the main source of NA within CNS (Aston-Jones, 2004). The widespread network of LC-derived adrenergic projections exerts a powerful modulatory influence on cortical and sub-cortical circuit (Chandler et al., 2019). Nonetheless, the adrenergic innervation of the striatum and globus pallidus is sparse (Aston-Jones, 2004) in line with lower quantities of NA (Fitoussi et al., 2013).

2.4. Morphology of monoaminergic neurons

Beyond the number of cell bodies located in each monoaminergic center, there is the issue of the axonal branches and the shape of the fibers. It is now well demonstrated that monoaminergic fibers have an extremely broad axonal development, with numerous “en passant” synapses, which are potential sites of release (Descarries et al., 1991; Descarries and Mechawar, 2000). Matsuda et al (2009) succeeded to label one dopaminergic neuron from the SNpr and follow its projection to the striatum (Matsuda et al., 2009). It is remarkable that one neuron innervates a large part of the striatum, crossing different territories of the striatum and interacting with different functional sub-division of the striatum, namely the matrix and the striosomes. The reconstruction of 5-HT neurons projection from the DRN is even larger (Ren et al., 2019).

Neurochemists described by the end of the 60’s and 70’s the existence of distal pools of release away from releasing sites using pharmacological compounds (Glowinski, 1975). The anatomical part of these neurochemical data came some years ago with

the evidence that some varicosities, despite housing vesicles of exocytosis, cannot release their content (Liu et al., 2018). It has been described for DA axons of the striatum only as we know, which could explain why the metabolism of DA in the striatum is so low compared to other brain regions (see below). It is therefore unknown if the data by Liu et al. (2018) are applicable for DA neurons in extrastriatal territories, and for 5-HT and NA neurons in general.

3. Biochemistry of monoaminergic systems

3.1. Biochemical organization of monoaminergic neurons

The biochemical organization of monoaminergic neurons is complex, like other systems of neurotransmission, but it has received considerable attention. The first complexity comes from the fact that neurons are considered as polarized, from the somatodendritic parts to the axons. But when we address the biochemical function of the neurons, this polarization vanishes towards compartments capable of both synthesizing and releasing the neurotransmitter to various degrees (Ludwig et al., 2016). The somatodendritic release of monoamines has been shown in 1981 for DA (Cheramy et al., 1981), and for all monoaminergic neurons (Adell et al., 1993; Adell and Artigas, 1998; Hery and Ternaux, 1981; Pudovkina and Westerink, 2005; Quentin et al., 2018; Westerink et al., 1987). It is a fundamental mechanism participating in the regulation of the activity of the neurons and can also participate to neurotransmission.

Figure 1 reports a general organization of the anabolic and catabolic pathways of monoaminergic neurons. The metabolism involves both monoaminergic neurons and cells (neurons and/or glial cells) within their vicinity. Catecholamines DA and NA share the initial involvement of cytosolic tyrosine hydroxylase (TH) in their biosynthesis to give endogenous L-DOPA. L-DOPA is decarboxylated into DA by aromatic amino acid decarboxylase (AADC) in the cytosol of catecholaminergic neurons. DA is entering the vesicles of exocytosis via the vesicular monoamine transporter type 2 (VMAT2) thanks to the acidic gradient generated by an ATPase pump (Justice, 1993). The pH of the vesicles being acidic, protects catecholamines from auto-oxidation (Mosharov et al., 2009; Umek et al., 2018). In NA neurons, the cytosolic synthesis of NA is presumably minimal via cytosolic, soluble DA-β-hydroxylase (DBH) (Issidorides et al., 2004; Olschowka et al., 1981). The synthesis of NA mainly occurs in the vesicles of exocytosis themselves due to the presence of soluble and membrane-bound DBH

in the vesicles (Rush and Geffen, 1980) and DA itself. Thus, NA neurons could release DA as has been exemplified in numerous *in vivo* studies (Devoto et al., 2014, 2005; Kempadoo et al., 2016). The synthesis of 5-HT occurs from the mono-oxygenation of tryptophan into 5-hydroxy-tryptophan (5-HTP) by tryptophan hydroxylase 2 (TPH2) and its decarboxylation into 5-HT by AADC, all steps occurring in the cytosol (Mosienko et al., 2015). 5-HT is then transported into the vesicles of exocytosis via VMAT2. The anabolic pathways of monoamines show that monoaminergic neurons do not have strong biochemical identity because they share the AADC and VMAT2 steps.

Meanwhile, it is now well documented that the exogenous administration of the precursors 5-HTP or L-DOPA - aromatic amino acids entering CNS and all cells via the transporter LAT1 - promotes synthesis and release of 5-HT from catecholaminergic and DA from 5-HT neurons (De Deurwaerdere et al., 2017; Jackson and Wightman, 1995; Navailles et al., 2010; Stamford et al., 1990).

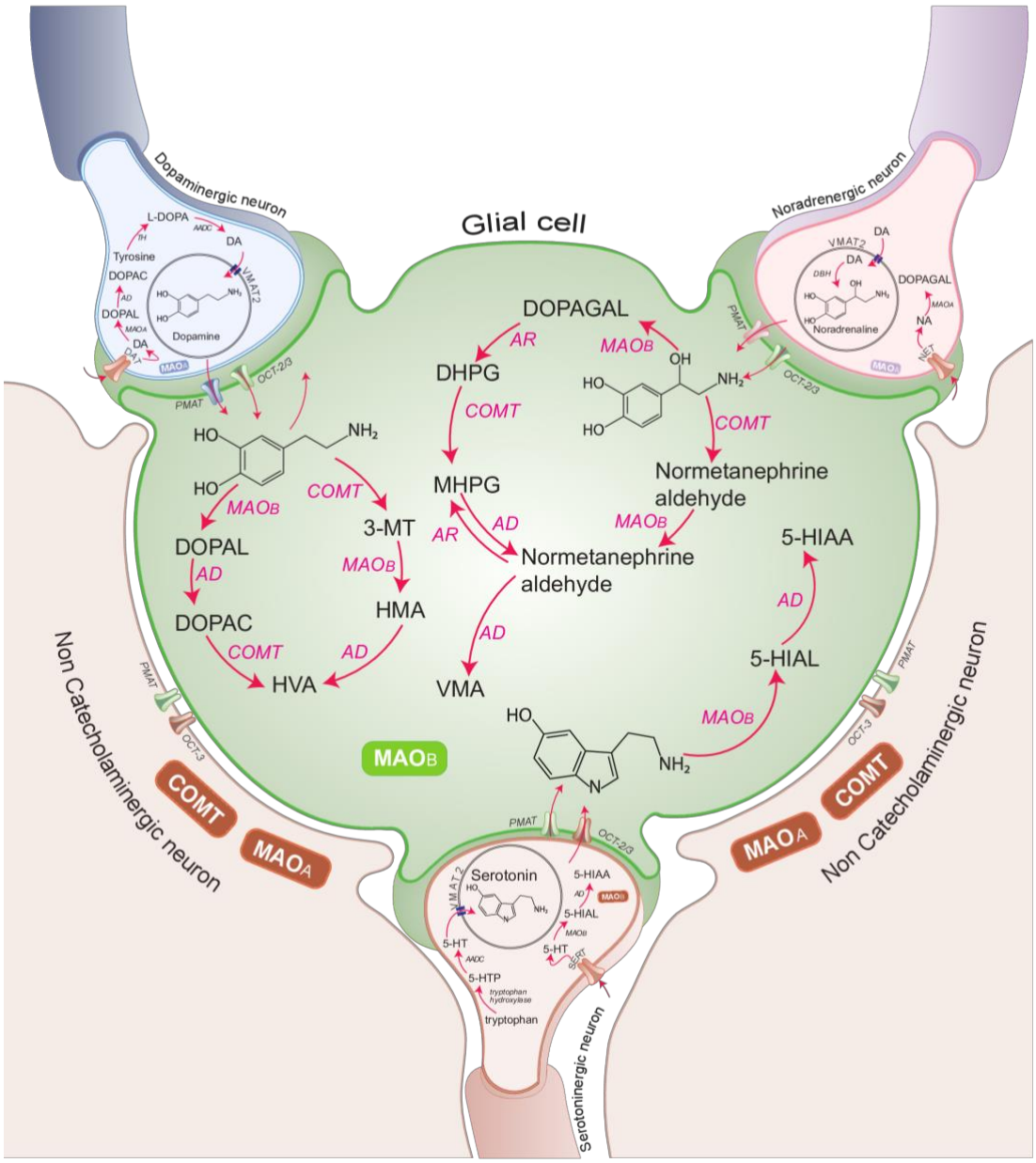

Figure 1 – Metabolic pathways of monoaminergic neurons. This drawing represents terminals of dopamine (DA) (top left), noradrenaline (NA) (top right) and serotonin (5-HT) neurons surrounding a glial cell and non-catecholaminergic neurons. L-amino acid decarboxylase (AADC); L-3,4-dihydroxyphenylalanine (L-DOPA) tyrosine hydroxylase (TH); Monoamine oxidase A and B (MAOA, MAOB); 3,4-Dihydroxyphenylacetaldehyde (DOPAL); Aldehyde dehydrogenase and reductase (AD, AR); 3,4-Dihydroxyphenylacetic acid (DOPAC); Catechol-o-methyltransferase (COMT), 3-Methoxytyramine (3-MT); homovanillic acid (HVA); dopamine beta-hydroxylase (DBH). 3,4-methoxyphenylacetaldehyde, (DOPEGAL); 3,4-dihydroxyphenylglycol (DHPG); 3-Methoxy-4-hydroxyphenylglycol (MHPG); vanillylmandelic acid (VMA); 5-hydroxytryptophan (5-HTP); 5-hydroxyindole acetaldehyde (5-HIAL); 5-hydroxyindoleacetic acid (5-HIAA); vesicular monoamine transporter 2 (VMAT2); DA transporter (DAT); 5-HT transporter (SERT); NA transporter (NET); plasma membrane monoamine transporter (PMAT); organic cation transporter 2 and 3 (OCT-2, OCT-3). (VMAT2); DA transporter (DAT); 5-HT transporter (SERT); NA transporter (NET); plasma membrane monoamine transporter (PMAT); organic cation transporter 2 and 3 (OCT-2, OCT-3).

The degradation of biogenic amines is complex, it involves several enzymes and cell types. Among these, monoamine oxidase (MAO) plays a central role in regulating amine levels within the brain. The enzyme exists in two isoforms, MAO-A and MAO-B, which catalyse the oxidative deamination and produce hydrogen peroxide, ammonia and the corresponding aldehyde. Catecholaminergic neurons mainly contain MAO-A, whereas 5-HT neurons are rich in MAO-B at terminals. DA, NA and 5-HT can be degraded by MAO-A (Di Giovanni et al., 2016; Ramsay, 2013). Another enzyme catechol-O-methyltransferase (COMT) can also metabolize DA and NA (Eisenhofer et al., 2004). COMT is not present in DA terminals in rodents (Kaenmaki et al., 2009; Myohanen et al., 2010; Schendzielorz et al., 2013), implying that glial cells or other neurons reuptake extracellular DA in tissues where the clearance of DA is low (Figure 1). COMT is also involved in the degradation of metabolites generated by MAO activities. The final product from these two distinct catabolic pathways is homovanillic acid (HVA) for DA metabolism and vanillyl mandelic acid (VMA) for NA metabolism (Eisenhofer et al., 2004).

Hence, DA is metabolized to DOPAL by the activity of MAO-A and to 3,4-dihydroxyphenylacetic acid (DOPAC) by aldehyde dehydrogenase (AD) enzymes (Goldstein et al., 2015, 2013; Liu et al., 2014). It is then converted to HVA by COMT. Likewise, NA is metabolized to VMA by the activity of MAO-A, AD, and COMT. The NA metabolites 3-methoxy-4-hydroxyphenyl ethylene glycol (MHPG) and 3,4-dihydroxyphenyl ethylene glycol (DHPG) are produced from the activity of MAO-A followed by aldehyde reductase (Eisenhofer et al., 2004). 5-HT is metabolized to 5-hydroxyindole acetic acid (5-HIAA) by MAO-A and AD.

3.2. The biochemical index of monoamines: raw data and indirect indexes of turnovers

One may wonder what is the meaning and the origin of the indexes that are measured using different biochemical approaches. Table 1 reports basal values reported for DA, 5-HT, and NA using either in vivo intracerebral microdialysis to ascertain extracellular levels, or post-mortem measurement to ascertain tissue content in a brain region. The approximate data are taken from numerous studies to give a rough idea on the difference between brain regions whenever they exist (Abercrombie et al., 1989; De Deurwaerdere et al., 1998; Devroye et al., 2017; Fitoussi et al., 2013; Gobert et al., 2004, 2000, 1998; Gresch et al., 1994; Gros et al., 2021; Huang et al., 2012; Li et al., 2007; Marty et al.,

2023; Mlost et al., 2024; Navailles et al., 2011; Shannon et al., 1986). The extracellular levels, corresponding to continuous release from terminals are minimal. Even though extracellular levels of DA are higher in the striatum, a high level of sensitivity is required to measure it in dialysates. The contribution of these extracellular levels to the whole measurement of DA in post-mortem striatal tissue is considered to be negligible (Pregeljc et al., 2019). The proportion of DA in the cytosol would correspond to 3% while the main contribution to the whole tissue content should come from the vesicles of exocytosis. These predictions come again from DA content in striatal tissue. It is likely different in extrastriatal territories as well as for 5-HT and NA because their metabolism (turnover) is higher compared to DA in the striatum. Extracellular levels of DA are extremely low in the prefrontal cortex, but they are high compared to extracellular levels of DA in the striatum (10 times lower), relative to post-mortem tissue DA content in each tissue (at least 50 times lower). The differences, in addition to a faster metabolism in the cortex compared to the striatum, are related to the possible participation of NA terminals (Devoto et al., 2005), and the lower density of reuptake DA sites, namely the norepinephrine transporter (NET), and the DA transporter (DAT, negligible in the cortex) (Holloway et al., 2018).

Table 1 – Extracellular and tissue concentration of monoamines and indexes in different brain regions.

	Striatum	NAc	mCPF	hippocampe
DA				
Extracellular DA (pg/µL)	0.72	0.44	0.06	0.02
Tissue DA	8,000	4,000	50	20
Tissue DOPAC	900	800	100	20
DOPAC/DA	0.12	0.2	2	1
5-HT				
Extracellular 5-HT (pg/µL)	0.12	0.15	0.14	0.14
Tissue 5-HT	200	250	150	250
Tissue 5-HIAA	1,000	1,000	100	10
5-HIAA/5-HT	2	1.8	2	2.2
NA				
Extracellular NA (pg/µL)	0.02	0.02	0.06	0.04
Tissue NA (pg/mg)	100	200	250	300

The metabolism for a monoamine varies across brain regions as exemplified by the ratios DOPAC/DA [sometimes (HVA + DOPAC/DA)] and 5-HIAA/5-HT (Bannon et al., 1981;

Commissiong, 1985; Ebinger et al., 1987; Fitoussi et al., 2013; Johnston and Moore, 1983; Shannon et al., 1986) whose functional value is more important than the tissue neurotransmitter itself to assess metabolic activity (Eisenhofer et al., 2004). This statement might not be that evident if we look at correlative analyses, which is in part the purpose of this review. These metabolic indexes are often named turnover, a biological process including the balance between synthesis and catabolism of monoamines (Eisenhofer et al., 2004; Glowinski, 1975). The turnover stricto sensu is less studied *ex vivo* or *in vivo* as it needs specific analyses mainly relying on radiolabelled compounds. So, the ratios are indirect indexes of the turnover only. Again, the same index such as DOPAC/DA could have different meaning in the striatum and in extrastriatal tissue. In the striatum, the origin of DOPAC would be primary related to DA terminals themselves in basal conditions in rodents (Justice, 1993; Sharp et al., 1986; Zetterström et al., 1988). The reasons for lower knowledge in extrastriatal tissues is related to the difficulties in monitoring low quantities of DA and metabolites reliably. Moreover, the DA-based pharmacology (psychostimulants, DA agonists, DA antagonists) that enabled researchers to better understand striatal DA terminal function and activity are less relevant in several extrastriatal tissues due to the low levels of DAT and D2 autoreceptors (Jiao et al., 2003). Solely this argument should justify broader analyses of tissue content for neurotransmitters and metabolites across the CNS as the regulatory mechanisms operating on the activity of monoaminergic neurons in CNS are presumably different in different brain regions.

3.3. The neuron and the gland

The varicosities of monoaminergic neurons interact with local partners that can be cell bodies, glial cells, and afferent neurons/dendrites. The biochemical activity of monoaminergic terminals is modulated by these partners (heterologous controls) and the autoregulatory mechanisms (homologous controls) that maintain the set point of the activity of the varicosities. The biochemical modulations of the activity of terminals can be independent of the electrical activity of cell bodies as simply exemplified by the ability of synaptosomes to synthesize and release neurotransmitter content in exocytotic and non-exocytotic manner (de Belleroche and Bradford, 1980). These considerations are important in view of correlating tissue content of neurotransmitters between brain regions. While the homologous controls keep the activity at similar levels, the heterologous controls can change according to the conditions. It is possible that changes

in correlations profiles between brain regions could correspond to variations of heterologous controls, rather than changes in homologous controls that would change the quantities of neurotransmitter/metabolite. It is remarkable that the full lesion of DRN/MRN 5-HT neurons do not change the striatal tissue content of DA three weeks after the lesion (De Deurwaerdere et al., 1998, 1995), despite the multiple evidence showing that 5-HT may modulate the activity of DA neurons (Alex and Pehek, 2007; De Deurwaerdere and Di Giovanni, 2017; Kapur and Remington, 1996; Soubrie et al., 1984). In fact, the electrolytic lesions (MRN or DRN, or both) modified the “utilization” of DA (DOPAC/DA) sometimes in an opposite manner between the cortex and the NAc (Hervé et al., 1979a; Herve et al., 1981). Thus, numerous homeostatic processes occur to maintain the tissue levels of DA, which supposes that the same levels in two distinct conditions involve distinct mechanisms. Similarly, amino acid neurotransmitters are involved in multiple metabolic pathways. While their concentration is maintained in each compartment, the relationships with substrates and products of enzymes evolve according to conditions. Similar levels in two conditions do not imply no change of the parameter, a reason why correlative approaches could be informative.

4. Practical and technical measurements of post-mortem data, with possible caveats

4.1. Origins of neurochemical variability

In quantitative approaches, the variability can be attributed to multiple factors, starting with the analytical devices. Analytical procedures are by necessity extremely rigorous whenever quantification of the tissue content or the in vivo extracellular measurement is required to minimise this variability. The variability of the data also comes from biological factors. The steps of post-mortem tissue analysis and the measures taken to avoid non-biological variability of the data are depicted in Figure 2.

4.2. Detection of compounds

Quantification of tissue monoamines and metabolites requires sensitive and stable analytical devices. The two criteria are checked daily using standards solution. Stability also implies a reproducible injection procedure, to minimize injection volume error, or the dispersion of the sample before the HPLC columns. The injection volume error is usually low with autosampler, or by manual injection by a trained person. This can be verified by the variability of consecutive standards, which should be less than 5% with manual

injectors. A more complicated source of variability is confounding peaks in the chromatogram. The chromatographic conditions are prepared with a core recipe (fixed concentration of the phosphate or citric acid buffer, fixed concentration of EDTA, usually 0.1 mM, and fixed concentration of methanol). The concentration of the pairing ion (for instance sodium octyl sulfate) and the pH are included in the initial mobile phase, but these two factors are used to adjust the elution time of the compounds to achieve a clear separation between peaks, allowing the signal to reach a stable baseline between peaks (Chagraoui et al., 2019a). The quality and purity of the different constituents is important to minimize contaminations of different origins. Contaminants can also be compounds present in tissue samples. In fact, most HPLC conditions can detect several compounds that are not considered because their endogenous concentrations are supposed to be low or even negligible. When we measure DA, DOPAC, or HVA tissue contents in regions where their endogenous levels are extremely low like the hippocampus or the cortex (Table 1), a higher gain is required. Particularly at higher gains, negligible concentrations of electrochemically sensitive compounds can induce a signal. In our conditions, L-DOPA, 5-HTP, and adrenaline to cite a few induce strong electrochemical signals in standards, but their concentration is low or negligible (adrenaline in the brain). At a higher gain, we can measure low but reproducible quantities of L-DOPA or 5-HTP (5 to 50 pg/mg of tissue). For this reason, the correct separation of a sample in the initial conditions should include the determination of the elution time of other compounds in the sample, measured or not (Marty et al., 2023). Another example is the confounding elution time between 5-HT and 3-MT. While this problem is minimized in extrastriatal regions due to the lower abundance of 3-MT compared to 5-HT, it must be considered in striatal regions. Either we succeed to separate 5-HT and 3-MT, or we fix a low potential of oxidation for 5-HT that is not sufficient to oxidize 3-MT, and then a higher potential on another electrode for 3-MT, implying an electrochemical detection system equipped with several electrodes and detectors (Chagraoui et al., 2019a). Once the chromatographic conditions are satisfactory, with all these criteria in mind, calibration curves are performed with 3 to 5 distinct concentrations. It is better to perform calibration curves within the expected range of tissue concentrations for each the compound. Thus, the calibrations curves should be different for the striatum and extrastriatal regions. Calibration curves are essential not only for determining the actual quantities of compounds in a sample but also for identifying the usable portions of the curve (linear segments with a minimum

regression coefficient, R^2, of at least 0.98). These curves enable the determination of detection limits, quantification limits, and saturation points.

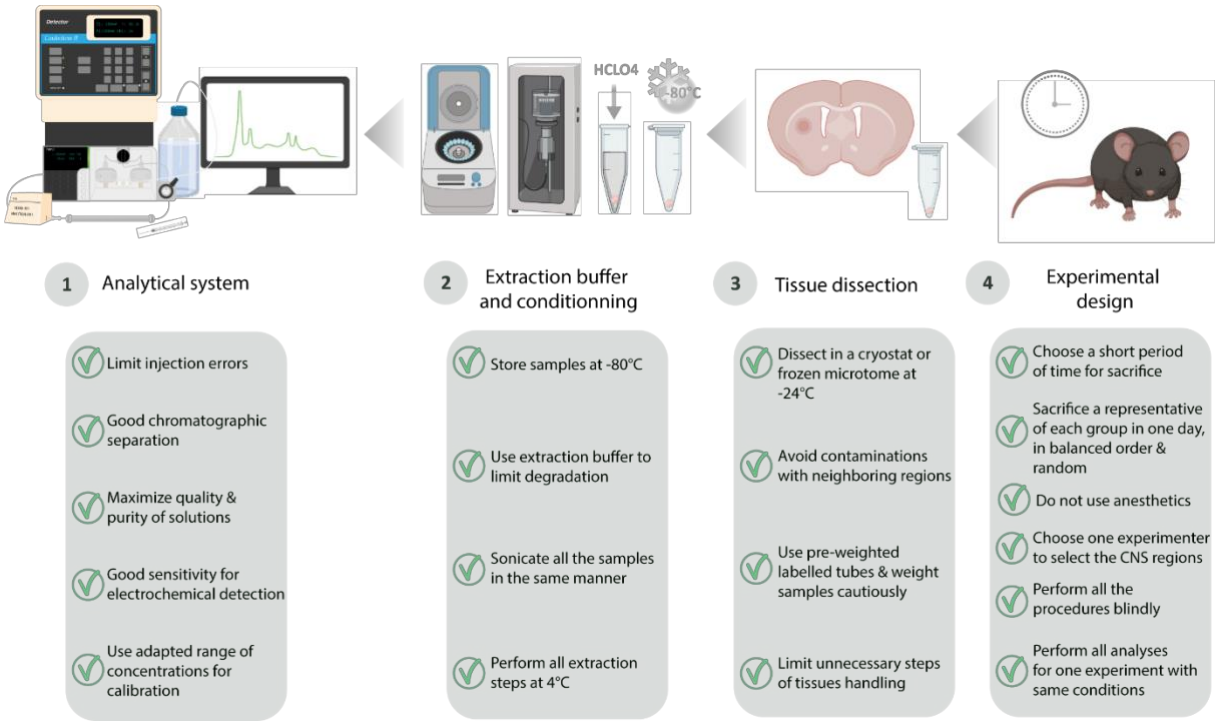

Figure 2 – The sources of variability in the quantification of post-mortem parameters. The figure reports good practices to minimize the variability in considering different steps of the process including the analytical part (1), the extraction (2), the tissue dissection (3) and the experimental design. The arrows show the time course of the experiment including the experimental design, the dissection of the tissue, the extraction of the compounds and the analytical system.

4.3. Extraction buffer, and tissue conditioning

The metabolism of monoamines and classical neurotransmitters is fast, and sensitive to anaesthetics. Thus, the sacrifice of the animals should occur without anaesthesia whenever subtle changes of monoamines quantities are expected. There are two possibilities after the extraction of the CNS: either the CNS is directly dissected, preferentially on a cold plate, and the selected pieces of tissues are frozen (cold isopentane, or liquid nitrogen), and placed in labelled tubes to be kept at -80°C; or the brain is directly frozen to be kept at -80°C pending subsequent dissection (see below). In all cases, within an experiment, the procedures should be the same for all animals.

The procedure of extraction of the compounds from the tissue is essential. At variance with peripheral tissues, the extraction of monoamines from CNS tissues can be achieved using perchloric acid (HCLO4, 0.1 N), without absorption/filtration. The use of acidic milieu is required to limit the activity of the enzymes, and the auto-oxidation of monoamines, particularly L-DOPA, DA, and NA. Other extractions buffers are used, all of them conferring antioxidant properties and/or inhibition of enzymes. Sonication, to destroy cells, should be the same between samples, particularly as it can heat the tissue. There is also the possibility to use magnetic balls to destroy the tissue, which limits experimenter discomfort and unwanted alterations produced by sonication. All procedures including the subsequent centrifugation should be performed at 4°C.

4.4. Dissection of the tissue

As indicated above, the CNS regions of interest can be directly dissected after the removal of the CNS. The pieces of tissue are usually quite large (several mg), which allows for their direct weighing just before being frozen. The main limit of this approach is the risk of taking neighbouring structures and the low number of dissected brain regions obtainable. This is likely the cause of regularly reported elevated levels of DA in the cortex (above than 100 pg/mg of tissue), and sometimes in the hippocampus, likely from the presence of striatal tissue or mesencephalic tissue in the sample. In fact, the quantities of tissue DA in the striatum are 100 to 1000 times higher compared the quantities measured in the cortex or the hippocampus respectively (Table 1). Thus 1% of striatal tissue can screw up the correct determination of DA quantities in the hippocampus or the cortex. Since this kind of regional contamination is unexpected, it can vary between samples in the same experiment, without implying outliers, but inducing variability.

Another approach involves dissecting the brain using a cryostat or frozen microtome, with the procedure performed at around -24°C (Fitoussi et al., 2013; Saavedra, 1977). This method offers several advantages, it allows precise control over the size of the tissue 'punches', enabling the collection of smaller tissue samples, including sub-regional areas within brain regions, and facilitates the analysis of a much larger number of brain regions. In the hands of a trained manipulator, this technique helps limit the punching of neighbouring regions, thereby reducing for instance the contamination of striatal tissue with cortical regions, amygdala, septum, globus pallidus, and other surrounding areas. Once a piece of tissue is selected, it is placed in a pre-weighed and labelled tube in the

cryostat. Since frozen dissection is less temporally sensitive, it also allows for the collection of photomicrographs of the selected brain regions. However, there are two caveats with this approach. First, the pieces of tissue are small, between 1 and 3 mg. It is almost impossible to weigh them directly without melting, presumably altering metabolism. The weight of the piece of tissue is thus determined by the difference between the filled and the empty tube. The same balance must be used, and tubes wiped quickly and cautiously to remove any drop of water or ice which would be heavier than the piece of tissue itself. The second caveat of this punching procedure is related to the multiple steps of tissue handling, which could have an impact on neurotransmitters metabolism and degradation. For instance, in all studies we performed with this approach, we could not obtain a ratio DOPAC/DA below 0.2 in the dorsal striatum while it should be closer to 0.1 (Table 1).

4.5. Experimental design and Randomisation

To reveal the existence of biological effects of low magnitude, it is required to manage numerous biases that could influence the dataset. Sex, age, strain, or life conditions are among the evident conditions that are classically managed. The time of sacrifice is another possible bias because the concentration of monoamines can vary during the circadian cycle (Cagampang et al., 1993; Hery et al., 1977; Menon et al., 2019; Rueter and Jacobs, 1996). Thus, choosing a short window of time for sacrifice limits the impact of the circadian rhythm in the dataset. As the experimental groups can be large, this may require the experiment to last several days. It is recommended to have representative of each treatment groups each day, in a randomised and balanced order. This minimizes the impact of a “day effect” that could impact the dataset if all representatives of one experimental group (one independent variable) are done the same day. To further accord with a rigorous scientific method, the same experimenter should select the CNS regions of interest either freshly or using a cryostat. Finally, the experimenter should be blind of the experimental groups from dissection to handling samples. Because the quantities of compounds of interest are different between brain regions, it is preferable to analyse the regions of the experiment separately.

A scientific approach is important in quantitative and semi-quantitative methods because the quantification is relative, and not absolute. This means than one structure should be analysed with the same analytical devices, and from the same batch of dissected regions. Increasing the number of observations (increasing the n) after several weeks or months

is rarely satisfactory, as the added values could differ by 10-20% compared to the initial controls. Such a variability comes from all steps of the procedure (mostly life conditions, sampling procedures, and other chromatographic/detection conditions). It can be managed with intracerebral microdialysis as the data is largely expressed relative to the basal values obtained before a treatment within the same animal. Post-mortem data, lacking a temporal dimension, provides a single value for a given region, making the expressing of data as a % of baseline impossible (control animals comprising another group in an unpaired experimental design).

4.6. Meaningful variability

In summary, the origin of the variability of the data as expressed by the mean standard deviation (SD) or standard error of the mean (SEM) is already a matter of multiple sources. This expression of the data as mean +/- SD is convenient to include all types of variabilities, from technical to biological (Butler et al., submitted). These variabilities must be addressed to leverage an approach towards multivariate analyses. Indeed, while the expression of as mean or median for one group masks the individual values, the correlative and multivariate approaches give more importance to each individual value within the group. It would be thus annoying to attribute a biological meaning of a correlation while it could come from an injection error, or an aberrant sampling location.

5. Correlative and multivariate analyses of CNS neurotransmitters

5.1. Rational to change the paradigms

The monoaminergic systems exhibit biological responses through their interaction across the brain (Fitoussi et al., 2013; Hensler et al., 2013). Knowledge of the biological functions and complex interactions between neurochemical systems is limited, and currently does not sufficiently consider the widespread nature of their axonal branches. Dynamic approaches assessing the function of monoaminergic neurons, such as electrophysiology or microdialysis, are interesting to address mechanisms within a single brain region. However, they do not necessarily represent the situation in other brain regions. It is postulated that changes monoamine tone in one brain region can have local or distal influences on the activity of the other monoamines. This has been exemplified in numerous, dynamic studies without giving a wide-brain overview (De Deurwaerdere and Di Giovanni, 2017; Guiard et al., 2008; Hensler et al., 2013). The

interplay between monoamines likely occurs in several CNS territories during behaviour. Post-mortem data can also be used to address at rest the biochemical distribution of monoaminergic systems over large territories to study inter-individual differences in multiple dimensions of behaviours.

Correlative analysis offers the possibility to study interaction between neurotransmitters and/or their metabolism at wide brain scale. A correlative approach may also inform about qualitative changes of monoamine tissue contents that could occur independently from quantitative changes. Whenever quantitative changes of neurotransmitters/metabolites are detected in the tissue, correlations can offer additional information about the qualitative nature of these changes hidden in the expression as the mean +/- SD (Butler et al*., submitted).*

5.2. Is there a default mode neurochemical network?

We have performed several studies using correlative approach of neurotransmitter content. The pattern of correlations for monoamines and/or their metabolite was studied, in most cases at rest, without conditions other than a drug, or genotype. In our expertise, there are a few correlations across the brain in vehicle treated/naive rodents in resting conditions (Bharatiya et al., 2020; De Deurwaerdere et al., 2020, 2020; Dellu-Hagedorn et al., 2017; Fitoussi et al., 2013; Marty et al., 2023; Puginier et al., 2019). Other groups developing such kind of analyses also report low number of correlations in a limited number of brain regions (Midzyanovskaya et al., 2020; Mlost et al., 2024). We also found a low level of correlations of monoamines in cerebroid ganglion, pedal ganglion, and gonads in *Perna Perna* taken along the Moroccan Atlantic coast (Klouche et al., 2015). The density of correlations barely reaches 10% of the total number of possible correlations for any monoamine or metabolite, often lower. The pattern of correlations is not reproducible between experiments, so this approach would be devoid of quality controls, except for the quantitative values. For instance, in the two experiments concerning the effects of the 5-HT_{2C} receptor agonists WAY-163909 and lorcaserin, the experimental conditions were approximately the same (Chagraoui et al., 2019b; De Deurwaerdere et al., 2020; Di Giovanni et al., 2020; Whitestone et al., 2019). There was a low, similar number of correlations in vehicle-treated rats between the two experiments, but the pattern of these “basal” correlations was slightly distinct for all neurotransmitters (see Figure 3).

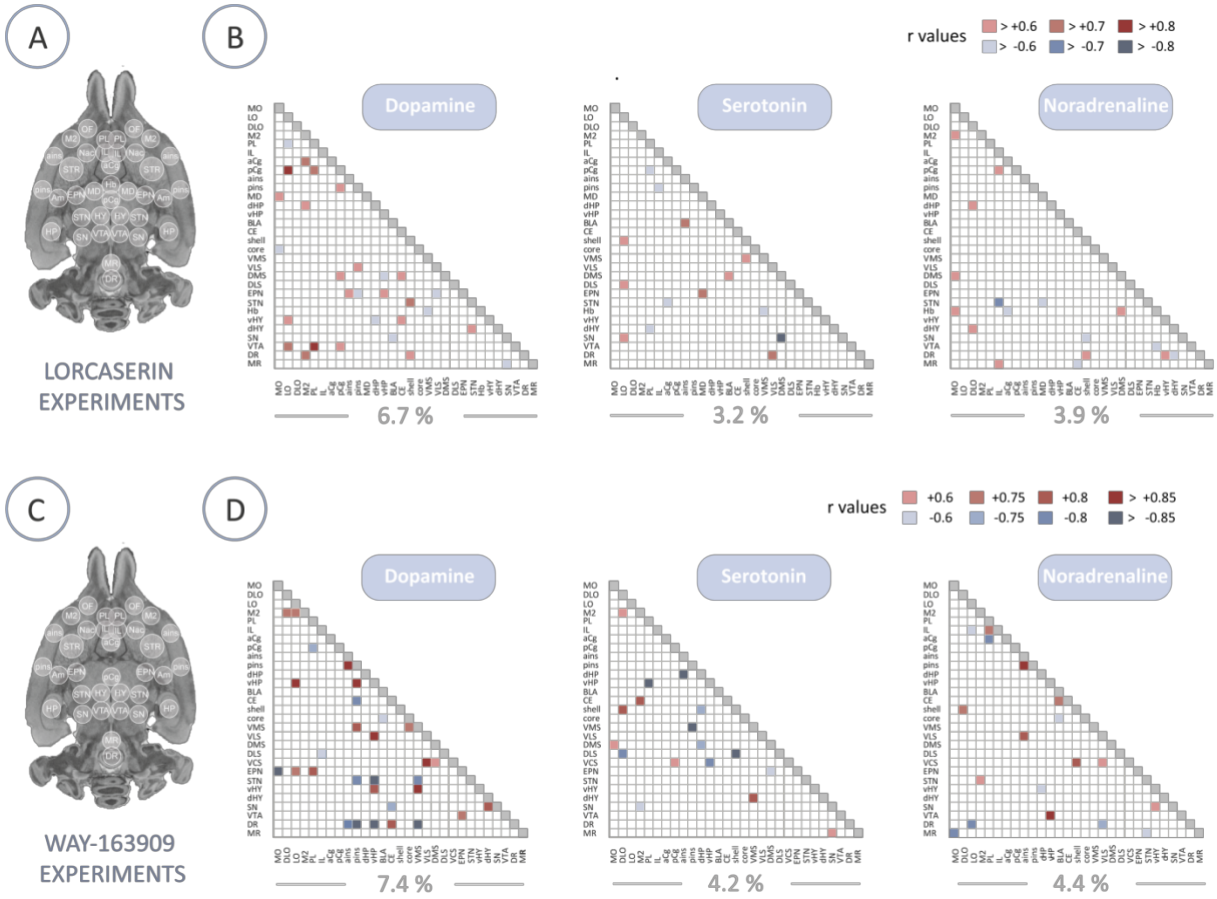

Figure 3– Neurochemical correlations of monoamines in resting conditioning in vehicle-treated rats from two distinct experiments relative to lorcaserin (De Deurwaerdère et al., 2020) and WAY-163909(Chagraoui et al., 2019). Approximate punches localization for the 30 and 29 brain regions. (B, D) Correlative maps reporting existing, significant correlations (Pearson's r coefficient) for the tissue content of dopamine, serotonin, and noradrenaline (pg/mg) between the different brain regions sampled in vehicle-treated rats. Each colored box corresponds to a significant correlation ($p < 0.05$; red, positive; blue, negative), and the color intensity represents the strength of the correlation (r values). The percentages of significant correlations for each system are indicated below the correlative maps. Orbitofrontal cortical regions (OF) : medial orbital cortex (MO), lateral orbital cortex (LO), Dorsolateral orbital cortex (DLO); Motor cortex M2; Prelimbic cortex : PL; Infralimbic cortex (IL); Anterior cingulate cortex (aCg); Posterior cingulate cortex (pCg); Anterior insular cortex (ains), Posterior insular cortex (pins); Nucleus acumbens (Nac) : shell and core; Striatal regions (STR) : Dorsomedial striatum (DMS), Dorsolateral striatum (DLS), Ventrolateral striatum (VLS), Ventrocaudal striatum (VCS); Amygdala (Am) : Basolateral nucleus (BLA), Central nucleus (CE); Habenula (Hb); Mediodorsal thalamic nucleus (MD); Entopeduncular nucleus (EPN); Subthalamic nucleus (STN); Hypothalamus (HY) : dorsal parts (dHY), ventral parts (vHY); Hippocampus (HP) : dorsal anterior parts (dHP), ventral posterior parts (vHP); Substantia nigra (SN); Ventral tegmental area (VTA); Dorsal raphe nucleus (DR); Median raphe nucleus (MR).

5.3. Expression of the data and Quality controls

Before discussing the validity of a correlative approach, we discuss the way of representing these correlations. In the initial submission of the first article (Fitoussi et al., 2013), we drew figures reporting the established links for all three monoamines alone or in combination between regions as it could be seen in a recent article (Mlost et al., 2024). One of the reviewers indicated that it might be a convenient way of reporting the data, but it could mask the functional aspect of monoaminergic systems in the CNS: these systems are chaotic in essence, and the correlative links might not be clearly expected. Upon the advice of this inspired reviewer, we display the full maps reporting the coefficient of correlations that are associated with statistical significance. As discussed above, the maps are indeed full of blank boxes in most cases for monoamines in resting conditions. We take this as a criterion even though the profile at rest is not reproducible.

There are two other possible tips, which are not proof of concepts. First, when combining the correlations between neurotransmitters, the proportion of correlations is sometimes higher in the same brain region (Chagraoui et al., 2019b; Fitoussi et al., 2013). The occurrence of correlations between monoamines has also been reported in the human striatum (Hortnagl et al., 2020). It is an interesting finding that suggests possible correlations between extracellular levels whenever multiple neurotransmitters are measured. In a very recent article, it has been shown in the cortex of rhesus macaque that extracellular levels of neurotransmitters monitored by microdialysis and tandem mass spectrometry could correlate differently according to the cognitive demand (Mayer et al., 2025). Second, it is always possible to determine existing correlations between a neurotransmitter and its metabolite(s) in the same region. It never indicates full, positive correlations for all CNS regions, but the proportion of correlations between parent neurotransmitter and metabolite can concern 50% of brain regions (Dellu-Hagedorn et al., 2017; Marty et al., 2023). These two points are not criteria though, as a correlative link can evolve according to the status of the animals. For the interaction of neurotransmitters themselves, the experiment showing distinct profiles depending on the cognitive status is an important information that suggests that the link between transmitters can vary as a product of organism context. For the link between parent compound-metabolite, it can be modified as previously reported (Gros et al., 2021; Marty et al., 2023). In an unpublished study, we measured the

quantities of monoamines and metabolites in the spinal cord of developing pups submitted, or not, to brief motor training. The quantitative values for monoamines and metabolites were the same between the two groups. However, DA and DOPAC contents strongly correlated in trained pups, but not in controls (Quilqars, Bertrand, De Deurwaerdère, unpublished).

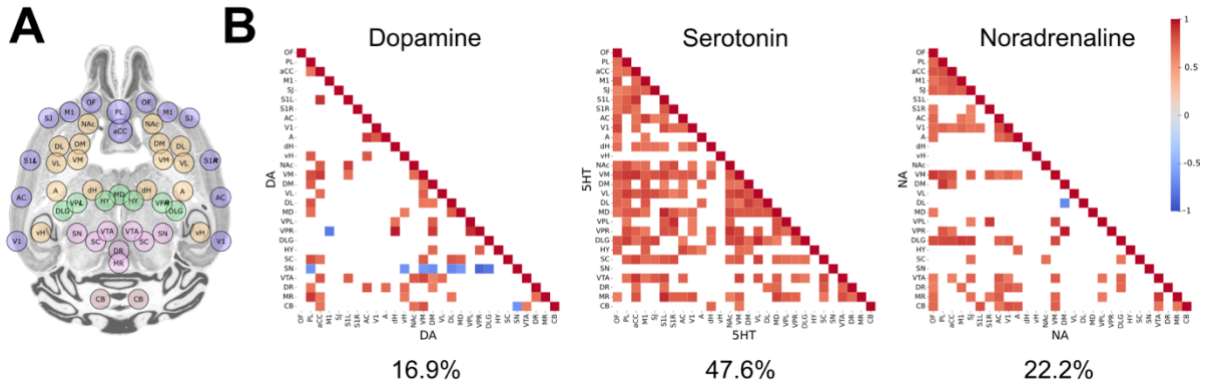

Figure 4 – Neurochemical connectivity of **monoamines** following exploratory behaviour in vehicle-treated mice, data adapted from previous work (Butler *et al., submitted*). OF (orbitofrontal cortex), PL (prelimbic cortex), aCC (anterior cingulate cortex), M1 (primary motor cortex), SJ (somatosensory cortex jaw), S1L/R (left/right primary somatosensory cortex), AC (auditory cortex), V1 (primary visual cortex), A (amygdala), dH/vH (dorsal/ventral hippocampus), NAc (nucleus accumbens), DM/DL (dorsomedial/dorsolateral striatum), VM/VL (ventromedial/ventrolateral striatum), MD (medial dorsal nucleus), VPL/R (left/right ventral posteromedial nucleus), DLG (lateral geniculate nucleus), Y (lateral hypothalamus), SC (superior colliculus), SN (substantia nigra), VTA (ventral tegmental area), DR (dorsal raphe nucleus), MR (median raphe nucleus), CB (cerebellum).

5.4. Connection or connectivity?

Among the quality controls that could be evoked to ensure the validity of the dataset would be the relationship between the neurotransmitter content at terminals and cell bodies. For instance, it would be tempting to speculate that the physical link between the SN and the striatum would favour the occurrence of correlations for DA and its metabolites between the two brain regions (same group of neurons). Such correlations are not reported. In fact, it has been shown for a long time that the regulatory mechanisms operating at the level of the striatum and the SN on DA content and metabolites, marked by a distinct ratio DOPAC/DA, are different even though it corresponds to the same group of neurons (Argiolas et al., 1982). Thus, the correlative link of biochemical parameters between the regions housing cell bodies and their terminal fields is not a criterion at all, as also exemplified by the usual lack of

correlations between 5-HT in the DRN and the numerous brain regions it innervates (Figures 3 and 4). We recently confirmed this aspect in reporting lack of specific correlations for NA between punches encompassing the LC and other parts of the brain (Gros et al., 2021). A refinement of this approach would be to study separately the left and right hemispheres. Indeed, in most cases, lateralized regions are combined, which could limit the occurrence of correlations if there are lateralized regulation, as suggested by studies separating left and right sides (Mlost et al., 2024).

Correlating neurotransmitter contents across the brain could therefore correspond to the characterization of connectivity within a neurotransmitter system or between neurotransmitter systems rather than anatomical connection. As an example of functional connectivity between brain regions, we could evoke the presumably opposite role of cortical DA upon subcortical DA function (Deutch, 1993; Latagliata et al., 2023; Louilot et al., 1989; Tassin et al., 1978). We reported once negative correlations between the infralimbic cortex and the shell of the nucleus accumbens in a cohort of 35 Wistar rats (Fitoussi et al., 2013), but this negative correlation is not systematically reported in the other experiments.

The question of what is behind an identified correlation in terms of biological meaning is still under question. The study of tissue monoamines in *Perna Perna* throughout different stages of the reproductive cycle was very interesting on that matter (Figure 4). Indeed, the organization of the pattern of correlations between NA, 5-HT, and DA levels in cerebroid ganglia, pedal ganglia, and gonads throughout the cycle corresponded to the description of what was supposedly occurring, from the control of gonad NA function at rest to the control of gonad 5-HT function for the release of gametes (Klouche et al., 2015). The meaning of some negative correlations could also be coherent with endocrine controls of NA or 5-HT content in the gonads. The dramatic changes in the reproductive cycle and the profile of correlations in polluted sites could be attributed to dramatic alteration of monoamines functions together with the inclusion of mussels at different stages, the distribution being less clear-cut than in the non-polluted site (Klouche et al., 2015).

Thus, more than physical connections, the study with correlations would inform on connectivity, a connectivity at rest that is still elusive for neurotransmitters, and more informative for the ratios DOPAC/DA and 5-HIAA/5-HT in rodents.

5.5. Lack of clear correlation profile: inter-individual differences?

Correlative approaches, and multivariate analyses rely on individual values. Apart from the variability coming from non-biological sources as discussed above, individual differences in a cohort likely play a role in the lack of systematic profile of correlations at rest. Although this has not been specifically addressed, our database indicates that the composition of the cohort is a factor conditioning the pattern of correlations. In a cohort of 35 wistar male rats, all the animals were characterized by their behavioral performances in six tests addressing impulsive/compulsive dimensions of behavior (Dellu-Hagedorn et al., 2018). For each test, three groups were composed a posteriori: performant, intermediate, and non-performant. The intermediate group was discarded and the monoamine profiles between performant and non-performant rats were compared, i.e. the extreme phenotypes within the cohort. The composition of the performant and non-performant groups was distinct for each of the 6 tests, indirectly showing that maladaptive response in one test does not imply maladaptive response in all tests (Dellu-Hagedorn et al., 2018). We reported changes in tissue 5-HIAA/5-HT, 5-HT, DOPAC/DA, and DA between performant and non-performant rats for each test, sacrificed one month after the last behavioural test. We are still processing these data, but the preliminary analysis indicates that the pattern of the correlations for the ratios is completely distinct between the two cohorts in the “risk taking” test (Butler et al., submitted). Acknowledging that all the data from these animals were initially combined for the purpose of studying correlations for monoamines (Fitoussi et al., 2013) or their metabolism (Dellu-Hagedorn et al., 2017), it suggests that the inter-individual differences are playing an important role in the outcome of correlations profiles. Consequently, in resting conditions, one cannot expect a specific profile.

Since there would not be specific profile of correlations characteristic of vehicle-treated animals, it implies that changes of correlation profiles obtained between two or three cohorts such as transgenic mice versus wild type or after pharmacological drugs versus vehicle require cautious interpretation. In two independent experiments, the 5-HT_{2C}R agonists lorcaserin and WAY-163909 reduced the number of correlations for

DA tissue content and increased the number of correlations for NA and 5-HT (Chagraoui et al., 2019b; De Deurwaerdere et al., 2020; Di Giovanni et al., 2020). On the other hand, the number of correlations for the ratio DOPAC/DA was dramatically reduced by lorcaserin, and slightly increased by WAY-163909 (De Deurwaerdere et al., 2020; Whitestone et al., 2019). Both 5-HT_{2C}R agonists had a few though distinct quantitative effects, except for an increase in the tissue content of DA in the central amygdala.

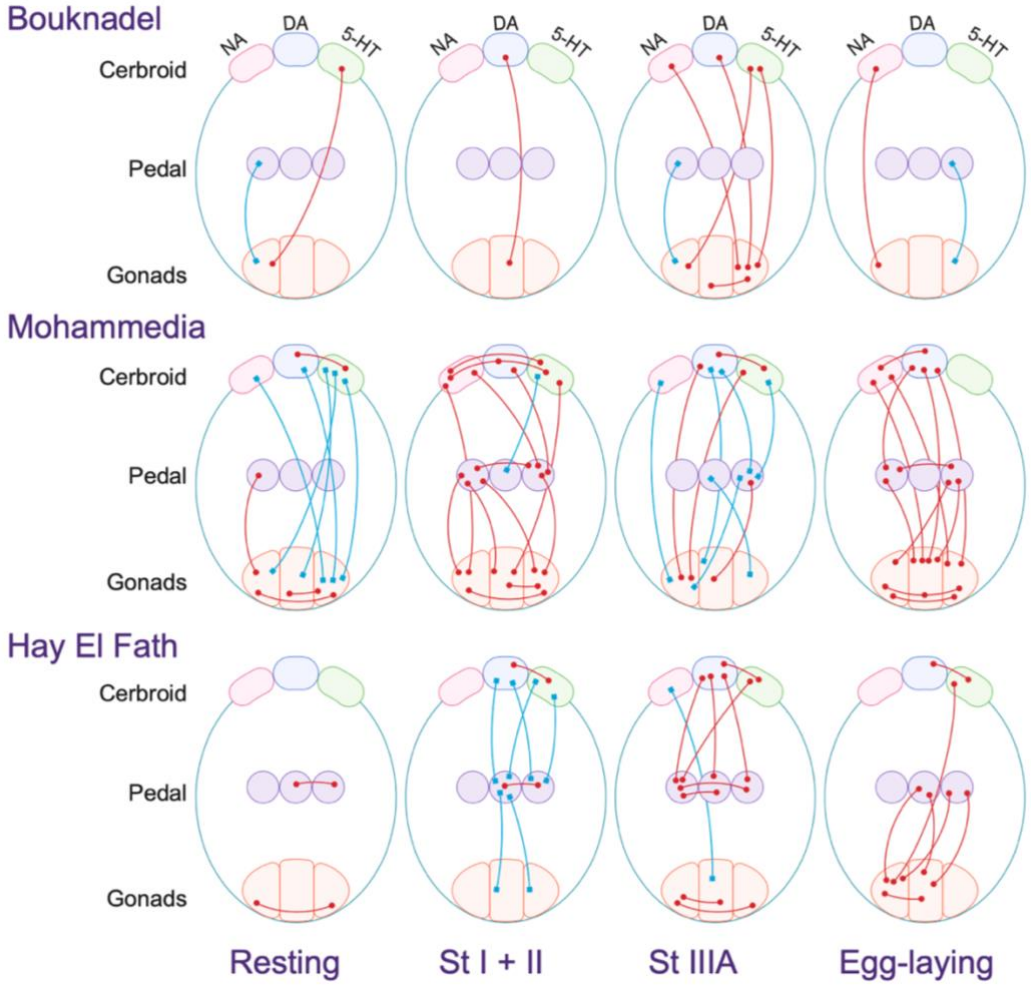

Figure 4 - Tissue monoamine connectivity in *Perna Perna.* Correlation analysis of NA, DA, and 5-HT content between organs at different stages of the sexual cycle of Perna perna at the Moroccan sites of Bouknadel, Mohammedia and Hay El Fath. The different stages (Resting; StI + II; St IIIA and egg-laying) are approximate. The Spearman's rank order correlation test was used to establish correlations ($p < 0.05$) between individual measures. These correlations were either positive (red) or negative (blue); adapted from (Klouche et al., 2015).

5.7. Resting conditions or activated conditions?

It has been shown for years that mice submitted to open field or electric foot shock stress exhibited an increase in the cortical ratio DOPAC/DA (more DOPAC, less DA) right after the exposure (Hervé et al., 1979b; Tassin et al., 1980). It has been shown latter that it corresponded to an increase in extracellular levels of DA, also associated with an increase in NA (Abercrombie et al., 1989; Feenstra, 2000; Gresch et al., 1994). Whatever the existence of this robust effect in rodents, one may wonder what the consequences of this effect are in other parts of the brain. It is the purpose of correlative analysis.

The study of classical psychedelics would benefit from leveraging unconditioned exploratory behaviour, modified uniquely by psychedelics, to further an understanding of their mechanism of action (Halberstadt and Geyer, 2018). Such a situation was interesting for us to determine the extent to which the correlative maps of neurotransmitters were sensitive to the environment of the animal immediately prior to sacrifice. We studied the effect of the 5-HT_{2A}R agonist TCB-2 and the role of 5-HT_{2A}R in its effect using the 5-HT_{2A}R antagonist MDL-100,907. After having monitored the head twitches to determine if the treatments were efficacious, we changed the flooring for the 10 last minutes of the experimentation. We substituted the transparent floor for a novel flooring made up of different textures and colours to promote forced exploratory behaviour in mice. We were surprised to discover that the number of correlations, mostly all positive, in vehicle-treated mice was tremendously high for 5-HT content across the brain (Figure 4). This was also true for DA and NA which additionally showed a clear regional organization. We also found extremely high level of positive correlations for amino acids including GABA, aspartate, and glutamate with the highest density (>60%) of correlations for glutamate. When combining pairs of neurotransmitters across the brain, the profile obtained was organized whenever DA or NA were included, suggesting that these two systems were playing an important role in triggering the responses to the novel environment.

TCB-2 dose-dependently reduced the number of correlations for all neurotransmitters and suppressed the organized pattern of correlations whenever DA and NA were included. MDL-100,907 alone also dramatically reduced the number of correlations. With TCB-2 (high doses) or MDL-1009,07, there was an increased occurrence of

negative correlations. At variance with TCB-2, MDL-100,907 did not induce major quantitative effects on the concentrations of tissue neurotransmitters, as expected from previous studies (De Deurwaerdère et al., 2021; De Deurwaerdere and Di Giovanni, 2017). The dramatic decrease in the connectivity within and between neurotransmitter systems with 5-HT_{2A}R ligands suggest that 5-HT_{2A}R plays a role in the organization of neurotransmitter systems in response to novel environment.

Thus, it is interesting to conceive experimental situations mobilizing neurotransmitters systems to better address the mechanism of action of the psychoactive drugs. In another, unpublished experiment, we studied the effect of the typical antipsychotic haloperidol on monoamines tissue content. However, the density of correlations was extremely low in vehicle-treated animals. It therefore seems pertinent to address the effect of potential antipsychotic drugs (haloperidol, 5-HT_{2C}R agonists) and probably other classes of medicine in more complex experimental settings.

5.8. Extension of the connectivity: graph theory

Recent data in the lab showed that the connectivity between regional neurotransmitters can be dramatically affected by distinct experimental conditions. Moreover, the reduction of total the number of correlations with TCB-2 or MDL-100,907 gave unique profiles. To better investigate and eventually describe this neurochemical connectivity implemented graph theory.

The output of correlation analysis is a correlation matrix of r values (Pearson or Spearman), which can be filtered for significance *i.e.* $p<0.05$. Most commonly this is then represented as a heat map called a correlogram and compared visually (Figures 3 and 4), or by making additional heatmaps of the difference between two correlograms. Differential correlograms are most common in the field of neuroimaging. For instance, it is largely used in fMRI to approximate blood flow highlighting regions of alteration in a subject (patient) following some treatment (Carhart-Harris et al., 2016, 2012) although this approach is limited to the comparison between two conditions. Further developing on the methods to display, analyse and conceptualize correlative analysis of neurochemicals, graph theory was applied to build networks from correlation matrices.

A network or graph, in graph theory, is a mathematical structure used to model relations between objects (nodes). In the case of post-mortem neurochemistry, we are modelling the relation between any two brain regions' tissue content of a compound or pair of compounds. Each brain region sampled is a node in the network, which is connected to other nodes via edges, significant correlations. Additional information can also be represented in different types of networks. Weighted networks allow for a value to be ascribed to each edge, allowing the r value (or p value) of a correlation to be represented in the network. Correlations between pairs of neurotransmitters may be represented in directed multi-graphs, where OF DA may correlate with NAc 5-HT, but the reverse is not true. Once created these networks have specific parameters, most simply conceived is network density, as discussed above is simply the percentage of edges present out of all possible edges. Another basic property of a network is the average node degree, the average number of edges for a node in that network. However more complex parameters take more nuanced information into account, such as the weigh or direction of edges. Additionally, network parameters such as clustering coefficient, characteristic path length, global efficiency, or local efficiency can be extracted to provide insights into the overall connectivity and efficiency of the network. These parameters may offer a quantifiable method to distinguish difference between treatments, particularly in the case of neurochemistry where the generation of several networks is possible with relatively few compounds (analysing within and between neurochemicals).

The first caveat here is normalisation of networks. Within the same neurochemical experiment each treatment group should have the same regions, and therefore the same nodes. However, this is not necessarily true for the number of significant correlations, which can be altered pharmacologically as shown in Figure 5 (Butler *et al., submitted*). For comparisons of more complex network dynamics a normalisation for the network density is required. The concern here is the dismissal of biologically relevant correlations, as filtering on p or r value may not be a good measure of biological significance.

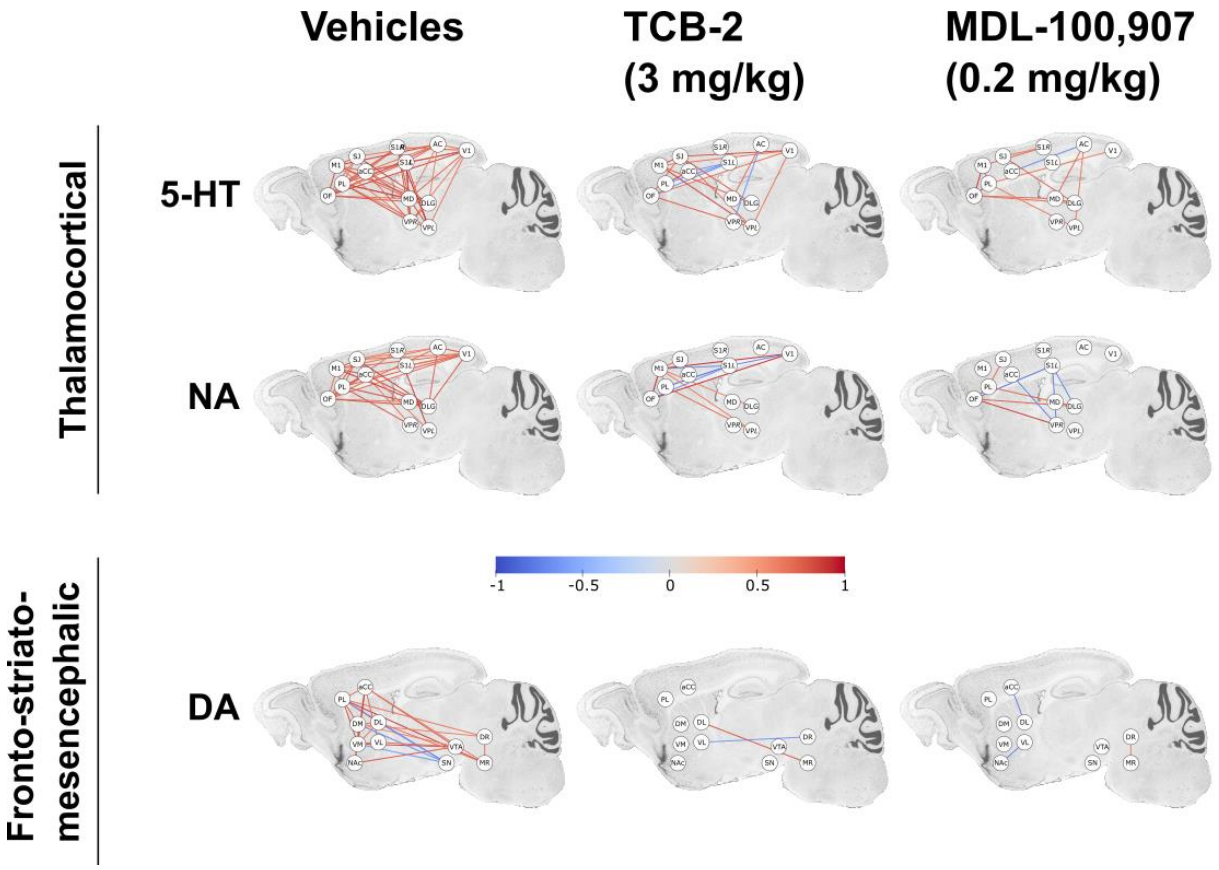

Figure 5 – Neurochemical connectivity in thalamocortical and fronto-striatal-mesencephalic regions, for serotonin (5-HT), dopamine (DA), and noradrenaline (NA) systems in vehicle-treated mice (Butler *et al., submitted*). Connectivity reduced by agonist (TCB-2) and antagonist (MDL-100,907) of the 5-HT$_{2A}$R (Butler et al., *submitted*). OF (orbitofrontal cortex), PL (prelimbic cortex), aCC (anterior cingulate cortex), *IC (insular cortex)**, M1 (primary motor cortex), SJ (somatosensory cortex jaw), S1L/R (left/right primary somatosensory cortex), *S1L6L/R (left/right primary somatosensory cortex layer 6)*,* AC (auditory cortex), V1 (primary visual cortex), A (amygdala), dH/vH (dorsal/ventral hippocampus), NAc (nucleus accumbens), DM/DL (dorsomedial/dorsolateral striatum), VM/VL (ventromedial/ventrolateral striatum), MD (medial dorsal nucleus), VPL/R (left/right ventral posteromedial nucleus), DLG (lateral geniculate nucleus), Y (lateral hypothalamus), SC (superior colliculus), SN (substantia nigra), VTA (ventral tegmental area), DR (dorsal raphe nucleus), MR (median raphe nucleus).

The application of graph theory in neuroscience is a rapidly growing field, offering a powerful framework for understanding the brain's complex systems (Bullmore and Sporns, 2009; Fornito and Bullmore, 2015). Caution is warranted when interpreting network parameters across different data types. We hypothesized that the visually apparent regional organization of correlations between monoamines could be described by the variability of connections (correlations) associated with each node (region). Specifically, we posited that an organized system would exhibit greater variability in connections, with some regions showing high correlations and others

exhibiting little to no correlation. A metric to quantify this variability is the standard deviation (SD) of node degree. However, the SD of the node degree is heavily biased by the network density or the total number of correlations. Making this metric of limited use in cases where the density or average node degree is not consistent between treatments. Acknowledging that normalising based off a ranking of significant p or r values has its caveats.

6. Conclusion

We addressed in this article the possibility to use postmortem quantification of neurotransmitters and metabolites to study brain wide connectivity of neurotransmitter systems. These studies being sensitive toward the variability of the individual values composing a cohort, we have cited several sources of technical variabilities that could impact correlations without biological significance. The approach offers a unique characterization of the structure and relations of neurotransmitter systems under various conditions. At rest neurochemical characterization may reveal the organization accompanying specific behavioural traits.

For the study of the mechanism of action of drugs, it also has interest to determine altered neurotransmitter connectivity, especially in the absence of quantitative modifications. Our recent study indicates that specific environmental conditions could be appropriate for further studying the mechanism of action of drugs, as a mobilized neurochemical system may be more sensitive to pharmacological manipulation. Furthermore, the last experiment suggests the environment before death can have consequences on the connectivity of neurotransmitters, which may allow for the characterization of neurochemical systems mobilized for different tasks or environmental demands in the brain. The limit of the approach is that there are apparently no specific profiles of correlations in resting conditions, acknowledging that the number of correlations is usually low. As far as biological explanations are concerned in the lack of reproducible profiles, we posit that the inter-individual differences of animals composing a group largely influence the outcome of correlative links, particularly at rest.

Acknowledgements

This work was supported by grants from the University of Bordeaux, the University Claude Bernard Lyon 1, and the French research organisms INSERM and CNRS. J.J.B. and C.A. were fellowship recipient from the Ministère de la Recherche et de l'Enseignement Supérieur. The authors wish to thank the GPR brain of the University of Bordeaux and notably the subgroups Adapsy and Inhibition for their support.

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Summary Art VI

With specific requirements, postmortem tissue quantification (which can be combined with other quantitative techniques such as quantitative autoradiography for receptors) can be effectively applied to study the relationships between neurotransmitters and metabolites in the brain. This would correspond to a neurochemical connectivity rather than anatomical connections, as physical monoaminergic links between regions (example: the nigrostriatal DA pathway) does not imply the existence of interaction for DA between the striatum and the SN (discussed below). Since monoamines and amino acid transmitters are subjected to homeostatic processes, involving several enzymes and transporters, the same tissue quantity (mean +/- SD) in two conditions does not necessarily imply an absence of differences. This should encourage the exploration of more subtle neurochemical modifications, which can be indirectly assessed through correlative analyses with metabolites or other neurotransmitters. It seems particularly relevant in the case of monoaminergic systems to catch some subtle, balanced regulation across the brain. Yet, additional data are required to better understand the meaning of these correlations and to develop additional analysis of large datasets.

Art VII *(submitted)* –Neurochemical assessment of tissue levels of neurotransmitters: from quantification to correlative networks.

Questions and purpose of the review

The viewpoint addresses the reasons why neurochemists did not consider multivariate and correlative analyses in the past. It is likely related to the fact that quantitative measurements influenced by stringent conditions. The variability in the measurements has not been necessarily conceived as a biological fact, acknowledging that analytical devices could play a non-negligible role in this variability.

Neurochemical assessment of tissue levels of neurotransmitters: from quantification to correlative networks

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Author Contributions

⊥J.J.B. and A.C: Equal contribution.

Notes

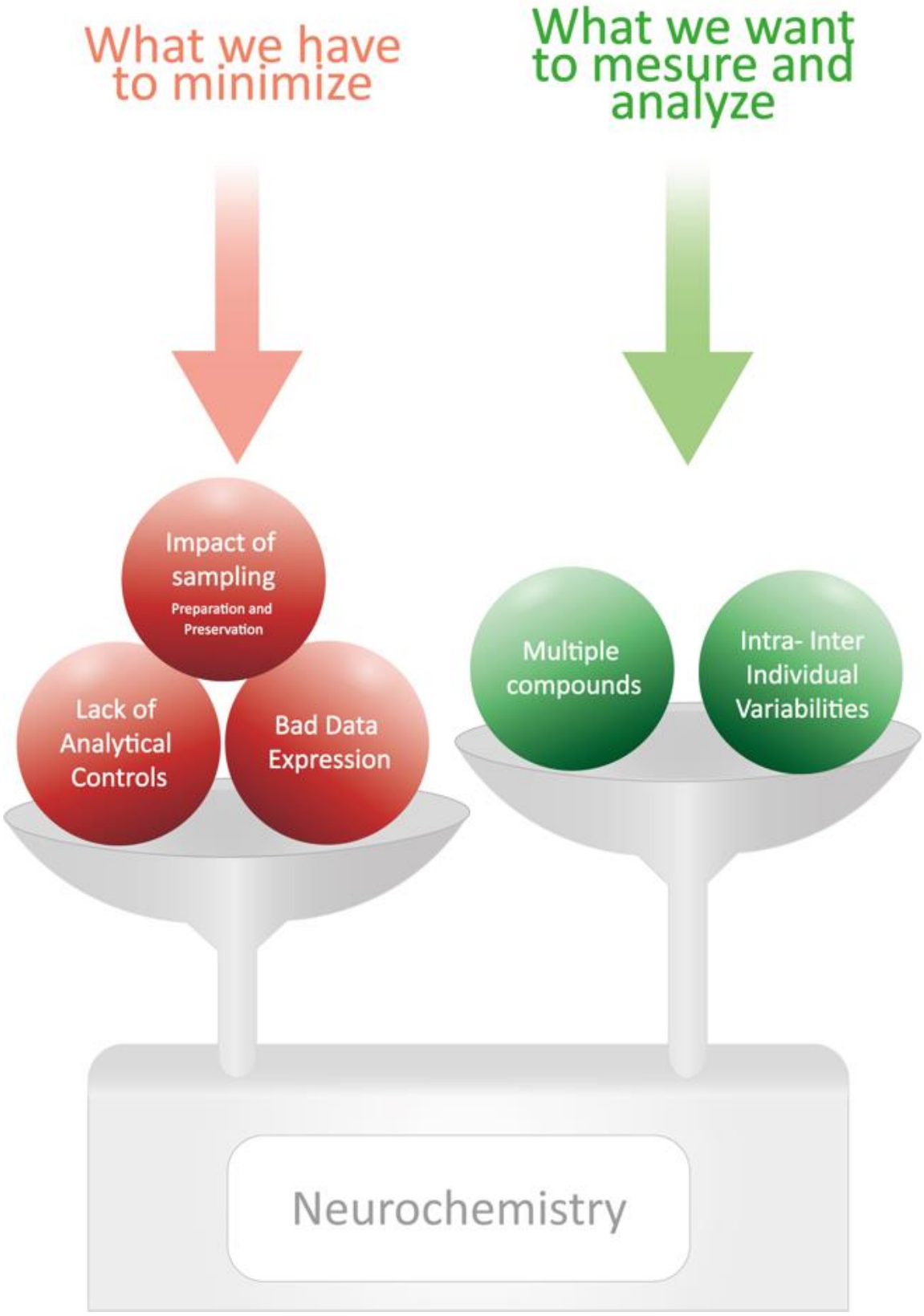
The authors declare no competing financial interest.

Abstract

The post mortem measurement of tissue neurotransmitters is an interesting technique to address the gross biochemical activity. Its primary limitation is a lack of temporal resolution, although this is mitigated by enhanced spatial resolution, compared to *in vivo* methods. This neurochemical data is quantitative and requires no complex transformation, making it ideal to analyse the neurochemical connectivity of the biochemical signals between brain regions. These correlative approaches of quantitative measurements are fundamentally based on the variability of the data, an underdeveloped analysis in neurochemistry. One of the main reasons, as discussed in this viewpoint, is that neurochemists recognize that variability in quantitative data stems not only from the biological variability such as inter-individual differences, but also from other factors such as analytical devices. There are several ways to reduce variability caused by analytical and experimental biases through well-designed, precise protocols, allowing for the study of meaningful biological variability, such as inter-individual differences between subjects.

KEYWORDS: neurochemistry, variability, multivariate analysis, inter-individuality, brain

Graphical Abstract



Quantifying tissue neurotransmitters permits the assessment of tissue quantity across the central nervous system (CNS). It is classically used in different species for addressing changes of tissue levels of neurotransmitters under different experimental conditions. Used in the past as a mere proxy of neurotransmitters release, the advent of “in vivo methods” (push-pull cannula, intracerebral microdialysis, voltammetry, biosensors) offered the possibility to monitor extracellular levels of neurotransmitters with a temporal resolution. These techniques are invasive, limiting the number of sampled regions. *In vivo* methods favour temporal resolution over a spatial resolution that would permit the assessment of cooperative activity of neurotransmitters systems across multiple brain territories. Thus, post-mortem methods, which permit the collection of a higher number of regions, open the possibility to address the cooperation of neurotransmitter systems across the brain, an interregional neurochemical connectivity. This neurochemical connectivity may address several pertinent questions in neurochemistry, such as the study behavioural traits.

To illustrate this point with monoaminergic systems, several years ago we completed an experiment focused on the inter-individual differences in the impulsive/compulsive behavioural dimension in 35 male Wistar-Han rats. Based on their performance in six distinct behavioural tests, animals were grouped according to their behavioural phenotype for a given test (Dellu-Hagedorn et al., 2018). Figure 1 concerns animals’ performance in the emergence test to evaluate spontaneous risk taking. Rats were separated into two groups: risk-averse (0 RISK), animals which hid in dark compartment, and risk-taking (RISK), animals which spontaneously explore open and bright areas (Figure 1A). One month after the final behavioural test rats were sacrificed and 20 brain regions sampled. This data revealed a quantitative reduction of the metabolic index of serotonin and dopamine in a few brain regions (Figure 1B).

The field of quantitative neurochemistry has been shy to develop multivariate analyses perhaps Likely as it relies on the heterogeneity of the data. In quantitative approaches, there is a profound consciousness that the variability can be multi-factorial, as the analytical procedure is extremely rigorous whenever concerning quantification of the tissue content or *in*

vivo measurement. It requires sensitivity and stability of analytical devices that are checked daily, reproducible injection of samples in the analytical system, verification and management of possible contaminants, and calibration curves, the quintessence of quantitative methods that additionally inform on the lower and upper limits of quantification. Moreover, it also requires reproducible and efficient procedure of sample extraction including proper measurement of sample weight or volume (or protein content), determination of the anatomical level of the in vivo probe or the extracted tissue, anticipation of the degradation of the eluents, and similar conservation between the samples. For this reason, neurochemists are well aware that the origin of variability in the data, expressed as a mean ± standard deviation (SD) or standard error of the mean (SEM), is a product of multiple factors.

To what extent is the variability in this ± error margin due to the analytical procedures? The analytical factor can be minimized, not dismissed, in the total variability of well-designed protocols, acknowledging the need for continuous technical amelioration of analytical systems. The analytical factor can sometimes explain outliers. That said, the biological factor in neurochemical data variability also has multiple aspects. Beyond variables like sex, strain, age, regimen, and housing conditions that are usually managed, there is also the circadian rhythm, the location of the probe or tissue dissection, and the spontaneous inter-individual differences. The time of sampling is a bias that can be managed by fixing a short, identical time frame for all animals in the study. Therefore, in a well-controlled study the two main factors of variability should be the strict location of the sampled tissues, and the inter-individual differences.

The location of the tissue can be a huge source of variability, error, and outliers. The CNS distribution of monoamines, as well as transmitters and factors in general, is heterogeneous in the mammalian brain. This heterogeneity is particularly evident for dopamine, with significant variations observed between different brain regions, and even within a single region such as the striatum (Fitoussi et al., 2013). Similarly, noradrenaline shows extreme heterogeneity in its distribution within the hypothalamus (Kvetnansky et al., 1983). Neurochemists can establish standardized methodologies within the scientific method to reduce location bias in tissue

sampling within an experiment. For instance, a single experimenter can consistently extract brain regions of interest using defined landmarks, maintain uniform sample sizes, and document any traces left by the punch procedure or dissection, performed over consecutive days in a randomized order. One of the interests in being highly rigorous in sampling, preparation, and analytical procedures is the obtaining of data with meaningful biological variability in the data. As a complementary, unpublished analysis of our risk-averse and risk-taking rats, we studied the correlation between the indexes of dopamine and serotonin across the sampled regions using the Pearson's coefficient (Figure 1B). This subsequent analysis showed that significant correlations were more distributed among subcortical areas in "0 RISK" rats and cortical areas in the "RISK" rats, with the surprising lack of correlations engaging the dorsolateral striatum in the latter cohort. This correlative analysis gives additional information between the two cohorts that could not be anticipated from the comparisons of their mean ± SEM. Recent work including this correlative analysis reported several changes in connectivity between the left and right parts of the striatum, the nucleus accumbens and the frontal cortex for monoamines and metabolites in a rat model of osteoarthritis (Mlost et al., 2024). Additional analytical approaches such as principal component analysis, discriminant statistics, decision trees, or graph theory to cite a few could be implemented the analysis of these neurochemical data. The pertinence of these analysis will depend on the scientific rigour of the quantification, as discussed above, to meaningfully interpret the findings. For instance, in the study of Mlost et al. 2024, the values for dopamine and its metabolites reported in the cortex were possibly contaminated by striatal tissue during dissection as dopamine tissue content was high, over 200 pg/mg, and the index of turnover correspondingly low, around 0.2 (similar to the striatum). As little as 5 to 10% striatal contamination is sufficient to overwhelm cortical dopamine content.

Beyond the neurochemical aspects needed to obtain reproducible signals, the quantitative assessment of chemicals requires standardized procedures grounded in experimental and scientific methods to eliminate biases that could obscure variability due to inter-individual differences. The analysis of quantitative neurochemical data enables the validation of

biological values without requiring complex signal transformations, providing a string foundation for both quantitative and multivariate analyses. Here, we have outlined the main challenges in collecting neurochemical data where variability holds biological relevance. We also discussed the correlative techniques developed in our lab, which leverage this variability to gain deeper insights into the neurochemical interactions between brain regions and systems within the CNS. This expanded approach to neurochemical analysis holds significant translational value, particularly as it advances our understanding of evolutionarily conserved systems, such as monoamines, across multiple species.

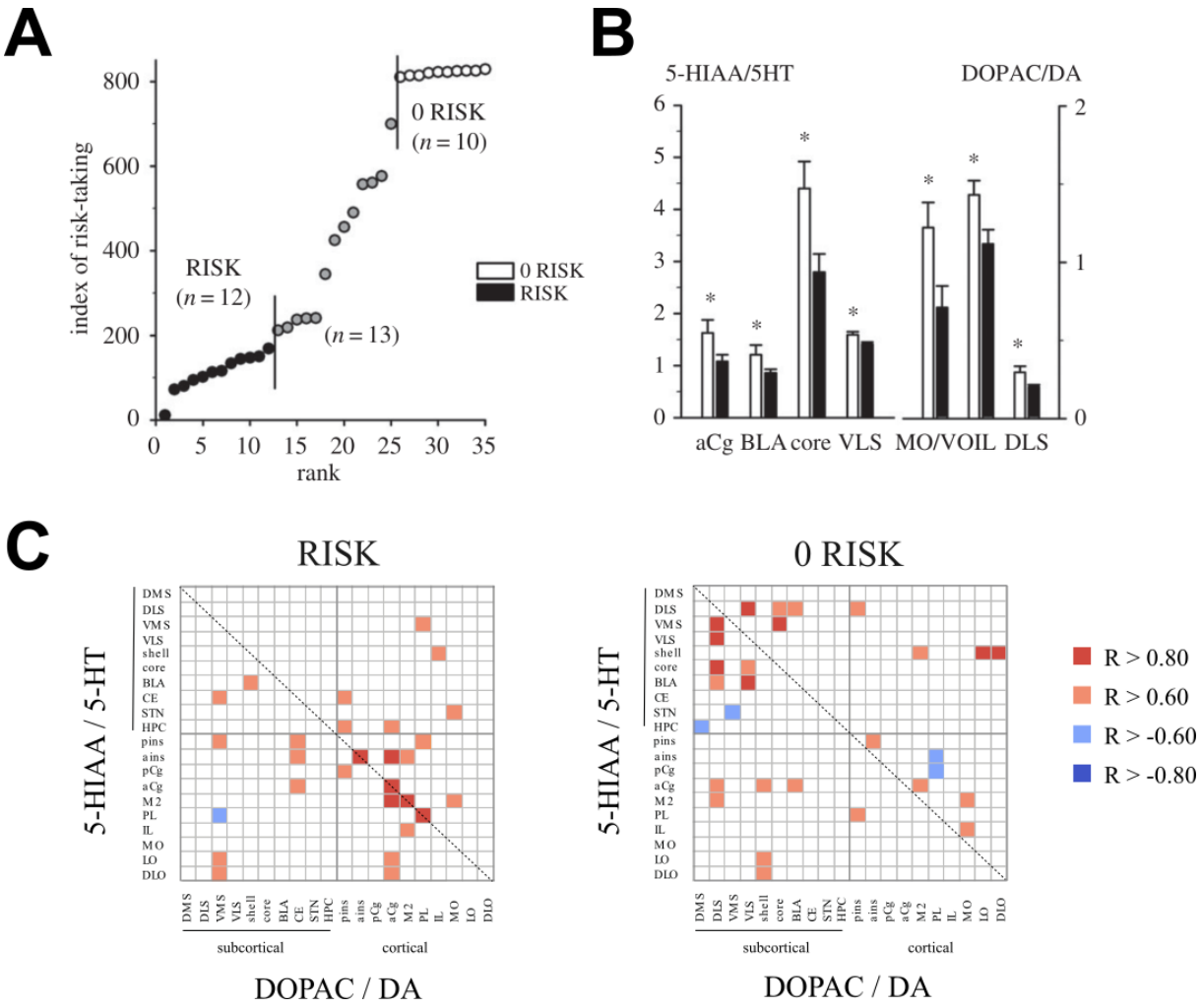


Figure 1 - Inter-individual differences of risk-averse and risk-taking male Wistar-Han rats. **A** Individual animal performance in the emergence task used to distinguish animals into RISK and 0 RISK groups. **B** Significant regional modifications of serotonin (5-HIAA/5-HT) and dopamine (DOPAC/DA) indices of turnover between RISK (white) and 0 RISK (black) rats. Values correspond to mean ± SEM, significance indicate * for $p<0.05$ following unpaired t test (Dellu-Hagedorn et al., 2018)(A and B, reproduced with the kind permission of the Royal society). **C** Unpublished correlative analysis of the neurochemical dataset for RISK and 0 RISK rats. Correlograms report significant Pearson's correlations between dopamine and serotonin indices, the R value is indicated by the colour, either positive (red) or negative (blue). *Abbreviations: dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA), dorsolateral orbitofrontal cortex (DLO), lateral orbitofrontal cortex (LO), medial orbitofrontal cortex (MO), ventral orbitofrontal cortex (VO), infralimbic cortex (IL), prelimbic cortex (PL), motor cortex (M2), anterior cingulate cortex (aCg), posterior cingulate cortex (pCg), anterior insular cortex (aIns), posterior insular cortex (pIns), hippocampus (HPC), subthalamic nucleus (STN), central amygdala nucleus (CE), basolateral amygdala nucleus (BLA), ventromedial striatum (VMS), ventrolateral striatum (VLS), dorsolateral striatum (DLS), dorsomedial striatum (DMS).*

Acknowledgements

This work was supported by grants from the University of Bordeaux, the University Claude Bernard Lyon 1, and the French research organisms INSERM and CNRS. J.J.B. and C.A. were fellowship recipient from the Ministère de la Recherche et de l'Enseignement Supérieur. The authors wish to thank the GPR brain of the University of Bordeaux and notably the subgroups Adapsy and Inhibition for their support.

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Summary Art VII

The viewpoint indicates some rules belonging to the scientific method to overcome possible biases. It indicates that multivariate and correlative analyses could go beyond the simple expression of the mean +/- SD to report maps of correlations for neurotransmitters and metabolism. The viewpoint alerts that the variability of the analytical devices apart, the biological variability can have different forms overwhelming the inter-individual differences. Among them, an approximate sampling of the tissue could dramatically change the concentrations, and the main marker for this being DA, close to the limit of quantification in cortical territories or the hippocampus, and 100 to 1000 more concentrated in the striatum, respectively. For this reason, we discarded two brain regions in the TCB-2 experiments: the anterior insular cortex, and the deep layers of the somatosensory cortex. Others did not, the risk being that the described correlations correspond to the magnitude of the striatal pollution in cortical samples(Mlost et al., 2024).

Discussion

Statement

The purpose of the discussion is to move beyond the discussion of the articles and more broadly elaborate on the two main contributions of the thesis: the role of 5-HT_{2A}R in modulating neurochemical organization in response to a novel environment, and the development of code for analysing neurochemical datasets. The relevance and application of this work toward an understanding of consciousness is also discussed.

The value of correlative analysis of quantitative neurochemistry

The quantitative analysis of neurotransmitter content and/or their metabolites in brain extracts is laborious. The index itself does not represent neurotransmission and it is often regarded as a meagre, poor index in the field of monitoring brain neurotransmitters. Our study was to boost what at the time was embryonic thought, based on a growing set of experimental data from the lab, that the postmortem approach could approximate the connectivity of neurotransmitter systems at a brain wide scale. The thesis started in that context, obtaining additional arguments that this approach was potentially informative. The idea of changing the flooring for the last 10 minutes of the experiment was, in part, to challenge the organization of brain neurochemical systems, which corresponded to a better way to apprehend the actions of psychedelics (Halberstadt & Geyer, 2018). The results were highly surprising, as the novel flooring was associated with an unprecedented degree of 5-HT correlations between brain regions, reaching approximately 50%, while previous work in the lab under resting conditions had not surpassed 10%. But the most surprising result was the sharp and distinct brain organization of the correlations for the catecholamines NA and DA. These data add new evidence for the regionally distinct engagement of brain modulatory systems associated with a novel environment/exploration.

Shortly after arrival in the lab, upon conception of this experiment, a new type of analysis was set up dedicated to the detection of amino acids, and notably glutamate, GABA, aspartate, glycine, and taurine. The findings corresponded to the known distribution of these compounds in the brain (Donzanti & Yamamoto, 1988; T.-H. Kim et al., 2014) although such a broad analysis across the brain has not been done. We

only have unpublished in-house data concerning the correlative maps of amino acid across some brain regions. Similarly to monoamines, there are very few correlations in resting conditions. It was therefore of particular interest to observe a high correlation density for amino acid neurotransmitters exposed to the textured floor.

Brain connections or connectivity?

The meaning of one correlation between two brain regions is elusive whatever the neurotransmitter(s) considered. As discussed in the methods paper (*Art VI*), the physical connection between the SN and the striatum via the nigrostriatal DA pathway could favour the occurrence of correlations between DA content in the SN and striatum, but it is not at all the case in resting conditions (Gros et al., 2021; Marty et al., 2023). Following exploratory behaviour, we have even found negative correlations between striatal and nigral DA. Similar considerations can be raised with 5-HT content in the DRN and terminal fields of DRN neurons. On the other hand, as stated in the introduction, several brain territories receive 5-HT afferences from both the DRN and MRN (Steinbusch, 1981). Thus, without excluding connections, the occurrence of correlations could correspond to emerging properties between neurobiological networks.

Regarding DA neurons, it is not so puzzling that nigral and striatal DA content do not correlate, as the regulatory mechanisms operating in each tissue have been shown to be different for a long time, despite originating from the same groups of neurons (Argiolas et al., 1982). Thus, the postulated emerging properties that could favour the occurrence of correlations in the striatum could come from somewhere else, including the cortex, the thalamus, and possibly adjacent territories of the NAc and striatum. The hypothesis of Harber et al. that the basal ganglia are made up of ascending loop from the shell of the NAc to the dorsolateral striatum could explain some of the reported correlations (Haber et al., 2000), as previously anticipated (De Deurwaerdere et al., 2020). This argument must be counterbalanced by the fact that one brain region in our experiment includes the right and the left side for most structures. If there are lateralized correlations, as we reported for the somatosensory cortex, and the ventroposterolateral thalamus (Figure 12), or as recently reported in the NAc (Jeanblanc et al., 2024), these lateralized effects could be confounding factor. Such

effects may impair the accurate determination of whether physically connected systems exhibit correlations. Additional data with appropriate brain regions are warranted to further address this possibility.

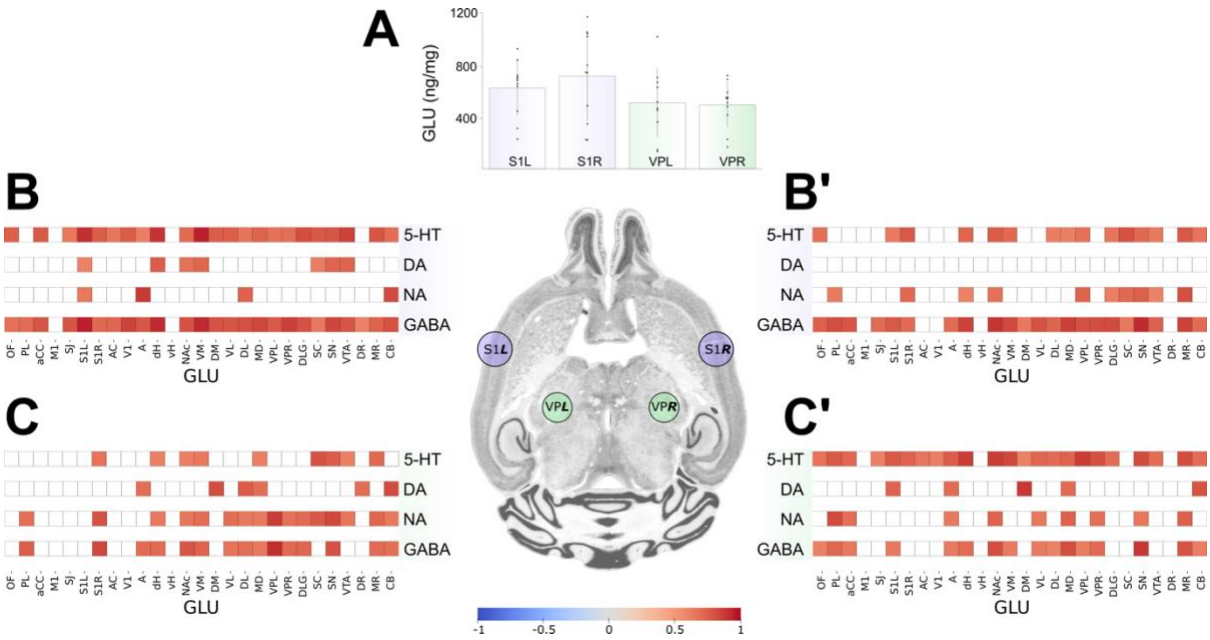

Figure 12 - Lateralised correlative differences in vehicle-treated mice following exploration.

A The quantitative tissue level of glutamate (GLU) in the left and right primary somatosensory cortex (S1L, S1R) and the ventral posteromedial nucleus (VPL, VPR), reported as mean ± SD. **B-C** Significant Pearson correlations between neurotransmitters 5-HT, DA, NA and GABA and GLU tissue quantity in the left (**B, C**) and right (**B', C'**) somatosensory cortex and ventral posteromedial nucleus, respectively.

The tissue index parameters and the correlative aspects

The ratios DOPAC/DA or 5-HIAA/5-HT are often used as reference postmortem markers of activity because they are indirect index of the turnover of the neurotransmitters DA and 5-HT, respectively. In our study, these ratios, with the addition of 3-MT/DA, were used to indicate, quantitatively, that TCB-2 was altering the activity of both 5-HT (reduction of the ratio in most brain regions) and DA (reduction in striatal sectors) neurons. We did not specifically comment on correlations of these ratios, as the profile did not follow those of the parent neurotransmitter, or metabolites, which were similar as briefly shown in the first article with 5-HIAA. On the other hand, in most previous studies published in the lab, obtained following resting conditions, correlations of the ratios were apparently more informative in terms of regional

organisation (Gros et al., 2021; Puginier et al., 2019). This could be further studied in the context of the TCB-2 experiment, or in previous pharmacological experiments in resting conditions dealing with the 5-HT_{2C}R agonists lorcaserin and WAY-163,909 (Chagraoui et al., 2019; De Deurwaerdère, Ramos, et al., 2020). We cannot exclude that the different indexes acquire pertinence depending on the context of the experiment, i.e. resting versus activated conditions.

Correlative analyses and the cohort.

There is a point that is important to keep in mind when performing correlative analyses: the results depend on the cohort of animals composing the group. Thus, beyond technical problems that could bias correlation patterns (contaminations, aberrant location of the punch, bad chromatographic conditions and so on), the inter-individual differences are an important factor shaping the correlative maps. Working on the first dataset produced in the lab in male Wistar rats (Fitoussi et al., 2013), we found that the correlative maps concerning the ratios DOPAC/DA and 5-HIAA/5-HT were different according to the behavioural performance in the impulsive/compulsive tasks (Butler et al., *submitted a*). All these animals had the same life, without any drug injection, but the grouping as performant or non-performant indicated unique correlative patterns. In our experiment, the change of flooring was associated with a strong level of correlations, and we believe that it is related to the experimental situation. Yet, the mice in the other groups might not have perceived the change of flooring in the same way, due to their treatment or interindividual differences. As discussed in the articles, there are still organized patterns of correlations, and fewer occurrence of negative correlations in the low dose TCB-2 group, or in the combination group. Thus, it is likely that the disruption of the correlations induced by TCB-2 and MDL-100,907 alone is related to a diminished or altered perception of the flooring and the behavioural consequences. Nonetheless, the study would have been ameliorated with the addition of a group without the change of the flooring to definitively attribute the high level of correlations to the flooring. This is currently under study (see below).

Psychedelics, 5-HT$_{2A}$R, and neurotransmitters

The objective of our study was to determine the impact of a 5-HT$_{2A}$R agonist on neurotransmitter systems in the brain. Unfortunately, we cannot affirm that the results we obtained on the pattern of correlations with TCB-2 are related to 5-HT$_{2A}$R involvement. The most striking result was the powerful effect of MDL-100,907 alone on the organization of neurotransmitter systems in response to the change of flooring, which occurred without major quantitative effects.

TCB-2

TCB-2 is a fair 5-HT$_{2A}$R agonist that has some resemblance to DOI in terms of affinity and efficacy at human 5-HT$_{2A}$Rs (McLean et al., 2006). It is considered as a preferential 5-HT$_{2A}$R agonist, acknowledging that its pharmacological profile is unknown, and that a few data in the literature indicate that it could trigger mechanisms beyond 5-HT$_{2A}$R stimulation (Di Giovanni & De Deurwaerdère, 2018). Importantly, it was also available. Thus, the experimental design included a dose response that was discussed previously and the 5-HT$_{2A}$R antagonist MDL-100,907. TCB-2 (tested only with the dose of 3 mg/kg) induced effects that were dependent and independent of 5-HT$_{2A}$R stimulation. The 5-HT$_{2A}$R-dependent effects were on head twitches, in line with previous data (Fox et al., 2010; Halberstadt et al., 2013), and some neurochemical markers like the decreased ratio 3-MT/DA in the striatum, the increase in all three monoamines in the anterior cingulate cortex, and some other neurochemical effects. The 5-HT$_{2A}$R-independent effects (MDL-100,907-insensitive) were reported on the decrease in the 5-HIAA/5-HT ratio in several brain regions and presumably on the increase in locomotor activity. The latter effect contrasts the data of Halberstadt et al. (2013), although our experimental procedure differs significantly, in arena size, timing of novel environment exposure and environment itself. There is some ambiguity in our results where the statistical analysis did not reveal any difference despite tendencies, a result of high variability and/or low magnitude of effect (weak statistical power). This concerned several neurochemical data, the preference for the orange floor, and the reduction in climbing, and sniffing.

The existence of mechanism of actions beyond 5-HT$_{2A}$R stimulation implies that we cannot affirm that the reduction of correlations induced by TCB-2 is related to 5-HT$_{2A}$R stimulation, or to other pharmacological targets. Consequently, the correlation profiles

obtained on all neurotransmitters upon the combination of TCB-2/MDL-100907 are almost uninterpretable in terms of pharmacology. These profiles confirm that TCB-2 acts on other targets as they are very different from MDL-100,907 alone.

Additional data and consideration

There are two important points pending additional analyses. The first one concerns the impact of the floor that should be addressed as an independent variable. The second is to confirm whether other psychedelic drugs can disrupt the organization of neurochemical systems across the brain.

We completed another experiment using 3 doses of DOI (0.03, 0.3, and 3 mg/kg), following forced exploration with the novel flooring. Additionally, this experiment addresses the influence of the flooring change with either the solvent or the dose of 3 mg/kg DOI. As expected, DOI induced head twitches at 0.3, 3 but not 0.03, allowing us to distinguish “psychedelic-like” effects in the dose response. This is consistent with several data in the literature (Fox et al., 2010; González-Maeso et al., 2007). The dissection has been done with some improvements (32 brain regions), but the neurochemical analysis was unfortunately blocked by the bad conditions in the new building (since September 2023), particularly the ultrapure water. The second experiment was performed only on the textured floor and concerns the psychedelic 5-MeO-DMT, injected at 0.5 or 5 mg/kg. Again, the dose of 5, but not 0.5 mg/kg increased head twitches. The brains are still pending the dissection.

5-HT_{2A}R antagonism

The 5-HT_{2A}R antagonists have a few or no effect in resting conditions (De Deurwaerdère & Di Giovanni, 2017) but they acquire properties under some conditions, and notably antipsychotic properties (Schmidt et al., 1995). Our condition with the novel floor is not a resting condition, but MDL-100,907 was almost devoid of effects on neurotransmitter content in the quantitative analysis. This does not necessarily suggest unaltered neurotransmitter release as postmortem data include several origins for a compound and is much less sensitive than the extracellular levels monitored by microdialysis.

Reduction of correlations with 5-HT_{2A}R agents: a working hypothesis

The disruption of correlative maps observed with TCB-2 and MDL-100,907 suggests that 5-HT_{2A}R play an important role for the coordination of neurotransmitter systems in response to forced exploration. The blockade of 5-HT_{2A}R with MDL-100,907 may dampen sensory integration and reduce cognition. Although does not clearly act on monoaminergic neuron activity, in contrast with TCB-2 which decreased the index of 5-HT turnover across the brain. TCB-2 reduced the ratio 3-MT/DA via a 5-HT_{2A}R-dependent effect. This indirectly suggests that exploration mobilizes the activity of mesencephalic DA neurons. In line with this possibility, the pattern of correlations for DA was highly organized in vehicle-treated animals, and when combined with other transmitters like 5-HT, glutamate, or GABA, the resulting correlative maps were regionally organized according to DA. A similar comment is retained for NA neurons, which seem to structure correlative maps, although we do not have clear evidence that TCB-2 quantitatively alters the activity of NA neurons.

The lab, in agreement with several authors, has published on numerous occasions that 5-HT_{2A}Rs exert a state-dependent facilitatory control on the activity of mesencephalic DA neurons (Butler, Ricci, et al., 2024). It is therefore puzzling that TCB-2 would inhibit NAc and striatal DA tone. This could potentially correspond to a 5-HT_{2C}R-dependent effect, known to reduce the electrical activity of DA neurons (De Deurwaerdère & Di Giovanni, 2017), but it was blocked by MDL-100,907, suggesting a 5-HT_{2A}R-dependent effect. It is noteworthy that the state-dependent, excitatory role of 5-HT_{2A}Rs on DA release and metabolism has been studied in the context of pharmacology to better understand the mechanism of action of psychostimulants, opiates, or antipsychotic drugs (De Deurwaerdère & Di Giovanni, 2017). However, this role has not been explored in the context of behaviours such as exploration. We could tentatively propose that the psychedelic, by acting on 5-HT_{2A}R, reduces the brain mechanisms at the origin of the increase in DA neuron activity for exploration: anxiety, salient stimuli, awareness, attentional load, or novelty. The anterior cingulate cortex likely has an important role as TCB-2 enhanced the tissue content of all monoamines in a 5-HT_{2A}R-dependent manner.

The idea that various brain mechanisms might be disrupted by psychedelics during exploration corresponds to the initial proposal of Adams and Geyer (1986) recalled in

Halberstadt and Geyer (2018). Exploratory behaviour, in which an animal explores its environment to develop a cognitive map (Berlyne, 1966), may be altered by psychedelics at various levels, including sensory and cognitive processes. Our correlative behavioural analysis suggested that the animals in the vehicle-treated group 'perceived' the 4 quadrants in a different manner, even though they spent the same amount of time, on average, in each quadrant. There were positive correlations between the time spent on the orange floor and all neurotransmitters, at contrast with the with the white and hessian floor. These correlations concern 4 (GABA) to 9 (DA) regions for each neurotransmitter, rarely the same regions. These correlations were not seen in TCB-2-treated animals, who spent more time on the orange floor and less on the white floor. These data suggest that the exploration, as measured by time spent on each texture, has regionally distributed relations to the different neurotransmitter systems. The altered exploration observed, with preference for a specific texture, may be a consequence of the altered perception in TCB-2-treated mice, perhaps reflected in their altered neurochemical connectivity.

Psychedelics, 5-HT_{2A}R and therapies

Our study focused on the acute effects of one psychedelic that will probably have no development in the clinic. Nonetheless, we can consider some facts that enter the current debate on psychedelic mechanisms and propose some experiments that would help to address these hypotheses.

The first fact is that we report widespread effects in the brain, not only on the connectivity, but also on quantitative data even if their magnitude is low. As anticipated, the action of psychedelics is large and concerns several brain territories. The origin of major depressive disorder or anxiety is not well known. Often viewed as defect processes in the hippocampus and/or prefrontal cortices, depression can be seen also as a loss of excitability of various neurobiological networks including sensory territories (Harrison et al., 2019). Thus, by directly acting and profoundly altering numerous biological networks at the same time, psychedelics could have a fast and broader action compared to SSRIs. The circuits of anxiety are also complex (Millan, 2003) and a powerful action on the circuits could account for the fast onset of anxiolytic responses in patients. It is noteworthy that the clinical use of psychedelics is

conditioned by setting up specific conditions (set and setting) favouring the benefit of these compounds (Hartogsohn, 2016; Pahnke, 1970; Studerus et al., 2012).

It has been proposed that the amplitude of the psychedelic experience may condition the therapeutic effect of psychedelics (Barrett & Griffiths, 2017; Ko et al., 2022; Roseman et al., 2018). Although it remains to be determined if the psychedelic-experience is required to trigger the antidepressant/anxiolytic effects. Numerous preclinical data report that an antidepressant effect could be achieved with compounds that stimulate 5-HT_{2A}R, like lisuride, ICH-7026, or DM506, compounds that do not induce the HTR in mice (Arias et al., 2024; Cao et al., 2022; González-Maeso et al., 2007). Consequently, we could compare the neurochemical profile of psychedelic versus non-psychedelic psychoplastogens several days after their administration in animals presenting depressive-like symptoms. The question then becomes whether the experiments should be conducted using a specific brain activation method, such as the novel flooring, or under resting-state conditions.

Intimately linked to the above comments, it is still not understood why a 5-HT_{2A}R agonist acquires psychedelic properties. Numerous studies suggest that it is related to their biased agonism property at 5-HT_{2A}R (González-Maeso et al., 2007; Rodriguiz et al., 2021; C. L. Schmid et al., 2008), which is often observed for 5-HT_{2C}R as well (Berg et al., 1998). In any case, our data suggest that the action on 5-HT_{2A}R from a full agonist (TCB-2) to an antagonist/inverse agonist (MDL-100,907) might profoundly affect the connectivity of neurotransmitter systems in the brain. Even at a low, sub-psychedelic (non-HTR-inducing), dose TCB-2 altered neurochemical connectivity imposed by the environment. Thus, we postulate that biased agonists at 5-HT_{2A}R could have profound effects on integrative properties of the brain without inducing psychedelic-effects.

The part on which we cannot comment is the neuroplasticity. Indeed, numerous studies are linking the anti-depressant like activity of psychedelics to neuroplasticity including the raise of neurotrophic factors like BDNF. The field is growing fast. This link is even further stressed by the recent finding that LSD and psilocin are positive modulators of the TrkB receptor, thereby favouring BDNF actions (Moliner et al., 2023). In addition to those effects, our data suggest specific actions on monoaminergic

transmission. Given the key role of monoaminergic systems in maintaining coherence within and between neurobiological networks through their widespread modulatory functions, it is likely that the action of psychedelics also depends on their ability to alter monoaminergic neurotransmission. The changes in monoaminergic transmission likely endure at least several hours after psychedelic administration as some of these psychedelics are desensitizing 5-HT_{2A}R and possibly other 5-HTR such as 5-HT_{2C}R (Berg et al., 2001; de la Fuente Revenga et al., 2022; Sharp & Collins, 2023). Thus, in addition to neuroplasticity, modification of monoaminergic transmission could participate to the longer duration of antidepressant effects compared to the non-selective NMDA channel blocker ketamine (Matveychuk et al., 2020; Singh et al., 2020). Again, this hypothesis requires specific experiments that are currently underway in the lab.

Our comments in the field of the anti-addictive effects of psychedelics particularly with respect to alcohol or tobacco addiction will be similar. The widespread action of psychedelics could disrupt mechanisms directed towards the object of addition. In terms of connectivity, the most obvious point is that the acute administration of a psychedelic disrupts brain connectivity, and our hypothesis is that this relies on an action on DA and NA neurons, which seems to have a primary role in coordinating neurotransmitter systems. It is one interesting argument, which must be counterbalanced by the increasing knowledge of the state of addiction. Indeed, it is difficult to predict the action of psychedelics because the relationships between neuronal systems are modified by addictive drugs. For nicotine, it has been shown that the relationships between VTA DA neurons with the amygdala, the prefrontal cortex, or the lateral habenula changed after chronic treatment with nicotine(Nguyen et al., 2021; Pierucci et al., 2022). Moreover, addiction might not be considered only via the DA angle, but more as a complex affliction sustained by numerous dysregulation processes (Koob & Moal, 1997), including a lower responsiveness of the PFC. Thus, the dampening action of 5-HT_{2A}R ligands on the coordinated response of neurotransmitter systems, presumably via an action on DA neurons could occur with other mechanisms, notably in the PFC. In any case, neuroplasticity could be involved to in the preservation of psychedelic mediated brain wide modifications.

The connectivity of neurotransmitter systems, as described here, could undoubtedly provide new insights into the 5-HT/DA interaction in the context of drug addiction. In this field, the control of DA release in the NAc has always been a gold standard parameter to determine the suitability of putative treatments to reduce drug of abuse properties. Yet, it did not work with 5-HT_{2A}R or 5-HT_{2C}R ligands. For instance, while 5-HT_{2C}R agonists reduced the reinforcing properties of a variety of drugs of abuse including psychostimulants (e.g. amphetamine, cocaine), opiates, or nicotine (Howell & Cunningham, 2015), they did not modify the increase in DA extracellular levels induced by psychostimulants (Cathala et al., 2015). Evidence that DA in the NAc is not a sufficient measure to characterise addictive behaviour. Similarly, while 5-HT_{2A}R antagonists demonstrate pre-clinical anti-addictive properties (Cunningham et al., 2013; Howell & Cunningham, 2015), they did not modify the increase in DA release induced by morphine (Porras et al., 2002). The concept behind these data is that 5-HT via its receptors can refine DA transmission independently from an action on DA release. Exploring the connectivity of neurotransmitter systems could yield intriguing perspectives that are valuable for further developments in this field.

From neurochemical data to coding in Python and database

Among the deliveries of this thesis is the elaboration of a code for the analysis neurochemical datasets. The implementation of a code or software is indispensable for high throughput techniques such as fMRI or EEG. While this was not traditionally the case in neurochemistry, correlative neurochemical analysis can now generate large volumes of data. For instance, in the study by Fitoussi et al. (2013), there were 35 rats, 20 brain regions, 6 monitored compounds, and two ratios, resulting in the management of approximately 5,600 data points. The experience with the SNORD115 mice was smaller, with approximately 1,500 parameters, but it was the occasion to start building a Python script dedicated to handling postmortem neurochemistry. The TCB-2 experiment required than handling of ~42,000 parameters, excluding ratios, and the behavioural data.

At its core, it allows for statistically correct and reproduceable handling of outliers, determination a parametricity and quantitative statistical analysis based of the afore-mentioned data characteristics and experimental design (i.e. factors, number of

groups). This basic functionality includes the plotting of mean ± standard deviation. This functionality alone has added a level of data inspection that was previously laborious, at best. It also expands the possibility of exploration of these datasets, compelling all significant quantitative effects one can search for all compounds modified in cortical regions for instance. Beyond the generation of classic ratios, all possible ratios and their significant modification can be calculated and inspected, while this kind of obscure analysis may be uninteresting within a single experiment, particularly under human inspection, it is an area in which AI implementation may be of particular interest, particularly in the context of a neurochemical database.

Although as discussed, these datasets and the corresponding code were conceived to move beyond this regional catheterisation of tissue quantity. With a sound data structure, the implementation of multivariate analysis such as principal component analysis, or graph theory is accessible. The latter has been a large focus of this thesis, inspired from the connectivity described when correlating neuroimaging signals between regions. The application of graph theory in neuroscience is a rapidly growing field, and understandably so. It offers a powerful framework for understanding the brain's complex systems and allows a new dimension of relationality to be addressed (Bullmore and Sporns, 2009). Most simply it provides an excellent way to characterize correlation matrices, allowing easy inspection withing a network (i.e. the extent to which any brain region correlates), or between networks (i.e. how densely correlated a network is). However, this is the tip of the iceberg, graph theory is in its own right a complex mathematical field and its application in specific context in science warrants particular attention. In our work thus far, it has allowed for the characterization of gross neurochemical system connectivity, reflected in the density of between compound networks in Article IV (Butler *et al., in prep a*). Speculatively we have also posited that standard deviation of significant correlations for each region in a network may offer a quantification of the organisation so clearly observable in correlograms including DA or NA. However, this kind of inter-network comparison warrants serious consideration on normalisation, as discussed in Art VI (Butler *et al., in prep b*).

Lastly a dedicated neurochemical analysis code has permitted the correlation of single region neurochemistry with other datasets, in our case the exploratory behaviour

described in the TCB-2 experiments. The novelty here lies in the number of regions and compounds with which this can be done, although with this comes the need to filter as manual inspection alone will be too laborious. The parameter on which filtering would be best done are currently under development. Our data suggest a distributed role of neurotransmitter systems in the brain, supported by the correlation of a behaviour with the several compounds across several regions.

Ultimately the code is a prerequisite to neurochemical correlative analysis of this scale and is being developed with the intention to be made publicly available with the neurochemical database discussed in Art V (Butler, Parrot, et al., 2024). This availability of both the previous work from the lab, for which correlative analysis was not done, and the code to do such an analysis, will make available a considerable amount of unexplored data. Taking a long view, this open-source database and analysis favours the implementation of AI for meta-analysis of perhaps obscure recurrences in the data. We believe that with time this database and analysis will help to better address function and interaction of monoaminergic systems together with other neurotransmitters (Butler, Parrot, et al., 2024).

A comment on consciousness

To circle back to where we started this thesis, and perhaps one of the most interesting features of psychedelics, their ability to profoundly alter consciousness. Although exactly what consciousness is modern science can only speculate on, if we take it simply as phenomenological experience, we can then assume that animals do have a phenomenological experience and that it is very likely altered by psychedelics. Which, if you have seen animals on psychedelics might be inclined to believe, given the irregular or backward walking in rodents under some, but not all psychedelics, crooked swimming in tadpoles or peculiar webs woven by spiders under the in influence of their own psychedelic-experience (Samorini, 2002).

Our data would support that perception, in mice, is altered following psychedelic TCB-2. Perhaps the best evidence for this comes from the correlation of neurotransmitters across the brain with the percentage time spent on each textured floor. Vehicle animals, with no mean difference in time spent on each floor, recurrently had positive

correlations with the orange flooring, not the others. Mice who received TCB-2, two cohorts reviving either 3 or 10 mg/kg, had a marked place preference for this flooring, although the positive correlations with neurotransmitter tissue levels were no longer present. This was juxtaposed by the white floor, in which no positive correlations were observed in vehicles, and TCB-2 treated animals avoided (Art IV: Butler et al., *in prep a*). In vehicle animals there was some kind of association with the orange floor and lack of with the white, TCB-2 perhaps altered the perceptual experience (paralleled by a desynchronisation of neurochemical systems) and ultimately their exploratory behaviour. Previous data have reported that visual, tactile, and auditory sensory modalities are altered by the psychedelics 25I-NBOMe in a dose-dependent manner (Miliano et al., 2019). The place preference for the orange flooring may be due to its own perceived properties (interesting, safe) or may reflect a reduced need to explore or 'neophobia' as described previously in rodent psychedelic behaviour (Halberstadt & Geyer, 2018).

If this is the case, we could further speculate that neurochemical systems, particularly monoamines may offer a neural correlate of consciousness. However, one may also wonder if alteration of physiological processes could participate in the effects of psychedelics. Psychedelics have complex peripheral effects. They may change body temperature (Fox et al., 2010), possibly acting on brown adipose tissue (Ootsuka & Blessing, 2006), modulate microvascular activity in the brain (Cohen et al., 1999), and act on arterial blood flow (Buchborn et al., 2020). In their review, Dos Santos et al. suggest, based on numerous human studies, that hallucinogens increase introspection and positive mood by modulating brain activity in the fronto-temporo-parieto-occipital cortex (Dos Santos et al., 2016). Psychedelics being presumably able to alter hemodynamics on their own (Buchborn et al., 2020), it seems important to address this factor in technics measuring hemodynamic variations like fMRI. At this stage, we cannot exclude metabolic effects induced by psychedelics favouring desynchronization of brain activities.

In our experiment, the measurement of tissue amino acids does not reflect extracellular levels (µM range, negligible), rather intracellular concentrations and the metabolism (Gobert et al., 2011). The concentrations of amino acids were not modified by TCB-2 for most amino acids (even others we measured) but the correlations were

clearly reduced for all of them, including amino acids that have no known transmitter functions like alanine. Thus, the next step on this dataset will be to address the changes of metabolic activity that can be studied by correlations or multivariate analysis. In the future, some metabolic markers such as blood glucose could favourably complete the neurochemical data sets.

The altered connectivity between brain regions connectivity is reported with several technics (EEG, fMRI) and now neurochemical quantitative markers suggesting that it could be an important mechanism participating in the modification of state of consciousness that psychedelics induce. In fact, the application of different psychedelics (LSD, TCB-2, psilocin, DMT) on PNs of layers V-VI of the somatosensory cortex *in vitro* had complex effects on electrical properties of these neurons (work done in Larkum Lab). Thus, the altered perception induced by psychedelics could be sustained by a top-down process. Taken individually, each of these parameters falls short of capturing the intricate dynamics of our conscious or phenomenological experience. However, the profound shifts induced by psychedelic compounds, combined with the collective application of scientific models of brain connectivity across various levels, may pave the way toward a deeper understanding of the mechanisms underlying phenomenological experience.

Conclusion

In this thesis, using postmortem tissue indexes, we provide evidence that forced investigatory behaviour in mice enhanced the connectivity of neurotransmitter systems (monoamines and amino acids) in the brain. This response, presumably structured around catecholaminergic systems, is disrupted by both the psychedelic agent TCB-2 and the 5-HT_{2A}R antagonist MDL-100,907. These data suggest an important role of 5-HT_{2A}R in the response of mice to a new environment and provide another view on the study of neurotransmitter systems in the brain. A view pertinent to further the understanding of the mechanism of action of psychedelics on consciousness, mood disorders, and addition.

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