Where Does it Stem From?

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Functional Neuroimaging of Monoaminergic Brainstem Nuclei in Altered States of Consciousness: Translational, Diagnostic and Therapeutic Implications

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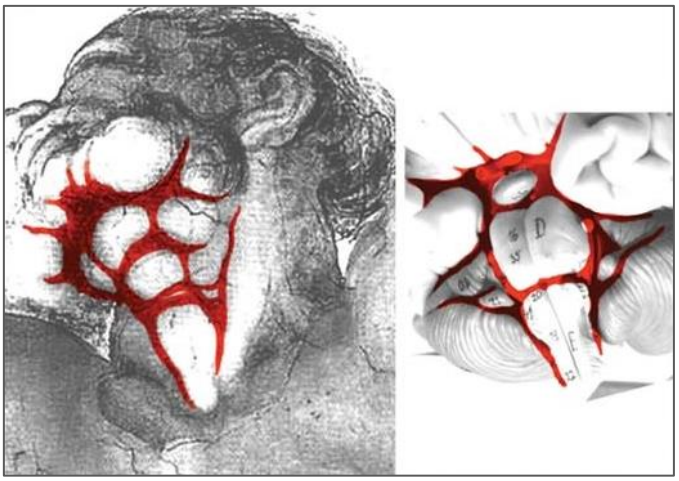
Separation of Light from Darkness (1512)

Michelangelo di Lodovico Buonarotti Simoni, known as Michelangelo (1475-1564)



Outline transcription from ***Concealed Neuroanatomy in Michelangelo's Separation of Light From Darkness in the Sistine Chapel*** by Suk & Tamargo (2010; *Journal of Neurosurgery*)

Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration, except as declared in the Preface and specified in the text. It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution, except as declared in the Preface and specified in the text. I further state that no substantial part of my thesis has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. The word count of 58,201 does not exceed the prescribed word limit for the relevant Degree Committee.

Preface

A large group of researchers, clinicians and technicians across many different studies have contributed to collection of data analysed in this thesis. At the beginning of each chapter I report a short preface to state respective contributions to each study in recognition of the importance of transparent collaborative research.

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Table of Contents

Acknowledgements

As you set out for Ithaka | hope your road is a long one | full of adventure, full of discovery.

[From: "Ithaka", by Constanin Cavafy, Collected Poems, Princeton University Press, 1975]

Many a seasoned academic would concur with Cavafy, and say that a PhD is a journey – though no one can say through what unexpected landscapes and places one might venture. On the expedition that the research for this thesis has been, many a more experienced explorer, insightful mapmaker, adventurer, and travelling companion have made all the difference; for which I want to express my deepest gratitude. Firstly, my thanks are to Dr Emmanuel Stamatakis, who helped chart much of the course to this Ithaka, but also let me run wild in scientific exploration. His dedication to science, the department and his students, nurturing of my scientific skill and confidence, and sense of 'true-north' have let me become an independent and well-oriented explorer in systems neuroscience, neuroimaging and beyond. Equally, I want to extend my thanks to Prof David Menon, whose unequalled and comprehensively mapped knowledge of manifold fields surrounding this work and their horizons, borne out of his deep dedication to research and clinical work, has been greatly inspiring and instructive – and whose kindness of letting me observe Neurocritical Care Unit work was transformative for the path I have taken.

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A merry band of itinerant fellow voyagers in my lab ensured that this journey kept on-course, and that excitement and inspiration were never lost. Among them, the most heartfelt thanks are due to my dear and most special friend Dr to-be Alexander R. D. Peattie. He ensured that fine conversations and glasses were enjoyed along this seaway – and is a true renaissance-man who always manages to capture fundamental discovery as the flavour of scientific, intellectual and personal journeying, be that on four wheels, on windy tides or in thought. I trust that this friendship will last long past this and well into the next journeys to come, unto the breach. Similarly, Dr to-be Peter Coppola, whose love for the true depths of neuroscience and philosophy is infectious, continuously reminding oneself that

scientific treasure is never found at the surface but far below - who has been a marvellous partner-in-crime in the fight against cortico-centrism. Also to Dr Andrea Luppi for never shying away from a scientific locking-of-horns, discovering in the process how complementary our work is, and for being a special collaborator in the quest of promoting neuromodulation. Finally, further thanks must go to Dr to-be Isaac Kelleher Unger, for his towering knowledge of neuroimaging, but also for reminding everyone that on a sea voyage, there is always a beach nearby, that needs enjoying just as much as exploring. Similarly, there are important refuges where one may want to find shelter in storms and ill-weather, to continue charting the rest of ones personal and academic voyage. Among these thanks need to be to Anna Sephton, Thomas Zoeller of the Dudelsackakademie, the Sherrington Society, and to its offspring. The bonds with all of the people mentioned above are special ones I treasure, ones only shared adventure can hone.

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Lastly, much as Cavafy would ask of the traveller, I am grateful not just to the people who came on the journey with me and shared the path, but also to the journey and adventure in and of itself – for having been able to form part of the greater collaborative and trans-generational adventure and enterprise that is Science. I feel deeply privileged to have been a part of this, and believe we need scientific questions *and* answers these days more than ever. As my scientific inspiration, the late Carl Sagan would have said: These are some of the things that hydrogen atoms do – given 15 billion years of cosmic evolution.

ASC	Altered State of Consciousness
ARAS	Ascending Reticular Activating System
CSF	Cerebrospinal Fluid
CONN	Connectivity Toolbox
DAN	Dorsal Attention Network
DCM	Dynamic Causal Modelling
DMN	Default Mode Network
DNMS	Diffuse Neuromodulatory System
DOC	Disorders of Consciousness
DR	Dorsal Raphe
EC	Effective Connectivity
EEG	Electroencephalography
FC	Functional Connectivity
fMRI	Functional Magnetic Resonance Imaging
FWE	Family-Wise Error
GLM	General Linear Model
GM	Grey Matter
LC	Locus Coeruleus
LIS	Locked-In Syndrome
LSD	Lysergic acid diethylamide
LSN	Large Scale Network
MCS	Minimally Conscious State
MnR	Median Raphe Nucleus
MNI	Montreal Neurological Institute
MRF	Mesencephalic Reticular Formation
PET	Positron Emission Tomography
RSN	Resting State Network
SPM	Statistic Parametric Mapping
TBI	Traumatic Brain Injury
UWS	Unresponsive Wakefulness Syndrome
VTA	Ventral Tegmental Area
WM	White Matter

Abstract

Altered states of consciousness (ASCs) provide models through which the necessary and sufficient neural underpinnings for consciousness can be characterised. Indeed, the study of ASCs through human functional neuroimaging has identified large-scale cortical networks (such as the default mode network; DMN) whose disintegration is common across both pharmacological transient and pathological chronic ASCs. However, these cortico-centric network accounts have largely been unable to fully characterise fundamental neurobiological processes which may underpin disruptions of consciousness and/or network changes. Instead, such work has mainly been performed in preclinical animal experiments and in lesion studies, which have identified that brainstem neurotransmitter nuclei – among them in particular the monoaminergic source nuclei and transmitter systems – are consistently implicated in both pharmacological and pathological perturbations of wakefulness/consciousness. Thus, the dysfunction of these nuclei could underpin human ASCs and network changes, and their assessment might correspondingly hold translational, diagnostic and therapy-informing utility.

To address this central question, in this thesis I used resting state fMRI recordings to analyse how the dopaminergic ventral tegmental area (VTA) and the serotonergic raphe nuclei are affected in pharmacological (propofol sedation, lysergic acid diethylamide & psilocybin administration) and pathological (disorders of consciousness, DOC) contexts, and how these findings relate to network-level impairments, as well as previous animal findings concerning the same nuclei.

In Chapter II, I assessed how functional connectivity of the VTA is altered across both propofol sedation and DOC patients. I found that in both sedation and DOC, VTA connectivity is disrupted from the main node of the DMN in the precuneus/posterior cingulate cortex, revealing a connectivity impairment universal to both ASCs. The extent of this VTA connectivity impairment was associated with DMN disconnectivity, as well as behaviour and/or outcome measures. Furthermore, I found that this connectivity could be upregulated by a dopaminergic agonist.

Chapter III further developed the findings of the previous chapter by aiming to integrate these with a pre-existing DOC disease framework called the anterior forebrain mesocircuit, which regards thalamic impairment as central to DOC pathoyphysiology. I found that VTA-thalamus connectivity is also impaired in DOC patients, but that this was undetectable in seed-to-voxel contrasts due to a sub-group of patients showing control-like connectivity levels. Patients who showed such preserved VTA-thalamus connectivity also were found to be responsive in an fMRI volitional task (Tennis task), and the greater this VTA-thalamus connectivity strength was, the less impairment of whole brain

complexity of the fMRI signal was observed, which supports the concept that thalamic and mesocircuit impairment in DOC might be at least partially mediated through dopaminergic dysfunction.

Chapter IV focussed on psychedelic ASCs, which are elicited by serotonergic psychedelic drugs, such as lysergic acid diethylamide (LSD). I found that serotonergic raphe nuclei connectivity is powerfully disrupted in acute LSD administrations – strikingly once more to the posterior DMN node in the precuneus/posterior cingulate. The extent of this disruption was again associated with DMN disintegration, and showed causal mediation effects on hallmarks of the psychedelic experience. I also demonstrated that the cortical ratio of 5HT2A receptors over 5HT1A receptors might be driving this raphe uncoupling effect. Based on these results and pre-existing literature, I hypothesised that this observed connectivity disruption in acute psychedelic administration may have adaptive sub-/post-acute effects on raphe functionality that might underpin psychedelic therapeutic effects, e.g. in depression.

Chapter V tested said hypothesis of psychedelic-induced sub-/post-acute raphe changes in two depression cohorts who received psilocybin. In data-driven analyses, I found that only post-acute connectivity of the raphe to the precuneus was associated with strength of depression symptom reduction across both trials. Psychedelic treatment induced significant sub- and post-acute upregulations of raphe-precuneus coupling, whereas SSRI treatment did not. Additionally, raphe-precuneus connectivity enhancements also positively covaried with DMN integrity and were accompanied by increases in spontaneous raphe BOLD activity. This provides preliminary evidence that a post-acute upregulation of raphe functionality might underpin psychedelic anti-depressant effects.

Altogether, these studies demonstrate that monoaminergic nuclei dysfunction might be a hallmark common to various different ASCs – and that functionality of these brainstem sources of non-classical transmitters might plausibly be associated with behavioural, clinical and network-level phenomena associated with perturbed consciousness. This provides various necessary translational bridges between preclinical and clinical work, and hints at the possibility of non-invasive neurotransmitter systems-level assessments with diagnostic and therapeutic utility.

Chapter I | Introduction

Introduction

1.1 Overview

A doctoral thesis should open with an operational definition of the topic it concerns. In the case of consciousness however, a comprehensive definition has evaded millennia of enquiry – as most attempted answers to "what consciousness is" end up becoming accounts of "in what terms consciousness can be circumscribed". The arguably simplest of these circumscriptions is that "to be conscious is to have subjective experience" (Koch et al., 2016). Although the inherence of subjectivity in this account led some to suggest that epistemic studies of consciousness are impossible, work by David Chalmers (1995) and others clarified that consciousness research truly needs to address two different types of problems: namely *hard* problems of understanding the nature of said subjective experience, and *easy* problems which concern bio-physical substrates and associated mechanisms that underpin consciousness. These *easy* problems are amenable to empirical scientific study, and are as such the focus of the work in this thesis (Koch et al., 2016). Because consciousness is of primary importance for epistemology, intrinsically linked to the human sense of personhood and existence, it is clear that to develop an in-depth understanding of the biological substrates that underpin it is highly relevant for scientific and clinical contexts. As such, multiple scientific and clinical sub-disciplines have conducted work that has identified consciousness' neurobiological substrates – the disruption of which changes, diminishes, or erases experience.

It is these disruptions of normal consciousness, i.e. pharmacologically- and pathologically-induced states of altered consciousness (ASCs), which have been instrumental in the research effort to characterise the fundamental consciousness-underpinning processes in the nervous system. In particular, in this thesis I employ the perspective that assessing ASCs can identify what processes are necessary and/or sufficient, and are thus 'rate-limiters', for consciousness and associated brain function. To this end, this introductory chapter will outline and describe the pathological and pharmacological ASCs used in this thesis, namely disorders of consciousness (DOC), anaesthesia/sedation, and psychedelic experiences – capturing a diversity and spectrum of consciousness alterations. To assess these states *in vivo* in humans, various forms of neuroimaging

have been instrumental, which have characterised the function of the brain as organised into large-scale networks. The impairment of these networks in ASCs is the arguably most replicated hallmark across pathological and pharmacological consciousness perturbation. However, despite their wide replication, these large-scale macroscopic perspectives have in isolation not produced actionable mechanistic and neurobiologically-plausible frameworks of consciousness.

I submit that for such perspectives it is imperative to integrate the network- and cortico-centric human *in vivo* neuroimaging accounts with insights from both preclinical animal and human lesion studies. Indeed, a central hallmark identified across both pathological and pharmacological states in such preclinical and lesion work, is that the integrity and function of the brainstem and neurotransmitter nuclei situated in pons and midbrain is fundamental for normal consciousness. These highly-localised brainstem neurotransmitter nuclei project globally throughout the brain to provide their signalling molecules, and are thus well-positioned to have central roles for large-scale brain processes. Among them, particularly the monoaminergic brainstem nuclei and transmitters have been consistently implicated in maintenance of animal wakefulness, as well as the putative mechanisms associated with human pharmacological and pathological ASCs (Brown et al., 2010, 2018a). While *structural* brainstem lesions have previously been linked to ASCs in humans, I propose that transient or chronic dysfunction (i.e. *functional* 'lesions') of monoaminergic nuclei may bring about ASCs and associated large-scale neural phenomena.

To this end, the experimental work in this thesis seeks to use an easily-implementable, translationally-motivated, and clinically-feasible approach in functional magnetic resonance imaging (fMRI) to assess the functionality of monoaminergic nuclei in both pathological and pharmacological ASCs *in vivo* in humans. This aims to translate, scale-up and contextualise more microscopic/mesoscopic insights from animal models to the predominantly macroscopic perspectives in humans, to enable the characterisation of mechanistic frameworks and actionable targets in ASCs.

1.1.1 Consciousness in the Clinical Setting

In the clinical setting, a two-dimensional reference space to capture consciousness has been formulated, (first by Laureys and colleagues, 1998, and subsequently iterated in many forms e.g. Bayne et al., 2016a, 2016b, 2017; Edlow, Sanz, et al., 2021 and others). Fundamentally, this clinical framework postulates that two main dimensions characterise human conscious states: *awareness*, which is synonymous with the perceptual *content* of consciousness (i.e. quality and qualia of first-person experience; Northoff & Heinzel, 2006) and *arousal* (also called wakefulness; Brown, Purdon, & Dort, 2011) which is synonymous with the *level* of consciousness. In normal, waking consciousness both awareness and arousal are high and closely associated, but in natural, pharmacological and pathological perturbations away from this state these two dimensions can become disrupted and disentangled (see Fig.1). These disentanglements are the aforementioned altered states of consciousness (ASCs), which provide the main models through which neurobiological correlates of consciousness are typically studied in humans. In the following section, the pathological and pharmacological ASCs included in this thesis and its analyses are introduced and described.

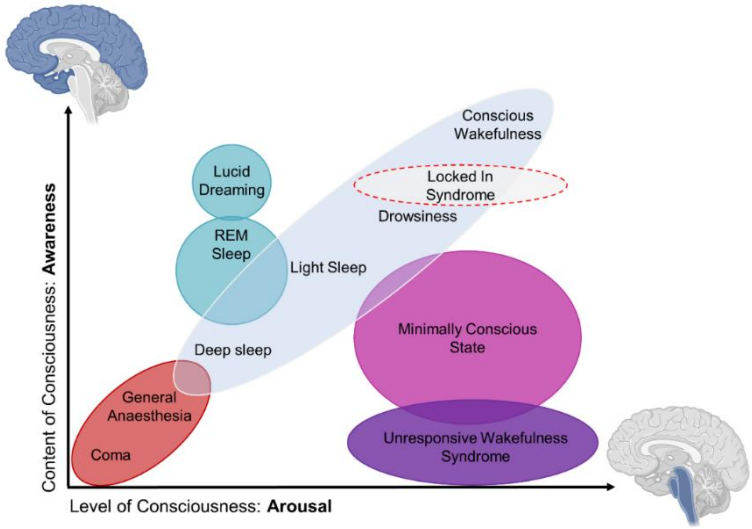

Fig. 1: Annotated two-dimensional consciousness space, adapted from original by Laureys *et al.* (2005, figure adapted). Classically, it has been assumed that contents of consciousness (i.e. *awareness*) are mediated by cortical function, whereas wakefulness (i.e. *arousal*) is putatively set by brainstem activating systems. The coloured circles denote different transient or chronic deviations from normal conscious wakefulness, highlighting that the close association between arousal and awareness as seen in normal waking consciousness can become disentangled, giving rise to altered states of consciousness. Note that whereas in full anaesthesia and coma (red) both arousal and awareness are low, in DOCs (purple/magenta) arousal is – in terms of clinical phenomenology – present, instead being classified as isolated lack of awareness. Therein, moving up along the y-axis is thus a move up the strata of DOC as detailed in-text.

1.2 Pathological States of Altered Consciousness: Disorders of Consciousness

Pathological alterations of consciousness can occur as the result of various forms of traumatic, hypoxic-ischaemic, neurodegenerative and even viral (such as COVID-19) injury (Edlow, 2021a; Edlow, Claassen, et al., 2021; Gosseries et al., 2011). The resultant conditions of loss of or inhibition of consciousness are as an umbrella term referred to as 'disorders of consciousness' (DOC), which are understood along a spectrum of severity of consciousness disruption. Briefly, in reference to the arousal-awareness framework (see Fig.1), at the most severe end of this spectrum is *coma*, in which both arousal and awareness levels are low (Laureys et al., 2004). Those who survive coma commonly emerge into *unresponsive wakefulness syndrome* (UWS, also known as *vegetative state*) – a pathology in which some arousal is preserved in absence of awareness (of self and environment). Patients who do display detectable but atypical signs of fundamental awareness over and above a measurable arousal level are grouped into *minimally conscious state* (MCS). In classical stratifications of DOCs, the only pathology that sits above MCS is the *locked-in-syndrome (LIS)* in which there is full preservation of awareness and arousal (and therein consciousness) in a quadriplegic and anarthric body (Edlow, Claassen, et al., 2020; Kondziella et al., 2020).[1] Altogether, the incidence of DOC cases remains very difficult to establish, with estimates ranging from two per million, to 61 per hundred thousand people (Giacino et al., 2018a; Løvstad et al., 2014). In part, this difficulty arises from the inherent complexity of diagnosing a DOC out of the many different behavioural phenotypes – which is reflected in the variety of bedside behavioural evaluations for DOC, as introduced in the following section.

1.2.1 Behavioural Diagnostic Tools for DOC in the Clinic

While the conceptual distinctions between different DOCs are in theory relatively clearcut, differential diagnosis is highly challenging. The longest-serving diagnostic tool used in DOC is the *Glasgow Coma Scale* (GCS; Teasdale & Jennett, 1974) designed for various forms of brain injury. It

[1] Today, the precise sub-classification of DOCs remains subject to debate, with the upper-bound at times being regarded as including the *confusional state* of those emerging from MCS, and new behavioural and imaging paradigms allowing the identification of *cognitive-motor dissociation* (CMD). In CMD functional cognition is present under full paraplegia (Bayne et al., 2017; Edlow, Claassen, et al., 2020; Kondziella et al., 2021). Later paragraphs cover this in greater depth.

entails three subscales which assess *eye-opening*, *verbal* and *motor* functions, which are used in intensive care settings to differentiate coma from brainstem death (see following section). The GCS has however not shown reproducible utility for differentiating finer graded diagnoses such as UWS and MCS from coma. This might be due to it lacking explicit and separate measures of arousal and awareness, and the covariance of all its sub-scales with overall motor function, which is in many cases indistinguishable from reflexive behaviours (Bodien et al., 2021; Mehta & Chinthapalli, 2019). Nevertheless, differential and acute diagnosis of coma *vs* brainstem death is in itself highly valuable for DOC care, and the minimal required training and its quick administration have made the GCS a widely-adopted tool for the purposes of informed patient stratification.

A measure that was developed to directly address the issue of differential diagnosis of the DOC sub-strata, is the *Disorders of Consciousness Scale* (DOC-Scale). As opposed to the GCS, the DOC-Scale rates behavioural responsiveness on a spectrum (i.e. measuring continuous, rather than binarized variables) across four main domains: *auditory-language*, *somatosensory*, *visual* and *gustation*/*olfaction* (Pape et al., 2014; Weaver et al., 2019). As a result of its multimodal organisation, the DOC-Scale is resource-intensive, requiring lots of equipment, ranging from ice-chips, over toothbrushes to family photographs, as well as necessitating extensive training for physicians (Seel et al., 2010). Despite this making it untenable for day-to-day clinical practice, it advanced upon the GCS and other scales in exploratorily incorporating prognostication of DOC through a mile-stoning of behavioural recovery (Pape et al., 2009).

Finally, the *JFK Coma-Recovery-Scale Revised* (CRS-R) has become the main tool for the differential diagnosis of DOC patients (Giacino et al., 2004). It consists of 23 hierarchically-organised items that span *auditory*, *visual*, *oromotor*, *communication* and *arousal* categories. The scoring subdomains aim to enable the distinction of motor function on the one, and overt cognition on the other hand. The lowest items on each subscale reflect reflexive activity, while the highest items reflect cognitively mediated activity (Bodien et al., 2016a), with hallmark behaviours scored as either present or absent to empower inter-rater and test-retest reliability. The CRS-R has found wide use for differential diagnosis of DOCs (Sattin et al., 2015), and has relevantly parametrized and elaborated on the original

arousal-awareness framework (Kondziella et al., 2021). Nevertheless, recent work has revealed that *covert cognition* such as in cognitive-motor dissociation (CMD) is still not diagnosable when using the CRS-R in isolation (Bodien et al., 2016b; Fernández-Espejo & Owen, 2013; Satpute et al., 2019). As such, despite the CRS-R being considered the gold-standard, its linear scales and approach might, despite recording graded responses, be insufficient for comprehensive DOC diagnostics. As a result of the above considerations, it is now widely appreciated that behavioural diagnostic tools need to be integrated into multimodal batteries with neuroimaging to empower precise diagnosis and prognosis (and potentially personalized treatment choices; Edlow, Barra, et al., 2020) across acute, subacute and chronic types of DOC, as was recently formalized in the Advanced Classification of Consciousness Endotypes (ACCESS) framework (Kondziella et al., 2021).

1.2.2 Moving through the DOC diagnostic process and landscape

Enabled by the above-mentioned bedside diagnostic tools, patient assessment following a brain injury aims to principally place patients along the DOC spectrum. Firstly, it is established whether the patient is brain-, i.e. in the United Kingdom and United States of America brain*stem*-dead, by testing for the absence of any and all brainstem reflexes (corneal, pupillary light, oculocephalic, oculovestibular, gag and cough) including inability to breathe self-sufficiently (apnea; Giacino et al., 2018b; Kondziella et al., 2020; Perri, 2014). Importantly, brainstem-death is regarded as the final state in which a patient has irreversibly lost the capacity to regain consciousness – and is thus the ultimate absence of consciousness, not a DOC (Ercole et al., 2020; Varelas, 2016).

Consequently, the most severe type of DOC is coma, a state which is characterised by continuous, deep, eyes-closed unresponsiveness to any form of external or spontaneous stimulation, often associated with large-scale brainstem and thalamic lesions, and more rarely diffuse bi-hemispheric cortical damage (D. B. Fischer et al., 2016; Golaszewski, 2016; Hindman et al., 2018a). However, brainstem reflexes are preserved in coma patients (Brown et al., 2010; Perri, 2014), thus leading to coma to be demarcated in the diagnostic reference space of the CRS-R by absence of purposeful motor activity and absolute lack of overt cognitive function and sleep-wake cycles. Following

traumatic brain injuries, patients may stay in this state for approximately half a month, before passing away or alternatively showing diagnostic signs consistent with UWS or MCS (Brown et al., 2010; Helbok et al., 2022; Laureys, Boly, et al., 2009).

The emergence from coma into UWS (formerly also referred to as vegetative state; VS) is characterised by the recurrence of eye-opening, sleep-wake cycles and reflexive motor responses to certain stimuli as assessed with the CRS-R (Laureys, 2005; see Fig.2). These responses are however typically inconsistent and fundamentally not interactive, indicating that although some level of intact arousal is present, absence of overt cognition and self- and environmental awareness is predominant (Giacino et al., 2014a; Vanhaudenhuyse et al., 2018). If the condition persists for longer than 3 months in non-traumatic and longer than 12 months in traumatic aetiology cases, the condition is classed as chronic (Giacino et al., 2014b).

Instead, patients in MCS display detectable, but atypical signs of fundamental awareness of environment, and traces of cognitive functions. In assessment with the CRS-R, these patients show directed basic motor behaviour and eye-tracking, which is considered representative of an MCS- diagnosis; whereas those diagnosed as MCS+ show emotional responses to emotive stimuli, basic vocal communication attempts/ability, and especially binary command understanding (Bodien et al., 2020; Thibaut et al., 2020). The MCS+ evidence is regarded as requiring some level of overt cognitive function compared to UWS and MCS- (Naccache, 2018; see Fig.2). In this lower severity stratum of MCS, brain lesions usually show less brainstem and thalamic involvement than for UWS (Hindman et al., 2018b; Parvizi & Damasio, 2001, 2003; Silva et al., 2010). At the upper bound, the confusional state (sometimes seen as identical with emergent MCS; eMCS) is characterised by the patient regaining the ability to functionally handle and interact with objects, thus having fully regained their motor function, but still showing significant impairments in the overt cognition domain.[2]

[2] eMCS and the confusional state are difficult to distinguish because de-coupling general cognitive dysfunction following brain injury from consciousness-associated cognitive deficits is challenging. The confusion assessment protocol aims to enable this (Bodien et al., 2020; Sherer et al., 2005).

When strictly considering cognitive function *per se*, above the previously-mentioned conditions sits the locked-in-syndrome (LIS), which as mentioned before are anarthric and quadriplegic patients who maintain full cognitive functions and consciousness (Khanna et al., 2011; Laureys, Owen, et al., 2004; Smith & Delargy, 2005). The lesions associated with this condition again typically involve the brainstem, however specifically the ventral pons' motor nuclei associated with voluntary and respiratory muscle control (Barbic et al., 2012.; Hocker & Wijdicks, 2015; Sciacca et al., 2019; E. Smith & Delargy, 2005) – thus likely sparing neurotransmitter brainstem nuclei. Importantly, to enable the diagnosis of LIS, a patient still needs to display some residual level of purposeful motor function to compensate for their aphonia and otherwise-paralysed body (Khanna et al., 2011).[3] When such stable communicative movement (typically preserved oculomotor behaviour) cannot be observed, patients cannot be differentiated from complete LIS (CLIS; Edlow, Claassen, et al., 2020).

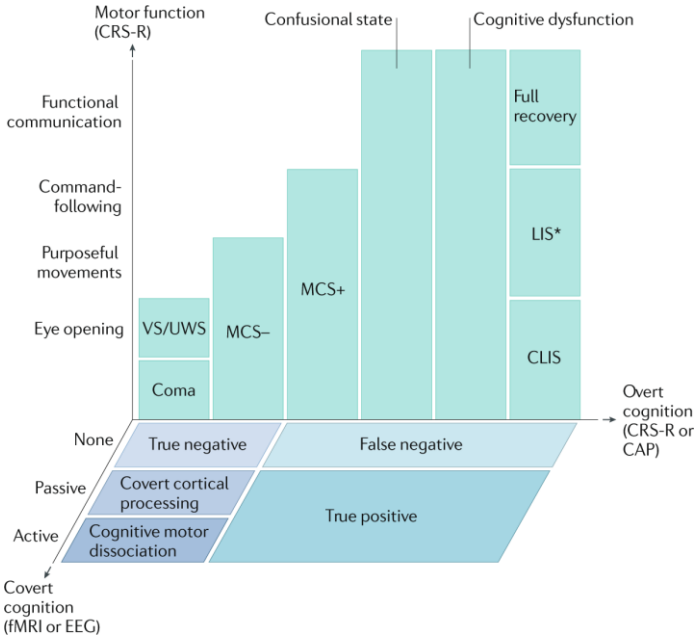

Fig. 2: Multidimensional assessment of consciousness utilising behavioural and neuroimaging data (figure reproduced from Edlow et al. 2021, under CC-BY 4.0 license)**.** Re-emergence of motor function is not necessarily co-occurrent with re-emergence of overt cognitive function along the recovery trajectories in DOC patients. Using the CRS-R, these two general dimensions can be assessed with relative distinguishability. However, cognitive capacities in DOC patients are often very difficult to ascertain due to the general paucity of reproducible intentional behaviours. As such, CRS-R based assessments for the overt cognition domain can – and should be – complemented where possible with neuroimaging paradigms that can detect covert cognition, such as the Tennis and/or Spatial Navigation tasks (see later section; Edlow, Claassen, et al., 2020; Owen et al., 2012).

[3] The LIS-sufferer Jean-Dominique Bauby (1952-1997) powerfully raised public awareness for LIS and DOC when he published his autobiography *Diving Bell and the Butterfly*, an account of his life in an anarthric and quadriplegic body with full consciousness – which he wrote by blinking his left eye to signal single letters to a nurse with a letter-board.

The difficulty in accurately diagnosing cases in absence of reproducible behaviour can have devastating ethical consequences. This makes it imperative that clinical examinations include complementary assessments of brain structure and function through human neuroimaging, which I describe in a later section. As formalized in the recent ACCESS framework (Kondziella & Menon et al., 2022), especially for patients with DOC in whom no overt cognition can be ascertained with CRS-R, active functional neuroimaging paradigms such as the Tennis and Spatial navigation tasks as well as passive stimulation paradigms are advised to be incorporated (Bodien, Giacino, et al., 2017). If active responsiveness is established through brain signal modulation, diagnoses of unresponsiveness can be refined into cognitive-motor dissociation (CMD), or if just passive processing is observed into "covert cortical processing" (CCP) cases (Bayne et al., 2017; Edlow, Claassen, et al., 2020).

1.2.3 Treatment Strategies for DOC

Despite many advancements in the behavioural and neuroimaging assessment of DOCs, most treatment strategies remain largely experimental, as a unifying conceptual framework for both disease pathological and therapeutic mechanisms remains absent.

Nevertheless, broadly speaking, five classes of treatment approaches can be differentiated, namely electromagnetic, mechanical, sensory, regenerative, and most importantly, pharmacologic treatment (Edlow, Sanz, et al., 2021). While there have been some case reports of relatively successful electromagnetic, transcranial direct current, mechanical and sensory stimulations of certain central and peripheral nervous system targets (such as thalamic nuclei, Cain et al., 2022; and vagus nerve, Corazzol et al., 2017), no randomized controlled trials have yet been performed. As such, these strategies remain limited in terms of efficacy, reproducibility, and translatable clinical impact. This is owed principally to the lack of rational bases for the selection of anatomical targets to stimulate, the paucity of biomarkers to track subclinical treatment responses (Edlow, Claassen, et al., 2020), and the general diagnostic challenges highlighted above.

Ultimately, the main treatment strategy for DOC remain pharmacological agents which act principally on different neurotransmitter systems (Edlow, Sanz, et al., 2021; Luppi, Cain, Spindler, Gorska et al., 2021). The motivation for the usage of these transmitter-centric therapeutics derives from convergent evidence from a rich preclinical literature: transmitter tones are dysregulated in animal models of coma and/or post-TBI, which is also found in anaesthetic loss of consciousness (Brown, Purdon, & van Dort, 2011; Clauss, 2010, 2011; Kawa et al., 2015; McGuire et al., 2018; Opladen et al., 2016; Zheng & Tong, 2015). Indeed, lesion sites associated with coma are consistently found to mainly be coincident with brainstem neurotransmitter nuclei that provide those transmitters (Fischer et al., 2016; Golaszewski, 2016; see following section). Blood-based biomarkers have shown that transmitter concentrations are altered post brain injury, in particular the biogenic amines such as dopamine, serotonin, and noradrenaline, as well as the inhibitory gamma amino-butyric acid GABA (Clauss, 2010, 2011; McGuire et al., 2018). Importantly however, the unique mechanistic contributions of different transmitter systems to bringing about the neural and behavioural phenotype of DOCs remain unresolved (Luppi, Cain, Spindler, Gorska et al., 2021a).

Despite this lack of understanding these transmitter systems' unique contributions, some pharmacological interventions have shown promising results. The most striking evidence comes from dopaminergic drugs, the usage of which has been associated with better outcomes in various brain injury and DOC cohorts. Specifically, levodopa (Fridman et al., 2019; Matsuda et al., 2005), bromocriptine (Passler & Riggs, 2001), apomorphine (Fridman et al., 2009; Sanz, 2019; Sanz et al., 2019), and methylphenidate (Martin & Whyte, 2007) showed beneficial effects in open-label small scale trials and/or case reports – and amantadine demonstrated a substantial benefit after severe traumatic brain injury in a placebo-controlled randomized trial with 184 patients (Giacino et al., 2012; Lehnerer et al., 2017). Indeed, amantadine remains the only medication endorsed by the Food and Drug Administration (FDA) for off-label use in DOC treatment.[4] The mechanism by which dopaminergic drugs might contribute to behavioural recovery remains unclear, although a central theory is that such intervention might compensate for striato-pallidal hypofunction that causes

[4] This endorsement has been made despite the treatment effect not surviving beyond the washout phase, meaning that behavioural recovery at post-trial was indistinguishable between placebo and amantadine groups.

thalamus over-inhibition and thereby large-scale brain dysfunction (anterior forebrain mesocircuit model; Schiff, 2010). Most strikingly, it thus far has not been resolved whether DOC-relevant dopaminergic deficits might originate from the dopaminergic brainstem nuclei. I describe the relevant dopaminergic source(s) in greater detail in a later section.

Furthermore, inhibitory pharmacological agents, such as zolpidem, which act on $GABA_A$ receptors, have also shown 'paradoxical' recovery-associated effects in small samples as well as placebo-controlled trials (Noormandi et al., 2017; Whyte et al., 2009 & 2014). Despite their limited efficacy, these substances as well as other monoaminergic, particularly serotonergic and noradrenergic drugs (such as desipramine, amitriptyline, protriptyline and fluoxetine, and even psychedelics; Scott & Carhart-Harris, 2019) need to be assessed further to identify their potential in promoting recovery of consciousness and managing other brain injury symptoms (McGuire et al., 2018).

Altogether, pharmacological treatments hold arguably the greatest promise among DOC therapies. As expressed in a recent gap analysis by the Curing Coma Campaign (Edlow, Sanz, et al., 2021) however, key developments are required to allow evidence-based, successful and possibly personalized pharmacologic treatment in DOC: (**I**) Linking individual neurotransmitter/neuromodulator systems to large-scale brain and brain network function, (**II**) measuring neurotransmitter imbalances *in vivo*, ideally non-invasively, and (**III**) identifying biomarkers to predict and/or track treatment effects for individual patients (Edlow, Sanz, et al., 2021; Luppi, Cain, Spindler, Gorska et al., 2021). These deliverables, which are applicable to DOC and other ASCs and disease areas with neurotransmitter implications (Clauss, 2010, 2011) form the key motivators and aims of the experimental work carried out in this thesis. To this end, the *in vivo* functionality of brainstem neurotransmitter nuclei whose signalling molecules are implicated in ASCs should be assessed using functional neuroimaging in both pathological states such as DOC and also in comparable, pharmacologically-induced reversible ASCs. I introduce the main models of reversible ASCs below.

1.3 Pharmacological altered states of consciousness: Reversible and controllable 'model' states

Consciousness alterations are not only brought about by brain injuries, but can also be powerfully elicited by pharmacological agents. Many different classes of drugs can produce transient, reversible ASCs, the comparison of which to normal consciousness and pathological states is instrumental for the development of a mechanistic understanding of consciousness-specific processes. The two main pharmacological modalities used in this thesis are (I) anaesthetic and (II) psychedelic drugs.

1.3.1 General Anaesthetics – Acting through Neurotransmitter Systems

General anaesthesia (from ἀν "not" and αἴσθησις "sensation"; Miller, 2010) has become an indispensable clinical tool to allow patients to enter a state in which they can tolerate painful and unpleasant interventions, such as a surgical operation. As in DOC, consciousness can be disrupted to varying and graded extents with anaesthesia depending on the type and dosage of pharmacological agent used (Bonhomme et al., 2019a; Franks, 2008).

Original mentions of anaesthesia reach as far back as original writings of the Islamic physician Ibn Sīnā (980-1037) who in his *Canon of Medicine* described 'sedative inhalation' from a soporific sponge for surgical excision procedures. Eight-hundred years later, Humphry Davy (1778-1829) suggested the use of nitrous, and Henry H. Hickman (1800-1830) the use of carbon dioxide for the same purposes – both were largely ignored, or in Hickman's case even publicly ridiculed in *The Lancet* for performing "medical and surgical humbug" (Sprigge, 2005; West, 2014). Exposed in societal social settings to the intoxicating effects of ether, the dentist William Morton (1819-1868) began using it as an anaesthetic for his patients, and conducted the first public demonstration of its use at Massachusetts General Hospital in 1846 (Lew et al., 2018). Since then, anaesthesia has revolutionized everyday clinical practice – letting surgery evolve, as some commentators put it, from 'trauma and butchery' into a humane therapy (Brown, Purdon, & van Dort, 2011).

Today, the term 'general anaesthesia' refers to the varied co-occurrence of the induced states of unconsciousness, immobility, amnesia and analgesia, while preserving fundamental physiological

stability of a patient. Despite its continuous development over soon two centuries, and the routinely administration of many millions of general anaesthesiae every year, the fundamental mechanism of how anaesthetics bring about drug-induced unconsciousness remains, as Kennedy & Norman (2005) put it, one of the biggest unsolved mysteries in modern medicine.

Intriguingly, in a direct parallel to DOC pharmacological therapies, anaesthetic drugs are classified into five distinct classes, based solely and specifically on the neurotransmitter system (and/or associated receptor) they are thought to target, based on preclinical evidence (Brown, Purdon, & van Dort, 2011).

Alpha-2 adrenergic receptor agonists, such as clonidine and dexmedetomedine are imidazoles, which produce a state that qualitatively (and electro-physiologically) resembles non-REM type sleep, i.e. a pharmacologically-induced dreamless sleep (Guldenmund et al., 2017a). These are typically used for short latency sedative purposes, and maintain a relative level of respiratory function and ready arousability through a mechanism that strongly affects noradrenergic system function (Guo et al., 1996).

An additional class of substances that is also explicitly associated with a monoaminergic system are the neuroleptic anaesthetics, which are dopamine receptor agonists, such as haloperidol, butyrophenones, and droperidol (Brown et al., 2010, 2018b). Today the neuroleptics are only utilised as sedatives and adjuvants to other anaesthetic agents, as their consciousness-suppressing effect is insufficient for anaesthesic loss-of-consciousness. They are thought to globally affect dopaminergic signalling, which can bring about serious side effects (such as cataplexia and cardiac dysrhythmias), causing the U.S. Food and Drug administration to issue a black label warning for droperidol (Bissonnette et al., 1999; Brown et al., 2018b; Brown, Purdon, & Dort, 2011).

The action of neuroleptics on dopaminergic system signalling is partially shared with another class of anaesthetic drugs, the opioid receptor antagonists, which target μ-, κ-, and δ-type opioid receptors. These substances are strongly associated with analgesia, as their hallmark effect is the blockade of peripheral pain (Egan, 2019). As such, the natural (morphine, codeine, papaverine), synthetic (methadone, meperidine, fentanyl, alfentanil, sugentanil, remifentanil) and semi-synthetic

(hydromorphone) opioids are typically used in postoperative settings, or as sedative adjuvants surgery, given their non-hypnotic profile (Hewson et al., 2019; Lang et al., 1996; Smith et al., 1994). Furthermore, N-methyl-D-aspartate (NMDA) receptor antagonists such as ketamine are also considered anaesthetics, with strong analgesic and hypnotic effects in the clinic. As ketamine does not share the addictive potential of opioids like fentanyl, it is today used for analgesia and as an adjuvant for maintenance of hypnotic effects of other anaesthetic drugs (Brown et al., 2010, 2018a). Whereas higher doses are hypnotic, ketamine is referred to as a 'dissociative' anaesthetic because low doses have hallucinogenic effects (Kurdi et al., 2014; Marland et al., 2013). Both higher dose hypnotic and lower-dose hallucinogenic action remain mechanistically ill-understood.[5]

Finally, the most widely-used class of anaesthetics are the $GABA_A$ receptor agonists, such as propofol, sodium thiopental, methohexital, and etomidate. These drugs can induce full unconsciousness, which is suggested to occur by increasing broadscale inhibitory signalling – thus causing a brain-wide dis-facilitation, i.e. tipping the excitatory-inhibitory balance in the favour of inhibition. Nevertheless, effects on brainstem neurotransmitter nuclei are wide-spread in preclinical reports (Brown et al., 2010; Wang et al., 2016; B. Yang et al., 2011; Yip et al., 2013). With the exception of methohexital, to induce unconsciousness all of these drugs are administered as bolus doses, which within 30 seconds can render a patient wholly unresponsive. The thus pharmacologically-induced loss of consciousness is associated with irregular breathing patterns, meaning that patients are typically intubated (Brown et al., 2018a). Propofol, the most widely used of the anaesthetics, also causes an overall lowering of muscle tone, foregoing the requirement to co-administer a relaxant to pre-empt muscular spasms (Lundström et al., 2010). The state induced by $GABA_A$ agonists arguably most resembles the lower strata of the DOC spectrum, and is thought to derive from broad effects on brainstem 'arousal' neurotransmitter nuclei, breathing and motor centres (Brown, Purdon, & Dort, 2011). Although propofol-induced unconsciousness is sometimes referred to as 'anaesthetic coma' (Brown et al., 2010, 2018a), $GABA_A$ agonist administration across a spectrum

[5] Preferential high-affinity binding on inhibitory GABAergic interneurons has been suggested as a potential mechanism for hallucinogenic effects, by producing brain-wide broadscale excitation through the downregulation of inhibitory signalling (Sleigh et al., 2014).

of distinct dosages can create stable sedation states which can resemble DOCs such as UWS and/or MCS more than full anaesthetic coma (Ramsay et al., 1974).

Taken together, all anaesthetics are thought to exert their effects *via* primary action on particular neurotransmitter systems, the sources of which are mainly in the brainstem. This conceptual distinction of anaesthetics into five transmitter-specific classes is however generated effectively solely from preclinical animal experiments – as for long, *in vivo* assessments of the neurotransmitter systems and/or nuclei in humans were impossible. Given that there are still significant occurrences of intraoperative awareness (Mashour & Avidan, 2015), and that the incidence of post-anaesthetic delirium (especially in the elderly; Jin et al., 2020) is mounting, it is clear that to understand how brainstem transmitter nuclei react to anaesthesia in humans is of central importance. Indeed, to pass this translational impasse from animal insights to the human brain relates directly to the challenge of treating DOCs: as there might be final common pathways of transmitter-system dysfunction common to anaesthesia and DOC, and universal to consciousness-maintenance (Bonhomme et al., 2019a; Brown et al., 2010; Kelz et al., 2019). This need for a detailed understanding of the effects of drugs on brainstem transmitter systems in ASCs also applies to another type of pharmacological agent that causes wholly different ASCs, namely psychedelic drugs.

1.3.2 Psychedelic drugs – Serotonergic Agonists that produce Altered Conscious States

Another pharmacological modality to alter and perturb the conscious state are psychedelic drugs, such as the semisynthetic ergoline LSD (lysergic acid-N-N-diethylamide, first synthesized by Albert Hoffmann in the 1940s), the tryptamines psilocybin (4-phosphoryloxy-*N*,*N*-dimethyltryptamine, naturally occurrent in the *peyote* cactus) and DMT (*N*-*N*-dimethyltryptamine, the active ingredient in the Amazonian spiritual brew *ayahuasca*), and the phenethylamine mescaline (Nichols, 2016a).[6] Psychedelic substances have been used for millennia across many different hominid cultures in ritualistic and therapeutic contexts, but only recently re-emerged into mainstream scientific and

[6] There are many more synthetic serotonergic psychedelics, most of them discovered, synthesized, and personally 'sampled' for rigorous analysis by Alexander and Ann Shulgin. Their findings are summarised in the books *PiHKAL* and *TiHKAL* (*Phenylethylamines and Tryptamines I Have Known And Loved*).

clinical inquiry (Carhart-Harris & Goodwin, 2017; Chi & Gold, 2020; Muttoni et al., 2019; Nutt et al., 2020; Nutt & Carhart-Harris, 2021; Schenberg, 2018). All of the classical psychedelics are classified as *serotonergic* psychedelics, i.e. they have broad agonist action at a variety of serotonin receptors (and to lesser extent also other aminergic, i.e. dopaminergic and noradrenergic receptors) at substance-dependent affinities – with particular importance for signalling *via* the serotonin 2A receptor (5-HT2AR) to bring about hallmarks of the psychedelic experience (Ettrup et al., 2014; Madsen et al., 2019; Nichols, 2016b).

The term 'psychedelic' is a hybridised neologism of the Greek term ψυχή (psikhe, "mind, soul") and δῆλος (delos, "manifest, visible") as a reflexion of these substances' strongly mind-altering characteristics. As noted by the psychedelic research pioneer Daniel X. Freedman: *"One basic dimension compellingly revealed in [psychedelic] states is 'protentousness' – the capacity of the mind to see more than it can tell, to experience more than it can explicate, to believe in and be impressed with more than it can rationally justify, to experience boundlessness and boundaryless events, from the banal to the profound"* (Freedman, 1968). As such, psychedelic 'trips' as an ASC are associated with changes in interoception, affect, perception, cognition, and volition, (Nichols, 2012, 2016a).

The correspondingly complex subjective experience is difficult to measure purely quantitively. Despite this difficulty, the 11-dimensional ASC questionnaire has become the standard tool to quantify the psychedelic experience along the following 'experiential dimensions': experience of unity, spiritual experience, blissful state, insightfulness, disembodiment, impaired control and cognition, anxiety, complex imagery, elementary imagery, audio-visual synesthesia, and changed meaning of percepts (Dittrich, 1998; Studerus et al., 2010; see Chapters IV and V). Interestingly, psychedelic drugs and the experiences they elicit have shown promise in treatment of multiple mental disorders, ranging from alcohol addiction and anorexia, over major depressive disorder and treatment-resistant depression, to end-of-life anxiety (Andersen et al., 2021; Artin et al., 2021; Carhart-Harris et al., 2021a; Carhart-Harris, Bolstridge, et al., 2016; Carhart-Harris et al., 2017, 2018; Carhart-Harris & Goodwin, 2017; Chi & Gold, 2020; Galvão-Coelho et al., 2021; Kurland et al., 1967; Muttoni et al.,

2019; Nutt & Carhart-Harris, 2021; Palhano-Fontes et al., 2019; Psiuk et al., 2021; Schenberg, 2018; Vollenweider & Kometer, 2010; Weigmann, 2018). These effects appear fast and acutely upon single or double psychedelic administration, and last long into the chronic sub- and post-acute phase, performing as well or better than traditional treatments in associated disease areas, such as depression (Carhart-Harris et al., 2021a; Carhart-Harris, Bolstridge, et al., 2016; Carhart-Harris et al., 2018; Griffiths et al., 2016; Hamon & Blier, 2013; Muttoni et al., 2019; Willner et al., 2013). Although a serotonergic system deficit is at the heart of psychiatric disorders, such as depression, only few clinical research groups have proactively explored the clinical potential of serotonergic psychedelics (Cosci & Chouinard, 2019; Delgado, 2000; Goldberg et al., 2014; Hamon & Blier, 2013). This has compounded the fact that both acute and post-acute mechanisms-of-action are only rudimentarily and insufficiently understood, including a lack of knowledge regarding the effects of psychedelics on the serotonergic brainstem source nuclei (Carhart-Harris & Nutt, 2017; Hornung, 2012; Jasinska et al., 2012).

Overall, as psychedelics are not subjected to serotonin reuptake mechanisms, they might fully saturate 5HT2ARs, dysregulating cortical excitation (Winkelman, 2017), as signalling *via* 5HT2AR has been demonstrated to be necessary for psychedelic-induced ego dissolution (Preller et al., 2017). While current theories of psychedelic action do account for such cortical effects of psychedelics, they do not include how psychedelic-induced alterations of serotonin homeostasis might strongly affect the function of brainstem serotonergic source nuclei.

On a final note, psychedelic drugs highlight a limitation of the arousal-awareness framework. Psychedelic-induced ASCs are not easily placed onto the unidimensional, linear axes of the space depicted in Fig.1, as they can on the one hand not be considered to “heighten” awareness just because experiential aspects are altered, nor on the other as increasing arousal just because basic environmental connectedness is intensified (Carhart-Harris, 2018; Carhart-Harris et al., 2014). This lays bare that both arousal and awareness are altered in qualitative, rather than purely quantitative terms in psychedelic-induced ASCs – reinforcing that arousal and awareness and specifically their

interplay must be considered as containing qualitative sub-dimensions that might not be readily captured in linear scales (Satpute et al., 2019).

1.3.3 Neurotransmitter Systems are implicated across all common Altered States of Consciousness

Taking together the above sections on pathological and pharmacological ASCs, a universal common thread emerges, namely the implication of neurotransmitter system function – spanning from treatment approaches in DOC, over anaesthetic mechanisms, to psychedelic-induced ASCs. When assessed even more specifically, direct parallels are found particularly for the monoaminergic, i.e. dopaminergic, serotonergic and noradrenergic systems, the sources of which are all in the brainstem. Although it is thus of central importance to our neurobiological understanding of ASCs to understand how brainstem nuclei might be functionally affected in ASCs in humans, related research efforts have until lately not sufficiently begun to resolve this.

In the following section, I first review what neuroimaging techniques are used to study both structure and function of the brain in humans, specifically in ASCs. I outline the achievements and key findings made by this research, and how these have shaped modern-day concepts of ASCs through consistent implications of macroscopic phenomena in neuroimaging. I focus in particular on the most replicated finding from this research, namely large-scale brain networks (LSNs) and their disintegration in ASCs. While these LSN perspectives have been highly influential, I outline that cortical phenomena should be complemented with an understanding of brainstem (and particularly monoaminergic nucleic) influences – because these nuclei are ideally-positioned biologically plausible rate-limiters for LSN integrity and consciousness maintenance, due to their neuromodulatory function. As such, I briefly review the sum of preclinical and clinical evidence that points directly to monoaminergic nucleic function as involved in ASCs. Finally, I submit that to reconcile the cortico-centric research in neuroimaging and the preclinical neurotransmitter system evidence has the potential to close the most important preclinical-clinical translational gaps for a mechanistic understanding of ASCs – and that functional neuroimaging can finally address this using simple clinically-feasible approaches.

1.4 What Has Neuroimaging Ever Done for Us (in Consciousness Research)?

For ethical reasons, the human brain cannot normally be studied experimentally in the invasive fashion preclinical animal experiments allow. As such, the mainstay of studying the human brain in ASCs has been neuroimaging. In the following, I briefly introduce various neuroimaging modalities (electroencephalography, EEG; positron emission tomography, PET and magnetic resonance imaging, MRI), focussing in particular on MRI, the main mode of neuroimaging used in this thesis. After introducing and differentiating these methods of directly and indirectly detecting brain *function* as a product of either electrical or metabolic changes (and in MRI also *structure*), I summarise main findings made with these methodologies in ASCs, which have converged in particular on 'large scale networks' associated with consciousness and its alteration.

1.4.1 Assessing Brain Structure with Magnetic Resonance Imaging

Just over a hundred years after Wilhelm Roentgen received his Nobel prize for the seminal discovery of X-rays, Sir Peter Mansfield, and Paul Lauterbur received their own 2003 Nobel honours for work on enabling magnetic resonance imaging to become a mainstream scientific and clinical instrument for assessments of brain structure (Lauterbur et al., 2019).

At the foundation of MRI is the usage of two main tools, namely strong magnetic fields (and field gradients) on the one hand, and radio frequency wave pulses on the other. Different combinations of these allow high-contrast images of different brain tissues to be obtained. This is possible as brain tissue (as all organic matter) contains many free protons (i.e. hydrogen/H^+ ions). As a result of free thermal energy (per the second law of thermodynamics) these protons are continuously spinning in random directions when not under the influence of a magnetic field. But due to their magnetic charge, when a strong and stable magnetic field B_0 is applied to them by an MRI scanner, the protons begin to spin in a direction aligned with the field B_0. This alignment is parallel to B_0 for the majority of protons (low energy state), whereas a minority take on an anti-parallel (high energy state) direction. The spinning of protons is also referred to as "precession", and happens at a particular frequency called the *Larmor* frequency. Crucially, when a radio-frequency pulse (magnetic field B_1) at this precise spinning frequency is applied at an angle of up to 90° to B_0, the protons react by flipping from

'low' towards 'high' energy (i.e. perpendicular to B_0) state. When the radio-frequency pulse is no longer applied, the protons return to their original spinning orientation in the B_0 plane (i.e. from 'high' to 'low' energy state). The return to the low energy state is called relaxation, and inherently tied to release of energy in the form of magnetisation decay – and it is this decay that is detected as a change of voltage in the receiver coils of an MRI scanner. Importantly, different components of the relaxation time to return to the low energy state give us the opportunity to resolve different signal types in the human brain (Jenkinson et al., 2017; Vassiliou et al., 2018; Worthoff et al., 2018).

The T1 relaxation is the time it takes for the spins to release the radio-frequency excitation energy back into the environment (also called spin-lattice relaxation). Correspondingly, a T1-weighted image acquired with an MRI-scanner is characterised by contrast based on differences in relaxation time driven by different tissue compositions. This means that different tissues become distinguishable at high resolution, as e.g. protons in lipid-rich regions show quick relaxation (higher signal intensity, brighter), whereas water-rich regions appear as lower intensity regions due to much slower relaxation. The T1 scan is correspondingly the chosen tool for purely anatomical and structural assessments of the brain (Worthoff et al., 2018). Instead, T2*-weighted images detect the local magnetic field inhomogeneities surrounding the protons as they drop out of the perpendicular orientation brought about by application of the transverse radio-frequency pulse B_1 (relative to the simultaneously applied B_0), decreasing local transverse magnetisation. The possibility to detect the field inhomogeneities brought about by the T2* relaxation forms the basis of the blood-oxygen-level-dependent (BOLD) signal at the heart of fMRI as a means of detecting brain function (Khanna et al., 2015). As the main tool employed in this thesis, the BOLD signal and fMRI are considered in greater detail further below, after introducing two slightly older modes of measuring brain function, namely EEG and PET.

1.4.2 Electroencephalography

Electroencephalography (EEG), is one of the longest-standing modes of non-invasively detecting human brain electrical activity, originally developed by Berger and colleagues in the 1920s (Haas, 2003). Its ability to detect electrical activity is accomplished through electrodes (metal plates) placed

on a participant's scalp. Between each pair of electrodes, electrical potential differences can be detected, which are thought to be brought about by ion fluxes induced by large-scale 'in-concert' activity of neuronal populations (Michel & Brunet, 2019). As this change in electrical potential has to traverse neuronal, glial, cerebrospinal fluid, bone and skin layers before reaching the electrodes, EEG has to be understood as a procedure with a relatively low signal-to-noise ratio (SNR) and very low spatial resolution. However, as detection of potential changes (transient electrical dipoles) is very quick, EEG has a correspondingly very good temporal resolution. Clear and robust oscillatory patterns have been characterised by EEG, which occupy distinct frequency bands, namely: alpha (8-13Hz), beta (13-30Hz), delta (1-4Hz), theta (4-8Hz; Shackman et al., 2010). Because of its non-invasiveness and portability, EEG is widely used for the study of pathological and pharmacological states of altered consciousness (Boly et al., 2017; Koch et al., 2016; Rosanova et al., 2018; Sanz et al., 2021; Tononi et al., 2016). [7]

1.4.3 Positron Emission Tomography

The arguable inspiration for fMRI came from an earlier neuroimaging technique that measured metabolic changes in the brain, namely positron emission tomography (PET). The foundations for this method were laid by three main characters independently, namely Lassen, Sokoloff, and Petersen. Lassen used intracarotid injections with xenon-133 to measure cerebral blood flow and therein published the first picture of brain activity in *Scientific American* in 1978. However, Sokoloff had in fact years before developed autoradiographic methods to quantify cerebral metabolism in rats, using the ligand fluorodeoxyglucose (FDG, still used today; Sokoloff et al., 1977), while Petersen had measured CBF by injecting ^{15}O-labelled water (Petersen et al., 1988). These foundational pieces of work demonstrated the special properties of radioactive isotope-tagged ligands that maintain PET's great relevance today (Hooker & Carson, 2019; Carson, 2019): These ligands can freely diffuse across the blood-brain barrier and through the brain, while emitting positrons which give rise to pairs of gamma rays, that can in turn be detected by the PET scanner (Slough et al., 2016). This way, a

[7] It is worthy to note that its methodological cousin magnetoencephalography (MEG) utilises the same principles, but instead of directly detecting electrical dipoles, MEG measures changes in the magnetic fields brought about by electrical activity. MEG has thus-far only been used sparingly in the study of consciousness.

three-dimensional image of the distribution of the tracer throughout the whole brain can be obtained at a spatial resolution superior to EEG/MEG. Altogether, PET is an indirect measure of brain function, with very low temporal resolution, but reasonable spatial resolution. Its lower-cost relative, single photon emission computed tomography (SPECT) uses longer-lived radioligands (e.g. exametazime) to detect gamma rays, but has significantly lower spatial resolution (Raji et al., 2014). Importantly, both of these techniques' greatest strength, in particular for PET, lies in the fact that radioactive isotopes are readily conjugable to many ligands, meaning that many different aspects of brain systems, functions and processes can be visualised with it, such as the distribution of neurotransmitter transporters and/or receptors. Such receptor maps are critical adjuvants to create mechanistic perspectives in human neuroimaging that build on basic neurobiology and physiology, and are used to this end in this thesis. Although the preclinical-clinical translatability of ligand development is fast-paced, there remain various barriers to mainstream clinical usage of PET, chief among which are patient concerns about radioactivity, the necessity of injections, the long duration of scans, and ultimately prohibitively high costs (Slough et al., 2016).

1.4.4 Functional Magnetic Resonance Imaging

Despite being superior to EEG, PET still has a restrictively coarse temporal and spatial resolution for certain brain analyses requiring finer grain in both dimensions (Khanna et al., 2015). Motivated to find another indirect measure of brain activity, many separate research efforts converged in the development of BOLD functional magnetic resonance imaging (fMRI). Though underappreciated by many fMRI developers and users today, the seminal discovery that allowed for it to become the most widely used neuroimaging technique, was actually made by Linus Pauling in the 1930s. He demonstrated that oxygenation changes the magnetic properties of haemoglobin in blood – with oxygenated haemoglobin being *diamagnetic*, whereas deoxygenated haemoglobin is *paramagnetic* (Pauling & Coryell, 1936). This paramagnetic property means that where oxygen is being consumed, there are magnetic field inhomogeneities (as explained above for the T2* images), which can be detected by the MRI scanner signal coils (Glover, 2011; Worthoff et al., 2018).

This is why the signal used in fMRI is referred to as the Blood-Oxygen-Level-Dependent (BOLD) signal: The glucose that the brain requires as an energy source can only be metabolized in the presence of oxygen, which is provided through flow of oxygenated blood, i.e. blood saturated in *oxy*-haemoglobin. Given that oxyhaemoglobin is diamagnetic, it does not alter local magnetic field properties – but *de*oxygenated blood's paramagnetic state causes magnetic field inhomogeneities and thus a drop in the detectable signal for an fMRI scanner. Therefore, when there are regional metabolic changes, the ratio of oxyhaemoglobin-to-deoxyhaemoglobin is altered, with an oversupply of oxygenated blood flowing to areas with high metabolic demand (i.e. higher activity), which resultingly increases BOLD signal intensity in those regions. This association with activity was for a long time controversial, but many lines of convergent research have demonstrated that the BOLD signal is indeed strongly correlated with various measures of neuronal activity, such as local field potentials, gamma oscillations, and covaries very tightly with optogenetically-elicited neuronal activity (Logothetis, 2008; Kahn et al., 2013; Schmid et al., 2017). Because fMRI has a much greater spatial resolution than PET, it is the only mainstream neuroimaging technique available to assess the activity and functionality of smaller brain regions, such as the monoaminergic brainstem nuclei this thesis is concerned with.[8]

Both PET and fMRI methods have been utilized to analyse activity in task-based paradigms and during rest, and have brought forth the possibility to assess how particular brain regions co-activate. This approach for co-activation, called *functional connectivity* analysis quantifies how the measurable signal of brain regions correlates with one-another over a given time window. It has been highly influential in neuroscience, and transformed the field by highlighting that function is not necessarily localised *per se*, but can be distributed across *large-scale networks (*LSN; Friston, 2011; Park & Friston, 2013a; Smith et al., 2012). These LSNs are groups of different, spatially-distinct brain regions whose BOLD signal consistently correlates, and both task- and resting-state recordings have been instrumental in characterising them (Cole et al., 2014).

[8] In recent years, there has been an increase in the number of studies using functional *ultrasound* imaging, claiming that the spatial and temporal resolution is superior to fMRI, and that it measures blood-flow variations with greater specificity than the BOLD signal (Deffieux et al., 2021). This is an exciting prospect, particularly given the portability and low cost of ultrasound, but will need further replication. It is likely that ultrasound signal depth to subcortical areas such as the brainstem might be insufficient, and as such this technique is outwith the scope of this thesis.

1.4.5 Neural Correlates of Consciousness: Neuroimaging Evidence in Pathological and Pharmacological Altered States of Consciousness

In this section, I review main findings from functional neuroimaging in altered states of consciousness. After introducing task-based paradigms and how these have generated key insights in DOC and anaesthesia, I introduce how findings from 'resting-state' fMRI have been instrumental in highlighting consistent parallels across different ASCs in their effects on large-scale networks and associated key regions (Mashour & Hudetz, 2018).

1.4.6 Neuroimaging of Task-Based Activity: Establishing Responsiveness

In the early days of neuroimaging, using BOLD (or FDG-PET) signal increases as an indirect measure of stimulation or task-associated activity, formed the pillar of many 'localization' studies, which mapped cognitive functions to associated neural substrates (Khanna et al., 2015). A typical example of landmark work of this kind is by Kanwisher et al. (1997), who through a multitude of facial recognition tasks (juxtaposed to inanimate-object sorting tasks) characterised an area in the fusiform gyrus that showed consistently elevated fMRI activation when viewing faces – coining the term "fusiform face area". This work formed part of a greater cognitive neurosciences trend, which took a modular view of the brain in which certain functions map to certain brain regions, an approach that has also shown clinical utility, e.g. in the identification of epileptic focus sites (Goebel & Goebel, 2007; Sadjadi et al., 2021; Swallow et al., 2003).

The clear association of BOLD increases in certain brain regions in response to tasks or stimuli also opened up the possibility for work in behaviourally unresponsive DOC patients to explore establishing covert cognition in absence of overt behaviour: In a landmark research letter of their own, Menon et al. (1998) demonstrated that in a patient who was diagnosed as persistently vegetative (i.e. UWS) at 6 months after brain injury, PET activity was significantly heightened in the fusiform face area in response to pictures of familiar faces – thus indicating potential preservation of consciousness, masked by an inability to communicate (Menon et al., 1998). Using fMRI, this

possibility was further advanced by Staffen et al. (2006) and Di et al. (2007), who employed event-related fMRI to assess whether responses to a patient's own name being spoken by a familiar voice might elicit particular hemodynamic changes. Those patients who subsequently improved to MCS showed clear activations in higher order associative temporal cortex in response to their name – whereas those who did not, showed no subsequent improvement (Di et al., 2007; Staffen et al., 2006). These passive stimulation paradigms catalysed the realisation that brain-damaged patients diagnosed as 'vegetative' might often be misdiagnosed due to having covert consciousness. Because responding to ones name may be a somewhat automatic, non-attentive process without executive function requirements, some peers questioned the validity of these results as detecting truly 'conscious' responses (Nachev & Hacker, 2010) – thus also questioning the responsiveness in DOC patients (and anaesthetized people). The proposed solution to this problem was to use active *task*-based evaluations.

Today, it is established that when behavioural assessments (e.g. CRS-R, GOS-E) are used in isolation, misdiagnoses of patients as UWS/VS (who subsequently show signs of covert consciousness) can occur at rates of 15-20% (Wade, 2018). This troubling realisation was catalysed by task-based paradigms, with the main tasks employed in this way being the Tennis and Spatial navigation tasks developed among others by Boly et al. (2007). In these tasks, participants are asked to either imagine playing tennis (i.e. hitting a ball back-and-forth across to another player) or to imagine walking through their own home, from room to room. In their original work, Boly et al. (2007) observed that the imaginary arm movements (playing tennis) were associated with strong supplementary motor area activation in fMRI, whereas imaginary spatial navigation was associated with distinguishable parahippocampal, posterior parietal and lateral premotor activation. Due to their stable replicability, activity in response to these prompts might serve as a proxy for behavioural responsiveness by allowing a participant to wilfully and actively modulate their BOLD signal. The discriminability of Tennis *vs* Spatial navigation task fMRI signal formed the basis of the foundational experiments by Owen et al. (2006) which demonstrated that using this approach, not only can participants be identified as covertly responsive – but in fact some rudimentary communication with the patient can be established in response to yes/no questions (Coleman et al., 2007; Monti et al., 2009). While

absence of a Tennis and/or Spatial navigation task response in DOC patients does not necessarily demarcate full absence of consciousness/awareness, this tool has been crucial in initiating the paradigmatic shift towards neuroimaging as a key complementary tool in DOC patient diagnosis (Giacino et al., 2018b; Kondziella et al., 2020). The battery of elaborated and alternative tests that have been proposed and trialled in this context continues to grow, with particular interest in the usage of EEG due to its ready usability at the bedside (Bardin et al., 2012; Bekinschtein et al., 2011; Coleman, Bekinschtein, et al., 2009; Coleman, Davis, et al., 2009; Monti et al., 2013). Altogether, task-based paradigms have therefore shown great utility in the differential diagnosis of DOC.

1.4.7 Imaging the Resting Brain and the Emergence of Large-Scale Networks

Region-function mapping as a main trend across neuropsychology and neuroimaging was followed by a complementary view that advanced beyond modularity towards a central facet of brain organization: that interactions *between* and *within* large-scale distributed brain systems (i.e. multiple, spatially separate regions) might underlie the central nervous system's complex and flexible functionality (Engelhardt, 2014). These macroscopic scale functional units of spatially-distant regions have been coined *large-scale networks* (LSNs; Biswal et al., 1995; Bressler, 1995; Lewis & Akeju, 2017; Seeley et al., 2007; Smith et al., 2012). Their original characterisation was strongly catalysed by the discovery that during goal-directed tasks there is a group of brain regions that shows simultaneous task-induced de-activation (Andreasen et al., 1995). At the time, these deactivations were thought to possibly be 'unconstrained' baseline noise in fMRI paradigms, and as such not a neurophysiological process of interest. However, various meta-analyses demonstrated that this characteristic and stable set of regions is not just de-activating during tasks, but rather strongly co-actived during rest, and in fact also partially in some tasks (Mazoyer et al., 2001; Raichle et al., 2001; Shulman et al., 1997; Vatansever et al., 2015). This signature of resting activity as a fundamental neurophysiological process motivated detailed and systematic exploration of the brain through *'resting-state'* brain imaging with fMRI and PET.

The ‘resting state’ is a condition where a participant is instructed to lie in the scanner, without any task prompt, either with eyes closed or open, or fixed on a cross-hair – letting their mind wander, without thinking of anything in particular (Park & Friston, 2013a; Raichle, 1998). Raichle et al. (2001) demonstrated using PET imaging that a set of regions comprising the posterior cingulate/precuneus, medial frontal lobe, temporoparietal junctions (also called angular gyri) showed the greatest activity (and as such co-activation with one another) during rest in healthy participants. They confirmed that the activity in these regions was indeed an ongoing neurophysiological process in extension of the results of Andreasen et al. (1995), and coined these regions’ coactivation pattern at rest as the “default mode network” (DMN). Simultaneous work with resting-state fMRI had also found that low frequency fMRI oscillations in the BOLD signal showed synchronous activity of spatially distinct brain regions that closely resembled those found by Raichle et al. (Biswal et al., 1995; Friston et al., 1994). Formally testing the correspondence of these patterns to the PET results, Greicius et al. (2003) assessed whether ‘seeds’ placed in the same regions that PET had identified to be part of the default mode network (DMN) also consistently co-oscillated in terms of BOLD activity with one another. This approach, also known as *‘resting state functional connectivity’* has become a mainstay analysis method in the resting brain, and has countlessly replicated the spatial topography of the DMN found with PET in fMRI (Friston, 2011; Friston et al., 1994; Park & Friston, 2013a). Consistent with the PET findings, during a cognitively demanding task the DMN is typically found to de-activate. In fact, Greicius et al. (2003) also demonstrated that the DMN’s key ‘node’ (i.e. ‘seed’ region) in the posterior cingulate cortex showed anti-correlated BOLD signal behaviour to many other regions in the brain, which became referred to as the task-positive network in juxtaposition to the DMN. Although this anticorrelation of the DMN with task-positive network(s) is today no longer regarded as sufficient to capture the dynamic inter-relationship of the DMN with the rest of the brain (see e.g. Vatansever et al., 2015), this observation still gave rise to the working hypothesis of the DMN as the neural correlate of internal, introspective reflection (Davey et al., 2016; Horn et al., 2014; Raichle, 2015).

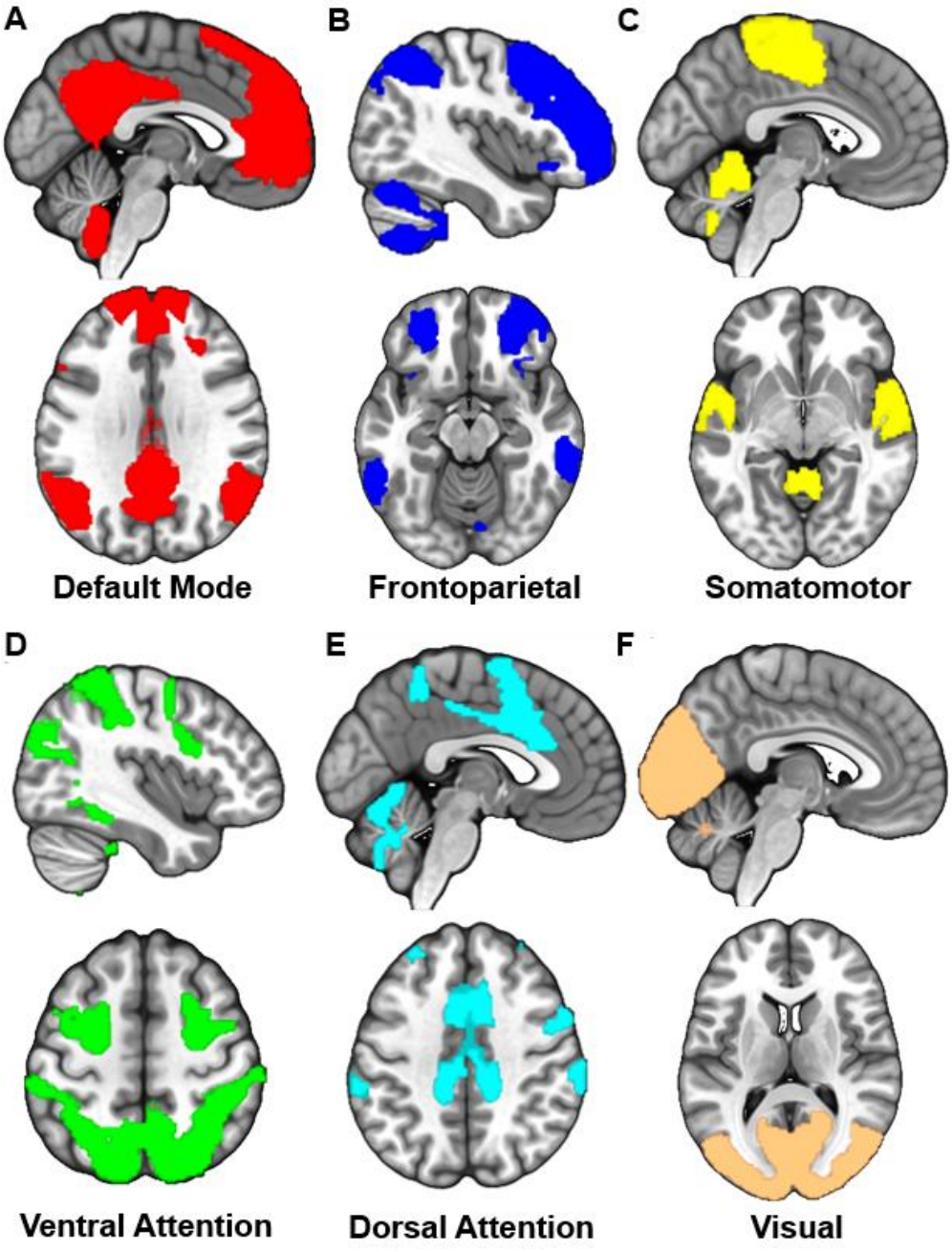

Fig. 3: Canonical Resting-State Networks as defined by Yeo et al. (2007). The limbic network is not shown. All networks A through F are often considered to be purely cortical (bar cerebellar components), although more recent work has refined network models to also include subcortical nodes, e.g. (Buckner & DiNicola, 2019; Li et al., 2021). Nevertheless, the previous cortico-centricity of network models over the past decades strongly influenced research in ASCs.

The recognition of large-scale networks that can be detected at rest, but also show task-specific relevance, greatly catalysed a shift towards network-level perspectives throughout cognitive and

clinical neurosciences. Many independent research efforts characterised strikingly similar functional organisations of the resting brain into networks of spatially-distant but co-activating regions (Barrett & Satpute, 2013; Breakspear, 2017; Bressler & Menon, 2010; Han et al., 2021; Liu et al., 2017; Marrelec et al., 2006; Menon, 2011; Park & Friston, 2013b). The canonical architecture of large-scale resting-state networks beyond the consistently observed DMN that have been characterized are the frontoparietal (executive control) network (FPCN), the salience (ventral attention) network (SN), dorsal attention network (DAN), as well as the somatomotor network (SMN), visual network (VN) and the limbic network (LN) (Yeo et al., 2011; see Fig.3). This approach of network-level, cortico-centric analyses and perspectives has dominated neuroimaging research (Alves et al., 2019) – and as such the work on resolving the neural correlates of ASCs (Koch et al., 2016).

1.5 Neuroimaging in Altered States of Consciousness Has Been Highly Cortico-Centric

Over 65% of all papers on PubMed that use neuroimaging to research consciousness take network-level perspectives.[9] Of these, most are focussed on networks that were long considered to be predominantly and sometimes purely cortical (Boly et al., 2012, 2017). A striking hallmark of altered states of consciousness that has emerged from this line of work is the disruption of the default mode network (DMN). Both with step-wise anaesthesia-induced loss of consciousness and with increasing severity of DOC, a progressive loss of the functional connectivity integrity of this large-scale network, associated with introspection and awareness is observed (Amico et al., 2017; Aubinet et al., 2018; Boly et al., 2009, 2012; Bonhomme et al., 2019b; Boveroux et al., 2010; Comanducci et al., 2020; Demertzi et al., 2017, 2013, 2019; di Perri et al., 2014; Giacino et al., 2014a; Golkowski et al., 2019; Gómez et al., 2013; Guldenmund et al., 2012, 2017b; Heine et al., 2012a; Kondziella et al., 2020; Martínez et al., 2019; Sanz et al., 2021; Schrouff et al., 2011; Soddu et al., 2012b; Vanhaudenhuyse et al., 2010).

[9] This is the result of a PubMed search taking the query "consciousness + neuroimaging" (n=3659) and the query "consciousness + network" (n=2386) and taking the direct percentage (Date: 22nd of March 2022).

1.5.1 Anaesthesia

Specifically, in anaesthetic-induced sedation, functional connectivity between the DMN and the FPCN (Hudetz et al., 2012, MacDonald et al., 2015) is disrupted and the within-network connectivity integrity decreased – whereas lower-order networks such as sensorimotor and visual networks maintain their connectivity during sedation (Martuzzi et al., 2011). Importantly, at mild sedative dosages of the anaesthetic propofol, intra-DMN connectivity strength remains relatively preserved, but proportionally to the increase of propofol dosage then decreases (Boveroux et al., 2010; Gómez et al., 2013; Sarasso et al., 2015; Schrouff et al., 2011). Indeed, along with this connectivity disintegration goes an increase of connectivity of the principal DMN node in the posterior cingulate cortex to regions it is not normally connected to, i.e. regions outside the canonical DMN, as demonstrated by Stamatakis et al. (2010). Cerebral blood flow (CBF) is the most strongly reduced in response to anaesthesia in the posterior cingulate and precuneus, which together form the posterior midline cortical nodes of the DMN (Demertzi et al., 2013; Vogt & Laureys, 2005). Conversely, strong increases in CBF in these regions and increased integrity in their connectivity to the rest of the DMN (Guldenmund et al., 2012, 2017b), are associated with re-emergence from anaesthesia, i.e. regaining of consciousness (Xie et al., 2011). Altogether these observations made with anaesthetic perturbation of consciousness led to the suggestion that strong effects of anaesthesia on the posterior cortical regions could be mechanistically involved in producing unconsciousness through impairing a putatively consciousness-supporting role of the DMN (Hudetz, 2012; Hudetz & Mashour, 2016; Mashour & Hudetz, 2018).

1.5.2 Disorders of Consciousness

Strikingly, these observations and theories have been strongly paralleled in DOCs, where resting-state neuroimaging is an ideal tool to overcome the overt lack of behaviour of patients. Early studies by Laureys et al. (1999) demonstrated that in vegetative state/unresponsive wakefulness syndrome, DOC patients' cortical glucose metabolism measured with FDG-PET was globally decreased by 40-50% throughout the cortex – but also that FDG uptake was most strongly impaired in the DMN nodes in the precuneus, posterior cingulate and angular gyri (Heine et al., 2012b; Laureys et al., 2004; Vogt &

Laureys, 2005). Indeed, these regions have proportionally greater glucose metabolism the better a patient's diagnosis and CRS-R score are (Thibaut et al., 2012), which has even shown diagnostic utility in distinguishing UWS from MCS (Stender et al., 2015).

Just as in anaesthesia, resting-state fMRI has mirrored and corroborated these PET findings, demonstrating that DMN intra-network connectivity is heavily impaired in DOC, but increases progressively when moving up the strata from coma to MCS+/eMCS ((Demertzi et al., 2017, 2013, 2014, 2015; Guldenmund et al., 2012, 2017c; Heine et al., 2012a; Martínez et al., 2019; Rosazza et al., 2016; Vanhaudenhuyse et al., 2010). The severe disruption of DMN intra-network connectivity and its connectivity to other cortical resting-state networks are arguably the most replicated and most influential finding in DOC research (Amico et al., 2017; Boly et al., 2009, 2012, 2017; Cauda et al., 2009; Cavaliere et al., 2016; Chen et al., 2021; Coulborn et al., 2021; Crone et al., 2011, 2013; de Pasquale et al., 2015; Demertzi et al., 2014, 2015; di Perri et al., 2013, 2016; D. Fischer et al., 2022; Guldenmund et al., 2012; He et al., 2014; Huang et al., 2014; Jain & Ramakrishnan, 2020; Koch et al., 2016; Luppi, Cain, et al., 2021b; Marino et al., 2016; Mazoyer et al., 2001; Norton et al., 2012; Ovadia-Caro et al., 2012b, 2012a; Peran et al., 2020; Silva et al., 2015; Soddu et al., 2012b, 2012a; Thibaut et al., 2012; Threlkeld et al., 2018; Vanhaudenhuyse et al., 2010; H. Wu et al., 2022; X. Wu et al., 2015; Xie et al., 2011). Firstly, these fMRI measures have demonstrated an intuitive, but crucial finding: in patients classed as brain(stem)-dead, the DMN is fully absent – whereas in UWS and MCS patients it is still found at varying degrees of impairment (Boly et al., 2009; Cauda et al., 2009; Vanhaudenhuyse et al., 2010). Similarly to PET, resting-state measures of DMN integrity (mainly from seeds in the precuneus and PCC) have been suggested to have potential clinical utility, as certain experimental approaches have been able to differentiate UWS/VS from MCS using fMRI – and some prognostic use has equally been suggested, with DMN integrity predicting functional outcome at 3 or more months post-injury in different cohorts (S. Chen et al., 2018; Norton et al., 2012; X. Wu et al., 2015). This suggests that the preservation of DMN integrity and/or its re-emergence may be of central importance to regaining normal wakeful consciousness, and that increases in connectivity co-occur with clinical improvement across the DOC spectrum. Nevertheless, it is important to highlight that these are only suggestions, and that in practice very few hospitals rely solely on rs-fMRI assessments

for differential diagnosis, as the connectivity patterns show far too much overlap between UWS/VS and MCS – but their general clinical utility is being explored.

DMN changes have been suggested as target biomarkers of therapeutic efficacy, e.g. when using transcranial direct current stimulation (Cavaliere et al., 2016) and other experimental interventions (Bodien, Chatelle, et al., 2017). More formally, DMN and frontoparietal control network impairment are suggested to be the neural substrates of impaired consciousness in theories such as the anterior forebrain mesocircuit model (Coulborn et al., 2021; Fridman & Schiff, 2022; Lant et al., 2016; Schiff, 2010). As mentioned earlier in the introduction, this model developed by Schiff and colleagues, centres on the idea that brain injury impairs striatal inhibition of the globus pallidus, which in turn causes a lack of thalamic outflow, causing brain-wide hypo-excitation and as such impairment of the 'frontoparietal' network (in this case both DMN and FPCN). Although many different sub-mechanisms within this model have been proposed, striatal inhibition of the globus pallidus is dopamine-dependent, meaning that dopaminergic drugs might alleviate DMN impairments. However, trials have largely not used biomarkers of DMN integrity, and none have tested the association of the DMN with transmitter source nuclei as a potential sub-clinical outcome measures – although suggestions have been made in the literature (Edlow, Barra, et al., 2020). Indeed, to resolve how certain neurotransmitters such as dopamine are involved in impairing cortical network function might greatly enhance pharmacological therapeutic frameworks, as recent work has demonstrated (Fridman et al., 2019). Altogether, the consistent implication of DMN and large-scale brain functional disruption in DOC has advanced the scientific understanding of consciousness disruption, but has inadvertently also created a heavily cortico-centric macroscopic focus.[10]

1.5.3 Psychedelics

The DMN importance in ASCs is rounded off by observations from psychedelic administrations. When healthy volunteers take psilocybin, LSD, DMT, or ayahuasca, consciousness is altered in a qualitatively entirely different way to DOC – yet these administrations are also characterised by a

[10] Indeed, even more macroscopic markers, such as the BOLD signal complexity across the whole brain are used, with decreases associated with impaired consciousness – most strongly in the precuneus/PCC (Luppi et al., 2019). I use and explain this marker in a secondary analysis in Chapter III.

sudden loss of DMN-integrity in resting-state resting-state fMRI (Atasoy et al., 2017; Carhart-Harris et al., 2012, 2014; Carhart-Harris, Muthukumaraswamy, et al., 2016a; Deco et al., 2018; Luppi et al., 2021; Millière et al., 2018; Nutt et al., 2020; Nutt & Carhart-Harris, 2021; Palhano-Fontes et al., 2015a; Parker Singleton et al., 2021; Preller et al., 2018, 2019; Sampedro et al., 2017; Tagliazucchi et al., 2016a; Viol et al., 2017). The extent of this disintegration of the DMN is correlated with the strength of ego-dissolution acutely experienced by a participant (Lebedev et al., 2015; Millière, 2017; Tagliazucchi et al., 2016b). What is more, the DMN not only shows disintegration in terms of within-network connectivity, but also shows strongly increased connectivity with normally non-DMN regions across many of these studies (Tagliazucchi et al., 2016b). Strikingly, (1 day to 3 weeks) after the psychedelic administrations and experiences which encompass acute DMN disintegration, in the post-acute phase, there is actually greater DMN integrity than at baseline (i.e. before psychedelic administration; Carhart-Harris, Bolstridge, et al., 2016; Carhart-Harris et al., 2012, 2018; Palhano-Fontes et al., 2015b; Roseman et al., 2018). This effect has become referred to as a drug-induced "DMN reset", the strength of which has been suggested to underpin therapeutic benefits in psychedelic treatment of treatment-resistant and major depressive disorder patients (Carhart-Harris et al., 2017). Given this psychedelic-induced increase in DMN integrity, some have speculated that psychedelics might be a feasible experimental treatment for DOC patients.[11] Altogether, psychedelic-induced consciousness-alterations therefore have strong effects on the DMN – which is neuro-pharmacologically plausible, as the DMN overlaps with precisely those regions of the cortex that are the richest in the 5HT2A receptor (Beliveau et al., 2017a; Madsen et al., 2019).

[11] It is the author's perspective that the complexity of ethical implications will for the foreseeable future make such work impractical. Nevertheless, the possibility of a strong monoaminergic intervention in these patients does present a possibly uniquely suitable opportunity, with lessons from psychedelic studies potentially applicable to DOC pharmacological strategies. This is revisited in the following Chapters and final Discussion (Chapter VIII).

1.5.4 Cortical Network Dysfunction is a Hallmark Feature of Altered Consciousness – But What Underpins It?

In conclusion, across different pathological and pharmacological ASCs, neuroimaging research has provided clear evidence of dysfunction of large-scale cortical networks, particularly the DMN and its main nodes. What however brings these network level changes about from a mechanistic point of view? It is striking that while classifications of anaesthetic drugs, deficits and treatment mechanisms for DOC, and psychedelic experiences all point to the involvement of particular neurotransmitter systems, these have largely not been assessed *in vivo* in humans as a potential basis of network dysfunctions.

The need to assess this relationship is even inherent in the awareness-arousal framework. Whereas based on network level findings cortical functionality was seen as the neural substrate for contents of consciousness (i.e. awareness), the arousal dimension has been associated with the brainstem and its nuclei. While this conceptual distinction is intuitively appealing, it is not only likely a strong oversimplification, but strictly speaking a theoretical non-sequitur. The behavioural and clinical tools used in consciousness research can only quantify the level of arousal (i.e. 'level' of consciousness). This consequently diminishes claims in the literature that correlations between CRS-R or GCS scores and DMN integrity identify the DMN and cortical function as the "substrate of awareness" – as the respective measures are in principle restricted to observable arousal. Importantly however, I do not suggest that network-level phenomena (such as DMN integrity) should be seen as correlates of solely arousal either. Instead, I submit that large-scale network biomarkers contain both awareness *and* arousal dimensions – through the specific influence of brainstem neurotransmitter systems on the cortex.

Historically, brainstem-derived arousal was long oversimplified into a conceptual "on/off" signal for the cortex – whereas today it is understood that arousal signalling is enormously complex and underpinned by the diverse brainstem neurotransmitter nuclei, and specifically their neuromodulatory influence (Avery & Krichmar, 2017; Shine et al., 2019). This knowledge is largely founded on

preclinical and lesion studies, which were not widely translated into *in vivo* human neuroimaging, thus foregoing the opportunity to reconcile the macroscopic network-level and more microscopic neurotransmitter-system levels of explaining ASCs.

To address this, the following section further introduces the brainstem as the seat of arousal-relevant neurotransmitter nuclei. I briefly review their recognised importance which spans from antiquity to today through examples of preclinical, anatomical, and structural imaging work. I specifically highlight that the neuromodulatory function of neurotransmitter nuclei could account for network-level dysfunctions. Lastly, I lay out how this thesis will address neurotransmitter function across different ASCs using recently available, simple and clinically-feasible tools.

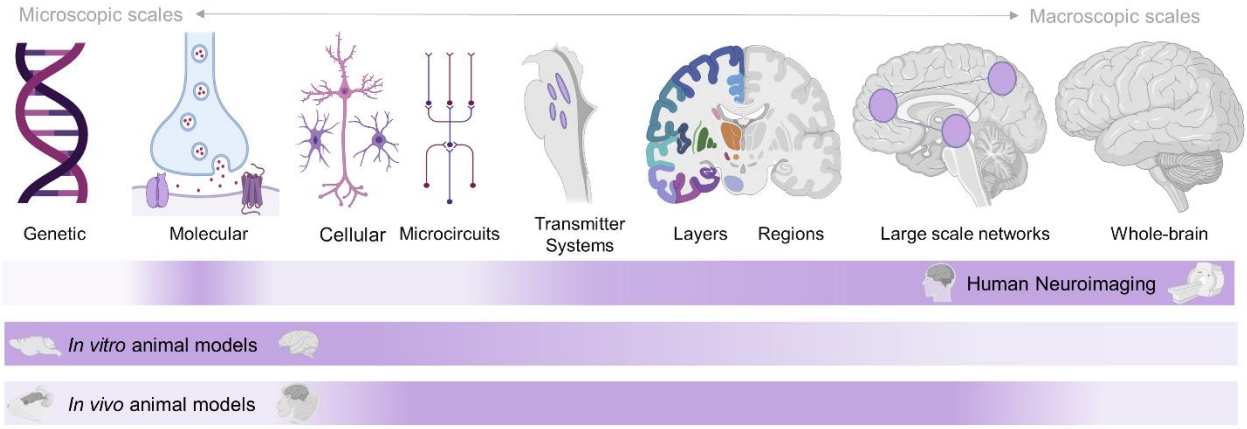

Fig. 4: Conceptual overview of levels of analysis to be considered in consciousness research across the microscopic-to-macroscopic spectrum. Colour bar saturation indicates capability of a technique to address the systems-level denoted above. Human neuroimaging has produced macroscopic network biomarkers and identified certain regions/layers which are associated with consciousness maintenance and altered states of consciousness. However, for any inquiries at more microscopic scales, research typically defers to animal models in which experimental manipulations (DREADD, optogenetics, lesion approaches) allow for direct mechanistic investigations. While some of the insights gained from preclinical work can be tested in humans *in vivo* (e.g. by using pharmacological approaches), there has been a striking paucity of translational neuroimaging work that develops paradigms to test the connection between micro- and macroscopic insights. The area in which there is the most consistent overlap across *in vitro* and *in vivo* approaches are the brainstem neurotransmitter systems, making them a key translational interface to bridge micro- and macroscopic accounts of ASCs.

1.6 The brainstem and arousal – lessons from antiquity to today

Throughout the above sections, the brainstem and its neurotransmitter nuclei have been highlighted as being implicated in consciousness and across many ASCs. Most generally, brainstem death is synonymous with brain death, whereas any preservation of brainstem-reflexes and thus -function is synonymous with life (Ercole et al., 2020) – highlighting that this area is fundamentally important to nervous system functionality. This primacy of the brainstem for consciousness and its perturbation is not a recent discovery, but possibly one of the oldest lines of research for understanding the human brain.

Indeed, long before the term 'neuroscience' was coined, Galen of Pergamon (130– 210), a noteworthy proponent of the encephalocentric theory (i.e. that the 'mind' [psyche] resides in the brain), was studying the brain's anatomy and neurophysiology through animal vivisection (Baloyannis, 2016). In his work *On Anatomical Procedures,* he described that lesions between the first and third vertebrae cause tetraplegia, cessation of breathing, coma, and ultimately death (Raach, 1962). He explicitly described how this region he called *basis* was the only brain region where upon application of pressure a clear and deep stupor occurred to the animal. While pressure removal reversed this, applying a small cut in this region meant that the animal did not re-emerge into its 'natural condition' (Cucu et al., 2021; Acar, 2005), leading him to conclude that this was the seat of basic 'vegetative functions' in the human body (Baloyannis, 2016). After Galen, official prohibition of dissections hindered progress in a general understanding of the brain and brainstem. However different intellectuals with anatomical and physiological interest, among them people such as Leonardo da Vinci and Michelangelo Buonarotti, broke such laws and advanced the study of the brainstem. Of Michelangelo it is even controversially claimed that in the Sistine Chapel fresco *Separation of Light from Darkness,* he despondently hid a precise rendering of the brainstem at the level of the neck of God.[12] Jean Pierre Flourens (1858), who was familiar with Leonardo's drawings of the brainstem, rekindled Galen's idea of vegetative functions as arising from the brainstem but importantly was the first to suggest that different brainstem sub-systems serve different functions. Through vivisection and

[12] This is the fresco from which an excerpt is presented at the beginning of this thesis. A neuroanatomical perspective on this suggestion of 'concealed neuroanatomy' within these frescos was published by Suk & Tamargo (2010) in *Neurosurgery*.

lesion studies in rodents, pigeons and stray dogs, he characterised a diminutively small area in the brainstem, less than a millimetre in diameter, which he coined *"le noid de vie"*, the node of life – as any damage to this area caused sudden death (Cucu et al., 2021). Together with many others, this work laid the basis for the characterisation of the brainstem as key to maintaining wakefulness in non-human and human animals, and also to the subsequent characterisation of neurotransmitter specific nuclei and pathways contained within it as the wakefulness systems-level effectors (Satpute et al., 2019).

In the modern age, further lesion studies (both *post mortem* and *in vivo*) have allowed causative associations to be made between neuroanatomical deficits and consciousness deficits, which have continued to advance the case for brainstem nuclei involvement for consciousness. Severe impairments, or full and irreversible loss, of consciousness in humans almost always involves brainstem lesions, specifically in the pons and midbrain. Indeed, in patients with brainstem stroke, Parvizi and Damasio (2003) characterised that whenever a patient did not show coma, the lesions had spared the pons and midbrain – whereas whenever bilateral lesions within the paramedian brainstem centred on the pons and midbrain occurred, a diagnosis of coma was made (Parvizi & Damasio, 2003). This applies to bilateral diencephalic lesions too, where some level of brainstem impairment typically co-occurs (Fischer et al., 2016; Golaszewski, 2016). Indeed, in recent work by Hindman et al. (2018) it was observed that only when lesions extend into the brainstem, particularly pons and midbrain, are consciousness and arousal impairments observed. Supporting this pivotal importance of the brainstem further, work by Merker et al. (2007) demonstrated that people with severe cases of hydrencephaly who are thus born without a cortex still show signs of consciousness, making them posit that only normal brainstem and subcortical functionality might be required for 'core' consciousness (Aleman & Merker, 2014; Merker, 2007). Conversely, it has been suggested that coma is a structural disconnection syndrome, wherein the structural impairment of projections from the brainstem to the rest of the brain/cortex withdraws key signalling from the brainstem transmitter nuclei that is critical to nervous system function and 'core' consciousness (Edlow et al., 2012).

1.6.1 The Brainstem is Made Up of Different Neurotransmitter Nuclei with Neuromodulatory Function

Although it is outside the scope of this thesis to review how the different transmitter-specific brainstem nuclei were characterised, in the context of ASCs it is nevertheless important to highlight that Moruzzi & Magoun (1949) were arguably the first to formally suggest that influence of the brainstem "reticular formation" might serve to 'activate and arouse the cortex' to produce wakefulness. This was based on systematic observations in cats using barbiturates and experimental lesions in the brainstem reticular formation that caused broadscale behavioural and EEG impairment in cats. Strikingly, they also rightly hypothesized that tonic, pacemaker-like activity of this area might provide a background influence required for normal wakefulness, and replicated and extended this work to monkeys (French et al., 1952, 1953; Magoun & Rhines, 1946; Moruzzi & Magoun, 1949). While these pieces of work identified that wide-ranging afferents to the cortex and thalamus make the brainstem ideally-positioned to have large-scale influence over whole-brain function, brainstem arousal signalling was in this work still seen as an effectively binary signal "switching on" the cortex.

Today it is established that neurotransmitter nuclei (adjacent to the mainly glutamatergic reticular formation coined by Moruzzi and Magoun) are most consistently implicated in arousal and wakefulness/consciousness regulation. These are relatively homogenous in terms of the transmitter being synthesized there, released *via* their cortical and thalamic projections, and span cholinergic and specifically monoaminergic (i.e. serotonergic, dopaminergic and noradrenergic) phenotypes. To distinguish them from the reticular formation, these nuclei became referred to as the "diffuse neuromodulatory system" (Olszewski & Baxter, 1982), as a reflection of their far-ranging and brain-wide projections *via* which they provide non-classical transmitters. Indeed, it is their neuromodulatory function that might be key to consciousness maintenance – as the dysfunction of this process and the nuclei is one of the most likely candidates to underpin large-scale network dysfunction in ASCs and to thus be a 'rate-limiter' of consciousness-relevant processes.

1.6.2 The Concept of Neuromodulation – an Ideal Biological Rate-Limiter

Neuromodulation is a nervous system signalling process that is fundamentally different and distinct from classical synaptic point-to-point neurotransmitter signalling. Indeed, fast excitatory or inhibitory signalling such as in glutamate and GABA neurotransmission functions *via* fast, short-latency ionotropic receptor mechanisms, and occurs invariably across a synaptic cleft (thus point-to-point; Harris-Warrick & Johnson, 2010; Marder et al., 2014). Instead, neuromodulatory signalling is underpinned by transmitters that are all synthesized in the brainstem and released *via* brain-wide projections throughout the brain. This wide-ranging release occurs both in a diffuse manner (referred to as volume transmission), meaning that these signalling molecules are provided in a one-to-many architecture, as well as *via* various synaptic connections as well (Avery & Krichmar, 2017; Marder, 2012). This diversified architecture is thought to enable both pathway-specific and global effects to be closely associated with neuromodulatory systems (Liu, Goel, and Kaeser, 2022).

Their mode of signalling is also different, as neuromodulators signal exclusively *via* metabotropic G-protein coupled receptors. The G proteins of these receptors are coupled to second messenger cascades. These set in motion various cellular modification processes that can change the electrochemical and synaptic properties of neurons. This process' reliance on second messenger systems makes it slightly slower than classical neurotransmission (nanoseconds to milliseconds for neurotransmission *versus* seconds to minutes for neuromodulation; Marder, 2012; Marder et al., 2014). The change in synaptic and electrochemical properties ultimately alters the input-output relationships of neurons, microcircuits and large-scale networks (Avery & Krichmar, 2017; Marder, 2012; Marder et al., 2014; van den Brink et al., 2016; Shine et al., 2018, 2019). This process is fundamental for the central and peripheral nervous systems, as it allows the same structural circuits/connectome(s) to have many different input-output configurations through neuromodulatory tweaking – thus surmounting the problem of one-circuit-one-function which would necessitate human brains of enormous, and physiologically unviable sizes (Marder, 2012). Therein, the provision of neuromodulatory transmitters and their (once more) one-to-many signalling architecture *via*

metabotropic receptors is indispensable for normal function of the brain – including large-scale networks.

Strictly speaking, only the monoaminergic brainstem nuclei can be classed as fully neuromodulatory, as their transmitters signal solely *via* metabotropic receptors (which is not true of acetylcholine) and arise only from within the CNS (which is not true of opioids and histamines). When this particular profile of monoamines is considered together with their consistent and specific implication across DOC, anaesthetic and psychedelic contexts, it is clear that ASCs might be effected and/or influenced by changes in monoaminergic source nuclei: the dopaminergic ventral tegmental area (VTA), the serotonergic median and dorsal raphe nuclei (MR and DR), and the noradrenergic locus coeruleus (LC).[13] These monoaminergic nuclei are active in a range of 0.2 to 10Hz, largely *via* autonomic pacemaker conductances and cortical feedback loops, which creates a baseline 'tone' of their transmitter throughout the brain (Avery & Krichmar, 2017; de la Cruz et al., 2021; Marder, 2012; Marder et al., 2014). Upon some external demands, such as cognitive tasks or others, there can often be transient upregulations in nucleic activity, which are referred to as phasic signalling. Such phasic "transients" in transmitter tone and nucleic activity are the subject of many research domains e.g. in motor control (Miles & Sillar, 2011), impulsivity (Dalley & Ersche, 2019; Dalley & Robbins, 2017), and pathologies such as schizophrenia (McCutcheon, Abi-Dargham and Howes, 2019). However, as also posited originally by Moruzzi & Magoun (1949), phasic processes are highly unlikely to be involved in consciousness maintenance as it is – or should be – arguably one of the most fundamental tonic processes or phenomena in central nervous function.[14]

As such, tonic activity of these monoaminergic nuclei related to wakefulness has been characterised extensively in animal experiments that followed Moruzzi & Magoun's original work. I review key

[13] Due to the ongoing debate about the actual location and best means of localisation of the noradrenergic locus coeruleus, I only consider this source nucleus (LC) secondarily in this thesis. See e.g. Ye et al. (2021) *versus* Betts et al. (2019) and Giorgi et al. (2022) for disparate localisations *in vivo*.

[14] Phasic transient upregulations of nucleic function have however been linked to sleep-wake transitions in animals and have begun to be assessed for similar transitions in humans too. I touch upon this in the following section, however see also Luppi, Spindler et al. (2021).

pieces of such evidence separately in chapters where appropriate, but also briefly introduce these areas and the preclinical perspective on their importance for consciousness here.

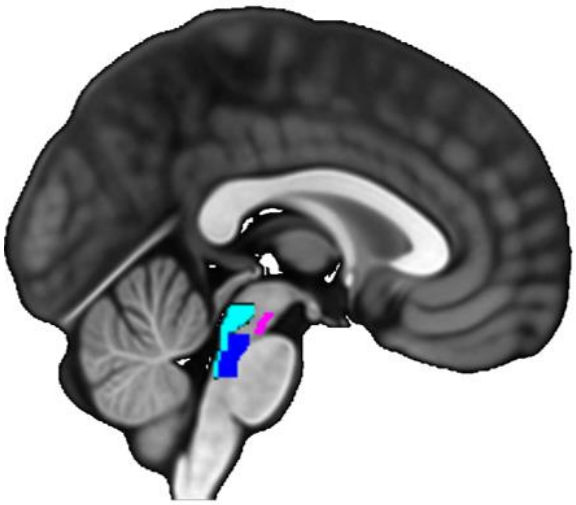

Fig. 5: Main monoaminergic nuclei that are considered in this thesis. Ventral Tegmental Area (Dopamine; magenta), Dorsal Raphe (Serotonin; Cyan), and Median Raphe (Serotonin; Dark Blue). All of these nuclei project globally throughout the cortex, providing their respective transmitter to relevant receptor sites in both 'volume transmission' modes and via synaptic connections, but with signalling consequences that are distinct from classical point-to-point fast neurotransmission, due to their pairing to second messenger cascades that have slower and one-to-many mapped effects on synaptic and electrochemical properties of neurons and networks. Rendering on the sagittal plane of the MNI-152 template image to highlight pontine/midbrain location of these nuclei.

1.6.3 Preclinical Evidence Supports the Importance of Brainstem Monoaminergic Nuclei: Implications of the Dopaminergic Ventral Tegmental Area

The ventral tegmental area provides dopamine to the whole of the cortex *via* the mesocortical pathway.[15] The neurons resident in this area (~300,000 cells) are highly active during wakefulness,

[15] The mesolimbic pathway also provides dopamine to the striatum and thalamus, as well as other subcortical structures. Indeed, recent work has suggested that another wakefulness-relevant portion of dopaminergic

but not during non-rapid-eye-movement (non-REM) sleep in animals (Eban-Rothschild et al., 2016). Indeed, Palmiter and colleagues have demonstrated that when mice are dopamine-deficient they show a behavioural phenotype they coined "behaviourally unconscious", reminiscent of sedative/vegetative states – but retroviral restoration of dopaminergic signalling reversed these behavioural deficits (Palmiter, 2011). Indeed, both optogenetic (Eban-Rothschild et al., 2016) and pharmacological VTA activation (Oishi et al., 2017) acutely promote wakefulness in rodents. Critically, electrical, optogenetic (Taylor et al., 2016) and even non-invasive ultrasound (Bian et al., 2021) stimulation of the VTA (but not substantia nigra, the other main dopamine source nucleus) can even fully wake animals from propofol anaesthesia (Solt et al., 2014). Instead, lesions with 6-hydroxydopamine which ablate the VTA lengthen post-anaesthetic recovery times (Zhou et al., 2015). Both methylphenidate- and dextroamphetamine-induced reversal of propofol anaesthesia in rats is suggested to act primarily *via* a dopamine-dependent mechanism (Kenny et al., 2015; Solt et al., 2011). Supporting this centrality of dopamine, dopamine transporter (DAT) knockout mice in whom dopaminergic tone is greatly heightened, have much longer wake periods than wildtype animals (Wisor, 2018; Wisor et al., 2001). Altogether, these convergent preclinical findings implicate dopaminergic signalling from the VTA as a tonic regulator of wakefulness, and strongly suggest that dopaminergic system tonic function is key to consciousness maintenance. Despite all of this evidence, the VTA's function has never been assessed in human altered states of consciousness, and its relationship to cortical networks was until very recently uncharted (see Chapter II; and Li et al., 2021).

1.6.4 Implications of the Serotonergic Raphe Nuclei

The raphe nuclei are the serotonergic (5-hydroxy-tryptamine; 5-HT) brainstem source nuclei, among which the median and dorsal raphe provide most of the serotonin throughout the cortex. The neurons in the raphe (<175,000 cells), have been found to show high activity during wakefulness, low activity during non-REM sleep and no activity during REM sleep (Jacobs & Fornal, 1999; Mcginty & Harper, 1976; Trulson & Jacobs, 1979). Indeed, optogenetic activation of these nuclei while a rat is sleeping

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neurons exists in the ventrolateral periaqueductal gray, which also has global projections throughout the cortex (George et al., 2019; Li et al., 2018; Ntamati et al., 2018).

can wake the animal from non-REM sleep to full wakefulness (Moriya et al., 2017), and localised Ca^{2+} injections into the raphe fully suppress all sleep in rats (Cui et al., 2016). Similarly, the systemic administration of various 5-HT receptor agonists (5HT1A, 5HT2A and 5HT1B) all produce acute arousal, increasing wakefulness. These nuclei's activity is instead again inhibited by various anaesthetics, whereas e.g. after isoflurane anaesthesia upregulations in their activity are involved in arousal promotion (McCardle & Gartside, 2012; Yang et al., 2019). Intriguingly however, various psychedelics also all powerfully inhibit the raphe to nearly full cessation of firing, resembling the raphe state during REM sleep (Aghajanian et al., 1968, 1970; Aghajanian & Marek, 1999; Cohen & Grossman, 2020; Foote et al., 1969; Hornung, 2003; Rogawski & Aghajanian, 1981). Although this might be mechanistically-relevant for understanding psychedelic acute mechanisms, as well as their long-term effects in treatment of pathologies such as depression (see Chapters IV and V), the many different yet complementary accounts of the whole-brain relevance of serotonin function often do not consider the brainstem sources of serotonin at all (e.g. Carhart-Harris & Friston, 2019). Similarly to the ventral tegmental area, the brainstem raphe nuclei's putative importance for ASCs and consciousness *per se* – such as their response to psychedelics – has never been assessed *in vivo* in humans.

1.6.5 Functional Neuroimaging of Monoaminergic Nuclei as a Recent and Relevant Translational Opportunity for Understanding ASCs

This preclinical evidence suggests that monoaminergic brainstem nuclei are potential mechanistic "rate-limiters" for consciousness. This makes the assessment of these nuclei *in vivo* in humans a key translational opportunity to better understand ASCs. From a theoretical standpoint, as neither PET nor EEG or other mainstream neuroimaging techniques have sufficient spatial and temporal resolution, resting-state fMRI is the best-suited neuroimaging method to make assessments of brainstem nuclei tonic functionality, and importantly also to contextualise nuclei function and network function (i.e. to approximate neuromodulatory consequences). Until recently however, research only rarely considered these nuclei, due to a variety of reasons.

Firstly, the cortico-centricity of research agendas created acquisition priorities in neuroimaging that meant that fields-of-view often did not capture the brainstem at sufficient height and detail to be able to cover the whole cortex (Sclocco et al., 2018). Furthermore, 3 Tesla (3T) scanning which has lower gray-white matter contrast than high-field 7 Tesla (7T), does not make it possible to visually delineate all nuclei in a transmitter-specific fashion *in vivo*, and to obtain strong signals from deep brain layers (Sclocco et al., 2018). As such, previous assessments had to limit themselves to drawing physiologically non-relevant spheres on scans as regions-of-interest (ROIs), placing them in brainstem locations where transmitter systems were believed to be (Bär et al., 2016), or to use PET derived ROIs suffering from the techniques' limited spatial resolution (Beliveau et al., 2015). Although such studies therefore were methodologically confounded, they still importantly demonstrated that different brainstem areas show different patterns of activity and connectivity (Bär et al., 2016; Beissner et al., 2014). The key limitation of these previous studies has recently been at least partially overcome through the creation of *ex vivo* histology- (and complementary automatic segmentation-) based atlases that can be used in neuroimaging reference spaces (Bianciardi et al., 2015, 2016, 2018; Edlow et al., 2013; Snider et al., 2020). Especially atlases based on histology can create physiologically-relevant ROIs that are selective for the relatively homogenous transmitter expression in a given brainstem area, providing the previously absent tooling to allow the assessment of independent transmitter nuclei. These *ex vivo* atlases can be used with 7T and 3T resting-state fMRI, with the latter having smaller spatial resolution, but also less field inhomogeneities and noise than 7T (Sclocco et al., 2018). Although future work will have to resolve whether either field-strength is ultimately preferable for the assessment of brainstem nuclei, very recent work has shown that findings in brainstem-cortical connectivity have great translatability between 7T and 3T, reinforcing that 3T is well positioned to assess these nuclei – which is especially important for clinical environments where only 3T is routinely available (Singh et al., 2022). Although these nuclei templates have only been available for a relatively short time, great progress has been made in mapping the relationship of brainstem nuclei and associated transmitter systems with the rest of the brain and particularly cortex using resting-state fMRI.

Above all else, the ventral tegmental area (VTA) and raphe have both been found to be strongly connected to the default mode network in 7T high-resolution resting-state fMRI – with the VTA in fact being the most strongly connected out of all histologically-characterised brainstem nuclei (Li et al., 2021). Recapitulating its importance for consciousness, in emergence from anaesthesia, the VTA shows the greatest upregulation in global resting-state connectivity (Nir et al., 2019). Correspondingly, monoaminergic influence might be an of underpinning large-scale networks, as emerging evidence also supports: Catecholaminergic bolus administrations change large-scale network interactions, and a catecholaminergic reuptake inhibitor administration downregulates integrity of large scale networks in a spatially heterogenous fashion (along an antero-posterior gradient) in resting-state fMRI (Pfeffer et al., 2021; van den Brink et al., 2016; Shine et al., 2019). This strongly suggests that tonic monoaminergic modulation may be directly associated with the topography and integrity of cortical networks (Shine et al., 2019), and that the disruption of function of these brainstem centres could therefore plausibly underlie network disintegration in ASCs (Pfeffer et al., 2021; Satpute et al., 2019).

On the basis of the above findings, it is intuitive that loss of *structural* projections between brainstem nuclei and the cortex is a hallmark of coma (Edlow et al., 2013), and that the most consistently coma-associated brainstem lesions occur in a region which at rest shows connectivity to some DMN nodes (Fischer et al., 2016). Indeed, brainstem nuclei functional connectivity follows their known structural connectivity as assessed in preclinical tracing studies and with high resolution tractography in humans (Satpute et al., 2019), which captures connections to higher order cortical nodes across many networks (Edlow, 2021b; Edlow et al., 2012). Finally and importantly, very recent evidence suggests that resting-state fMRI measured connectivity deficits of brainstem nuclei relate to *de facto* deficits of transmitter levels assessed with fluid biomarkers (McCarty et al., 2021).

1.7 Aims and Hypotheses of this Thesis

In line with these observations, Satpute et al. (2019) have suggested that the functional activity and connectivity of brainstem nuclei might capture *dynamic recipes* of cortical neuromodulation – which are primarily responsible for wakeful arousal, and also for affective and other traits. Elaborating on

this idea, I submit that a disruption of monoaminergic neuromodulatory nucleus function in particular, captured in changed functional connectivity of corresponding source nuclei, might be involved in human ASCs.

The fundamental question at the centre of this thesis therefore is: *"Are effects on the function of brainstem monoaminergic nuclei associated with both pathological and pharmacological ASCs, as well as their established large-scale network biomarkers?"*

To assess the monoaminergic brainstem nuclei using resting-state functional MRI serves to address multiple central gaps in our understanding of consciousness and its biological bases, namely:

(**i**) To bridge preclinical-clinical and microscopic-macroscopic translational divides on the importance of brainstem nuclei for consciousness maintenance (Luppi, Cain, Spindler, Gorska et al., 2021);

(**ii**) To formulate neurobiologically-plausible mechanisms for network- and consciousness impairments with diagnostic and therapeutic relevance for DOC (Edlow, Claassen, et al., 2020), as well as other neurotransmitter-related disorders; and

(**iii**) To establish whether resting-state fMRI can provide a clinically-feasible, non-invasive tool to assess neurotransmitter system function *in vivo*.

To this end, this thesis will use the ventral tegmental area (VTA), and median and dorsal raphe nuclei (DR and MnR; other nuclei as controls where appropriate) from the *ex vivo* histologically-characterised Harvard Ascending Arousal Network atlas (HAAN; https://www.nmr.mgh.harvard.edu/resources/aan-atlas; Edlow et al., 2012) to examine how monoaminergic brainstem centres are affected across both pathological and pharmacological consciousness perturbation. This atlas was chosen as it remains the sole histologically and thus neurotransmitter-system specific template of these nuclei.

All of the following experimental chapters are prefaced by a literature review with studies-of-interest to formulate research questions and hypotheses.

Chapter II seeks to identify whether the dopaminergic VTA is impaired in propofol-induced sedation and DOC patients, in line with the multitude of preclinical and clinical implications of this nucleus in pathological and anaesthetic states. The hypothesis is that VTA functional connectivity is similarly perturbed across both pathological and pharmacological ASCs, and that these connectivity alterations might be associated with disruptions of the DMN in a fashion consistent with neuromodulatory function. I begin with this simultaneously data- and hypothesis-driven analysis due to the clearcut implications from the literature, and to establish whether resting-state fMRI can provide preliminary translational bridges via *in vivo* biomarkers of dopaminergic system dysfunction in ASCs.

Chapter III elaborates on findings from the previous chapter, and takes considerations of VTA function into the context of this nucleus' interplay with the anterior forebrain mesocircuit model, specifically its main node in the thalamus. Based upon dopamine's demonstrated relevance to conscious state in drug trials and preclinical experiments, the hypothesis here is that VTA connectivity with the thalamus might also be disrupted in DOC patients. Explicitly, on the basis of recent work by Fridman et al. (2019), I hypothesise that a preservation of VTA interplay with the thalamus might correspond to maintenance of a consciousness-relevant level of dopaminergic function for the mesocircuit, in turn likely associated with thalamic facilitation of the cortex and responsiveness in DOC patients. Taking sum of Chapter II and III, I propose an extension of the mesocircuit model that includes influences of dopaminergic neuromodulation as an explicit contributor to conscious state.

Chapter IV further advances the idea of monoaminergic dysfunction for ASCs, by assessing how the psychedelic LSD acutely affects the serotonergic raphe nuclei. Utilising the same framework as previous chapters in this thesis, here I hypothesise that the strong serotonergic effects of psychedelics might selectively affect raphe functionality in a way that is associated with both established network-level and experiential phenomena of psychedelic ASCs. Given the clearcut pharmacological targets of LSD, I also introduce the usage of serotonin receptor PET maps to resolve a neurobiologically plausible mechanism for connectivity effects on the raphe. Finally, I suggest that the powerful

dysregulation of acute functionality of the raphe might be involved in a post-acute 're-setting' of raphe functionality that could partially underpin therapeutic effects of psychedelics.

In Chapter V, I assess the sub- and post-acute effects of the psychedelic psilocybin on the raphe nuclei, and whether these might be consistent with such a raphe 'reset' and associated with its anti-depressant properties. Specifically, I take a clinico-translational view that assesses raphe connectivity's efficacy-monitoring and diagnostic utility in depression treatment with psilocybin (and *versus* a classical serotonin reuptake inhibitor; escitalopram). In line with suggestions from Chapter IV, the working hypothesis here is that because depression is characterised by a serotonergic deficit that raphe functionality might be impaired in this pathology – but that psilocybin might be able to alleviate related connectivity and/or activity deficits. Finally, I take sum of Chapters IV and V to propose a refined preliminary model of psychedelic action across acute, sub- and post-acute phases that explicitly incorporates the serotonergic brainstem source nuclei as contributors to therapeutic effects.[16]

In **Chapter VII**, I discuss the conclusions that can be drawn from these pieces of experimental work.

[16] In an **Appendix Experimental Chapter VI**, I explore the utility of the brainstem nuclei connectivity perspective in a timely disease area with wide-ranging brainstem and arousal implications: Long-COVID. As this is however not strictly (though still according to some, such as original Plum & Posner's, 2003 criteria) classifiable as an ASC, this is provided as an Appendix Experimental Chapter, which has a self-contained discussion. This chapter introduces a metric aimed at quantifying individual participant impairments in nucleic connectivity against normative data, and an approach to quantify individual transmitter system contributions to Long-COVID symptoms. See the COVID research impact statement of this thesis for reference.

Chapter II

2.1 Preface and Overview

In this first experimental chapter, I seek to establish whether the multitude of reports on dopamine and its source nucleus in preclinical animal experiments and in emerging clinical work in DOC have a neuroimaging manifestation in impaired functional connectivity of the human VTA. I assessed this in separate cohorts involving pharmacological (propofol sedation) and pathological perturbation of consciousness (i.e. DOC) to delineate whether these different ASCs share a common connectivity impairment of the VTA, which might subserve widely-reported network-level impairments. This chapter thus provides an initial test of transmitter-specific brainstem connectivity analyses for the purposes of providing translational bridges between preclinical and clinical insights aiming for more mechanistic frameworks for ASCs.

The contents of this chapter have been published as:

Spindler et al. Dopaminergic brainstem disconnection is common to pharmacological and pathological consciousness perturbation. *Proceedings of the National Academy of Sciences (PNAS) 118*(30).

A team of researchers and clinicians at the University of Cambridge and the Wolfson Brain Imaging Centre (WBIC) performed the data collection, specifically Manktelow A.E., Finoia P., Sahakian B.J., Williams G.B., Allanson J., Pickard J.D., Menon D.K., Stamatakis E.A.. I performed all data analyses myself and wrote the text myself, with revision from all co-authors.

Dopaminergic Ventral Tegmental Area Connectivity is Disrupted in both Pharmacological and Pathological Consciousness Perturbation

2.1.1 Summary

Clinical research into consciousness has long focused on cortical macroscopic networks and their disruption in pathological or pharmacological consciousness perturbation. In both disorders of consciousness (DoC) and anaesthesia, these cortico-centric perspectives have shown diagnostic utility – but have remained unable to characterise which neurochemical systems may fundamentally underpin consciousness alterations and the associated large-scale network impairments. Instead, preclinical experiments in non-human animals have long implicated the dopaminergic ventral tegmental area (VTA) in the brainstem. Although small-scale and randomized-controlled trials in DoC have reported efficacy of dopaminergic drugs, and thus equally point to a dopaminergic mechanism, the VTA has not been studied in human perturbed consciousness. To bridge this translational gap between preclinical subcortical and clinical cortico-centric perspectives, in this chapter I assessed functional connectivity changes of the histologically-characterised VTA from the HAAN atlas, using fMRI recordings of pharmacologically- (propofol sedation, n=24) and pathologically-perturbed consciousness (DoC patients n=22). Both cohorts showed VTA disconnection from the precuneus and posterior cingulate (PCu/PCC), a main node of the default mode network (DMN), widely implicated in altered consciousness. Strikingly, across both cohorts, the stronger VTA-PCu/PCC connectivity was, the more the PCu/PCC functional connectome resembled its awake configuration, suggesting that disruption of VTA-mediated neuromodulation might underpin commonly observed DMN. In line with this assertion, a connectivity increase towards healthy control levels was observed only in those DoC patients who behaviourally improved at follow-up assessment. To test whether the VTA-PCu/PCC connectivity can be affected by a dopaminergic agonist, I also demonstrated that methylphenidate significantly increased this VTA-PCu/PCC connectivity in a separate cohort without DoC. Together, I argue that these results characterise an *in vivo* dopaminergic connectivity deficit common to reversible and chronic consciousness perturbation which bridges preclinical and clinical work, through allowing the association of dopaminergic VTA function with the widely-established macroscopic network alterations in anaesthesia and DoC.

2.1.2 Introduction

Delineating the neural underpinnings of perturbed consciousness is of basic scientific and clinical importance, both to understand its reversible suppression during sedation/anaesthesia (Mashour & Avidan, 2015), and to allow informed options to be formulated for patients with chronic disorders of consciousness (DoC)(di Perri et al., 2014). Striking parallels between these pharmacological and pathological perturbed states of consciousness have been characterized using human neuroimaging, which converge in particular on the DMN. This prominent large-scale network progressively loses functional connectivity integrity with increasing severity in DoC (di Perri et al., 2016; Vanhaudenhuyse et al., 2010), and also with increasing depth of anaesthesia (Guldenmund et al., 2017; Liu et al., 2015). Key nodes of the DMN in the posterior cingulate cortex (PCC) and precuneus (Stamatakis et al., 2010; Stender et al., 2015) show disrupted brain-wide functional connectivity, associated with loss of consciousness or severity of consciousness impairment across both pharmacologically- and pathologically-perturbed consciousness (Luppi et al., 2019). These macroscopic phenomena captured with human neuroimaging have demonstrated some diagnostic and prognostic value for DoC patients, and form empirical bases for contemporary theories of consciousness (Carhart-Harris, 2018; Demertzi et al., 2019; di Perri et al., 2016; Threlkeld et al., 2018; Tononi et al., 2016). However, these cortico-centric perspectives have remained inherently unable to address whether specific neurochemical systems may mediate perturbed consciousness and the associated macroscopic network changes. The identification of these neurochemical drivers of consciousness is paramount to understanding amenable therapeutic targets in DoC and anaesthetic mechanisms – and as such for the formulation of an integrated clinical account of consciousness.

To resolve this neurochemical dimension, preclinical work has – based on converging findings across a wide range of experimental animal work utilising anaesthetic drugs and lesion approaches – focused on the brainstem neurotransmitter nuclei. The transmitter systems studied in this context range from orexin and histamine, all the way to acetylcholine and the biogenic amines (serotonin, noradrenaline and dopamine; Brown et al., 2010). Among them, in particular the dopaminergic system has emerged

as a candidate neurochemical driver of consciousness, due to its consistent implication in both preclinical animal studies and clinical DoC contexts. Preclinically, Palmiter and colleagues demonstrated that dopamine-deficient mice show a phenotype they coined "behaviourally unconscious", reminiscent of sedative/vegetative states – but retroviral restoration of dopaminergic signalling reverses these behavioural deficits (Palmiter, 2011). The source of the relevant dopaminergic signalling was subsequently identified to be the ventral tegmental area (VTA), the main dopaminergic brainstem nucleus: Both optogenetic (Eban-Rothschild et al., 2016) and pharmacological VTA activation (Oishi et al., 2017) acutely promote wakefulness in rodents. Critically, electrical, optogenetic (Taylor et al., 2016) and even non-invasive ultrasound stimulation of the VTA (but not substantia nigra) can reverse the sedative effects of propofol (Solt et al., 2014; Li et al., 2022) – whereas lesions to the VTA lengthen recovery times following propofol anaesthesia in rodents (Zhou et al., 2015). Even methylphenidate-induced reversal of propofol anaesthesia in rats is suggested to act primarily *via* a dopamine-dependent mechanism (Kenny et al., 2015; Solt et al., 2011). Altogether, these convergent preclinical findings implicate dopaminergic signalling from the VTA as a tonic regulator of wakefulness, implicated in consciousness disruption.

While equivalent experiments cannot be carried out in humans due to their invasiveness, the relevance of dopamine has equally been revealed in clinical settings through reports of beneficial effects of various dopaminergic agonists in DoC patients. These drugs include levodopa (Matsuda et al., 2005), bromocriptine (Passler & Riggs, 2001), methylphenidate (Martin & Whyte, 2007), and amantadine (Giacino et al., 2012; Lehnerer et al., 2017); with further promising results from past and ongoing small-scale trials of apomorphine (Fridman et al., 2010; Sanz et al., 2021), and madopar (Saleh et al., 2022). The corresponding and long-standing idea of dopaminergic dysfunction in DoC is corroborated by a recent study which used 11C-raclopride PET imaging with concomitant bolus administrations of dopaminergic drugs to resolve that a presynaptic dopamine release deficit is found in MCS patients (Schiff, 2010; Fridman et al., 2019). Despite this clear implication of the dopaminergic system in clinical research, which parallels the preclinical implications of dopamine, work in humans has left unanswered whether pathological and pharmacological consciousness perturbations may arise due to

impaired function of the main dopaminergic nucleus, the VTA. Consequently, the characterization of VTA function in humans with consciousness perturbations holds critical translational potential, and could strongly enhance ongoing dopaminergic drug trials.

Explicitly, dysfunction of the VTA is a key candidate to underpin macroscopic network alterations consistently observed in states of lowered consciousness: as dopamine is a neuromodulator (Avery & Krichmar, 2017; Marder, 2012; Shine et al., 2019). As laid out in the Introduction (Chapter I), neuromodulation is the process by which diffusely and widely released non-classical transmitters, such as dopamine, can alter intrinsic electrochemical and synaptic properties of neurons (Marder, 2012) and thereby change input-output relationships at all scales of brain organization (Shine et al., 2019; van den Brink et al., 2019). This allows the finite number of anatomical connections in the brain to integrate into diverse meso- and macroscopic functional configurations (Marder, 2012; Shine et al., 2019). As dopamine is diffusely released from the VTA throughout the cortex (both through volume transmission and synaptic connections; Avery & Krichmar, 2017), dopaminergic neuromodulation is a strong candidate to mediate macroscopic network-level effects. This has been demonstrated for catecholaminergic manipulation more broadly in healthy individuals using fMRI (van den Brink et al., 2016; Shine et al., 2018), and is corroborated specifically for dopamine by the observation that posteromedial D2/D3 receptor occupancy measured with [18F]-fallypride PET is associated with whole-brain DMN integrity in healthy individuals (Nagano-Saito et al., 2017). Critically, these dopaminergic effects have not been tested in pharmacological and pathological consciousness perturbation despite the many dopamine implications, nor have they been associated with the dopaminergic source nucleus, the VTA. Conversely, rather than representing the loss of a crude “activating signal” (Satpute et al., 2018), VTA dysfunction could reflect altered neuromodulatory environments, which may precipitate neural phenomena such as DMN disintegration in perturbed consciousness (Satpute et al., 2019). Indeed, very recent work has demonstrated that the VTA is the subcortical node with the strongest connectivity to the DMN (Li et al., 2022). As such, delineating how VTA functional connectivity might be altered in ASCs holds unique potential to translationally scale-up whether VTA functional impairment as revealed in animal

experiments might relate to the consistently replicated *in vivo* human neuroimaging findings of disintegrated cortical networks.

In this chapter, I consequently explore how this dopaminergic nucleus behaves in perturbed consciousness in rs-fMRI data from healthy volunteers undergoing propofol sedation (n=24) and patients with chronic disorders of consciousness (DoC, n=22), using the anatomically- and histologically-validated VTA region-of-interest from the HAAN atlas (Edlow et al., 2012). Explicitly, I hypothesized (i) that alterations in VTA functional connectivity would occur across both reversible and pathological perturbation of consciousness, (ii) that these alterations should relate to whole-brain connectivity, information content changes and behaviour / outcome, and (iii) that VTA functional connectivity should be affected by a dopaminergic agonist.

2.2 Materials and Methods

Participants and Data Acquisition

20 healthy controls and 23 adults who were classified as in 'unresponsive wakefulness syndrome' (UWS) (n=9) or 'minimally conscious state' (MCS) (n=14) in line with current clinical guidelines, were scanned using a Magnetom 3T Tim-Trio (Siemens Healthcare, Erlangen, Germany) at the Wolfson Brain Imaging Centre, Addenbrooke's Hospital, Cambridge. Structural T1-weighted acquisitions were made using a fast MPRAGE sequence (TR=2300ms, TE=2.47ms, 150 volumes at 1x1x1mm^2 resolution). Functional resting-state scans used an echo-planar interleaved descending sequence consisting of 32 slices (TR=2000ms, TI=900ms, TE=30ms, flip angle=78°, 3x3x3.75mm^2 resolution), with a scan duration of 10 minutes. These 23 DoC patients were selected from a larger overarching dataset (n=71). These were all recruited from specialised long-term care centres. Patients required a DOC diagnosis, written informed consent of participation from their legal representative/surrogate decision maker, and capability of being transported to Addenbrooke's Hospital (Cambridge UK), to be invited to this study. Exclusion criteria included any medical condition that made participation unsafe (decision made by clinical personnel blinded to study aims) or any unsuitability for the MRI scanner environment (e.g. non-MRI-safe implants), significant pre-

existing mental health problems, or insufficient English premorbid language ability. Patients spent a total of five days (including arrival and departure days) at Addenbrooke's Hospital. After admission, each patient underwent clinical and neuroimaging testing. Patients were not sedated at time of scan. Coma recovery scale-revised (CRS-R) assessments were recorded at least once on the day of scanning, with periodic additional assessments on the remaining days of admission. Some of these patients enrolled in a larger ongoing observational follow-up study.

The subset of n=23 was selected for its suitability for brainstem connectivity analysis in the present study, based on strict inclusion criteria: (1) a lack of large focal brain- (i.e. more than 1/3 of one hemisphere) and in particular brainstem damage as assessed by a neuroanatomical expert blinded to the patient's diagnosis, (2) excessive head motion during resting state scanning (i.e. greater than 3mm in translation and/or 3 degrees in rotation), and (3) failure of segmentation and normalization during preprocessing. From this selection, one DoC participant was excluded after preprocessing, due to unsuccessful image co-registration. There were no significant differences in age between the healthy control cohort (35±11.448) and DoC patients (39.4±16.5) ($t(37.506)=-1.022$, $p=0.313$). Among the subset of 22 patients, seven had follow-up rs-fMRI scans with Coma Recovery Scale Revised (CRS-R) assessments (see Appendix, Table CII.M).

All clinical investigations were conducted in accordance with the Declaration of Helsinki and all relevant ethical guidelines. Ethical approval for testing patients was provided by the National Research Ethics Service (National Health Service, UK; LREC reference 99/391).

A subset of the participants who underwent propofol sedation were reported by Adapa and colleagues who describe acquisition and sedation protocols in detail (Adapa et al., 2013). Briefly, in total twenty-six healthy volunteers without history of neurological disorders (11 male), with mean age of 34.2 (range: 19–52) were briefed on the procedures and potential side effects of propofol sedation. Their scans were performed at the Wolfson Brain Imaging Centre, Cambridge UK on a Magnetom Tim Trio 3T (Siemens Healthcare, Erlangen, Germany). T1-weighted structural acquisitions were performed using a fast MPRAGE sequence (TR=2250ms, TE=2.99ms, TI=900ms, flip angle=9°, at 1x1x1.mm^2 resolution). Functional resting-state was acquired for 10 minutes using an EPI sequence in six separate sessions consisting of 32 interleaved, descending slices (TR=2000ms, TI=900ms, TE=30ms,

flip angle=78°, 3x3x3.75mm² resolution). Two participants had to be excluded, one due to unsuccessful scan co-registration and the other due to scans being distorted at brainstem level.

Sex	Age	Months post injury	Aetiology	Diagnosis	CRS-R	Arousal subscore
M	21	45	TBI	MCS+	11	2
M	46	48	TBI	UWS	7	2
M	57	14	TBI	MCS-	12	2
M	55	15	Anoxic	UWS	5	1
M	47	4	TBI	MCS	10	2
M	36	34	TBI	UWS	8	2
M	17	46	Anoxic	UWS	11	2
F	38	13	Anoxic	MCS	11	2
M	29	68	TBI	MCS+	10	2
M	23	4	TBI	MCS	7	2
F	70	11	TBI	MCS	9	2
F	30	6	Cerebral Bleed	MCS-	9	2
M	22	5	Anoxic	UWS	7	2
F	62	7	Anoxic	UWS	7	2
M	46	10	Anoxic	UWS	5	2
M	21	7	Anoxic	MCS	11	3
M	67	14	TBI	MCS-	11	2
M	46	23	TBI	UWS	9	2
F	55	6	Hypoxic	UWS	7	2
M	28	14	TBI	MCS	8	2
M	22	12	TBI	MCS+	10	2
F	28	8	ADEM	UWS	6	2

Table M1: Demographic information for patients with Disorders of Consciousness. UWS = Unresponsive Wakefulness Syndrome, MCS = Minimally Conscious State. Diagnoses were made considering the entire clinical record, instead of CRS-R alone. MCS− indicates that patients display visual fixation and pursuit, automatic motor reactions (e.g. scratching, pulling bed sheet) or localisation to noxious stimulation. MCS+ classification indicates that patients consistently and repeatedly, followed simple commands or intelligibly verbalised. Patients classified as MCS showed such behaviour but only intermittently . CRS-R is the highest score recorded by attending physician for the day of the scanning session. CRS-R scores were collected at least once on the day of scanning with periodic additional assessments on remaining visit days.

In the sedation experiments, the sessions of resting-state recordings corresponded to dosage levels of the anaesthetic agent propofol administered via target-controlled infusion (TCI) with an Alaris PK infusion pump (Carefusion, Basingstoke, United Kingdom). The levels recorded were: No sedation (Control; 0µg/ml), mild (Ramsey Level 2, 0.6µg/ml), moderate (Ramsey Level 3, 1.2µg/ml) and a recovery period (46). 10 minutes for equilibration of plasma levels was allowed after each administration. Then, 5-minute rs-fMRI scans were performed. The order of whether moderate or mild dosage was administered first was randomized to control for homeostatic attuning effects. Two trained anaesthesiologists were on site for all recordings, performing propofol administration and Ramsey Alertness/Sedation Scale assessment before and after each scan, monitoring all common physiological parameters using an MR-compatible monitor (Precesss, InVivo Corp., USA), observed participants in the scanner, and took blood samples (2x1ml) per condition for chromatographic analyses.

Following the resting state scan, at each sedation level a semantic judgement task was collected. The experimental procedure is also detailed by Adapa et al. (2013). Briefly, the task sessions were 5.5min in duration and made up of alternating 30s blocks of words and acoustically matched non-speech buzz or noise stimuli. Stimuli, in blocks of 8 followed by 6s interim-silences, were presented with stimulus onset asynchrony (SOA) of 3s in silent intervals between scans. Participants were instructed to identify with a button press whether heard words referred to living or non-living objects, and whether non-speech auditory stimuli were noise- or buzz-type. To assess explicit memory formation during each sedation levels, subjects were upon full recovery presented with familiar (i.e. previously heard; targets/signal) and unfamiliar (not previously heard; distractor/noise) items. Using the signal detection theory (SDT) model, consisting of two normal distributions, one representing signal and one representing noise, we calculated the most commonly used measure of sensitivity d' (d prime), as an approximation of cognitive conscious access (Goldmund et al., 2019). d' is the standardised difference between the means of signal and noise distribution, i.e. the higher d' is, the more readily signal is detected as the subject is better able to consciously distinguish old and new items. D' is used in correlations with VTA connectivity to ascertain the behavioural relevance of changes in

dopaminergic connectivity. Ethical approval was obtained from the Cambridgeshire 2 Regional Ethics Committee. Written informed consent was obtained prior to any experiments from all participants.

The TBI patients without disorders of consciousness who received methylphenidate were a subset (n=12, mean age 34.4±12.8) of patients from the larger dataset (n=15) which Manktelow et al. (2017), Moreno-Lopez et al. (2017), and Dorer et al. (2018) describe in detail. The sub-selection of n=12 was based on the same strict inclusion criteria set out for the DOC scans (see above), with scans from three patients not meeting these. These data were acquired using the same sequences as the DoC patients and their control cohort, but only for 5 minutes and 20 seconds (see above) on two different research visits separated by 2-4 weeks. TBI patients received 30mg MPh (30mg dose visually indistinguishable from lactose placebo) either on their first or second visit, randomized using a latin square design. Dosages were based on comparable doses used in previous studies in healthy participants as well as NICE guidelines for medication in adults (www.nice.org.uk). These patients received no other catecholaminergic/dopaminergic agents in the period between research visits. On the visit, 75 minutes were allowed after MPh administration, to ensure that peak plasma levels of MPh were reached. After these 75 minutes the volunteers completed an MRI scan which included task and resting-state fMRI as well as structural image acquisitions (see Manktelow et al., 2017). Written informed consent was obtained from all participants and/or legal surrogate decision makers prior to any experiments/scans.

Sex	Age	Months post injury	Aetiology	Lesion Description	GCS
M	27	25	TBI	Haemmorhagic contusions in bilateral frontal lobes	7
M	53	32	TBI	Right subarachnoid haemmorhage and subdural haematoma	14
M	49	27	TBI	Haemmorhagic contusion left lentiform nucleus	8
F	55	17	TBI	Subarachnoid haemmorhage in left frontoparietal cortex	12
M	29	14	TBI	Haemorrhagic contusions in left temporal lobe/basal ganglia/thalamus	5
M	19	32	TBI	Subarachnoid haemorrhage in left interpeduncular fossa	7
M	21	39	TBI	Multiple petechial haemorrhages, obliterated basal cisterns	3
M	36	11	TBI	Epidural haematoma right temporal lobe	6
M	26	25	TBI	Intraventricular haemorrhage	7
F	34	41	TBI	Intra-cerebral haemorrhage & right temporal/parietal contusions	NA
M	43	7	TBI	Right subarachnoid haemorrhage and subdural haematoma	10
F	21	9	TBI	Unavailable	NA

Table M2: Demographic information for TBI patients who received methylphenidate. Lesion diagnostic description was made by neurologist and/or neuroradiologist. When NA or unavailable, injury occurred abroad with detailed records unavailable. GCS = Glasgow Coma Scale score at time of admission.

Spatial and Temporal Preprocessing

Preprocessing for all scans was performed using the CONN functional connectivity toolbox (19c; running in MATLAB (2018b, The Mathwork, Inc. Natick, Massachusetts, USA).

For spatial preprocessing, functional images were first slice-time corrected, centred to (0,0,0) MNI coordinates, realigned to correct for movement, and were subjected to identification of outlier scans for scrubbing (ART-toolbox within CONN, Whitfield-Gabrieli et al., 2018). Following this, the structural image was co-registered to the mean functional image and then segmented and spatially normalized to the Montreal Neurological Institute (MNI-152) template. Functional images were then normalised to MNI-152 template based on parameters obtained from structural normalisation and were finally smoothed with a 6mm Gaussian kernel at full width half-maximum (FWHM).

Temporal preprocessing employed masks for white matter (WM) and cerebrospinal fluid (CSF) produced by the structural segmentation to regress out physiological noise in the BOLD signal as it can otherwise influence functional connectivity estimates. This method called CompCor (Y. Behzadi et al., 2007) regresses out the first five principal components of white matter and cerebrospinal fluid

signals, movement parameters obtained from realignment, and their first-order derivatives – alleviating the need for global signal regression (GSR), which can equally perturb FC estimates (Almgren et al., 2020). Although it has been argued that the global signal can contain non-neuronal noise (Murphy & Fox, 2017), removing the global signal alters the global topography of functional connectivity (a key variable of interest in the present study), by mathematically mandating that 50% of correlations will have a negative sign (Murphy & Fox, 2017). Crucially, the global signal was also recently shown to contain cognition- and behaviourally relevant information (Li et al., 2019) and also information about the likely state of consciousness of a participant, across physiological, pharmacological and pathological perturbations (Tanabe et al., 2020). For these convergent reasons, and in line with previous work, I elected not to remove the global signal, instead opting for the rigorous denoising procedure offered by anatomical CompCor. Additionally, CompCor scrubs outlier scans (i.e. identifies and regresses out timepoints within the scan with framewise displacement and/or BOLD signal increases over a given threshold). Thereafter, our data were linearly detrended and filtered using a high-pass filter of 0.008 Hz.

Ventral Tegmental Area Primary Functional Connectivity analyses

The VTA region-of-interest used is from the "Harvard Ascending Arousal Network Atlas" (HAAN; Edlow et al., 2012). To the best of my knowledge it is the only publicly-available histologically-characterised and thus dopamine-specific ROI. Its faithful co-registration to the anatomical and functional scans was extensively assessed visually.

Functional connectivity – the temporal correlation of regional timeseries – is conceptualised to represent information sharing and dynamic cooperation (Friston et al., 2013), identifying spatially segregated functional units at global scales. Here, FC was calculated using CONN, in form of seed-to-voxel analyses for assessing effects in the whole brain. Temporal correlations for the dopaminergic VTA seed (and in secondary analyses for its 'downstream' targets) were computed from unsmoothed data (as per the CONN standard procedure to avoid spatial smoothing across the small brainstem nuclei) for all other voxels in the brain using a General Linear Model (GLM). The functional connectivity analyses produced seed-to-voxel parameter estimate images which were entered into

population-level analyses; using independent sample t-tests to compute differences from healthy controls for DOC patients and paired-sample t-tests for differences from awake state for the propofol dataset. We report results thresholded at voxel level $p<0.005$ (uncorrected) and cluster level $p<0.05$ (FWE-corrected for multiple comparisons), for a valid voxel-wise inference approach (Eklund et al., 2018).

Correlations of VTA connectivity with 'downstream' whole-brain connectivity of targets and behavioural variables

The clusters of altered VTA connectivity that were revealed in primary functional connectivity analyses were entered as seeds into subsequent seed-to-voxel analyses in their respective cohorts, to compute differences from awake controls. This was to reveal altered 'downstream' connectivities of VTA-targets. All connectivity clusters were extracted as binary masks. To test our hypothesis that VTA connectivity may have neuromodulatory effects on its targets (and resultant network architectures), we extracted eigenvalues of connectivity-strength (β-estimates from GLM) per participant per condition for both VTA ⇌ primary-target connectivity and target ⇌ 'downstream' cluster connectivity. To approximate whether there is a neuromodulatory relationship between VTA ⇌ target connectivity alterations and target ⇌ 'downstream' connectivity, we assessed how these two functional connectivity strengths covaried for patients and sedated volunteers respectively.

All correlations were performed using R-Studio (R Core Team, 2014), with ggplot2 (Wickham, 2016) for DOC patients and for propofol experiments with the rmcorr-toolbox using analysis of covariance (ANCOVA) to account for non-independence among the repeated observations (awake, mild and moderate sedation, and recovery) by statistically adjusting for inter-individual variability. For further statistical stringency, the "rmcorr" correlations were bootstrapped (n=5000) to conform to 95% confidence intervals, and correlations for DoC patients used Spearman's rank. As behavioural measures, we included the sensitivity index d' (d-prime) for the propofol sedation dataset and for patients with DoC the clinical bedside assessments of highest coma recovery scale revised (CRS-R; score and highest CRS-R arousal subscore at time of scan (Giacino et al. 2014). Based on CRS-R

improvement and clinical assessment I classified which DoC patients who had follow-up rs-fMRI scans, had respectively improved or deteriorated and calculated their VTA-PCu/PCC change (see Appendix CI, Table CI.1).

BOLD signal complexity as Effort to Compress (ETC)

Given the previous associations of signal complexity with conscious state in both pathological and pharmacological consciousness perturbation in the literature, I also assessed the Kolmogorov complexity of BOLD timeseries, using the measure of Effort-To-Compress (ETC), which relies on the lossless compression algorithm Non-Sequential Recursive Pair Substitution (NSRPS; (Ebeling and Jiménez-Montaño, 1980). ETC has been shown to outperform both Shannon entropy and the popular Lempel-Ziv compression algorithm when applied to short and noisy timeseries from dynamical systems (Nagaraj *et al.*, 2013). The timeseries used for ETC calculation were extracted as the average signal of voxels contained in ROIs of a finer parcellation of the cortex (200 parcels; Schaefer *et al.*, 2018) and subcortex (32 parcels; Tian *et al.*, 2020) to ensure homogenous ROI distribution. A value of ETC_{norm} (Nagaraj et al., 2013; more detailed methods in Appendix CII) was derived for each ROI's BOLD signal timeseries, per subject per condition, which was then averaged per person to obtain a whole-brain ETC value. For the propofol data, paired sample t-tests were employed, whereas two-sample t-tests were used to compare DoC patients with awake healthy controls in terms of whole-brain ETC measures. Finally, I performed correlations to assess whether levels of complexity (quantified by ETC) for the whole brain covaried with the level of VTA-target connectivity in DoC patients and in moderately sedated volunteers using the ggplot2 toolbox in RStudio.

2.3 Results

2.3.1 The VTA loses precuneus and posterior cingulate connectivity during propofol sedation and in Disorders of Consciousness

As involvement of the VTA in sedative mechanisms has been repeatedly demonstrated in preclinical models (Brown et al., 2011), I began by assessing the VTA's functional connectomic changes during propofol sedation. In awake volunteers, i.e. at baseline, the VTA showed functional connectivity to the precuneus, posterior cingulate cortex (PCC), brainstem, cerebellum, insula and hippocampus (Fig.6a, Table 1), consistent with previous reports by Bär and colleagues (2016). However, upon propofol administration, whole-brain contrasts between awake *vs.* mild (Fig.6b) and awake *vs.* moderate sedation (Fig.6c) showed that the VTA lost connectivity exclusively with a cluster in the precuneus and PCC. During recovery, when participants began to emerge from their sedated state, the connectivity of the VTA to these areas re-emerged (Fig.6d). Repeated-measures correlations showed that the connectivity strength of the VTA to the precuneus/PCC cluster was negatively correlated with participants' plasma propofol concentrations across all experimental conditions ($r=-0.53$ $p<0.001$; Fig.6e & 6f).

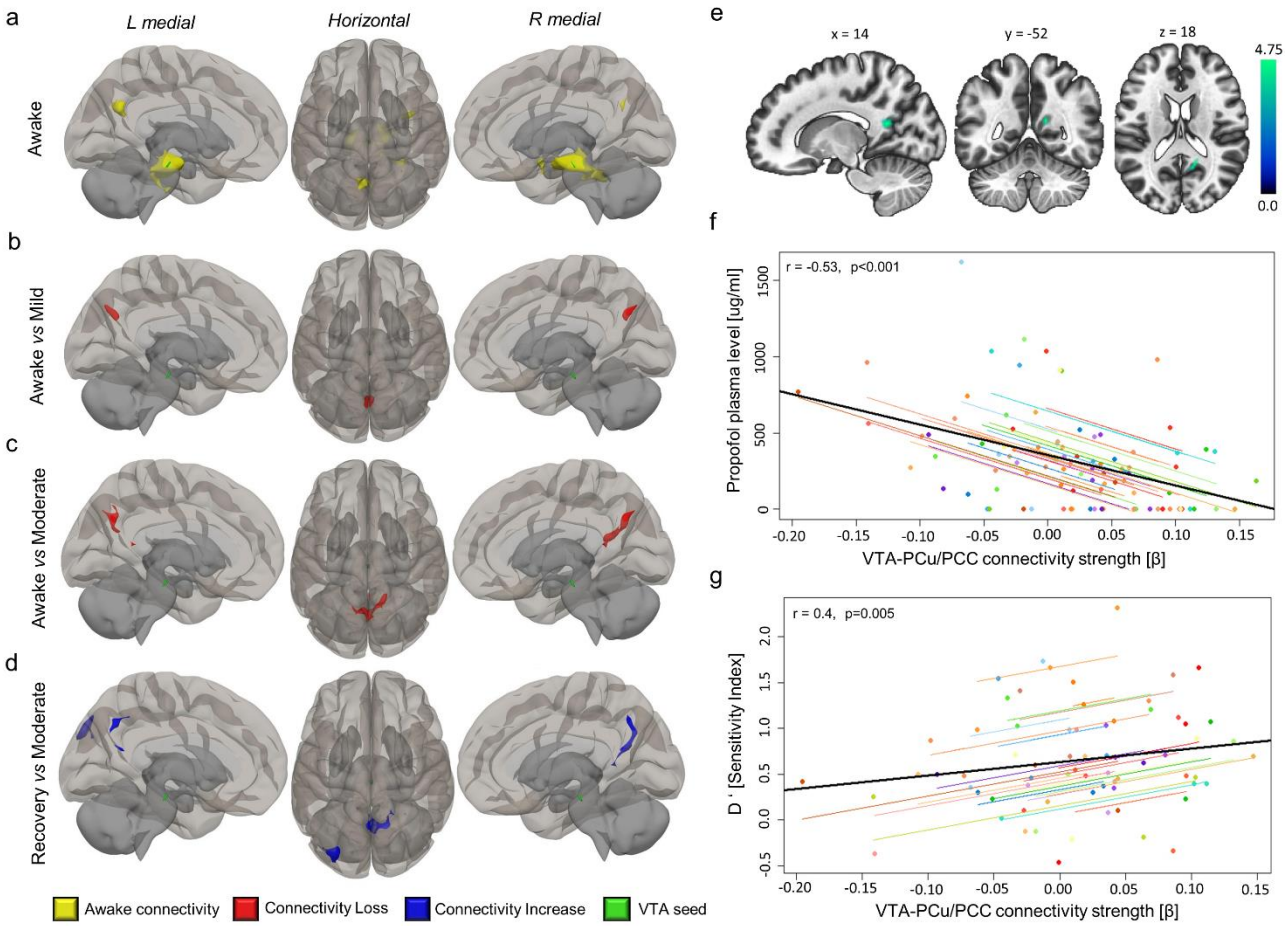


Fig. 6: Ventral tegmental area (VTA) disconnects step-wise, reversibly and dose-dependently from precuneus/PCC in propofol sedation. (a) In awake participants, the VTA ROI showed resting state functional connectivity to precuneus and posterior cingulate cortex, as well as hippocampal, insular, and cerebellar areas (see Table 1). Under mild (b) and moderate (c) propofol, the VTA showed a stepwise loss of connectivity specifically with precuneus and posterior cingulate. (d) In recovery, connectivity to precuneus and PCC was regained (blue). (e) Anatomical slice display, centred on peak MNI coordinates, of cluster from (c) which was used for extraction of subject-specific VTA-to-precuneus/PCC connectivity values. (f) The higher the effective propofol dosage was in participants' plasma, the more disconnected the VTA was from the precuneus/PCC cluster (i.e. lower connectivity) across all experimental conditions. (g) The strength of this connectivity was positively predictive of how reliably participants were able to discriminate novel and familiar stimuli from a semantic processing task performed at each sedation level, measured as the sensitivity index D'. Coloured dots in graphs are individual participants. Black line is overall regression line without participant variable. Statistical thresholds for connectivity changes were voxel-level $p<0.005$ (uncorrected) and cluster-level $p<0.05$ (FWE-corrected). Brains are in neurological orientation, i.e. 'L' is left.

The healthy control group used for comparisons with the DoC patients showed equivalent awake connectivity of the VTA, but displayed an additional connectivity cluster in the thalamus (Fig.7a). When DoC patients were compared to controls, the VTA showed reductions in functional connectivity with the precuneus and PCC, resembling the changes seen in propofol sedation (Fig.7b; Fig.6b & Fig.6c). In addition, we also found reductions in connectivity of the DoC patients' VTA to

mediofrontal cortex (another hub of the DMN; Buckner et al., 2019) and some increases in functional connectivity to subcortical regions and hippocampus (Fig.7b, Table 1). The clusters of VTA disconnection that were observed in both propofol sedation and DoC cohorts overlapped spatially in the precuneus and posterior cingulate (clusters hereafter: PCu/PCC) across both cohorts.

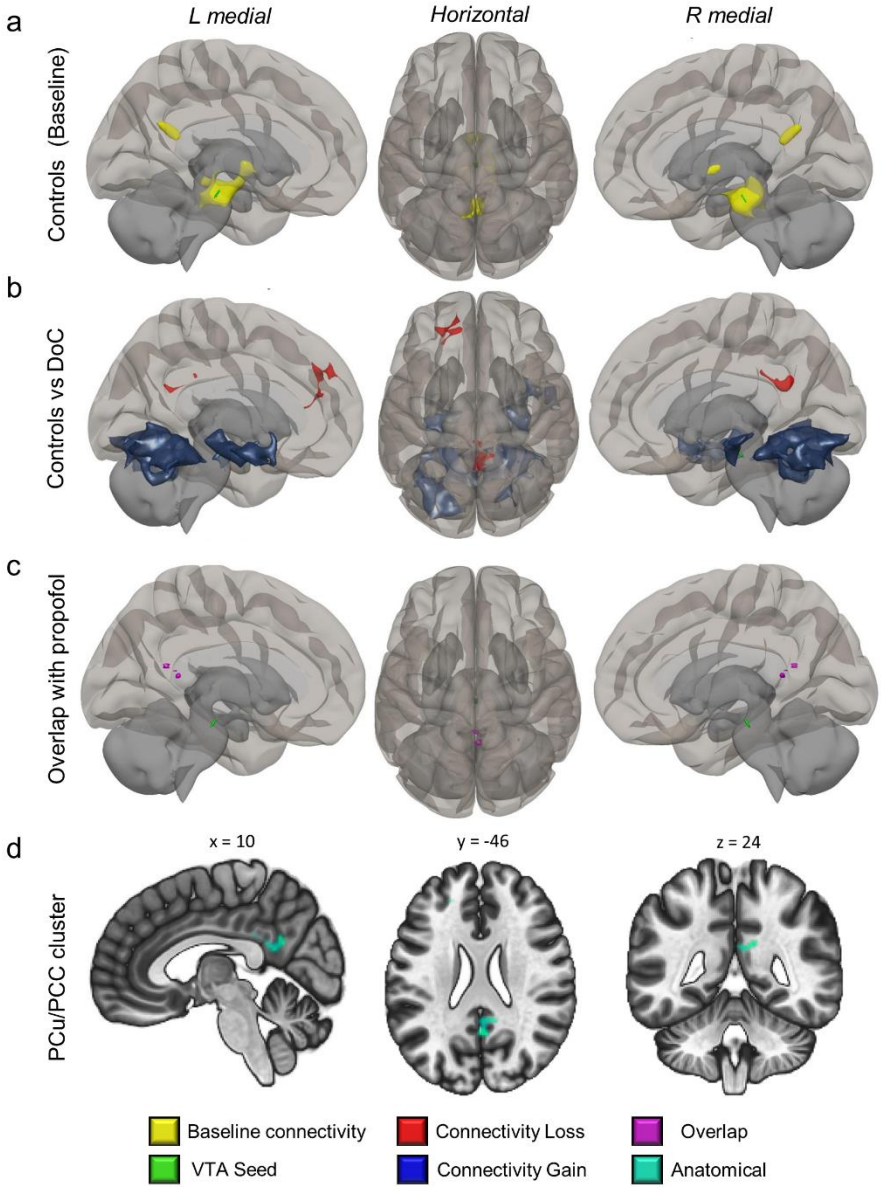


Fig. 7: Loss of VTA connectivity to precuneus and posterior cingulate in disorders of consciousness. (a) In the control cohort used for comparisons with the DoC patients, the VTA also showed precuneus and posterior cingulate connectivity, with an additional cluster in thalamus. (b) In a contrast of patients to these awake controls, VTA connectivity losses were observed with precuneal/PCC and mediofrontal regions, with concomitant subcortical gains (see Table 1). (c) At a lowered voxel threshold, the posterior disconnection clusters from sedation and DoC datasets spatially overlapped.(d) Display of posterior region from (b) on

Table 1: **Ventral tegmental area (VTA) awake connectivity and connectivity changes in Propofol sedation and Disorders of Consciousness compared to respective control conditions.** CONN atlas labels, peak MNI coordinates, cluster extent and FWE-corrected p-values are reported. DMN regions in **bold**. Awake connectivity was thresholded at p<0.001 voxel-level (uncorrected), and contrasts at p<0.005 (uncorrected) with p<0.05 cluster-level (FWE-corrected). ↓Loss corresponds to decreased functional connectivity, and ↑Gain to increased connectivity in comparison to control group.

Cohort	**Condition/ contrast**	***Δ connectivity change***	***Anatomical regions (CONN atlas)***	***Peak MNI Coordinates***	***Cluster size***	***Cluster p (FWE-corrected)***
Propofol Sedation	**Awake Volunteers**	-	Brainstem, Cereb45 l+r, Cereb6 r, Hippocampus l+r, pPaHC l+r, pTFusC r, TOFusC r, aPaHC l+r, Amygdala r	+00 -24 -18	1975	0.000
			Precuneus, PC	-04 -64 +26	192	0.000
			IC r, Amygdala r	+32 +00 -20	111	0.003
	Awake > Moderate (RL3)	↓Loss	**Precuneus, PC**	+14 -52 +18	424	0.000
	Awake > Mild (RL2)	↓Loss	**Precuneus**	+00 -68 +32	169	0.035
	Recovery > Moderate (RL3)	↑Gain	**Precuneus, PC**	+00 -56 +20	317	0.000
			sLOC l, OP l,	-36 -84 +38	213	0.009
Disorders of Consciousness	**Healthy Controls**	-	Brainstem, Thalamus l+r, pPaHC l+r, Cereb3 l+r, Cereb45 l	-02 -22 -18	1226	0.000
			Precuneus, **PC**	+04 -46 +18	268	0.000
	Awake > DOC	↓Loss	**FP** l, PaCig l	-22 +42 +22	235	0.036
			Precuneus, PC	+10 -46 +24	223	0.046
			LG l+r, OFusG l, Cereb1,45,6,8,9 l+r, TOFusC l+r, Ver45,6,7,10, OFusG r, pPaHC	-34 -80 -12	5204	0.000
	DOC > Awake	↑Gain	Hippocampus r, TP r, Amygdala r, PP r, aSTG r, pMTG r, IC r, aMTG r	+52 -02 -18	1149	0.000
			Hippocampus l, Brainstem	-14 -18 -18	523	0.000

anatomical slices centred on peak MNI coordinates. This cluster in (d) was used for connectivity strength extraction. Images are in neurological orientation, i.e. 'L' is left.

2.3.2 VTA-precuneus/PCC connectivity is associated with relevant behaviour in propofol sedation and in DOC patients

To answer whether the VTA's connectivity loss to PCu/PCC was associated with behaviour, I extracted β-coefficients from the general linear model for VTA-PCu/PCC connectivity and correlated these with relevant behavioural measures for each cohort. For the propofol cohort, I tested whether stimuli presented for a semantic decision task during the awake and sedated states were correctly identified as familiar or novel after recovery from sedation (see Methods). The participants' ability to correctly classify stimuli as novel or familiar was quantified as the sensitivity index d' (d prime), a proxy for explicit memory formation with conscious cognitive access (Bonhomme et al., 2019). We

found that the d' score covaried positively with VTA-PCu/PCC connectivity during propofol-based perturbation of consciousness ($r=0.4$, $p<0.001$, Fig.6g).

Similar behavioural measurements in patients with DOC are impossible. As an approximation of whether VTA-PCu/PCC connectivity may be related to behaviour or outcome, correlations of CRS-R ($r=0.2$, $p=0.373$) and its arousal subscore ($r=0.41$, $p=0.066$) did not show significant relationships. However, I more closely examined the patients within the sample who had follow-up scans (n=7, mean elapsed time 509±131 days). Among this subsample, two patients improved in terms of CRS-R and remain alive, whereas the remaining five further deteriorated and subsequently deceased. Although the small subsample-size makes meaningful statistics impossible, it is relevant to report that the two alive and improved patients showed a re-emergence of positive VTA-PCu/PCC connectivity coupling at their second imaging assessment, improving from negative VTA-PCu/PCC connectivity recorded at the previous visit. In contrast, the patients who deteriorated (lower CRS-R scores) and deceased after the imaging follow-up, had persistent and/or worsened negative VTA-PCu/PCC connectivity.

2.3.3 VTA-precuneus/PCC connectivity strength is associated with precuneus/PCC-whole-brain connectome disintegration

The areas of disconnection observed for the VTA were in the PCu/PCC and displayed resting connectivity to key nodes of the DMN in whole-brain analyses in both control cohorts (see Appendix CII, Fig. CII.1, Table CII.1). As DMN intrinsic connectivity, and its functional relationships with other brain regions, are commonly altered during perturbations of consciousness, I next asked whether changes in this large scale network could be driven by altered VTA-PCu/PCC connectivity – possibly reflecting altered neuromodulation of the main DMN node in the PCu/PCC precluding correct functional circuit assembly (Marder, 2012). Because neuromodulation *per se* cannot be measured with any single-modality neuroimaging technique, I decided to approximate a VTA neuromodulatory relationship by different means. To this end, I examined whether VTA-PCu/PCC connectivity strength in perturbed consciousness covaried with brain-wide connectivity alterations between the

PCu/PCC cluster and other brain regions (within and outside the DMN) in perturbed consciousness (for full rendering of 'downstream' brain-wide connectivity changes, see Appendix CII, Fig. CII.2).

In the case of propofol sedation, the PCu/PCC cluster from which the VTA disconnected showed no reductions in connectivity with any part of the brain that reached significance. However, it showed significant increases in connectivity to areas that are not part of the canonical DMN (see Appendix CII, Fig.CII.2b, Table CII.2). In repeated measures correlations across all sedation conditions, PCu/PCC connectivity to these predominantly visual, non-DMN, areas was significantly negatively correlated with VTA-PCu/PCC connectivity strength (r= -0.37, p=0.001; Fig.8).

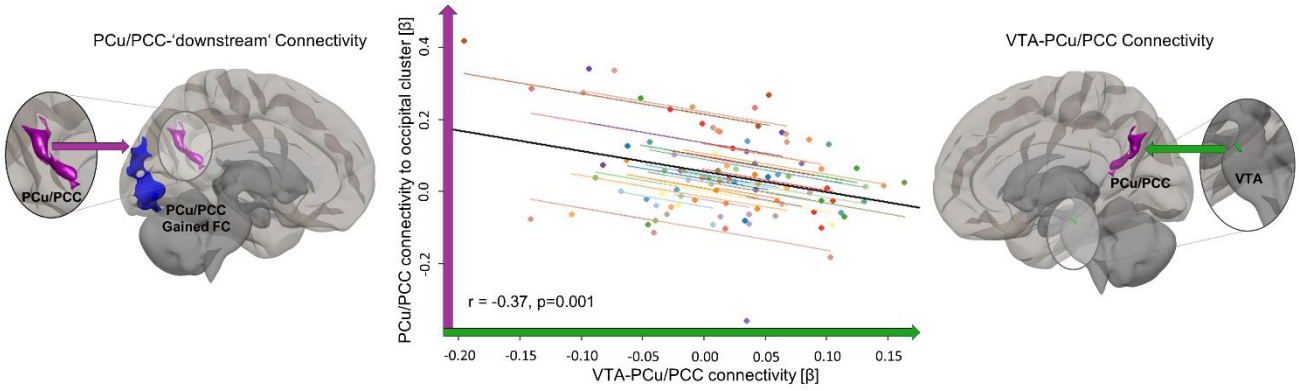


Fig. 8: VTA-PCu/PCC connectivity strength is associated with its PCu/PCC target's whole brain connectivity alteration in propofol sedation. Across all conditions in the propofol experiments, repeated measures correlations revealed that the stronger the connectivity (green arrow/axis) between the VTA (green ROI, magnified) and its PCu/PCC target (magenta ROI, magnified), the weaker the connection (magenta arrow/axis) between this PCu/PCC target and 'downstream' beyond-DMN occipital gains (blue cluster) was. 'Downstream' connectivity gains were characterised by using the PCu/PCC cluster originally identified in population-level contrasts of VTA connectivity (see Fig. 6c & Fig.6e) in new seed-to-voxel analyses (see Fig. S2b for 'downstream' seed-to-voxel analyses). Dot colour represents individual participant. Black line is overall regression line, ignoring the participant variable. All masks used for connectivity extraction were thresholded at voxel-level $p<0.005$ (uncorrected) and at cluster-level $p<0.05$ (FWE-corrected).

The PCu/PCC cluster in patients with DoC showed wide-ranging reductions in connectivity, predominantly with areas classically identified as DMN regions (Fig. 9a, red). Concomitantly, the PCu/PCC in the DoC patients also showed gains in connectivity to occipital regions (similar to changes seen with propofol sedation in healthy volunteers), with additional connectivity gains to sensorimotor, pre-central, postcentral and anterior cingulate cortices (Fig. 9b, blue). The strength of PCu/PCC connectivity to clusters with which it lost connectivity (red, Fig.9a) co-varied directly with

changes in VTA-PCu/PCC connectivity (r=0.5, p=0.017; red, Fig. 9a), indicating a potential modulatory role of VTA-PCu/PCC connectivity for intra-DMN connectivity integrity. The association of VTA-PCu/PCC connectivity with PCu/PCC connectivity to areas beyond the classical DMN did not reach significance in DoC patients (r=-0.35, p=0.11; Fig. 9b, blue). The exclusion of outliers in both correlations did not change the significance of either.

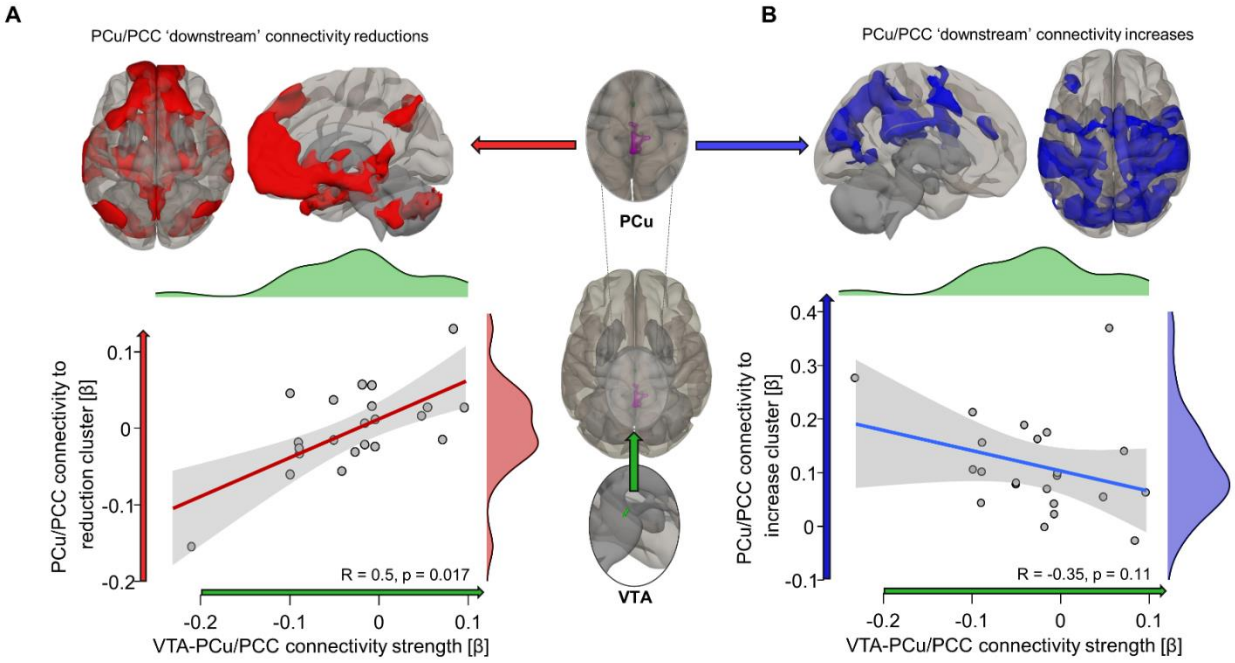


Fig. 9: VTA-PCu/PCC connectivity is associated with the strength of PCu/PCC connectivity to DMN areas in DoC patients. The VTA's connectivity strength to its PCu/PCC target (green arrow/axis) was positively associated with the PCu/PCC's own connectivity strength to 'downstream' DMN-centric areas with which it lost connectivity at population-level (red arrow/axis). Expressly, the more VTA-PCu/PCC connectivity was preserved for each patient, the more its PCu/PCC connectivity strength maintained a more awake-like DMN appearance. The negative association of VTA-PCu/PCC connectivity strength with PCu/PCC connectivity to cluster of increased connectivity (such as observed for propofol sedation) did not reach significance (blue arrow/axis). Correlations of connectivity strengths between respective seed and target masks used Spearman's rank. See Fig. S2a for 'downstream' seed-to-voxel analyses, from which masks used for β-value extraction were extracted at voxel-level $p<0.005$ (uncorrected) and at cluster-level $p<0.05$ (FWE-corrected) thresholds. 95% confidence intervals in grey.

2.3.4 Level of VTA-precuneus/PCC connectivity is associated with global BOLD signal complexity

The observation that PCu/PCC connectivity was associated with the level of VTA disconnection from the PCu/PCC corroborates that changes in dopaminergic neuromodulatory influence might impair PCu/PCC function. However, beyond DMN disintegration, also broad-scale reductions in BOLD signal complexity have consistently been associated with loss of consciousness, which some have suggested to possibly be underpinned by functionality of the precuneus/PCC (Luppi et al., 2019). Consequently, I hypothesized that global BOLD signal complexity may also be associated with VTA-PCu/PCC interplay, as altered neuromodulation of this cortical node might have global consequences. To test this, I assessed whether the strength of VTA-PCu/PCC connectivity may covary with global BOLD signal complexity, utilising a measure known as effort-to-compress (ETC), to estimate Kolmogorov complexity (a measure of unpredictability, or complexity) of the BOLD signal, for the whole brain (parcellated into 200 cortical and 32 subcortical regions; see Methods).

In both the DoC and propofol cohorts, significant decreases in average whole-brain complexity of the BOLD signal were observed when compared to awake control conditions (Fig.5, a.i & b.i). VTA-PCu/PCC connectivity strength was significantly associated with whole-brain ETC in both DoC patients (R=0.52, p=0.007, Fig.5a.ii), and the propofol cohort (R=0.35, p=0.046, Fig.5b.ii). These data suggest that dopaminergic neuromodulation may not just influence precuneal connectivity, but also global BOLD signal complexity. [17]

[17] In a secondary follow-up analysis in **Chapter III**, a multimodal average causal mediation model incorporates and demonstrates that VTA-PCu/PCC connectivity's effects on whole-brain ETC is mediated *via* its effects on PCu/PCC-DMN connectivity integrity in DOC patients.

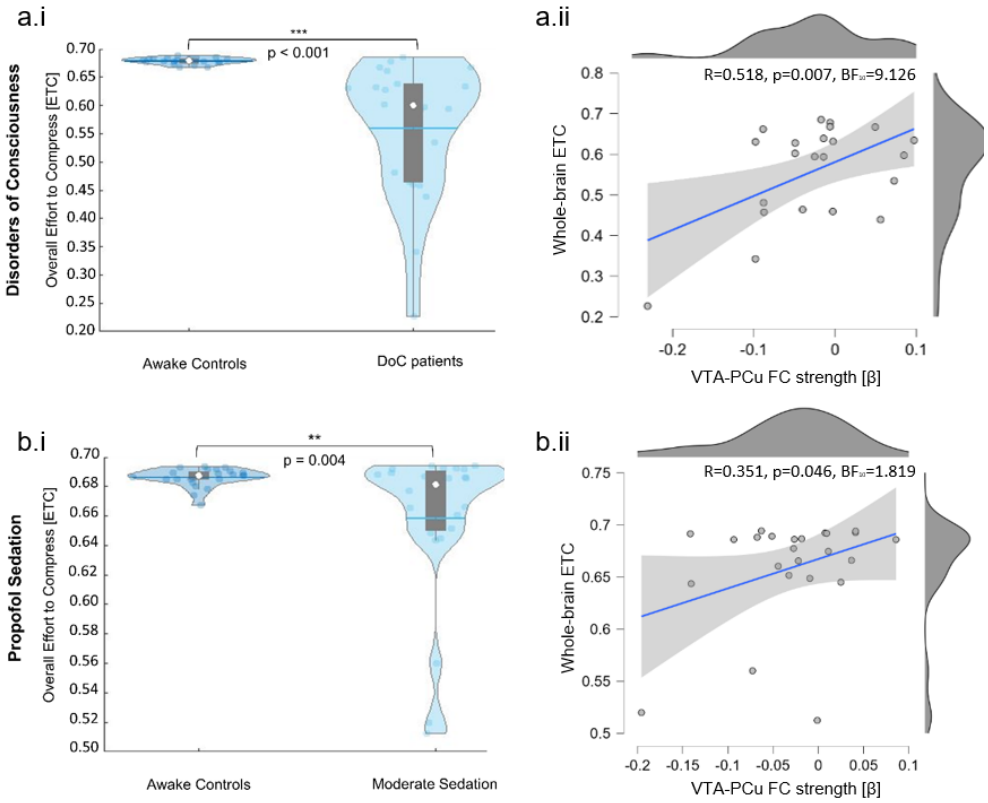


Fig. 10. Decreased whole-brain BOLD signal complexity is associated with strength of VTA-PCu/PCC connectivity. The whole-brain effort to Compress (ETC; complexity of BOLD signal timeseries) was significantly lowered in DoC patients (a.i) and sedated volunteers (b.i) compared to their respective control cohorts. The level of global ETC was significantly correlated with the strength of VTA-PCu/PCC connectivity in both cohorts (a.ii & b.ii) 95% confidence intervals in grey. Correlations were performed using the JASP toolbox interfaced with RStudio. FC = functional connectivity.

2.3.5 Methylphenidate increases VTA-PCu/PCC connectivity in a sample of traumatic brain injury patients without DOC

Finally, I hypothesized that if VTA-PCu/PCC connectivity is indeed a correlate of dopaminergic neuromodulation, it should be altered by a dopaminergic agonist. I tested this hypothesis in a separate cohort of traumatic brain injury/diffuse axonal injury patients (n=12) who did not have DOCs, but participated in rs-fMRI data collection in two separate sessions two to four weeks apart: once with a placebo, and once with administration of 30mg methylphenidate, a dopaminergic and noradrenergic agonist (see Methods). VTA connectivity to the cluster of PCu/PCC disconnection observed in DoC

patients (Fig.7) revealed that, as hypothesized, VTA-PCu/PCC connectivity was significantly higher ($t(11)$=-1.957, p=0.038) in the methylphenidate condition (M=0.065±0.018) compared to placebo administration (M=0.011±0.020; Fig.11).

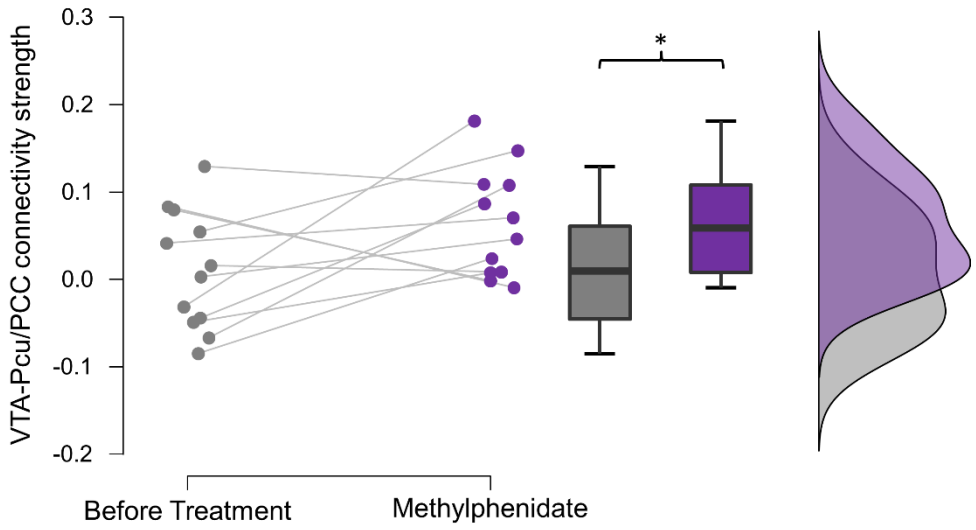


Fig. 11: Methylphenidate treatment significantly increases the level of VTA-PCu/PCC connectivity strength in a sample of traumatic brain injury patients. Note that the increase in connectivity was observed for all participants who had negative connectivity between the VTA and the PCu/PCC beforehand. * <0.05.

2.4 Discussion

When compared to healthy awake conditions, both pathological and pharmacological states of lowered consciousness demonstrate a substantial disruption of VTA connectivity to the PCu/PCC, a central node of the brain's default mode network. This *in vivo* evidence of a consciousness-relevant dopaminergic system dysfunction common to DoC patients and sedation is critically also associated with a loss of specificity in the PCu/PCC's whole-brain functional connectivity – the stronger VTA-PCu/PCC connectivity remained, the less the PCu/PCC's normal connectivity pattern was perturbed. This indicates that disrupted dopaminergic neuromodulation of this main DMN node may at least partially underpin the commonly-observed DMN disruptions and globally decreased BOLD complexity in human perturbed states of consciousness (di Perri et al., 2014; Mashour & Hudetz,

2018). I further demonstrate that this VTA-PCu/PCC connectivity strength can be modulated by the dopaminergic agonist methylphenidate.

In propofol sedation, a step-wise and reversible VTA-PCu/PCC disconnection was found, which is in keeping with a similar, but undiscussed, finding during dexmedetomidine (α-2 adrenergic agonist administration; Song et al., 2017). However, the present data link this change in connectivity to propofol plasma levels and behavioural measures of cognitive access. Above all however, I demonstrate that this VTA disconnection from the PCu/PCC is equally found in chronic DoC patients. This constitutes the first neuroimaging demonstration of a dopamine-specific parallel between pathological and anaesthetic-induced ASCs in humans.

In signalling terms, the PCu/PCC is rich in D1 (Kaller et al., 2017) and D2/D3 Dopamine receptors (Ishii et al., 2017). Although these receptors are widely expressed, indeed globally throughout the brain, there is a peak in the posterior cortex. Their presence suggests that dopaminergic release (both diffuse and potentially synaptic) in this brain area can change electrochemical and synaptic properties by signalling via these receptors. In turn, this suggests that the VTA functional disconnection may have physiological consequences: As preclinical models of chronic TBI (McGuire et al., 2018) and of anaesthetic treatment (Wang et al., 2016) variously show reduced dopamine levels, lowered VTA-PCu/PCC connectivity in rs-fMRI may coincide with or represent lowered dopaminergic tone, and thus altered modulation of targets. The fact that dopamine levels in rodents only elevate back to baseline in recovery from sedation (Wang et al., 2016), supports the assertion that the observed re-emergence of VTA-PCu/PCC connectivity in recovery from sedation could indeed indicate the re-stabilization of dopaminergic tone. This would be consistent with the recent suggestion that sudden increases in VTA connectivity and activity have a mechanistic influence in “active” emergence from propofol anaesthesia in humans (Nir et al., 2019). Despite the small sample size, the observed re-emergence of positive VTA-PCu/PCC connectivity only in those who improved and remain alive in our sample of DoC patients (in contrast to those who did not improve and subsequently deceased) suggests a possibly similar mechanism in DoC. Indeed, the recently demonstrated deficit of presynaptic dopamine release suggests that dopaminergic agents which effect behavioural improvements (Fridman & Schiff, 2014) may stabilize VTA functionality and connectivity and/or

compensate for the neuromodulatory disruption approximated in this chapter. The authors of the related study equally hypothesized the deficit revealed with [^{11}C]-raclopride-PET to arise from the brainstem, presumably the VTA (Fridman et al., 2019). As their analysis however restricted itself to dopamine in striatum and thalamus, they provided no data on the PCu/PCC. Based on the VTA's global projections, its release deficit should likewise affect cortical regions, such as the PCu/PCC, as structural connections to this area have been established in ex vivo HARDI tractography (Edlow et al., 2021b). As [^{11}C]-raclopride binding is dominated by high density of dopamine receptors in the striatum (Ishii et al., 2017), we suggest that fallypride and SCH23390 PET with concurrent rs-fMRI would provide a more suitable tool to explore whether alterations in cortical dopamine receptor binding in DoC patients are associated with our observed connectivity deficit (Elsinga et al., 2006). Preclinical work by Taylor et al. and Kenny and colleagues (Kenny et al., 2015; Taylor et al., 2013, 2016) has provided strong evidence of a link between the arousal effects of the VTA and the D1 receptor – whereas some recent work has instead linked VTA dopamine arousal effects to the D2 receptor (Oishi et al., 2017). Further preclinical studies and work in humans should therefore aim to tease apart whether these receptors have divergent or convergent functions in this context. Work carried out by Nagano-Saito and colleagues with ^{18}F-Fallypride PET and concurrent rs-fMRI has revealed that dopaminergic D2/D3-mediated modulation of the precuneus/PCC is functionally important, as D2/D3 receptor binding in posterior cortical regions (coincident with the PCu/PCC) is highly correlated to DMN intra-network connectivity strength in healthy adults (Nagano-Saito et al., 2017).

The present association of VTA-PCu/PCC connectivity with the PCu/PCC cluster's whole-brain connectivity changes, non-invasively replicates and extends these findings by Nagano-Saito and colleagues. In covariance with VTA-PCu/PCC connectivity strength, the PCu/PCC connectome becomes less specialised, evidenced by the fact that increases in connectivity occur with commonly anticorrelated, and decreases to normally correlated regions (compare Appendix CII, Fig.CII.3). Together, these observations suggest that the neurobiological mechanisms by which both sedative intervention and DoC pathology alter cortical brain-wide connectivity may be dopamine-centric.

Indeed, the present findings suggest that VTA-PCu/PCC connectivity may sustain the typical architecture of the DMN, putatively affecting how the functional connectivity profile of normal, waking consciousness can be orchestrated on the PCu/PCC structural connectome. This is further substantiated by the observation that the overall BOLD complexity's decrease is equally associated with the impairment of VTA-PCu/PCC functional connectivity. As this effect might be mediated *via* its effects on PCu/PCC whole-brain connectivity, these data reinforce that a dopaminergic neuromodulatory mechanism, centred on VTA-PCu/PCC connectivity, might impair this posterior hotspot's whole-brain connectivity, and consequently whole-brain function. Altogether, VTA disconnection might therein have 'diaschisis'-like consequences, wherein a *functional* (not necessarily the typically diaschisis-associated structural) impairment of the VTA might be the culprit and ultimate source for disruptions in the functionality of spatially distant cortical regions. If further work substantiates these connections of VTA and large-scale brain function with simultaneous PET and rs-fMRI, the simple seed-based rs-fMRI approach I present could be developed into a non-invasive and clinically-useful *in vivo* tool to identify consciousness-specific dopaminergic modulatory deficits – particularly as rs-fMRI has been suggested by both UK and US clinical bodies to be incorporated into clinical management of DoC patients (Giacino et al., 2018). This approach could therein show utility to stratify patients in diagnostic terms for the purposes of future clinical trials. In fact, the pharmacological dose-dependent downregulation of VTA-PCu/PCC connectivity upon propofol and its upregulation during methylphenidate administration, suggest that the monitoring of this connectivity pattern may hold additional value as a pharmacodynamic fMRI marker for ongoing and future drug trials. It is important to consider however that as the TBI patients who received methylphenidate did not have DoCs, the effect of VTA-PCu/PCC connectivity upregulation may only be able to act on functionally dormant/abeyant, but structurally intact VTA connectivity.

For clinical practice and research, this parallel between pharmacological and pathological consciousness disruption centred on the dopaminergic brainstem incentivizes a conceptual refinement to current theories of DoC. Elaborating on the idea of coma as a brainstem *structural* disconnection syndrome (Edlow et al., 2013), the more heterogenous strata of DoC may be best understood on a

spectrum of structural and functional brainstem disconnections, which may have diaschisis-like consequences on particularly important cortical network nodes. Critically, *functional* deficits may however be reversible as observed in recovery from propofol and in the two improved DoC patients whose VTA-PCu/PCC connectivity re-emerged – and may be amenable to therapeutic intervention as evidenced by this connectivity's increase during methylphenidate administration. Dormant, but structurally intact, brainstem neuromodulatory connections could therein be a key therapeutic target for dopaminergic and other pharmacological agonists (Edlow, Khanna et al., 2019) and/or provide targets for non-invasive ultrasound stimulation (Li et al., 2022; Cain et al., 2022). Future work will have to tease apart whether the VTA is only functionally abeyant but structurally intact in individual DoC patients, as this would differentiate treatment strategies (i.e. postsynaptic versus presynaptic mechanisms).

Above all however, such work should also recognise that – despite the striking parallel between propofol sedation and DoC centred on the VTA – this mapping of structure-function brainstem deficits must be extended to include brainstem nuclei of other transmitter phenotypes, given the sporadic successes of non-dopaminergic therapies in DoC (Fridman & Schiff, 2014; see Chapter VI). Expressly, individual patients may have equally individual brainstem nucleic connectivity (and thus transmitter-system) deficits. Even in our sample of DoC patients, brain-wide PCu/PCC connectivity analyses revealed disconnections from sites coincident with non-dopaminergic brainstem nuclei (overlapping with glutamatergic, cholinergic and serotonergic transmitter nuclei of the HAAN). Patients' neuromodulatory deficits may thus overall be more complex, and could vary relevantly from one to the other, both in terms of source nuclei as well as in terms of cortical and subcortical targets. Therefore, future work should explore whether patient-specific functional (and structural) connectome mapping might provide the most suitable predictive and treatment-tracking biomarkers for individuals (Edlow, Khanna et al., 2019), to provide a rational basis for precision-medical therapies in DoC. Importantly, beyond the interaction of brainstem nuclei with posterior elements of the DMN, it will also be important to contextualise these findings with anterior forebrain mesocircuit function, as this could help to re-define how complex brainstem "arousal signals" produce the synthesis of the preconditions of consciousness (Schiff, 2010).

Despite potential involvement of other nuclei in DoC more broadly, the consistency of VTA disconnection across pharmacological and pathological perturbation of consciousness in these cohorts – together with the wide-ranging pre-existing implications of dopamine – suggest that dopaminergic neuromodulation from the VTA may be a particularly important component of consciousness maintenance. Including *in vivo* biomarkers of the integrity of VTA and dopaminergic system function such as the connectivity demonstrated here could significantly enhance the ability of ongoing drug trials and existent theories of DoC to account for dopamine's efficacy in improving neural and behavioural symptoms of consciousness perturbation.

Various limitations of this work need to be acknowledged. Firstly, the present experiments lack the temporal resolution to delineate the temporal sequence of whether effects operate primarily at the VTA's level, rather than first occurring at cortical level. As such, it cannot be reliably disqualified that the pharmacological and pathological effects in propofol and DoC may in fact originate at the level of the PCu/PCC as previously suggested for perturbed consciousness (Mashour & Hudetz, 2018) – and may only thereafter affect the VTA. However, as this cortical region is thought not to project to brainstem nuclei, effects at the level of the VTA may still precede and could strongly influence those at cortical level. Additionally, the relatively short duration of scans and the resolution of 3T MRI imaging may limit certainty that VTA signal is reliably obtained (Sclocco et al., 2018). However, I accounted for motion, cardiac, respiratory, and physiological noise artefacts in our denoising procedure, and our laboratory has begun work to replicate these findings at 7T. Indeed, recent work has suggested that brainstem-cortical connectivity analyses have great translatability from 3T to 7T, suggesting inter-modality reliability (Singh et al., 2022). Finally, dysregulation of functional connectivity cannot yet conclusively be associated with a change in transmitter tone, despite the supporting findings from PET (Nagano-Saito et al., 2017), related preclinical observations (Solt et al., 2014) and my findings with methylphenidate suggesting so. Further work will need to use multimodal fMRI and PET techniques with various dopamine-receptor and -transporter specific ligands to close this knowledge gap.

In conclusion, this chapter provides the first demonstration of a functionally-relevant impairment in a dopaminergic source nucleus common to both pharmacologically and pathologically perturbed consciousness. Importantly, this provides a possible translational bridge between preclinical and clinical observations concerning dopamine's relevance for consciousness, as this connectivity's disruption can account for alterations in macroscopic network integrity. Therefore, VTA-PCu/PCC connectivity could be a non-invasive *in vivo* biomarker of dopaminergic system modulatory function required for consciousness with prognostic and diagnostic uses for DOC care and general brain injury settings. Furthermore, the upregulation of this connectivity by methylphenidate suggests that this non-invasive imaging technique could possibly be used to track subclinical pharmacological intervention effects. Altogether, this makes VTA-PCu/PCC connectivity a putative translational biomarker that satisfies key requirements set out for the advancement of pharmacological treatments in DOC (Edlow et al., 2021a, 2021c). This work thus preliminarily resolves a key aspect of brainstem-cortex interplay, which may hold the potential for accelerating the translation of insights and treatments from benchside to bedside, as required for a more mechanistic account of consciousness maintenance.

Chapter III

3.1 Preface and Overview

In the previous chapter I demonstrated that a disconnection of the VTA from the precuneus/PCC is common to both pharmacological and pathological consciousness perturbation, and that this might underpin large-scale default mode network dysfunction in DOC. However, as expressed in the discussion of Chapter II, dopaminergic modulation in DOC might be relevant well beyond the posterior cortical regions. Indeed, the arguable main target of dopaminergic ascending projections from the VTA is the thalamus. This targeting might be mechanistically relevant in DOC, as a popular and influential disease model of DOC is the 'anterior forebrain mesocircuit' hypothesis, which postulates that thalamus dysfunction is at the heart of DOC. In this model, thalamic outflow is viewed as a bottleneck for overall cortical, and especially frontoparietal network function. Although the seed-to-voxel analyses of the previous chapter did not find disconnections from the thalamus in DOC patients, in the following chapter I explored VTA connectivity to the thalamus through an ROI-to-ROI perspective in the same sample. Based on the findings from Chapter II and III, a proposal for an extension of the 'anterior forebrain mesocircuit' to more explicitly include VTA projections and relationships to thalamus and posterior cortical targets can be made.

The team of researchers and clinicians at the University of Cambridge and the Wolfson Brain Imaging Centre (WBIC) who performed the data collection is identical to the DOC researchers mentioned in the preface of the previous chapter, i.e. specifically Manktelow A.E., Finoia P., Williams G.B., Allanson J., Pickard J.D., Menon D.K., Stamatakis E.A.. I performed all data analyses myself and wrote the text myself, with revision from senior co-authors.

Disruption of VTA-thalamus connectivity in disorders of consciousness – dopaminergic hindbrain modulation of a *posterior* forebrain mesocircuit?

3.1.1 Summary

The thalamus has been suggested to be one of the major targets of dopaminergic neuromodulatory input from the VTA. This is of potential relevance for the anterior forebrain mesocircuit, a circuit model that aims to account for consciousness deficits in DOC – and suggests that dopaminergic hypofunction is associated with over-inhibition of the thalamus, causing overall cortical impairment. To assess whether connectivity from the VTA to thalamus might correspondingly be impaired in DOC, I re-assessed the patients from the previous chapter. I found that using an ROI-to-ROI approach there is indeed a VTA-thalamus disconnection compared to controls, but that this is masked by some DOC patients maintaining control-like levels. Furthermore, I demonstrated that this higher preserved VTA-thalamus connectivity is associated with maintained responsiveness to the Tennis fMRI task, suggesting that this interplay might be key to preserved responsiveness and thus covert consciousness. Finally, VTA-thalamus connectivity levels were also predictive of thalamic connectivity to the rest of the brain, and of overall BOLD signal complexity. Altogether, these findings support a contributing role of the VTA to thalamic functionality. Together with the previous chapter's results on the PCu/PCC, VTA impairments might affect overall mesocircuit functionality, motivating an extension of this circuit model to explicitly include brainstem ascending dopaminergic influences on both subcortical and cortical nodes.

3.1.2 Introduction

In order to develop successful treatment strategies for the continuum of disorders of consciousness (DOC), it remains critical to resolve underlying pathological causes of DOC, as these might provide potential targets for therapeutic intervention (Calabrò et al., 2016; Gosseries et al., 2011; Septien & Rubin, 2018; Thibaut et al., 2019). The arguably most universal pathophysiological feature of DOC is a large-scale loss of activity and metabolism across the whole cerebrum, which is thought to be

brought about by a broad *disfacilitation* (i.e. loss of excitatory activity) of neocortex, striatum and, most importantly, the thalamus (Edlow, Claassen, et al., 2021). This has been formalized into the now long-established 'anterior forebrain mesocircuit model' which provides a putative mechanistic framework for the general importance of cortico-cortical, striato-thalamic and especially thalamo-cortical interplay for conscious state. Especially the latter has been suggested to mechanistically underpin the re-emergence of consciousness after severe traumatic brain injuries (Fridman & Schiff, 2014; Schiff, 2010, 2008). In the forebrain mesocircuit, the fundamental arbiter of effects is the central thalamus, which is overinhibited by input from the globus pallidus interna (GPi). The reason for the GPi's over-inhibition of the thalamus is hypothesized to be a lack of cortico-striatal inputs to the GPi, which fail to downregulate its activity and resultant inhibitory input to the thalamus. This in turn would produce a lack of thalamic outflow, which causes large-scale disfacilitation, and as such – in a vicious cycle – further downregulation of cortico-striatal input to the GPi. The most consciousness-relevant consequence of the resultant over-inhibition of the thalamus in the anterior forebrain mesocircuit model has been suggested to be an impairment of anterior brain regions in the frontal cortex (Schiff, 2010). Given their participation in the frontoparietal network (a combination of FPCN and DMN), the re-emergence of improved function within the mesocircuit is thought to enable recovery from DOC by allowing the frontoparietal network to return to normal functionality (Bodien et al., 2017; Edlow, Sanz, et al., 2021). Altogether, this circuit-to-network account, sometimes coined the 'anterior forebrain mesocircuit-frontoparietal' model (Edlow, Claassen, et al., 2021), considers the ultimate rate-limiter for recovery of consciousness and network function to be the functionality of the central thalamus with large-scale network- and mesocircuit dysfunctions emanating from it.

Importantly, all of the regions in the mesocircuit model, most strongly among them the thalamus, are targeted by various ascending arousal pathways of neuromodulatory inputs from the brainstem (Fridman & Schiff, 2014, 2022). Their input is thought to be fundamental to normal thalamic function – and the brainstem and thalamus are both positioned at the centre of the 'cone of vulnerability' within the human brain, in spatial locations of maximal traumatic injury potential, and maximal injury sequelae for severe brain injuries (Bigler, 2021). Various pharmacological agents in DOC are

correspondingly thought to act, at least partially, by re-activating brainstem-thalamic interplay (Fridman & Schiff, 2022; Saleh et al., 2021). As described in Chapter II, the most consistent implications of successful treatment in DOC converge on dopaminergic drugs (e.g. levodopa, Matsuda et al., 2005; bromocriptine, Passler & Riggs, 2001; methylphenidate, Martin & Whyte, 2007; amantadine, Giacino et al., 2012 and Lehnerer et al., 2017; apomorphine, Fridman et al., 2010; Sanz, 2019; and now also madopar, Saleh et al., 2021). The corresponding long-standing idea of dopaminergic dysfunction in DoC (Schiff, 2010) might in part be due to a presynaptic dopamine release deficit centred on the dopamine D2-receptor rich thalamus, as PET and bolus dopaminergic drug administrations in Fridman et al.'s (2019) recent work have demonstrated. The likely hypofunction of the dopamine-synthesizing enzyme tyrosine hydroxylase and resulting release deficit was speculatively assigned to the VTA in their original publication as well (Fridman et al., 2019).

Indeed, the thalamus is the main target of dopaminergic ascending projections from the ventral tegmental area (VTA; Edlow, Sanz, et al., 2021; Edlow, 2021). *Via* D2 receptors, VTA neuromodulatory influence on the thalamus might be directly involved in setting the level of brain-wide thalamic 'facilitatory outflow' (Schiff, 2010, 2008). Strikingly, the VTA and central thalamus have both been identified as the two subcortical nodes that are the most strongly connected to the DMN (Li et al., 2021). Thus, a disruption of VTA-derived neuromodulation of the thalamus might act in synergy with its direct influence on the precuneal/PCC posterior DMN node (see Chapter II; Spindler et al., 2021) to impair forebrain mesocircuit function and whole-brain signalling in a consciousness-perturbing fashion. Despite the many preclinical and pharmacological treatment implications of dopamine for DOC, the VTA and its interplay with the thalamus are typically not included in the anterior forebrain mesocircuit model as areas-of-interest. I submit that to develop DOC frameworks and efficacious therapies it may be important to also resolve this brainstem-subcortical interplay to complement our understanding of brainstem-cortical interplay.

Consequently, in this chapter I aimed to characterise whether VTA-thalamus interplay in the same DOC patients as in **Chapter II** might be disrupted, to complement the VTA-PCu/PCC connectivity

results. I therefore specifically assessed whether (i) ROI-to-ROI VTA connectivity with the thalamus might also be perturbed in DOC, in a way that is not readily detectable at seed-to-voxel level (see Chapter II); (ii) whether preservation of VTA-thalamus connectivity might be associated with covert consciousness/responsiveness measured with the fMRI Tennis task; and finally (iii) whether dysfunction of VTA-thalamus interplay might impair thalamic functionality at the centre of the mesocircuit hypothesis.

3.2 Materials and Methods

Participants and Data Acquisition

The participants used in this chapter are identical to the DOC cohort that formed part of Chapter II. The subset of n=23 was selected for its suitability for brainstem connectivity analyses based on the same strict inclusion criteria as in the previous chapter, with additional screening for the absence of thalamic structural lesions. There were no significant differences in age between the healthy control cohort (35±11.448) and DOC patients (39.4±16.5) ($t(37.506)=-1.022$, $p=0.313$). All clinical investigations were conducted in accordance with the Declaration of Helsinki and all relevant ethical guidelines. Ethical approval for testing patients was provided by the National Research Ethics Service (National Health Service, UK; LREC reference 99/391).

Responsiveness to mental imagery tasks

Moving beyond overt behavioural responsiveness assessed with the CRS-R, patients from this DOC cohort were further stratified into two subgroups ('Tennis +ve' and 'Tennis -ve'). This was done on the basis of the Tennis task, which as introduced in Chapter I has been used in healthy and DOC populations to establish responsiveness during fMRI. Explicitly, the Tennis task involves motor imagery, wherein each patient was prompted to "imagine being on a tennis court swinging their arm to hit the ball back and forth with an imagined opponent" (Luppi et al., 2020b, Monti et al., 2010, Owen et al., 2006) over five cycles, with alternating 30s of imagery task, and 30s of rest. Each

block of mental imagery was cued with the spoken word "tennis", whereas rest blocks were cued with "relax". For each patient, classification into 'Tennis +ve' and 'Tennis -ve' groups was based on the results of univariate fMRI analysis conducted on motor imagery tasks. For each functional scan, a separate general linear model was used to contrast periods of rest and active imagery (Monti et al., 2010). If a patient's fMRI activation was significantly greater than at rest in task-relevant regions (voxel-level threshold of $z > 2.3$, cluster-corrected $p < 0.05$) during the 'Tennis' mental imagery task, this was taken as evidence that the patient was indeed responding to the task. This observation of wilful modulation of the BOLD signal was seen as indicative of the patient having covert awareness if previously designated as UWS/VS (masked by an inability to produce overt behavioural responses due to e.g. motor impairments), or reinforced observations of behavioural responsiveness if previously diagnosed as MCS

N = 8 patients met the classification criteria for such covert consciousness (i.e. 'Tennis +ve'), whereas n = 13 patients did not respond to this task, and therefore did not show this type of evidence of covert consciousness; the latter were thus designated as 'Tennis -ve', consistent with previous publications (Craig et al., 2021).[18]

Spatial and Temporal Preprocessing

Preprocessing was identical to the previous chapter, performed using the CONN functional connectivity toolbox (19c; Whitfield-Gabrieli & Nieto-Castanon, 2012), running in MATLAB (2018b, The Mathwork, Inc. Natick, Massachusetts, USA).

Ventral Tegmental Area ↔ Thalamus Functional Connectivity analyses

In addition to the VTA region-of-interest (ROI), an ROI of the whole thalamus was created from the left and right hemispheric thalamic ROIs from the CONN atlas, using the fslmaths function (using

[18] It is important to highlight that absence of fMRI BOLD-measured responsiveness does not constitute conclusive evidence of unconsciousness (MacDonald et al., 2015). Nevertheless, in this DOC cohort tennis responsiveness (i.e. Tennis +ve) constitutes the most reliable/relevant measure of conscious responsiveness and state.

FSL version 5.0.9; https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/) with loss-less addition. This choice of creating a broad and general thalamic ROI based on this particular atlas was made as there are disagreements for how and where the 'central thalamus' should be delineated in MNI-152 space, with many different parcellations available. As such, the creation of this large thalamus ROI was considered to definitely entail the central thalamus, and be general enough to allow comparisons with future more detailed parcellations of the thalamic nuclei (e.g. Pauli et al., 2021).

ROI-to-ROI functional connectivity was calculated using CONN, with parameter estimate images for VTA connectivity used to extract β-values for connectivity to the combined thalamic ROI. In a second set of analyses, the thalamus ROI itself was used as a seed (see below).

Correlations of VTA-thalamus connectivity with thalamus 'downstream' whole-brain connectivity

To assess a potential linkage between VTA-thalamus connectivity and thalamic connectivity impairment, the thalamus-whole brain connectivity alterations were also assessed. The thalamus ROI was used as a seed in seed-to-voxel connectivity analyses, and clusters that showed as significant disconnections in group-level contrasts (i.e. Controls > DOC patients) were extracted as binary masks ('disconnection masks'). To test our hypothesis that VTA connectivity may have neuromodulatory effects on the thalamus, I extracted eigenvalues of connectivity-strength (β-estimates from GLM) per participant per condition for both VTA ⇌ thalamus connectivity and thalamus ⇌ 'disconnection mask' connectivity. To approximate whether there is a neuromodulatory relationship between VTA ⇌ thalamus connectivity alterations and thalamus ⇌ 'disconnection mask' connectivity, I statistically assessed how these functional connectivity strengths covaried in DOC patients.

All correlations were performed using R-Studio (R Core Team, 2014), with ggplot2 (Wickham, 2016) and JASP (v.14.0.0.0), using Spearman's rank.

Effort-to-Compress (ETC) of BOLD signal as a relevant mesocircuit outcome measure

The anterior forebrain mesocircuit hypothesis stipulates that overall "facilitatory" signalling in the human brain is impaired in DOC (Schiff, 2010). Although there is no standard metric based on the BOLD signal that is seen as able to measure this facilitatory signalling *per se*, many previous associations of signal complexity with conscious state in both pathological and pharmacological consciousness perturbation have been reported in the literature (e.g. Luppi et al., 2019). As such, the complexity of the BOLD signal should capture some fundamental aspects related to facilitated *versus* disfacilitated states. Therefore, I also assessed the whole-brain Kolmogorov complexity of the BOLD signal in these patients, using Effort-To-Compress (ETC) as in the previous chapter. The subject-specific ETC values were entered into correlations VTA-thalamus connectivity in DoC patients, to delineate whether ascending dopaminergic connectivity to thalamus might correlate with this proxy of thalamic efflux. Subsequently, a causal mediation analysis was performed in JASP (v.14.0.0.0) to establish whether potential effects on whole-brain ETC might be mediated *via* thalamic brain-wide connectivity and whether this effect is a better predictor in the model than VTA-PCu/PCC connectivity.

Controlling for Statistical Power in smaller (sub)sample analyses

In this chapter (and throughout this thesis), statistical power was assessed for each analysis and sub-analysis using an *a posteriori* approach. This perspective was taken as the rarity of the utilised datasets (as here: Disorders of Consciousness) makes *a priori* determinations impractical because certainty of recruiting enough participants cannot typically be established prior to data collection.

In essence, statistical power of a hypothesis test refers to the probability that the test can correctly reject the null hypothesis (H_0) – i.e. determine the presence of a true positive result and decrease the likelihood of a Type II error (false positive). For the present analyses, I determined the effect size of differences or correlation strengths as respectively Cohen's d or Vovk-Sellke maximum p-ratios, and then utilised these in the statistical software solution G*Power to estimate the achieved post-hoc power (Faul et al., 2009). As per general guidance, >80% statistical power is regarded as a reasonable

level to aim for (Dorey, 2011). The results that contrast DOC patients with controls all were sufficiently powered, with N=22 controls and N=22 DOC patients at α=.05 (one-tailed) to detect effect sizes above Cohen's d of 0.89 (such as observed for the analyses in the previous chapter), as per G*Power (critical t=2.02108). However, the t-tests that split the DOC patients into subgroups of Tennis +ve and -ve were found to be underpowered for the effect sizes observed (see Discussion section). This motivated the approach of employing additional Bayesian statistical validation of the findings, which is from here on applied throughout all chapters of this thesis.

Frequentist statistical tests of the null hypothesis cannot conclude in favour of the H_0 but merely to its rejection, which is especially problematic in the above outlined instances of low statistical power, imposing prohibitive limits on what can be inferred from the data. To counteract this, I from here onwards also use Bayesian statistics (using the software package JASP v.14.0.0). Bayesian statistics enable inferences to be made with confidence on the absence of a difference (or absence of correlation or prediction): traditional concepts of type I and type II error do not apply to Bayesian statistics, where one instead determines the relative model evidences in favour of the null (H_0), or in favour of the alternate hypothesis (H_1), or that the evidence is indeterminate from the data available – also forgoing the issue of correcting for multiple comparisons. The parameter BF_{10} (known as Bayes Factor) that I report is a measure of the evidence in favour of H_1 over H_0. For instance, a $BF_{10} = 8$ means that there is 8 times as much evidence for H_1 than there is for H_0. A BF_{10} between 1 and 3 is regarded as 'mild', between 3 and 10 as 'moderate', between 10 and 30 as 'strong', and above 30 as 'very strong' evidence for H_1 over H_0. Such an approach is particularly useful in a small sample such as the DOC dataset at hand, as it more directly controls for false positives and negatives as they more routinely can arise from traditional frequentist analyses.

3.3 Results

3.3.1 Ventral tegmental area is disconnected from thalamus in disorders of consciousness

As shown in the previous chapter, at rest in healthy controls the VTA showed connectivity to the thalamus. However, seed-to-voxel connectivity analyses that contrasted these healthy controls and DOC patients did not reveal a significant lower connectivity of the VTA to the thalamus in DOC patients (see Chapter II and Spindler et al., 2021). Based on the fact that the thalamus is in the literature suggested to be a main target of dopaminergic innervation from the VTA and is central to mesocircuit function, I hypothesised that a VTA-thalamus connectivity deficit might still be occurring in these patients – but that this is undetectable at seed-to-voxel level.

In turn, an ROI-to-ROI approach was employed, running t-tests on extracted β-values from the GLM for VTA connectivity to the thalamus ROI. This revealed that DOC patients (M=-0.016±0.012 orange, Fig.12) did in fact have significantly lower VTA-thalamus connectivity strength than controls (M=0.022±0.009; green, Fig.11; t(40)=2.460, p=0.018; 'moderate' strength Bayesian support BF_{10}=6.130). This suggests that, albeit not as readily detectable with seed-to-voxel metrics, there is indeed an impairment of connectivity of this dopaminergic source nucleus to the thalamus, which may be of neuromodulatory consequence to thalamus functionality. Importantly, the raincloud plots reveal that a subgroup of DOC patients (blue, Fig.11) showed VTA-thalamus FC strengths that were equivalent to the control mean (grey, Fig.11), possibly explaining why thalamus disconnection was not as readily observable in the Chapter II's seed-to-voxel analyses.

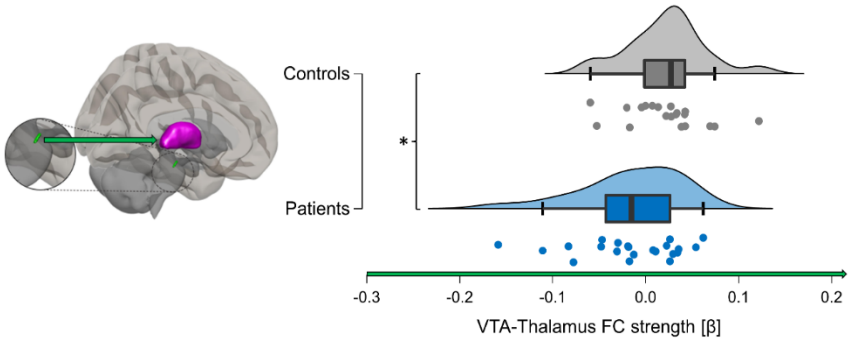


Fig. 12: Disorders of consciousness (DOC) patients have significantly lower connectivity between ventral tegmental area and thalamus than controls. A t-test between healthy controls (grey) and disorders of consciousness patients (blue) showed a significant difference in the level of VTA-thalamus connectivity (green arrow), extracted as β-values from the general linear model. Raincloud plot shows distribution density in histogram, horizontal boxplot with bold line highlighting the mean, with individual datapoints below. Of note, some patients showed connectivity strengths equivalent to or above the mean of controls. * indicates $p<0.05$.

3.3.2 Preservation of VTA-thalamus connectivity is associated with responsiveness in Tennis fMRI task

Based on the observation that some participants had VTA-thalamus connectivity at levels around the group mean of healthy controls (see Fig.12), it was hypothesized that participants with such preserved VTA-thalamus connectivity might also show 'control-like' preserved responsiveness in the Tennis fMRI task.

In concordance with this hypothesis, a Welch's t-test (performed as the assumption of equality of variance was violated) revealed that those patients who were classed as 'Tennis +ve' (Median=0.008±0.009) had significantly higher VTA-thalamus connectivity than those who were 'Tennis -ve' (Median=-0.029±0.017) (t(18.466)=-1.977, p=0.032; 'mild' Bayesian support BF_{10}=1.713; see Fig.13). Importantly, this discriminability was unique to VTA-thalamus connectivity, as no such difference was observed when comparing thalamus-wholebrain connectivity (t(11.783)=-0.724, p=0.242), or the previously-identified VTA-PCu/PCC connectivity (t(19.916)=0.081, p=0.532) between the Tennis +ve and -ve groups. These data thus suggest that dysfunctional coupling between the VTA and thalamus might be of primary relevance for whether patients maintain the neural properties required for responsiveness.

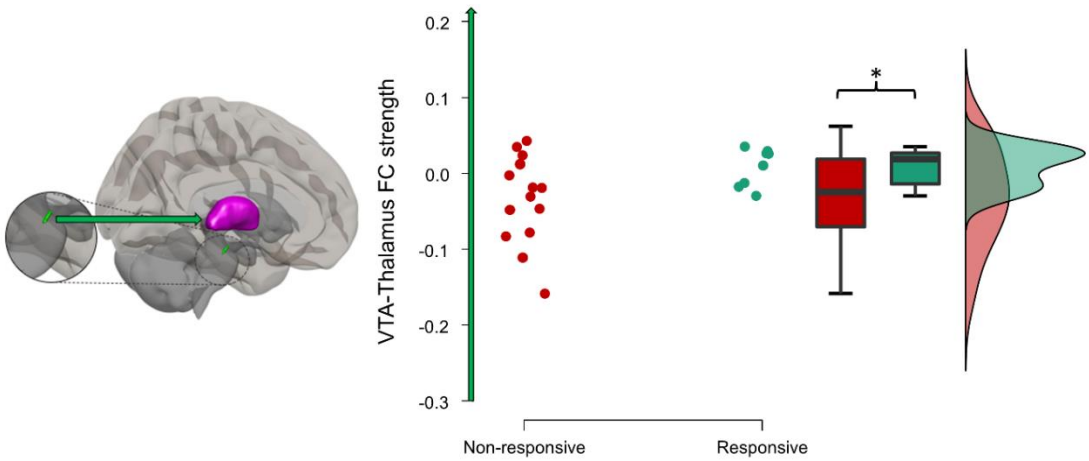


Fig. 13: VTA-thalamus connectivity strength is significantly higher in disorders of consciousness patients who are responsive to an fMRI motor task, representing detection of responsiveness and/or 'covert' consciousness. Rainbow plots showing the mean, distribution and individual datapoints of all DOC patients, separated into responders (-ve) and responders (+ve) to the fMRI Tennis motor task. Patients who showed significant motor activation during the motor task in the fMRI scanner (Tennis +ve), had significantly higher average VTA-thalamus connectivity, than those who did not show a response. * represents p<0.05

3.3.3 VTA-thalamus connectivity strength is associated in neuromodulatory fashion with thalamic connectivity and whole-brain BOLD signal complexity

The observation that VTA-thalamus connectivity is associated with responsiveness in these DOC patients, suggests that dopaminergic connectivity deficits might have exacerbating consequences on mesocircuit function through disrupting the thalamic rate-limiter (Moustafa et al., 2017). Consequently, I hypothesized that the VTA-thalamus disconnection might constitute an additional contributor to impairment of thalamic function within the mesocircuit model.

In seed-to-voxel whole-brain contrasts between controls and DOC patients, the thalamus was observed to have very wide-ranging whole-brain disconnections (see Appendix CIII; Fig.1SM; far outstripping the whole-brain functional connectivity losses of the precuneus/PCC observed in Chapter II). These thalamic disconnections from the rest of the brain might, at least partially, reflect altered dopaminergic neuromodulation from the VTA. To approximate whether this might be the case I assessed whether VTA-thalamus connectivity strength covaried with thalamus connectivity to these clusters of disconnection (red mask, Fig.14A, and Appendix CIII Fig.1SM). These correlations revealed a strong relationship between thalamic large-scale disconnections and residual VTA-thalamus connectivity ($R=0.510$, $p=0.008$, $BF_{10}=9.366$). Explicitly, this indicated that the more preserved VTA-thalamus connectivity remained in a given patient, the less impaired the thalamus-whole-brain connectivity was, thus supporting that a neuromodulatory relationship might be at play.

Additionally, the largest cluster of thalamic connectivity loss (red) was observed in the precuneus/PCC (2520 voxels, see Appendix CIII, Table CIII.1). I also found a strongly significant relationship between VTA-thalamus connectivity and the disruption of thalamus-precuneus/PCC interplay ($R=0.659$, $p<0.001$; 'extremely strong' Bayesian support $BF_{10}=96.87$, see Fig.14B). This suggests that thalamic function, underpinned by dopaminergic modulation, may not just be of relevance for *anterior* brain regions as postulated in the forebrain mesocircuit hypothesis, but may (as suggested in the previous chapter) extend to critical, consciousness-relevant, aspects of the *posterior* cortex, which are equally nodes of the frontoparietal network(s) (Buckner & DiNicola, 2019).

Furthermore, the mesocircuit hypothesis stipulates that downregulated thalamic outflow causes global disfacilitation. While there are no agreed biomarkers in the literature for how disfacilitation may be

reliably captured with rs-fMRI, I exploratorily used reduced information content in the BOLD signal measured as Effort-to-Compress (ETC). In DOC patients (M=0.560±0.026) Effort-to-Compress was indeed significantly lowered ($t(21.107)=4.627$, $p<0.001$), compared to healthy controls (M=0.679±0.001; see Fig.14C). This posed the corollary question whether greater preserved VTA-thalamus functional connectivity might be associated with better thalamic outflow and resultant facilitation levels, measured in global Effort-to-Compress. A strong correlation between VTA-thalamus connectivity and global ETC ($R=0.665$, $p<0.001$) was observed. Finally, to statistically resolve whether VTA-thalamus or VTA-PCu/PCC connectivity might underpin global ETC, I performed a two-predictor average causal mediation analysis, in which VTA-thalamus and VTA-PCu/PCC connectivity were both used as predictors, thalamus-'downstream' and PCu/PCC-'downstream' connectivity as mediators and global ETC as the outcome variable (for layout see Fig.14C). I found a significant direct effect of VTA-thalamus connectivity on global ETC, ($z=3.471$, $p<0.001$), which was not mediated *via* thalamic whole-brain connectivity, thus supporting the hypothesis of dopaminergic modulation of the thalamus potentially directly affecting thalamic function and outflow. VTA-PCu/PCC connectivity showed no significant direct, but a significant indirect, effect mediated *via* PCu/PCC-'downstream' connectivity strength ($z=2.442$, $p=0.015$) on global ETC. The effect size of this was however much lower than that of the direct effect of VTA-thalamus connectivity. This substantiates that preserved VTA-thalamic interplay might be a necessary condition for sufficient thalamic outflow and as such global facilitation to maintain consciousness-relevant BOLD signal information content.

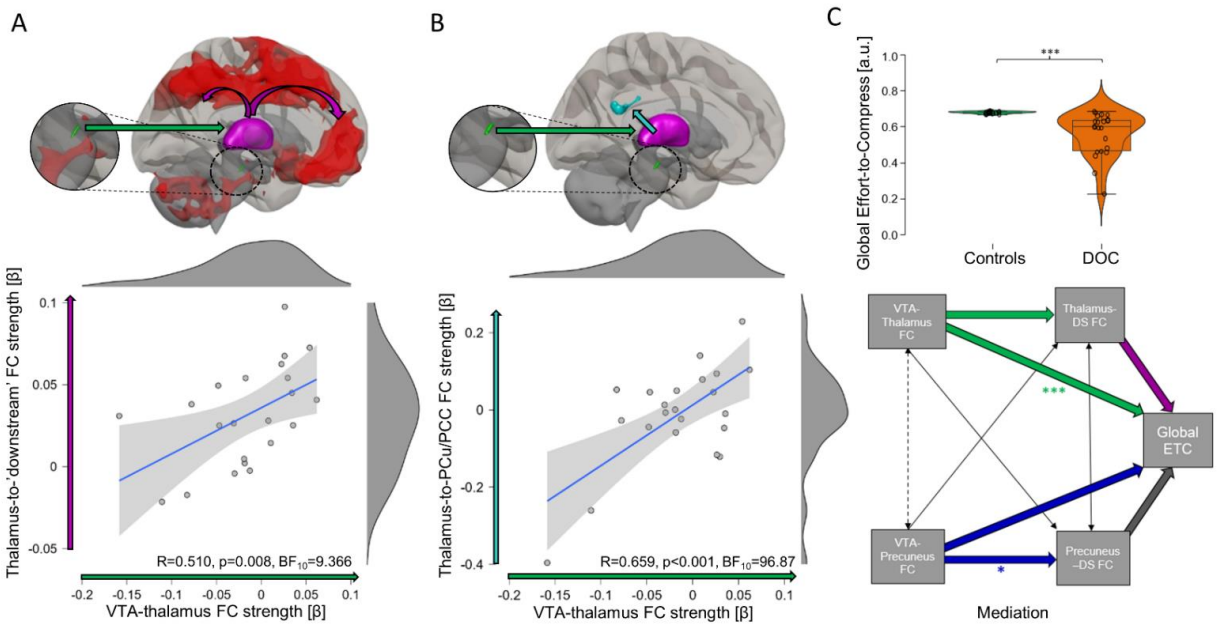


Fig. 14: VTA-thalamus connectivity strength is associated with both thalamus-whole-brain, thalamus-PCu/PCC connectivity and global BOLD signal complexity. (A) The more preserved VTA connectivity strength to the thalamus (green arrow) was, the less impaired thalamus connectivity strength (magenta arrow) to 'downstream' areas from which DOC patients showed group-level connectivity losses (red mask) was. (B) A similar positive association was found for VTA-thalamus connectivity strength (green arrow) and the connectivity of the thalamus to the PCu/PCC (cyan arrow). (C) There was a significantly lower global information content in the BOLD signal, measured as Effort-to-Compress, in DOC patients compared to the healthy controls. In a causal mediation analysis, VTA-thalamus connectivity (VTA-Thal FC in figure) in DOC patients was found to have a highly significant *direct* effect on the level of whole-brain ETC, whereas VTA-PCu/PCC connectivity (VTA Prec FC) had a significant *indirect* effect mediated *via* precuneal whole-brain connectivity (PCu glob FC). Correlations of connectivity strengths between respective seed and target masks used Spearman's rank. 95% confidence intervals in grey, overall regression line in blue. * indicates $p<0.05$, *** indicates $p<0.001$.

3.4 Discussion

This follow-up work to Chapter II provides the first *in vivo* evidence of a functional connectivity deficit between the VTA and thalamus in disorders of consciousness as captured with rs-fMRI. Firstly, this extends that not only VTA connectivity loss from cortical, but also from subcortical key nodes is associated with DOC. I found that VTA-thalamus interplay is impaired in DOC patients and associated with disrupted thalamic whole-brain connectivity and BOLD signal complexity. Importantly however, VTA-thalamus connectivity was preserved at control-like levels in those patients who showed significant supplementary motor activations in the fMRI tennis task, and were

thus deemed to have responsiveness/'covert' consciousness. Together with the results of Chapter II, these findings further substantiate the importance of VTA function for consciousness, and provide empirical evidence that both VTA-cortical (PCu/PCC) and VTA-subcortical (thalamus and mesocircuit) interplay might subserve functionality of the frontoparietal network relevant for DOC patient conscious state. I discuss these findings below, and in conclusion suggest an extension of the mesocircuit model to explicitly include the VTA as a substrate of dopaminergic influence on thalamic and posterior cortical function, and therein as a rate-limiter for mesocircuit operation.

The finding that VTA-thalamus connectivity is impaired in DOC patients when compared to healthy controls elaborates on various previous reports of brainstem functional disconnection from the whole-brain (Chen et al., 2018) and structural disconnection from thalamus in DOC (Snider et al., 2020). Given the thalamus' richness in D2 receptors (which parallels that of the precuneus; Ishii et al., 2017), this disconnection is likely to have physiological consequences, and may be a non-invasive fMRI neuroimaging correlate of the presynaptic dopamine release deficit characterised in the central thalamus by Fridman et al. (2019). A lack of dopaminergic modulation of the thalamus is likely to be causally involved in downregulating thalamic outflow, possibly acting in synergy with an already more GABAergic, rather than glutamatergic, transmission state throughout the mesocircuit (Fridman et al., 2019). Indeed, tonic dopaminergic neuromodulation can powerfully alter input-output relationships of thalamic neurons (Avery & Krichmar, 2017), with particular influence on tipping the excitation (glutamatergic) *versus* inhibition (GABAergic) balance in favour of excitation (Floresco & Tse, 2007). The effect of VTA-thalamus coupling on whole brain BOLD complexity I found in the mediation analysis might capture that dopaminergic modulation of the thalamus is a key player in determining thalamic outflow. Conversely, a state of disrupted VTA-thalamus interplay might be characterised by *hypo*-dopaminergic modulation of the thalamus, meaning that the response characteristics of the thalamus are such that the mesocircuit cannot function sufficiently to maintain consciousness – until this dopaminergic deficit is compensated. VTA-thalamus interplay may represent a neurobiologically plausible rate-limiter for how strongly the mesocircuit is disrupted,

providing a possible target and mechanism to explain the relative successes of dopaminergic drugs for re-initialising mesocircuit operation, most recently L-DOPA (Fridman & Schiff, 2022).

Conversely, greater preserved dopaminergic modulation of the thalamus might be captured by more preserved VTA-thalamus connectivity, enabling more healthy-like thalamic, and therein mesocircuit function. The association of VTA-thalamus coupling with how strongly the thalamus remains connected to a large extent of the brain, supports this assertion (Lee & Dan, 2012). Previous observations that brainstem-thalamus structural connectivity increases are associated with greater likelihood of emergence from traumatic coma (Snider et al., 2020) might therefore above all capture that VTA-mediated modulation of the thalamus is required for re-emergence and maintenance of consciousness. Importantly, this effect might act synergistically with direct VTA modulation of posterior DMN network nodes (such as PCu/PCC) to establish the cortical frontoparietal network-enabling modulatory conditions upon which thalamic outflow might act.

The result that patients with a greater residual level of VTA-thalamus connectivity are those who are responsive to the tennis-task in the fMRI scanner (Bayne et al., 2016; Fernández-Espejo & Owen, 2013) strongly suggests that preserved VTA function (and interplay with the thalamus) may sustain more normal function of the thalamus, mesocircuit and thus frontoparietal network in a behaviourally-relevant way. To however identify whether VTA disconnection from the thalamus does coincide with lowered dopamine receptor occupancy in the central thalamus, future studies should utilise concomitant rs-fMRI/PET approaches (informed by the demonstration of dopaminergic release deficit by Fridman and Schiff (2021), but using fallypride and SCH23390 to resolve subcortical dopamine signals, see Chapter II and VII).

Importantly, this highlights again that it is imperative for DOCs not to be oversimplified into syndromes referred to purely as “arousal without awareness” (e.g. Owen et al., 2010): particular arousal systems relevant to the conscious state, such as the VTA, may have residual preserved functionality in a given patient, meaning that they might be amenable to particular therapeutic interventions. Indeed, brainstem structural and functional connectivity analyses of this dopaminergic

source nucleus could prove clinically-feasible for personalised medicine approaches, by allowing the identification of which patient may be a likely responder to a particular dopaminergic therapy (expressly, e.g. whether presynaptic *versus* postsynaptic pharmacological intervention should be used, see Fig. 4). Indeed, if dose-dependent changes of VTA connectivity would be confirmed in addition to the observations made in the previous chapter with methylphenidate, this biomarker might allow pharmacodynamic monitoring of subclinical treatment effects (Edlow, 2021) – which are very likely to occur before behavioural signs of improvement, normal functionality of the mesocircuit, and of large-scale networks re-emerge (Ciurea et al., 2017; Edlow et al., 2020; Harris-Warrick & Johnson, 2010).

Taken together, the results of the present and previous chapter motivate an extension of the mesocircuit model. To best be able to generate, test and track mechanistic hypotheses with the help of the anterior forebrain mesocircuit model, it might be advisable to explicitly delineate a 'posterior forebrain' component centred on the precuneus/posterior cingulate[19], and to include both direct cortical and direct thalamic neuromodulatory influences from the dopaminergic VTA from a 'hindbrain-subcortical' component (see Fig. 4, purposely based on figure from Edlow et al., (2020) to enhance comparability and highlight additions). I propose that a dopaminergic deficit, as consistently implicated in previous preclinical and clinical research, and putatively captured by the present rs-fMRI analysis can adversely affect mesocircuit operation across all levels, specifically *via* its concomitant influence on posterior network nodes and the thalamus. Expressly, all key players in the mesocircuit (central thalamus and its outflow; frontal and posterior cortical network nodes) receive dopaminergic modulation from the VTA, thus lending physiological credibility to a rate-limiting function for dopaminergic modulation. This would make the VTA a key treatment target for DOC that could positively affect all mesocircuit nodes downstream of it. This might be accomplished through pharmacological means of upregulating dopaminergic tone (see Fig. 4), but importantly also through recently developed non-invasive ultrasound stimulation techniques. Ultrasonic stimulation of the

[19] While this was recently done in a review (Edlow, Claassen, et al., 2020), posterior components of the mesocircuit are very often not included in renderings and discussions across multiple publications.

thalamus itself has shown only mixed success in producing transient improvements in conscious state (Cain et al., 2021 & 2022). Strikingly however, in mice, the same stimulation technique applied to the VTA can fully wake up animals from propofol anaesthesia – thus achieving the same results as the electrical, optogenetic and pharmacological VTA stimulation experiments mentioned in Chapter II (Kenny et al., 2015; Solt et al., 2014; Taylor et al., 2013, 2016). These encouraging results suggest that if this method can be adapted for the human VTA, it might provide a feasible non-invasive tool to promote dopaminergic function, surmounting the issue of the extreme invasiveness of brainstem deep brain stimulation (Kundu et al., 2018). Again, for such treatment approaches, monitoring VTA-thalamus and VTA-PCu/PCC connectivity might allow the detection of treatment-induced efficacy-related changes, comparisons of pharmacological interventions *versus* other stimulation techniques, and possibly predictions of treatment response (Luppi, Cain, Spindler, Gorska, et al., 2021).

Altogether, dopaminergic modulation of the thalamus as the central 'relay station and gatekeeper' of the mesocircuit *via* the subcortical-hindbrain loop (Moustafa et al., 2017), might therefore act synergistically with direct dopaminergic influence on cortical targets (anterior and especially posterior forebrain components) to let the brain-wide modulatory environment for normal cerebral functionality and consciousness emerge *via* this extended mesocircuit architecture (Satpute et al., 2018; see Fig.15). To comprehensively resolve this model of subcortical-cortical interactions for consciousness, it will however also be important to iteratively include influences of other arousal-relevant brainstem nuclei beyond the VTA (and the often included pedunculo-tegmental nucleus), in particular other monoaminergic nuclei belonging to the diffuse neuromodulatory system (Olszewksi et al., 2013; see discussion in **Chapter VII**).

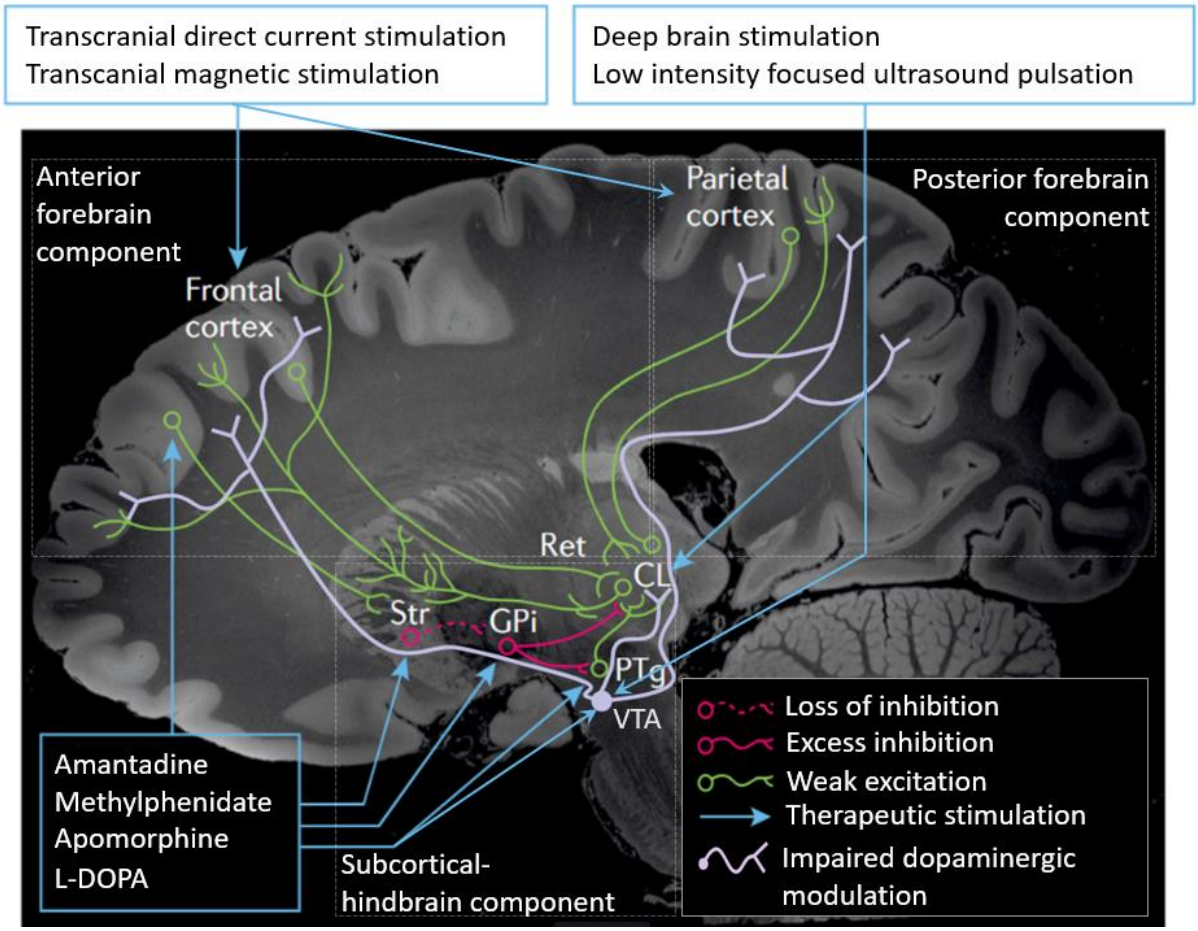


Fig. 15: An updated mesocircuit layout with posterior forebrain and subcortical-hindbrain dopaminergic components. The proposed updates to the mesocircuit model include the clear delineation of the 'posterior-forebrain' component as a key player, as well as the direct modulatory input from the ventral tegmental area to mesocircuit constituents such as the central thalamus (CL), and to cortical regions in the frontal and posterior components that mediate mesocircuit-derived frontoparietal network-level consequences. The fact that dopaminergic modulation from the VTA is dysfunctional in thalamic and posterior parietal regions (and likely also frontal aspects), highlights that dopaminergic neuromodulation constitutes a potential rate-limiting step for any therapies that are aimed at alleviating mesocircuit-centric deficits. As such, both pharmacological and invasive/non-invasive stimulation techniques should regard the VTA in particular, along with other aspects of the rostral brainstem (such as pedunculotegmental nucleus; PTg) as direct mediators and treatment targets in disorders of consciousness. Figure adapted from (Edlow et al., 2020) under a CC-BY 4.0 license.

Various limitations of the present chapter need to be acknowledged. Above all, the analyses of the DOC subgroups are of low statistical power, with small effect sizes suggesting that 1-β error probability sits at around 50% (0.509; critical t=1.7291). This is a problem that is inherent to the work with a rare patient sample who have heterogenous underlying pathologies (e.g. different brain injuries) for the same syndromic grouping (DOC). Nevertheless, this needs to be actively counteracted to decrease the probability of Type II errors, leading me to utilise additional Bayesian validation of my frequentist findings, which gives some confidence that the findings are valid enough to generate

relevant working hypotheses. Going forward however, it would be preferable to conduct *a priori* power analyses before/during the course of recruitment, in order to ensure that the effects in question are indeed detectable in the present sample – but given that these were followup analyses based upon pre-existing collected data and other analyses, this was not possible in this instance. Additionally, regarding low statistical power it is important to note that responsiveness is not entirely readily detectable using the Tennis task. As such, some patients may have been wrongfully included in either category of responsiveness or lack thereof. Such inadvertent mis-grouping would greatly increase noise when comparing the subgroups of this already small sample, and could lead to the smaller effect sizes observed. Despite making the best efforts to control for the caveats of low statistical power in the present analyses, it is critical to acknowledge that these analyses need to be replicated in an unrelated sample to allow for a validation of the working hypotheses suggested above. Equally, once more related to the matter of noise in the data, the thalamus ROI utilized here is not exclusive to the central thalamus, which thus may not capture only mesocircuit aspects of this subcortical region. Nevertheless, a physiologically-relevant and extensive thalamus ROI was favoured over a simple sphere, and its greater size seen to include the central thalamus, while being able to forego the inconclusive debate of this area's exact localization in the literature (e.g. Sieveritz & Raghavan, 2021). Given the very recent public availability of PET Dopamine D2 receptor maps, it would be advisable for future work to mask thalamic ROIs as used in this chapter, which could narrow down the physiologically-relevant sub-division of interest in the thalamus. As mentioned previously, it will also be important to extend these results to higher resolution imaging at 7T to ascertain the reliable detectability of relevant VTA and also sub-thalamic signal at 3T (Sclocco et al., 2018). The most recent work however strongly suggests good translatability and sufficient signal-to-noise ratios at 3T (Singh et al., 2022). Importantly, the data used here did not strictly have the ability to establish whether the VTA affects the thalamus 'first', or the striato-pallidic impairment of the thalamus the VTA. Nevertheless, given the clock-like activity of VTA neurons even in the absence of any input (due to pacemaker conductances; Avery & Krichmar, 2017; Khaliq & Bean, 2010), and the large body of findings establishing direct impairments of the VTA in DOC and animal models of anaesthesia, there is empirical support for a directionality of at least some effects flowing predominantly from the

VTA to thalamus. This however does not preclude potential bi-directionality and vicious-cycle effects of loss of thalamic feedback to the VTA (Tasserie et al., 2022). Additionally, the thalamus' proximity to various ventricles increases the potential relevance of confounding variables such as head motion – however I controlled for head motion, cardiac, respiratory, and physiological noise artefacts in the denoising procedure and found no relationship between FC estimates and these variables.

In conclusion, disruption of VTA-thalamus interplay is observed in DOC, and is likely to have neuromodulatory consequences for thalamic (and thus: mesocircuit) functionality in a way that could underpin preservation of responsiveness and 'covert' consciousness. In combination with my previous findings on VTA-PCu/PCC interplay, the work in this chapter suggests that VTA function as assessed with rs-fMRI can provide useful information to produce mechanistically-relevant extensions of existing disease models, and possibly help to inform therapeutic choices in DOC patients. This suggests that easily-implemented fMRI connectomics might therefore be suitable to begin to generate and test bi-directionally translational hypotheses (animal-to-human and human-to-animal; Luppi, Cain, Spindler, Gorska et al., (2021)) concerned with brainstem dysregulation in DOC and other ASCs.

Chapter IV

4.1 Preface and Overview

In the previous chapters, I demonstrated that altered/lowered states of consciousness are characterised by a dysfunction of the monoaminergic VTA, the dopaminergic source nucleus – and that re-emergence of dopaminergic system function might be crucial for regaining of functional consciousness and associated large-scale network (i.e. DMN) topography. However, dopamine is not the only monoamine implicated in consciousness alteration, as it is well-established that serotonergic psychedelic drugs bring about powerful changes of qualitative experience and conscious state, and are suggested to have various potential therapeutic uses. All classical psychedelics are serotonergic, meaning that they bind with high potency to serotonergic receptors to elicit their consciousness-altering effects. They therefore constitute a transient and reversible consciousness-altering pharmacological stimulus with reasonable transmitter-system-specificity. As the sole sources of serotonin in the brain are the raphe nuclei of the brainstem, these serotonergic substances provide an opportunity to test (i) the specificity of brainstem nucleic connectivity analyses for observing transmitter-system effects, but also to assess (ii) whether monoaminergic changes centred on the serotonergic nuclei (rather than dopaminergic sources) characterise the psychedelic ASC. In the following chapter, I use a cohort of healthy participants who received lysergic acid diethylamide (LSD) and were scanned in the acute phase after administration (rs-fMRI). I assess how the connectome of the raphe nuclei is affected by LSD and how putative effects relate to larger-scale cortical function – both of which reveal striking parallels to Chapter II. Importantly, I also introduce how positron-emission tomography receptor maps can be used in conjunction with brainstem connectomics to generate preliminary neurobiologically-plausible mechanistic hypotheses for *in vivo* psychedelic drug action.

The data for this chapter were collected by Leor Roseman, David J. Nutt, and Robin Carhart-Harris at Imperial College, London. I conducted all analyses and wrote the chapter and manuscript draft, and incorporated input from all co-authors (in addition to the above: Peter Coppola, Andrea I. Luppi, David K. Menon and Emmanuel A. Stamatakis).

Disruption of serotonergic raphe-cortex coupling upon LSD as a hallmark of acute psychedelic effects

4.1.1 Summary

Serotonergic psychedelic drugs, such as D-lysergic acid diethylamide (LSD), have shown great medico-therapeutic potential over the past decade. However, it is not completely understood how their phenomenology and macroscopic neural correlates emerge from their fundamental serotonergic neurochemical effects on the brain. Despite emerging consensus that these substances act *via* cortical serotonin 2a receptors (5-HT2AR), research in humans has – unlike preclinical animal work – left unanswered how the brainstem raphe serotonin source nuclei react to psychedelics. In this chapter, I demonstrate that in resting-state fMRI recordings of healthy volunteers LSD significantly disrupted the functional connectivity of serotonergic brainstem nuclei to posterior higher-order network nodes implicated in previous psychedelic research – specifically the precuneus, a key node of the brain's default mode network. The level of disruption of median raphe-precuneus connectivity was strongly associated with decreased functional connectivity within the default mode network, and greater subjective intensity of drug effects across multiple domains of the psychedelic experience. Most importantly, in pharmacologically plausible fashion, serotonergic brainstem disconnection from cortical regions is associated with relative regional 5-HT2AR density (5HT2AR/5HT1AR ratio), providing a potential linkage between cortical excitatory effects of LSD and disruption of brainstem nucleic connectivity. Altogether, this work therefore indicates that LSD-induced dysregulation of the neuromodulatory serotonergic brainstem source nuclei may be a mechanistic component of the acute action of psychedelics in humans.

4.1.2 Introduction

Lysergic acid diethylamide (LSD) is a synthetic serotonergic psychedelic drug that powerfully alters human conscious experience. Like other psychedelics, LSD is a non-selective serotonin-receptor agonist, whose psychological and potential therapeutic effects were widely studied in the nineteen-fifties, -sixties, and seventies (Kurland et al., 1967; Pahnke et al., 1970). However, the international prohibition of psychedelics effectively halted research with these substances until the turn of the century (Nutt et al., 2020). Nevertheless, in the last two decades, there has been renewed scientific and clinical interest in the medico-therapeutic potential of psychedelics, which has reignited clinical research in humans. Small-scale cross-sectional studies, and a more recent large-scale randomized controlled trial have since revealed LSD and other psychedelics to have putative beneficial effects in therapeutic areas ranging from eating-, alcohol and drug-use disorders, anxiety and depression, to post-traumatic stress disorder (Carhart-Harris et al., 2021; FA et al., 2006; Gasser et al., 2014; Spriggs et al., 2020, for review see Fuentes et al., 2020).

Based on methodological constraints, early research largely had to restrict itself to psychological assessment in humans, and a small number of surface and deep EEG recordings (Rodin & Luby, 1966), having to defer to non-human animals to assess effects on the brain by psychedelics. However, more recent work has been able to capitalise on advances in functional neuroimaging to allow the characterisation of *in vivo* neural correlates of psychedelic states in humans, using LSD, psilocybin and other psychedelic drugs (Carhart-Harris et al., 2012, 2013; Carhart-Harris, Muthukumaraswamy, et al., 2016b; Muthukumaraswamy et al., 2013; Palhano-Fontes et al., 2015a; Tagliazucchi et al., 2016b). These studies have largely taken macroscopic network and whole-brain perspectives, characterising marked reductions in the within-network connectivity of large-scale functional brain networks (Carhart-Harris et al., 2012; Carhart-Harris, Muthukumaraswamy, et al., 2016b; Palhano-Fontes et al., 2015a), accompanied by simultaneous global increases in between-network connectivity (Felix & Stefan, 2019; Müller et al., 2018; Tagliazucchi et al., 2016b). Particularly, high-level association cortices, which include central nodes of the default-mode (DMN; i.e. the precuneus and

posterior cingulate, and angular gyri), salience (i.e. anterior cingulate) and frontoparietal control (i.e. ventromedial prefrontal cortex) networks show decreased within-network connectivity, but increased whole-brain connectivity in response to various psychedelics (Felix & Stefan, 2019; Müller et al., 2018; Tagliazucchi et al., 2016b). Some differing methodologies have also found decreases in whole-brain connectivity, but nevertheless also observed increased whole-brain connectivity for the thalamus (Preller et al., 2019). Taken together, compromised modular, but enhanced global functional connectivity and acute disintegration of the DMN (and other canonical large-scale networks) are the main features of acute psychedelic action reported in the neuroimaging literature. The strength of these effects has variously been associated with acute psychedelic phenomenology, such as ego dissolution and visual hallucinations (Carhart-Harris et al., 2012; Carhart-Harris, Muthukumaraswamy, et al., 2016b; Felix & Stefan, 2019; Preller et al., 2019; Vollenweider et al., 1998).

Despite the emergence of these relatively reliable relationships between large-scale brain network changes and dimensions of subjective experience, further work is required to explain how these may be brought about by the fundamental neurochemical effects of psychedelics. The neurochemical common thread linking all classical psychedelics is their serotonergic nature, which has long been known from *in vitro* and *in vivo* animal work (Nichols, 2016). Indeed, the discovery of LSD precipitated the identification of serotonin in the brain and recognition of the structural similarity of LSD and serotonin and the action of the former on the serotonin system helped inspire serotonin-based theories of mental illness (Aghajanian & Marek, 1999). LSD binds with agonist effects to several of the many serotonin receptors with an appreciably high affinity, with its action at the serotonin 2a receptor (5-HT2AR) appearing to be critical for the induction of its characteristic psychological effects (Kim et al., 2020; Nichols, 2016; Preller et al., 2017). Indeed, subjective intensity of psilocybin is in dose-dependent fashion associated with displacement of the PET ligand [^{11}C]Cimbi-36 from 5-HT2ARs (Madsen et al., 2019). Although the role of agonist action at 5-HT2ARs in inducing acute psychedelic effects is thus relatively well-established, other serotonin receptors that are heterogeneously co-expressed with 5HT2AR, such as the 5-HT1A receptor

(5HT1AR; Beliveau et al., 2017b; Varnäs et al., 2004) are thought to play an additional role. As the 5-HT1AR's effects are predominantly inhibitory, this receptor is putatively functionally antagonistic to the 5-HT2AR's generally excitatory effects, which is mirrored in 5-HT1AR agonists, such as buspirone, decreasing the stimulant effects of psilocybin (Carhart-Harris & Nutt, 2017; Pokorny et al., 2016).

It however remains poorly understood how psychedelics' primary effects at postsynaptic serotonin receptors may affect endogenous serotonergic circuitry in humans – namely the median (MnR) and dorsal raphe (DR) nuclei of the brainstem (Hornung, 2003). These nuclei project widely through subcortex, thalamus and neocortex, diffusely releasing serotonin, via both volume transmission and synaptic connections. Acting *via* metabotropic neuromodulatory G-protein coupled receptors, serotonin can alter synaptic and electrochemical properties across all scales of brain organisation, allowing the diverse macroscopic functional configurations to be assembled on the anatomical connectome (Marder, 2013). The finely-attuned tonic serotonergic neuromodulation provided by the raphe nuclei is very likely to be perturbed by psychedelic action at the cortical serotonergic receptors (Avery & Krichmar, 2017). Disruption of raphe functionality is thus, at the least partially, likely involved in aspects of the acute psychedelic experience and established acute macroscopic network changes. Importantly, as a rate-limiter of serotonergic system function a raphe disruption may also be related to the sub/post-acute longer-lasting behavioural effects of psychedelics, which I explore in the following chapter (Griffiths et al., 2018; McGlothlin et al., 1967; Schmid & Liechti, 2018). However, first the acute effects of a psychedelic on the raphe nuclei need to be established, as these remain effectively unexplored in the human brain.

Meanwhile, in non-human animals the dysregulation of raphe nuclei activity by psychedelics has been well documented (Aghajanian & Marek, 1999). In various murine, rat and cat experiments, peripheral injections of LSD and related psychedelic drugs inhibit spontaneous firing in both the MnR and DR nuclei, and decrease levels of serotonin and its metabolism (Aghajanian et al., 1968; McCall, 1982; Trulson et al., 1984), which mirrors effects during REM sleep, the natural state that most resembles

the psychedelic experience (Kraehenmann, 2017). The observations in animals are consistent with a model of serotonergic system homeostasis being perturbed by psychedelics, in which inhibition of raphe operation could be caused either by 5-HT2AR-induced excitation of top-down cortical inhibitory feedback terminating on the raphe or directly on raphe nuclei cell bodies *via* drug-induced activation of inhibitory 5-HT1ARs (Winkelman, 2017). Irrespective of the causal mechanism, inhibition of raphe function would serve to decrease 5-HT efflux to effect a reduction in the concentration of endogenous ligand at relevant receptor sites, as a reaction to the psychedelic drug's dominant binding at post-synaptic targets.[20] Indeed, as non-psychedelic 5HT2AR and 5HT1AR agonists are not reported to have effects on the raphe and do not have hallucinogenic properties (Adams & Geyer, 1985; López-Giménez & González-Maeso, 2018; Marona-Lewicka et al., 2002; Mokler et al., 1983; Silbergeld & Hruska, 1979), effects mediated by the raphe nuclei might be a central feature of psychedelic drug-to-effect mechanisms.

As a result, in this chapter I explore how the serotonergic nuclei in the MnR and DR nuclei behave acutely under LSD administration using rs-fMRI data collected from non-drug-naïve healthy volunteers (n=15). This approach with rs-fMRI allows the raphe's acute reaction to LSD to be directly contextualised with the widely-established macroscopic whole-brain network-level phenomena brought about by psychedelics. Explicitly, I hypothesized that (i) alterations in raphe functional connectivity would occur under LSD administration, that (ii) these raphe connectivity changes may be associated in a neuromodulatory fashion with established large-scale network connectivity alterations, and subjective ratings of the LSD experience, and that (iii) in line with homeostatic transmitter system models, connectivity alterations induced by LSD may relate to regional ratios of excitatory *versus* inhibitory serotonin receptors (i.e., the 5-HT2A and 5-HT1A receptors).

[20] It has been argued that LSD at high concentrations outcompetes endogenous serotonin in binding to 5-HT2ARs, due to a higher binding affinity (Nichols, 2016).

4.2 Materials and Methods

Participants and Data Acquisition

The data in this study were originally published and collected for a study by Carhart-Harris et al. (Carhart-Harris, Muthukumaraswamy, et al., 2016b). The work was collected at, and sponsored by, Imperial College, London. It was approved by the National Research Ethics Service Committee London–West London and recruitment and data acquisition conducted in accordance with the revised declaration of Helsinki (2000), the International Committee on Harmonization Good Clinical Practice guidelines and National Health Service Research Governance Framework. Additionally, given the usage of psychedelics in the experimental setup, the research was conducted under a Home Office license for research with schedule 1 drugs.

In brief, participant recruitment was carried out *via* word of mouth. All participants provided written informed consent for their participation after receiving a full study briefing and screening for pre-existing physical and mental health issues. An electrocardiogram (ECG), routine blood and urine tests for recent drug use and/or pregnancy were performed, as well as a psychiatric interview. Participants also provided full disclosure of their drug use history. Prospective participants were subjected to key exclusion criteria. Participants had to be above 21 years of age; have no history of psychiatric illness and/or family history of psychosis; have had previous experience with a classic psychedelic (i.e. LSD, mescaline, psilocybin/magic mushrooms or DMT/ayahuasca); not have ingested any psychedelic drug within 6 weeks of the first neuroimaging session; not be pregnant; not have alcohol use above 40 units per week; or another medically significant condition making them unsuitable for the study.

The 20 recruited healthy volunteers underwent two scanning sessions, two weeks apart. During one session they were intravenously administered a placebo (10ml saline solution), and on the other an active dose of LSD (75 µg of LSD in 10-mL saline), in a randomised design. Resting-state fMRI (rs-fMRI) was collected in three seven minute scans with the participant's eyes closed. For the work in

this chapter, the first and third scan were concatenated as both were eyes-closed resting-state recordings, unlike session two which involved the delivery of a musical stimulus.

All imaging was performed on a 3T GE HDx system. Anatomical scans were acquired using fast spoiled gradient echo scans in an axial orientation, with a field of view = 256 × 256 × 192 and matrix = 256×256×129 providing 1mm isotropic voxel size (TR/TE = 7.9/3.0ms; inversion time = 450ms; flip angle = 20◦). The rs- fMRI acquisitions lasted 7 minutes and 20 seconds, and used a gradient echo planar imaging sequence (TR/TE = 2000/35ms, FoV = 220mm, 64×64 acquisition matrix, parallel acceleration factor = 2, 90◦ flip angle). Thirty five axial slices were acquired with interleaved slice acquisition order, at 3.4mm thickness with zero slice gap (3.4mm isotropic voxels). One participant aborted the experiment due to anxiety and four others had to be excluded from analysis due to excessive head-motion, leaving n=15.

Spatial and Temporal Preprocessing

Preprocessing for all scans was again performed using the CONN functional connectivity toolbox utilising statistical parametric mapping (SPM12; Wellcome Centre for Human Neuroimaging, London, UK) functions on a MATLAB (2018b, The Mathwork, Inc. Natick, Massachusetts, USA) platform.

For consistency throughout the chapters of this thesis, the spatial and temporal preprocessing was identical to the previous chapters: For spatial preprocessing, functional images were slice-time corrected, centred to (0,0,0) MNI coordinates, realigned to correct for movement, and were subjected to ART-based identification of outlier scans for scrubbing. Following this, the structural image was co-registered to the mean functional image and then segmented and spatially normalized to the Montreal Neurological Institute (MNI-152) template. Functional images were then normalised to the MNI-152 template based on parameters obtained from structural normalisation and smoothed with a 6mm Gaussian kernel at full width half-maximum (FWHM).

Denoising again employed the anatomical CompCor (aCompCor) method, utilising masks for white matter (WM) and cerebrospinal fluid (CSF) produced with structural segmentation in order to regress out physiological noise in the BOLD signal, as this can otherwise influence BOLD signal and

resulting functional connectivity estimates (Behzadi et al., 2007). The aCompCor method as implemented in CONN regresses out the first five principal components of WM and CSF signals, movement parameters obtained from realignment, and their first-order derivatives – alleviating the need for global signal regression.For these convergent reasons, and in line with previous work, I elected not to remove the global signal, instead opting for the rigorous denoising procedure offered by aCompCor. Finally, the data were linearly detrended and filtered using a high-pass filter of 0.008 Hz, in line with my previous work on the investigation of brainstem functional connectivity (Chapter II and III; Spindler et al., 2021).

Behavioural assessment

At the end of each scan, participants were asked to perform visual analogue (VAS) ratings. The scale range was from 0 (lowest) to 10 (highest) and assessed key experiential dimensions/subjective ratings of the LSD experience, namely: (i) *overall intensity* of the psychedelic experience, (ii) *complex imagery* (i.e. eyes-closed visions of objects, entities, landscapes etc.), (iii) *simple hallucinations* (i.e. eyes-closed visions of shapes, colours, geometric patterns etc.), (iv) *emotional arousal* (i.e. how emotional the participant felt, regardless of whether emotions were positive or negative), (v) *positive mood*, and (vi) *ego-dissolution* (i.e. a fading sense of self, ego and/or subjectivity).

The specific questions were phrased as follows: 1) “Please rate the intensity of the drug effects during the last scan”, with a bottom anchor of “no effects”, a mid-point anchor of “moderately intense effects” and a top anchor of “extremely intense effects”; 2) “With eyes closed, I saw patterns and colours”, with a bottom anchor of “no more than usual” and a top anchor of “much more than usual”; 3) “With eyes closed, I saw complex visual imagery”, with the same anchors as item 2; 4) “How positive was your mood for the last scan?”, with the same anchors as item 2, plus a mid-point anchor of “somewhat more than usual”; 5) “I experienced a dissolving of my self or ego”, with the same anchors as item 2; and 6) “Please rate your general level of emotional arousal for the last scan”, with a bottom anchor of “not at all emotionally aroused”, a mid-point anchor of “moderately emotionally aroused” and a top anchor of “extremely emotionally aroused”.

Serotonergic Raphe Nuclei Functional Connectivity analyses

To resolve the serotonergic nuclei's reaction to LSD in rs-fMRI, I used regions of interest (ROIs) of both the MnR and DR nucleus from the HAAN atlas (Edlow et al., 2012). The faithful co-registration of these ROIs to anatomical and functional scans was extensively assessed visually. Seed-to-voxel functional connectivity calculation followed the same procedures as outlined in Chapter II and III to assess the LSD-induced connectivity changes for the raphe with every voxel in the brain whole brain. The functional connectivity analyses produced seed-to-voxel parameter estimate β-images which were entered into group-level analyses in which placebo and LSD conditions were contrasted. For all seed-to-voxel analyses, the reported results are again thresholded at voxel level $p<0.005$ (uncorrected) and cluster level $p<0.05$ (FWE-corrected for multiple comparisons. The labels reported in Table 1 are the CONN atlas references to anatomical regions.

For the sub-analyses that assess the potential association of regional 5-HT receptor levels with connectivity changes of the raphe nuclei, receptor maps and seed-to-voxel parameter images were resampled using the Schaefer 1000 region parcellation (Schaefer et al., 2018). For each parcel I extracted the average receptor density and the functional connectivity difference between experimental conditions (Δ LSD - placebo) for the MnR and DR to each of the 1000 parcels respectively. The resulting vectors of receptor density and Δ-FC change were then entered into both frequentist and Bayesian regression analyses.

As frequentist tests of the null hypothesis cannot conclude in favour of the H_0 but merely to its rejection, I also use Bayesian statistics (using JASP v.14.0.0), which enable inferences to be made with confidence on the absence of a difference (or absence of correlation or prediction). Traditional concepts of type I and type II error do not apply to Bayesian statistics, where one instead determines the relative model evidences in favour of the null (H_0), or in favour of the alternate hypothesis (H_1), or that the evidence is indeterminate from the data available – also forgoing the issue of correcting for multiple comparisons. The parameter BF_{10} (known as Bayes Factor) that we report is a measure of the evidence in favour of H_1 over H_0. For instance, a $BF_{10} = 8$ means that there is 8 times as much

evidence for H_1 than there is for H_0. A BF_{10} between 1 and 3 is regarded as 'mild', between 3 and 10 as 'moderate', between 10 and 30 as 'strong', and above 30 as 'very strong' evidence for H_1 over H_0. Such an approach is particularly useful in a small sample such as the one at hand as it reduces the danger of false positives and negatives as it can arise from traditional frequentist analyses.

Cortical cluster 'downstream' whole-brain connectivity and Mediation analyses

The clusters that were found to be significantly disconnected from the MnR and DR in primary seed-to-voxel analyses were entered into additional seed-to-voxel analyses (also see 'neuromodulation' approach from Chapter I). First, these "disconnection-clusters" seed-to-voxel functional connectivity was assessed in the placebo condition. Brain areas found to be significantly connected at rest during placebo were extracted as separate binarized masks, as ground truths of unperturbed resting state network connectivity. The disconnection-clusters' connectivity change (i.e. Δ LSD - placebo) to these masks was then extracted. This was subsequently entered into a correlation analysis with the raphe-'disconnection-cluster' connectivity change.

In a secondary analysis, these disconnection-cluster↔mask connectivities were used as the mediator in average causal mediation analyses between raphe↔disconnection-cluster connectivity change and visual analogue scale (VAS) ratings of the psychedelic experience. Bootstrapping (n=10000) was used to obtain an additional non-parametric approximation of confidence intervals for the 2.5 and 97.5 percentiles. Frequentist correlation analyses were performed using RStudio (packages: "ggplot", "statcor" and "mediation") and Bayesian correlations were performed using the software JASP (v.14.0.0).

4.3 Results

4.3.1 LSD induces raphe seed-to-voxel connectivity changes

To establish whether LSD administration affects the function of the raphe nuclei, I began by assessing the MnR and DR functional connectome during placebo and thereafter by contrasting placebo and LSD conditions.

At rest during placebo, the median raphe (MnR) showed significant seed-to-voxel connectivity within the brainstem, as well as to precuneus and posterior cingulate cortex, thalamus, amygdala, lingual gyrus parahippocampal and hippocampal gyri, temporal regions and the cerebellum (see Fig.16a.i). Similarly, the dorsal raphe (DR) showed significant seed-to-voxel connectivity to the same regions, however its cortical connectivity was not as rostrally extended, but covered more of the amygdala, and reached to the temporal pole, pallidum and insula (see Fig.16b.i).

In contrasts of LSD administration with placebo, both raphe nuclei showed significant reductions in functional connectivity. The MnR nucleus showed losses in connectivity with the precuneus, thalamus, brainstem areas, lingual gyrus, and cerebellar regions (see Fig.16a.ii, Table 4). Similarly, the DR nucleus also showed losses in precuneus, cerebellar and vermis connectivity, with further connectivity decreases in the PCC and superior parietal lobule (see Fig.16.bii, Table 4). No significant increases in connectivity of either of the raphe nuclei were observed with LSD administration. It is worth noting that the posterior cortical regions from which both MnR and DR nuclei disconnected, coincide with regions in which decreased blood flow, activity and connectivity have previously been reported following psychedelic administration (Carhart-Harris et al., 2012; Carhart-Harris, Muthukumaraswamy, et al., 2016; Palhano-Fontes et al., 2015a), and are rich in the 5-HT2a receptor (Beliveau et al., 2017; compare Appendix CIV, Fig.CIV.1). This region is also adjacent to the area from which I found disconnections in propofol sedation and DOC patients in Chapter II.

To be able to ascertain the specificity of this effect to the serotonergic system, I also assessed seed-to-voxel connectivity of the ventral tegmental area and locus coeruleus as the other two monoaminergic nuclei. Both of these did not show any significant disconnections in seed-to-voxel analyses.

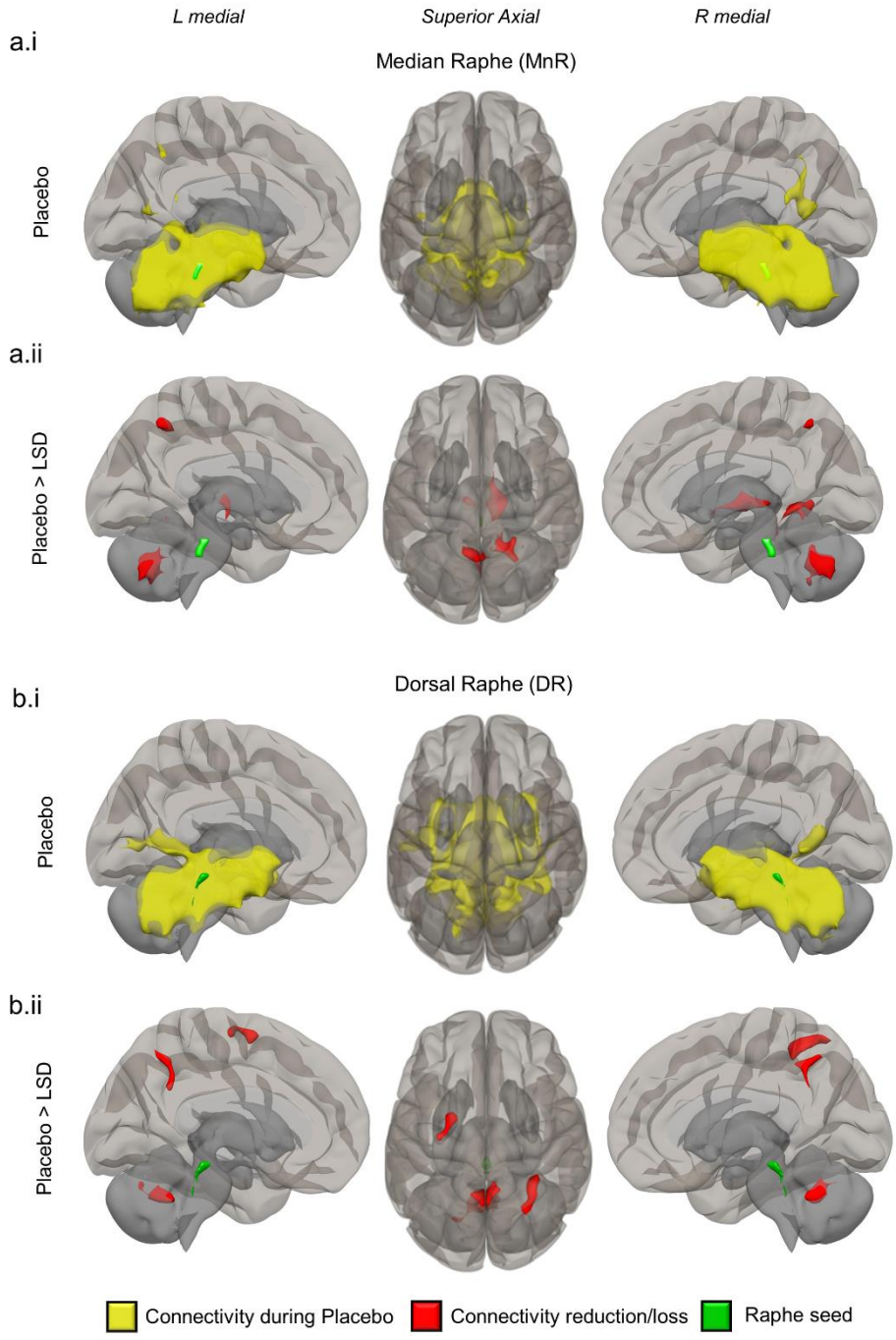


Fig. 16: Seed-to-voxel connectivity of Median and Dorsal Raphe is altered in response to LSD. (a.i) During placebo, the MnR showed significant resting connectivity to the brainstem, thalamus, cerebellum and various other subcortical targets, as well as the precuneus and posterior cingulate. (a.ii) In whole-brain contrasts of placebo>LSD, the MnR displayed resting state functional connectivity losses from precuneus and posterior cingulate cortex, as well as thalamus, lingual gyrus and cerebellar areas (see Table 1). (b.i) Similarly to the MnR, the DR showed significant intra-brainstem, cerebellar, thalamic, other subcortical, and posterior and temporal cortical connectivity during placebo. (b.ii) The DR also showed precuneal/posterior cingulate, cerebellar and superior parietal lobe connectivity losses in a contrast of placebo>LSD. No increases in functional connectivity of either of the raphe nuclei were found. Brains are in neurological orientation, i.e. 'L' is left. Results are at $p<0.005$ level (uncorrected) and $p<0.05$ cluster-level (FWE-correction). At more restrictive thresholds only the MnR showed significant disconnections.

***Table 1: Median (MR) and Dorsal Raphe (DR)* connectivity during placebo and connectivity changes in LSD condition.** CONN atlas labels, peak MNI coordinates, cluster extent and FWE-corrected p-values are reported. Seed-to-voxel connectivity contrasts were thresholded at p<0.005 voxel-level (uncorrected) with p<0.05 cluster-level (FWE-corrected). ↓Loss demarcates decreased functional connectivity in comparison to placebo condition.

Nucleus	Condition/ contrast	*Δ connectivity*	*Anatomical regions (CONN atlas)*	*Peak MNI Coordinates*	*Cluster size*	*Cluster p (FWE-corrected)*
Median Raphe	**Placebo > LSD**	↓Loss	*Precuneus (PCu)*	*-04 -56 +50*	*192*	*0.043*
			Ver6,7,8,9, Cereb1,2,7,8,9 l+r	*-06 -64 -34*	*744*	*0.001*
			Thalamus l+r, Brainstem	*+10 -30 +00*	*469*	*0.001*
			LG r, Ver45, Cereb45 r, pPaHC r	*+24 -58 +00*	*307*	*0.012*
Dorsal Raphe	**Placebo > LSD**	↓Loss	*Cer6,8,9 l+r, Ver6,7,8,9,10*	*-08 -56 -34*	*425*	*0.002*
			Precuneus (PCu), Posterior Cingulate (PCC)	*+00 -56 +48*	*281*	*0.023*
			SPL r, sLOC r	*+26 -62 +58*	*254*	*0.040*

4.3.2 Raphe-PCu disconnection is associated with precuneal-default mode network integrity and subjective ratings of the psychedelic experience

Given reports of disintegration of the default mode network (DMN) in previous neuroimaging studies of psychedelics, and particular involvement of the precuneus (Felix & Stefan, 2019; Palhano-Fontes et al., 2015a; Tagliazucchi et al., 2016a), I hypothesized that the observed raphe disconnection may represent an acute alteration in serotonergic modulation of this cortical node – which should consequently be associated with DMN network integrity.

To approximate neuromodulatory influence, I first performed placebo resting-state connectivity of the respective PCu clusters (from which the raphe disconnected during LSD administration, see Fig.16a.ii and 16b.ii). Both the MnR's and DR's PCu disconnection cluster was predominantly located within the Schaefer atlas precuneal region assigned to the DMN, but also partially the dorsal attention network (DAN; Schaefer et al., 2018). In concordance with this spatial location, their whole-brain connectivity patterns revealed by using the clusters as seeds mainly overlapped with the DMN, but also captured some smaller aspects of the DAN. I created masks of these PCu whole-brain placebo connectivities, which are referred to as DMN+. Using these masks, Δ LSD – Placebo connectivity changes of the PCu clusters to the masks were extracted, which were then entered into correlations with the strength of LSD-induced MnR and DR disconnection from PCu (Δ LSD – Placebo MnR-PCu FC). Strikingly, MnR-PCu connectivity strength was strongly associated with the PCu-DMN+

connectivity change (R=0.643, p=0.005, BF_{10}=13.47, see Fig.17a), indicating that the weaker MnR-PCu connectivity became after LSD administration, the less the PCu-seeded functional connectome resembled the DMN+ connectivity observed during placebo. For DR-PCu connectivity and PCu-DMN+ integrity, there was no significant correlation observed (R=0.107, p=0.705, BF_{10}=0.32).

DMN integrity has previously been associated with ego dissolution and intensity of the psychedelic experience (Carhart-Harris et al., 2016, 2012; Palhano-Fontes et al., 2015). Having established a link of MnR-PCu connectivity with DMN+ connectomic integrity, it was therefore hypothesized that – as a potential neuromodulatory rate-limiter of DMN+ integrity – MnR-PCu disconnection should also be associated with these two dimensions of the psychedelic experience. To statistically resolve how the interplay between MnR-PCu connectivity and DMN+ integrity may bring about the ego dissolution and intensity effects, I performed an average causal mediation analysis. As a first necessary step, it was found that MnR-PCu disconnection was significantly negatively correlated with intensity ratings of the psychedelic experience (R=-0.558, p=0.015, BF_{10}= 2.539, Fig.17b), and at trend-level with ego dissolution (R=-0.329, p=0.05, BF_{10} = 2.399; Fig.17c), which in the context of a mediation analysis constitutes the observation of significant 'total' effects. The subsequent causal mediation analyses used Δs (LSD – placebo) of MnR-PCu connectivity as the independent variable/predictor (see Fig. 17a, green arrow), intensity and ego dissolution as the outcome variables, and Δ PCu-DMN+ connectivity (LSD – placebo; magenta arrow Fig.17a) as the mediator variable.

A significant average causal mediation effect (ACME) was found, meaning that a significant proportion of the effect of Δ MnR-PCu FC on subjective ratings of intensity of the psychedelic experience was mediated through the PCu-DMN+ connectivity change (β = 30.96, CI=4.39:66.91, p=0.013). Similarly, a significant ACME was also found for ego dissolution (β = 58.09, CI=5.07:131.56, p=0.029). This indicates that both intensity and ego dissolution ratings of the LSD experience are putatively driven by the MnR-PCu disconnection, but that this effect is cortically mediated *via* PCu-DMN+ connectivity – thus suggesting that LSD-induced MnR-PCu connectivity

changes may, at least partially, causally underpin behaviourally-relevant DMN+ alterations that in turn underpin the psychedelic experience.

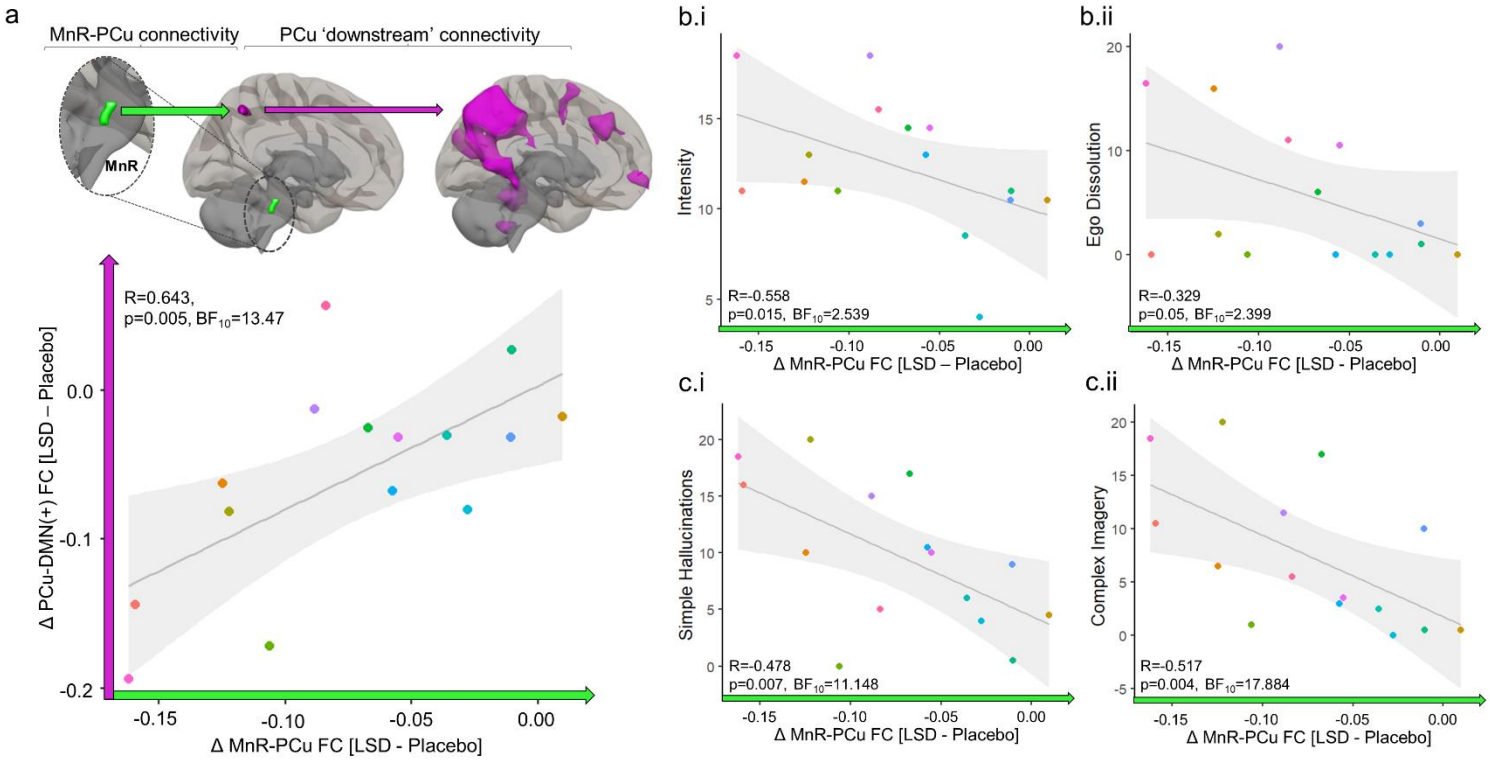


Fig. 17: Raphe disconnection from PCu is associated with precuneal whole-brain network integrity and subjective ratings of the psychedelic state/experience. (a) The strength of median raphe disconnection from precuneus (green arrow) was strongly correlated with LSD-induced connectivity loss of the precuneus cluster (magenta arrow) to 'downstream' areas it was normally functionally connected with during placebo. Bayesian support for this correlation was above the 'strong' cut-off. (b.i & b.ii) Behaviourally, the MnR-PCu connectivity loss was strongly associated with greater subjective ratings of the intensity of the LSD experience and at trend-level associated with ratings of ego dissolution. There was 'mild' Bayesian support for these correlations. (c.i & c.ii) The MnR-PCu connectivity loss was also strongly associated with hallucination and complex imagery ratings, with 'strong' Bayesian support for both of these correlations. Dot colour represents individual participant throughout the figure. Plots were made in RStudio, with both frequentist and Bayesian correlation strengths and significances extracted using JASP.

However, the precuneus is also thought to play a key role in visuospatial integration (Cavanna & Trimble, 2006; Schott et al., 2019). To produce a complete picture of the relevance of MnR-PCu connectivity I therefore also explored whether MnR-PCu disconnection may plausibly be associated with visual experience ratings, namely for 'Simple Hallucinations' and 'Complex Imagery' captured on the VAS. Δ MnR-PCu disconnection was strongly associated with both simple hallucinations (R=-0.478, p=0.007, BF_{10}=11.148; Fig.17d) and complex imagery (R=-0.517, p=0.004, BF_{10}=17.884;

Fig.17e). Importantly however, no causal mediation effect related to the PCu-DMN+ connectivity (as observed for intensity and ego dissolution) was found, indicating that a raphe-mediated effect underpinning the acute visual effects of LSD may either be more localised on the precuneus, or most likely related to specific PCu relationships with the visual system, rather than the DMN+.

No associations for DR-PCu connectivity with any of the subjective ratings and DMN integrity metrics were found. As the first requirement for mediation analyses was thereby not passed, no subsequent mediation analyses were calculated. Equally, as the VTA and LC as control regions did not show any PCu disconnection in the primary seed-to-voxel analyses, subsequent mediation analyses could not calculated.

4.3.3 Strength of raphe disconnection is associated with relative 5-HT2a receptor density of cortical regions

The significant cortical seed-to-voxel disconnection of both raphe in the precuneus occurred in 5-HT2AR rich areas when visually compared with high resolution PET maps for this receptor published by Beliveau et al (2017; see Appendix CIV, Fig.CIV.1). There is however also moderate to high expression of the 5HT1AR in these areas and a varied co-expression of 5HT2AR and 5HT1AR throughout the brain (Beliveau et al., 2017). Given the 5-HT2AR and 1AR's mutually antagonistic functional relationship (i.e., 2A receptor agonism has excitatory effects on host cells *vs* 1A's inhibitory effects), it is likely that serotonergic modulation may be most strongly perturbed in regions that are more vulnerable to excitatory LSD effects *via* 5HT2AR, without compensatory inhibitory effects *via* 1AR. As such in line with a model of homeostatic transmitter system function (Baumeister et al., 2014), I hypothesized that as a potential proxy of serotonergic modulation, LSD-induced raphe connectivity alterations with a given brain region may be most pronounced from regions of greater relative density of 5-HT2AR over 5-HT1AR. Higher 2A:1A ratios were therefore expected to result in a proportionally stronger functional connectivity alteration of the raphe nuclei (Δ LSD - placebo) from a given region.

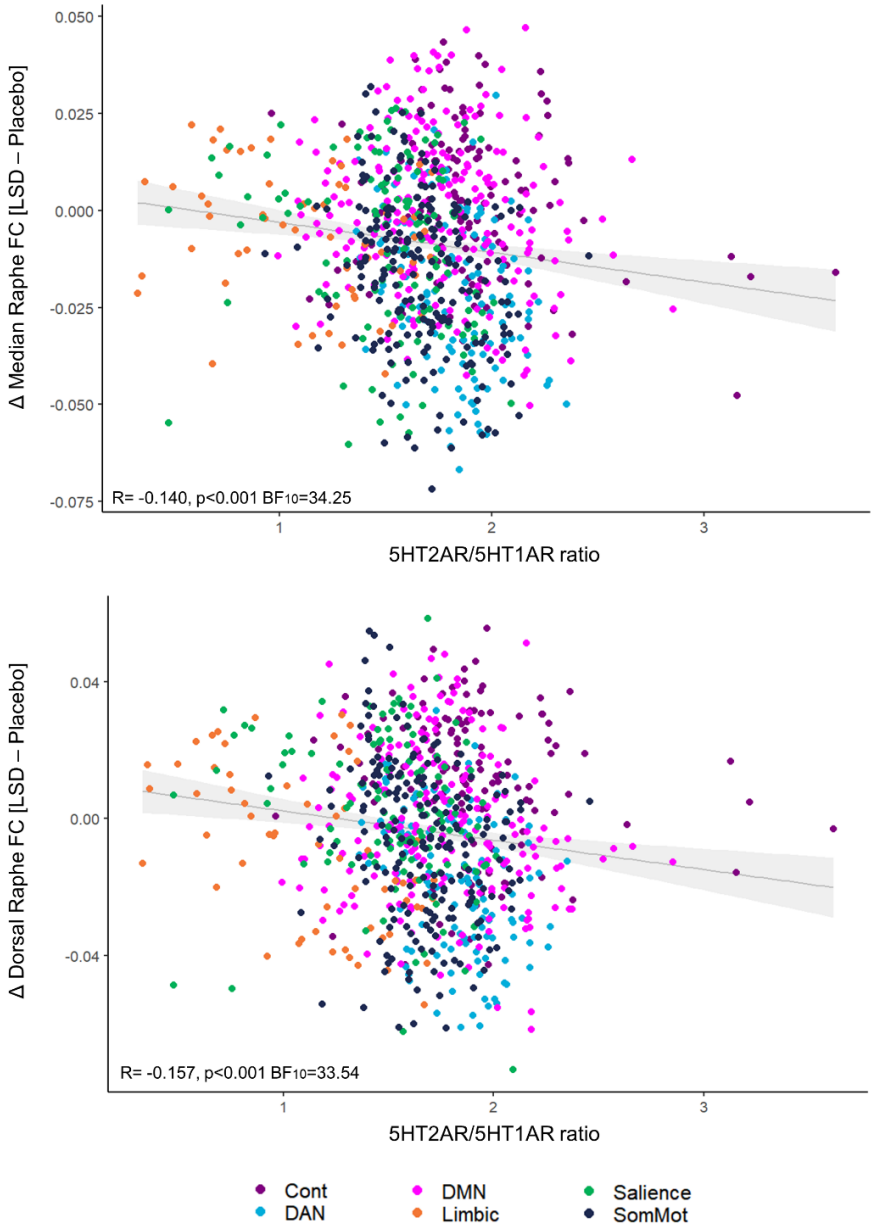


Fig. 18: Strength of LSD-induced raphe disconnection is associated with relative regional density of the 5-HT2A receptor. For both of the serotonergic source nuclei, the average level of regional disconnection observed in our sample (Δ connectivity change LSD-Placebo) was significantly associated with the relative density of 5-HT2AR (over and above 5HT1AR) in the region in question, meaning that higher relative regional 5-HT2AR levels were a predictor for stronger raphe disconnection from that region. Bayesian support for this predictive power of relative regional 5-HT2AR density for both Median and Dorsal Raphe disconnection was above the 'strong' evidence cut-off. Colours in graphs correspond to established labels of canonical resting state networks (Yeo et al., 2011; DMN = Default Mode Network; DAN = Dorsal Attention Network; ECN = Executive Control Network; LN = Limbic Network; SN = Salience Network; SMN = Somatomotor Network; as per the Schaefer 1000 parcellation. See Appendix CIV for graph including Visual Network, VN). Grey line is overall regression line with 95% confidence interval. Correlation rendering was made in RStudio, with both frequentist and Bayesian regression strengths, significances and support extracted using JASP v14.0.0.

To reflect regionally heterogenous expression differences of the 5-HT2AR and 5-HT1AR, I used said 5-HT receptor maps published by Beliveau et al. (Beliveau et al., 2017a), to extract average receptor densities for each of the 1000 regions provided in the Schaefer atlas. The resulting regional receptor densities were then entered into linear regressions as predictors of the average connectivity change (Δ functional connectivity LSD-placebo) of the MnR and DR nuclei averaged across subjects (see Fig.2a & b). Although the spread of connectivity change was wide, in concordance with our hypothesis, relative density of 5-HT2AR over 5-HT1AR was a significant predictor of Δ raphe connectivity for both MnR ($R=-0.140$, $p<0.001$, $BF_{10}=34.25$) and DR ($R=-0.157$, $p<0.001$, $BF_{10}=33.54$). Higher relative 5-HT2AR density (higher 2A:1A ratio) was therefore associated with stronger average raphe disconnection (see Fig.18a and 18b). This relationship remained significant both when including and excluding the visual network due to its extremely heightened 5-HT2AR but very low 1A density (see Appendix CIV, Fig.CIV.2). Overall, the significance of the frequentist regressions and Bayesian regression support above the 'strong' cut-off, corroborate the interpretation that raphe disconnection may – in biologically and pharmacologically plausible fashion – be a consequence of the strength of 5-HT2AR-induced excitatory effects on cortical regions.

4.4 Discussion

In this chapter, I provide novel *in vivo* evidence of an acute serotonergic system disruption in humans in response to the psychedelic drug LSD, and the first human fMRI evidence of altered serotonergic brainstem nucleic functioning under a psychedelic. Compared with placebo, LSD administrations were characterised by an acute decrease in raphe connectivity to subcortical and particularly cortical areas: both median and dorsal raphe showed specifically decreased connectivity to the PCu, a central node of the brain's default mode network, whose dysregulation has repeatedly been implicated in psychedelic administrations (Felix & Stefan, 2019). For the median raphe, the degree of this disconnection was associated both with decreases in DMN integrity in putatively neuromodulatory fashion, as well as with various subjective ratings of psychedelic effects. Importantly, I also

demonstrated that this serotonergic system disruption is neurobiologically and pharmacologically plausible in its association with relative regional 5-HT2AR density, the receptor *via* which psychedelics are thought to elicit their main excitatory and consciousness-altering effects. Taken together, these data thus suggest that the acute neural effects of LSD administration might not be characterised only by direct signalling *via* 5-HT2AR binding alone as previously suggested, but additionally through a marked dysregulation of the serotonergic brainstem source nuclei downstream of those cortical effects. In the following I discuss these acute effects on the raphe in the context of the existing literature.

To begin with, LSD administration is characterised by a significant connectivity disruption of both MnR and DR, with significant seed-to-voxel FC losses from particularly posterior cortical regions. This direct *in vivo* demonstration of an acute effect of LSD on raphe functioning in humans is a translational extension of the many preclinical animal experiments of the last century that reported that LSD (and other psychedelics) acutely affect activity of these serotonergic source nuclei (for review of early work: Jacobs & Trulson, 1979). This animal work revealed that both intravenous and local LSD administrations decrease raphe firing and spontaneous tonic activity (Foote et al., 1969; McCall, 1982). The functional disconnection observed here in humans may correspondingly coincide with a related lowering of serotonergic tone. This would also be consistent with a homeostatic model of serotonergic system function: Given LSD's purported higher affinity than serotonin itself for the 5-HT2AR (Kim et al., 2020; Nichols, 2012), heightened excitatory effects of LSD *via* this receptor should affect 'top-down' negative cortical feedback to the raphe to lower provision of serotonin. Likely v*ia* engaging local interneurons there, strong and almost full raphe inhibition is likely to occur as observed in animal studies (Aghajanian et al., 1968; Baumeister et al., 2014; Foote et al., 1969; McCall, 1982). Expressly, the function of this mechanism would be to curtail excessive 5-HT efflux and as such 'runaway' excitatory serotonergic modulation of the cortex.

This could explain the novel and biologically-intuitive finding that LSD-induced acute raphe disconnection is stronger the greater the 5-HT2AR:5HT1AR ratio in a brain region is. The more 5-HT2AR is available in a given region in absence of the functionally antagonistic inhibitory 5-HT1AR,

the more vulnerable said region is to greater LSD-derived excitatory modulation; this would thus proportionally more greatly disrupt the tonic homeostatic coupling between activity in that region and activity in the raphe nuclei to prevent excess excitatory neuromodulation. This model is supported by evidence from animal retrograde and anterograde tracing studies, as these have revealed that the raphe have brain-wide projections – but that the density of these projections is regionally heterogeneous Baumeister et al., 2014). Additionally, the observation that these projections are commonly bidirectional (i.e. brainstem-to-cortex and cortex-to-brainstem) provides the necessary architecture *via* which effects of LSD in cortical regions could alter cortical feedback to the raphe and in turn serotonergic tone provision in the suggested region-specific fashion (Avery & Krichmar, 2017; Baumeister et al., 2014).

It is likely that direct LSD binding to inhibitory (e.g. 5-HT1AR) raphe autoreceptors in the brainstem itself (Rogawski & Aghajanian, 1981) may play an additional role in LSD's effects (and possibly even more so in psychedelics such as 5-MeO-DMT with higher 5HT1AR affinity). Indeed, LSD also has a relatively high affinity for the 5-HT1A receptor (Nichols, 2016). Nevertheless, non-human animal recordings of LSD administrations together with the present analyses using 5-HT2A:1A ratio, suggest that LSD cortical excitatory effects *via* 5-HT2AR may be predominant drivers of the disruption of raphe nuclei function (de Gregorio et al., 2016). As such, effects of 5HT1AR binding directly in the raphe might act in synergy with the cortically-driven 5HT2AR-dependent effect to produce an overall disruption of raphe functionality.

The consciousness-altering consequences of changes in serotonergic neuromodulatory function may be most powerfully captured in the relationship of lowered MnR-PCu FC with precuneal-DMN+ connectivity disintegration. Significant projection tracts have been detected between the raphe nuclei and posterior cortical 'hotspots', coincident with the precuneus which is highly enriched in serotonin receptors (Behzadi et al., 1990; Ogawa et al., 2014). Therefore, tonic serotonergic signals likely modulate the precuneus' whole-brain connectomic (thus: default mode network) properties (Avery & Krichmar, 2017; Marder, 2012; Shine et al., 2019). Indeed, theoretical work by Georg Northoff and

colleagues has suggested that raphe-mediated serotonergic neuromodulation produces modulatory environments that favour the DMN in particular (Conio et al., 2019). The observation in this chapter that the individual severity of LSD-induced MnR-PCu disconnection is associated with DMN+ integrity provides some empirical evidence for this hypothesis, and may epiphenomenologically capture that LSD dominates post-synaptic serotonin receptors in cortical areas such as the precuneus, replacing and outcompeting serotonin.

The concomitant downregulation of the endogenous serotonergic tone while LSD occupies cortical receptors is likely to have vastly different qualitative consequences on cortical functioning than the normal, "clock-like" efflux of 5-HT. Indeed, the amount of LSD flooding the cortex is likely to far outstrip even the strongest of physiological phasic upregulations of serotonin provision – and importantly is acting globally, rather than selectively. As such, cortical functioning is effectively "hijacked" by LSD excitatory effects, with disrupted raphe connectivity being a central consequence – and together these processes cause macroscopic effects, such as the observed decreased within-DMN resting-state FC and decreased network modularity observed elsewhere (Tagliazucchi et al., 2016a). The PCu's network "hotspot" function and role as a transmodal association cortex may make it particularly vulnerable to brainstem-derived modulatory changes, as brainstem-PCu connectivity is also perturbed in sedation and DOC (see Chapter II). This may be explained by the fact that the precuneus area has undergone the greatest cyto-architectonic expansion in the evolution of the human neocortex, and contains one of the most complex neuromodulatory receptor and input landscapes throughout the brain (Bruner et al., 2017; Impieri et al., 2019; Paquola et al., 2019). This could indicate that even slight changes in tonic modulation of the precuneus might be of particular consequence for large-scale brain organisation.

The observation that a modulatory relationship with DMN integrity and psychedelic effects is found only for the MnR but not the DR may be explained by a much higher abundance of structural connections between MnR and posterior cortical regions than for the DR, as established in murine brains (Behzadi et al., 1990; Ogawa et al., 2014). The plausibility of this key role for the MnR is tentatively indicated by some older animal work that suggested the MnR to play a more pivotal role in

psychedelic action than the more-easily anatomically identified DR (Fink & Oelssner, 1981).[21] Importantly, the significant average causal mediation between MnR-PCu connectivity and intensity, as well as ego dissolution, *via* PCu-DMN+ integrity, implies a potential *causal* linkage between MnR-PCu connectivity and normal precuneal whole-brain connectivity. This is consistent with neuromodulatory theories that brainstem input to the cortex attunes network properties in a physiologically-relevant window (Avery & Krichmar, 2017; Shine et al., 2018, 2019). Conversely, dysregulation of raphe connectivity and local LSD effects together may drive network properties beyond window, causing the functional connectome to become heavily untethered from its structural underpinning, as previously observed by Luppi et al. (2021). To fully resolve the interplay between tonic serotonergic neuromodulation and its disruption by LSD however, a simultaneous PET/rs-fMRI approach with ligands such as that used by Madsen et al. (Madsen et al., 2019) will have to be employed, to assess whether dose-dependent displacement of a 5-HT2AR ligand upon LSD administration (or other psychedelics) is proportional to network disintegration – and whether changes in raphe FC accompany these effects. As an inception point, our results provide a framework that allows a working mapping between network effects, subjective experience and a relevant neuromodulatory effector system (the serotonergic raphe and serotonin receptors). Should further translational and back-translational research corroborate this work, raphe disconnection could become a useful, non-invasive, proxy of serotonergic modulation – which is as a process likely to contribute to therapeutic mechanisms and outcomes in psychedelic therapy (Carhart-Harris et al., 2021b).

Indeed, given the recency of the first randomized controlled trial of the psychedelic psilocybin *vs* a conventional selective serotonin reuptake inhibitor (SSRI) antidepressant (escitalopram; Carhart-Harris et al., 2021), the observation of acute effects of LSD on the raphe may be particularly useful for the generation of testable hypotheses of psychedelic treatment mechanisms. At first sight, macroscopic effects of SSRIs and psychedelics appear similar, with DMN hypo-connectivity in acute treatment and hyper-connectivity post-treatment, and both SSRIs and psychedelics are known to

[21] This easier anatomical identifiability might explain the overwhelming amount of experimental approaches concerned with the dorsal raphe nucleus, which typically do not mention and/or assess the median raphe.

strongly inhibit firing of the raphe acutely (Carhart-Harris et al., 2017; Klaassens et al., 2015; Kraus et al., 2014). The routes by which particularly the disruption of the raphe is incurred is however likely different between the two treatment modalities. SSRIs only slowly upregulate serotonin's global availability (Selvaraj et al., 2012), leading to the long lead-time until clinical effects are observed. Instead, the treatment effects with psychedelics are more sudden and long-lasting, with processes downstream of cortical receptor binding of psychedelics, such as the disruption of the raphe nuclei functionality, plausibly involved in the treatment mechanism. No direct comparison of especially the post-acute effects on the raphe nuclei has been performed in animals or humans, although SSRIs need to be taken at continuously high, or even escalating dosages, whereas with psychedelics one or two dosages can be sufficient for achieving remission in subjects as long as 6 months after treatment (Aday et al., 2020; Andersen et al., 2021; Carhart-Harris et al., 2018; Muttoni et al., 2019; Nutt et al., 2020; Ross et al., 2016). In accordance with allostatic theory, it could be that the great stressor of the acute psychedelic-induced raphe 'knockout' I observed here causes the serotonergic system to adaptively "reset" its functionality (McEwen, 2000; McEwen & Wingfield, 2003; Ramsay & Woods, 2014). This could provide a mechanism different of the continuous upregulation of serotonin at the postsynaptic level performed with SSRIs. I test this hypothesis across two datasets of depressive disorders in the following chapter (see Chapter V).

Numerous limitations of this work need to be acknowledged. Firstly, the specificity of the seeds being located to only serotonergic cells bodies of course remains an assumption, despite the histological work done to characterise them (Edlow et al., 2012), as inter-individual differences may exist and nuclei are not fully, but just predominantly homogenous for one transmitter type. For future studies, higher field strength scanning and specific acquisition parameters, such as multislice acquisition sequences would improve the resolution and confidence in this regard, aided further by additional molecular imaging (Puckett et al., 2018). Furthermore, some of the rs-fMRI alterations observed in this analysis may be associated with vasoactive properties of LSD. The intravenous mode of administration may also have affected outcome given its direct venous application and how it affects the pharmacokinetics of LSD (e.g., reducing onset and offset time). Additionally, different levels of

head motion between the treatment conditions may mean that off-target non-raphe effects may be inadvertently captured. However, I accounted for motion, cardiac, respiratory, and physiological noise artefacts in the denoising procedure and excluded those participants with excessive head motion, to combat the above issues, and found no correlation between head motion and FC estimates. Furthermore, the raphe nuclei's projections are well characterised in animal work, but less so in humans. Therefore, it is unclear whether the raphe structurally project to relevant cortical areas in humans, as they do in non-human animals – meaning that potential inter-species cyto-architectonic differences must be considered. Nevertheless, primate retrograde tracing and murine tracing studies suggest that there are raphe-PCu/PCC anatomical connections (Azmitia & Gannon, 1986; Behzadi et al., 1990), which together with the very high expression of serotonin receptors in the PCu/PCC gives confidence that raphe projection tracts to this region exist in human brains as well. Finally, the serotonin receptor PET maps were averaged per brain region for the present analyses, negatively impacting on resolution, but in an attempt to compensate for this a particularly high granularity parcellation was used. To advance on the present work, future studies could incorporate combined fMRI and PET techniques with various serotonin-receptor and -transporter specific ligands, as well as different psychedelic drugs, to progressively close these knowledge gaps and account for participant-specific serotonergic system differences.

In conclusion, Chapter IV provides initial evidence that the functioning of serotonergic brainstem source nuclei is disrupted by the psychedelic LSD in a manner that relates to recognised and replicated effects of the drug on network organisation and subjective experience. Raphe connectomics, like VTA connectomics (see Chapters II and III), can provide translational bridges between our preclinical knowledge of effects of a drug (inhibition of raphe in animals) and the macroscopic phenomena associated with the same substances in humans (network disintegration and experience ratings). Therein, brainstem transmitter-system connectivity analyses might provide *in vivo* biomarkers relevant for the study of certain pathologies and their suggested treatments. The following chapter takes preliminary steps towards the potential clinico-translational value of raphe connectomics.

Chapter V

5.1 Preface and Overview

At the end of Chapter IV, I suggested that acute psychedelic-induced raphe connectivity dysregulation might constitute a 'knockout' effect on the serotonergic system. As psychedelics have shown great clinical promise in the treatment of depression, a pathology often considered to arise from a monoaminergic (and specifically serotonergic) deficit, I hypothesized that such an *acute* psychedelic-induced 'knock-out' may precipitate a *sub-/post-acute* emergence of improved raphe function ('reset').

To complement Chapter IV and further establish whether this thesis' brainstem analysis approach has clinical utility, the following chapter took a stepwise, data-driven approach to (i) firstly establish diagnostic and treatment-tracking value of raphe connectomics in depression, and to (ii) secondly assess whether the related findings are consistent with a psychedelic-induced 'reset' of raphe functionality. In the broader context of this thesis, this aimed to establish whether strong pharmacological stimuli which cause *acute* transmitter system disruption might incur *post-acute* improvements in transmitter nucleus functionality.

The two datasets of depressed patients were collected by a large team led by our collaborators Leor Roseman, David J. Nutt, and Robin Carhart-Harris at Imperial College, London. Explicitly the two datasets were the neuroimaging subsamples of the open-label trial of psilocybin in treatment-resistant depression (Carhart-Harris et al., 2016; TRD-OLT) and the recent randomized controlled trial of psilocybin *vs* a classical SSRI (escitalopram) in major depressive disorder (Carhartt-Harris et al., 2021; MDD-RCT). I conducted all analyses and wrote the chapter.

Serotonergic raphe reset following the psychedelic altered state of consciousness is involved in alleviation of depression

5.1.1 Summary

Psychedelic drugs have fast-acting and long-lasting antidepressant properties. However, no systems-level mechanism for these effects that occur after single/double doses has been identified – and the linkage between acute and post-acute drug effects remains unclear. Motivated by findings from **Chapter IV** and the monoaminergic theory of depression, I hypothesized that successful depression treatment through psychedelics may after *acute* disruption of raphe functionality precipitate *sub/post-acute* improvement of serotonergic source nucleus function – i.e. follow a *knockout-to-reset* architecture. I tested this hypothesis in a stepwise, data-driven fashion using data from an open label psilocybin trial (n=15; rs-fMRI before and 1 day after dosing) in treatment-resistant depression (TRD-OLT) and the recent randomized double-blind controlled trial of psilocybin (n=22) *vs* escitalopram (n=20) in major depressive disorder (MDD-RCT; rs-fMRI before and 3 weeks after dosing). Strikingly, data-driven whole-brain correlation analyses of MnR seed-to-voxel connectivity with depression outcome revealed that only raphe-precuneus coupling at sub/post-acute timepoints is significantly associated with antidepressant effects: Psilocybin significantly upregulated raphe-precuneus coupling in successful treatment in the TRD-OLT and MDD-RCT cohorts, with the magnitude of connectivity enhancement being proportional to the extent of depression symptom reduction, as well as predictive of the longevity of treatment outcomes. Macroscopic efficacy-related phenomena, such as enhanced DMN integrity, were tightly related to how upregulated raphe-cortex coupling became after dosing. Finally, I also found that only psilocybin significantly increased spontaneous MnR BOLD activity. No connectivity, nor activity upregulation was observed for SSRI treatment, suggesting that this effect may be unique to psychedelics. Altogether, these robust acute and sub-/post-acute effects on the raphe provide empirical evidence that a raphe *knockout-to-reset* may occur in response to psychedelic treatment, and that increases in serotonergic source nucleus functionality may underpin the psychedelic antidepressant mechanism. In the broader context of this

thesis, these results suggest that raphe brainstem connectomics may have clinical utility that extends to beyond ASCs alone.

5.1.1 Introduction

Major depressive disorder (MDD) is the most prevalent mental illness across the globe, with over 300 million people affected, meaning that up to 6% of the global adult population suffer from this debilitating condition yearly (Bromet et al., 2011; Otte et al., 2016). The *Diagnostic and Statistical Manual V* (DSM-V) outlines that an MDD episode is characterised by hopelessness, anxiety, apathy, anhedonia, fatigue, lack of energy, as well as impaired cognitive function and multiple vegetative symptoms (Otte et al., 2016; Tolentino & Schmidt, 2018). Indeed, on the basis of these alterations, and patient reports of altered sensoria, perceptions, feelings of 'non-presence' and general environmental and interpersonal disconnectedness in their depressed state, recent work has suggested that depression might in fact have to be classed as an altered state of consciousness (Fuchs, 2013; Ouwersloot et al., 2020; Whiteley, 2021). Irrespective of whether such an ASC classification is ultimately advisable, MDD and other forms of depression are highly recurrent, and cause severe impairment of professional, personal and social lives, with an enormous societal cost associated with their prevalence (Malhi & Mann, 2018). Consequently, an in-depth understanding of the underlying mechanisms of depression is of utmost importance to produce effective treatments.

Although recent work has introduced the importance of inflammation (Bullmore, 2018), hypothalamic-pituitary-adrenal axis function (Keller et al., 2016), neuroplasticity (Egeland et al., 2015), as well as genetic (Flint & Kendler, 2014) and environmental factors in MDD (Teicher & Samson, 2013), the main theoretical framework for the underlying pathological cause of MDD has remained the monoamine hypothesis (Malhi & Mann, 2018). This hypothesis postulates that depression is underpinned by a deficit of monoamines at disease-relevant receptor sites throughout the brain – a deficit which antidepressant therapies serve to alleviate (Cosci & Chouinard, 2019; Delgado, 2000). This framework originated from the serendipitous observation that various antihypertensive drugs reduced total levels of monoamine neurotransmitters, which triggered major depressive

episodes in patients receiving these medications (Otte et al., 2016). This linkage was subsequently empirically supported by experiments using monoamine oxidase (the enzyme that metabolizes monoamines; MAO) inhibitors, as these had *anti*-depressant properties. This produced the 'first-wave' depression treatments that capitalised on MAO-inhibition (Cosci & Chouinard, 2019), followed by the 'second-wave' of the tricyclics which amplified largely serotonin and noradrenaline (sparing dopaminergic increases; Pereira & Hiroaki-Sato, 2018). Today, the five major mainstream classes of drugs to treat depression (serotonin or noradrenaline reuptake inhibitors, SSRIs/SNRIs; dual serotonin and noradrenaline reuptake inhibitors; selective MAO-A inhibitors; reversible MAO-A inhibitors; selective monoaminergic receptor stimulants) all remain focussed on this central monoaminergic paradigm, in particular on serotonin (Goldberg et al., 2014; Malhi & Mann, 2018). For almost all of the presently available drugs however, effects take a long time of continuous treatment (weeks) to manifest, and some forms of depression are highly resistant to these therapies – creating a clearcut need for faster- and longer-acting novel treatment alternatives (Jakobsen et al., 2020; Malhi & Mann, 2018).

Consistent with the importance of monoamines and in particular serotonin, a key alternative class of therapeutics that has (re-)emerged as a promising treatment modality for MDD (after initial work in this context in the previous century; Nichols, 2016; Nutt et al., 2020) are the classical serotonergic psychedelics (Andersen et al., 2021). Among them, particularly psilocybin (4-phosphoryloxy-N,N-dimethyltryptamine) has over the past decade received great attention in various open-label and randomized controlled trials for depression, thus far culminating in the United States Food and Drug Authority (FDA) granting it "breakthrough therapy" status (Galvão-Coelho et al., 2021; Muttoni et al., 2019). In depressed populations, psilocybin (and other psychedelics) show significant long-term beneficial effects for reducing depressive symptoms (Carhart-Harris et al., 2021a; Carhart-Harris et al., 2016; Griffiths et al., 2016; Grob et al., 2011; Palhano-Fontes et al., 2019; Ross et al., 2016). In contrast to conventional SSRI treatment, the therapeutic effects of psychedelics occur relatively quickly, and are long-lasting (Carhart-Harris et al., 2018; Palhano-Fontes et al., 2019), and the safety, tolerability and reproducibility of these drugs' effects are consistently reinforced in complementary

trials in healthy populations (Rucker et al., 2022). Possibly most importantly, the antidepressant effects of psychedelics are also evocable in populations that are resistant to multiple courses of conventional depression treatments (Carhart-Harris et al., 2016; Chi & Gold, 2020). Despite this evidence and resultant promise, the psychedelic treatment mechanisms underpinning depression alleviation are not resolved yet – hindering evidence-based conclusions as to their medico-therapeutic utility compared to traditional SSRIs in depression.

Given that both psychedelics and SSRIs are fundamentally serotonergic agents, the brainstem raphe nuclei are likely to be affected by their action – and might be centrally involved in their treatment mechanisms (Hornung, 2012; Hornung, 2003; Kosofsky & Molliver, 1987). As mentioned in Chapter IV, analyses of SSRIs and also psychedelics have revealed that both classes of drugs cause *acute* inhibition of the raphe in non-human animals – in the case of psychedelics even to the point of effective cessation of activity (Aghajanian et al., 1968, 1970; Aghajanian & Marek, 1999; Foote et al., 1969; Hajos et al., 1995; Rogawski & Aghajanian, 1981). However, animal studies have shown that serotonin system function is central to anti-depressant effects, ranging from the necessity for normal tryptophan hydroxylase function to the fact that electrical stimulation of the raphe shows anti-depressant effects in mice (Mosienko et al., 2012; Sachs et al., 2015; Teissier et al., 2015). As the time-frames for clinically detectable effects (much faster for psychedelics) and their pharmacodynamic and -kinetic profiles are very different from SSRIs (Nichols, 2016), it is feasible that despite similar *acute* effects, these two classes of treatment may have different longer-term *sub-* and *post-acute* consequences for serotonergic system function. In the case of psychedelics it is therefore particularly important to complement understanding of acute drug effects (as assessed for psychedelics in Chapter IV) with assessments of longer-term post-acute effects.

As posited at the end of the previous chapter, the observation of the acute raphe connectivity 'knock-out' during LSD administration poses an intriguing possibility: Could this acute effect on raphe function precipitate an adaptive 'reset' of serotonergic system/raphe function – i.e. improve raphe functionality in a depression-alleviating manner? Allostatic processes of this kind are common

throughout systems biology, wherein powerful acute perturbations of a system achieve "new stability through change" following initial perturbation (McEwen, 2000; Ramsay & Woods, 2014). An improvement or re-normalization of serotonergic source nucleus function would be ideally-positioned to alleviate the serotonergic deficit hypothesized to underpin depression – and would be a plausible therapeutic correlate to subserve long-lasting, positive clinical outcomes (Galvão-Coelho et al., 2021) downstream of the acute cortical effects of psychedelics, and could underpin other treatment-associated phenomena such as the DMN "reset" (Carhart-Harris et al., 2012, 2017). Preliminarily, this *knockout-to-reset* hypothesis is supported by previous work that has identified that strength of the acute psychedelic experience – which **Chapter IV** found to be associated with acute disruption of raphe connectivity – is a strong predictor of post-dosing long-term wellbeing (Roseman et al., 2018, 2019). Previous observations that raphe connectivity is disrupted in MDD and might have some diagnostic utility (Anand et al., 2019; Ikuta et al., 2017) further solidify that raphe connectivity should be assessed in MDD cohorts receiving psychedelic treatment. To assess whether a raphe connectivity deficit is alleviated in a treatment-relevant manner in the sub- and post-acute phases after psychedelic treatment would furthermore test clinical utility of raphe connectomics for diagnostic and treatment-monitoring purposes in serotonin-associated pathologies.

To this end, in this chapter I analysed data provided by our collaborators Carhart-Harris et al. (2021; 2016). The first dataset is from an earlier open label trial of psilocybin in a cohort of treatment-resistant depression patients (n=15; Carhart-Harris et al., 2016; hereafter: TRD-OLT), in which rs-fMRI was collected at baseline and just one day after the second treatment (25mg psilocybin; see Fig.18), thus allowing sub-acute effects of psilocybin to be characterised. The second dataset is from the first large-scale double-blind randomized controlled trial (hereafter: MDD-RCT) in major depressive disorder (Carhart-Harris et al., 2021) which compared the improvement in depression symptoms of a group receiving two single doses of psilocybin (2x35mg) in 3-week intervals against those of a separate group receiving continuous SSRI treatment (1st half: 10mg, 2nd half: 20mg) over a

6-week trial period (n=20 escitalopram arm, n=22 psilocybin arm).[22] All participants had rs-fMRI collected both before the trial (baseline) and after the full 6-week course (i..e 3 weeks after last psilocybin dosing, see Fig.19). thus capturing more post-acute effects of psilocybin (vs continuous SSRI).

Explicitly, I hypothesised: that (i) raphe connectivity and psilocybin- (and/or SSRI-) induced changes of this connectivity should be related to antidepressant effects. Secondly, (ii) that treatment-associated raphe connectivity changes might have neuromodulatory consequences for larger-scale network changes (DMN reset) consistent with observations in Chapters II and IV and the broader literature. Finally, to associate acute drug effects with post-acute changes in the raphe, I expected that (iii) acute psychedelic experience should predict post-trial raphe alterations.

5.2 Materials and Methods

Psilocybin Open-label trial in Treatment-Resistant Depression (TRD-OLT)

The first cohort assessed in this chapter is from an older open-label trial of psilocybin for treatment-resistant depression (TRD; Carhartt-Harris et al., 2016). The age range for recruitment of female and male participants was 18 to 80 years old, with all prospective participants subjected to a selection process that included the following key exclusion criteria. Participants had to have no family or personal history of psychosis; have no contraindications to undergoing fMRI or taking serotonin reuptake inhibitors; have had no previous escitalopram treatment (but were allowed to have had previous experience with psilocybin); not have a history of suicide attempts; not be pregnant; not have a suspected or diagnosed additional psychiatric illness.

Based on these inclusion criteria, 19 TRD patients were recruited to the open-label trial and attended a 1-day pre-treatment baseline session, which included eyes-closed resting-state fMRI and clinical

[22] Briefly, they found no significant difference between treatment groups in their primary outcome measure, the Quick Inventory of Depressive Symptomatology Self-Report (QIDS), although secondary measures such as the Beck Depression Inventory (BDI) slightly favoured psilocybin over escitalopram (see Carhart-Harris et al. (2021) for detail).

assessment. This was followed by two psilocybin therapy dosing days (DD), separated by a week: A low-dose of psilocybin (10mg) was orally ingested on DD1 and followed by a high-dose dose (25mg) on DD2 (see Fig.20). The follow-up fMRI and clinical assessment occurred one day after DD2, as opposed to the MDD-RCT data (primary dataset) where this was carried out three weeks after the last DD. The open label trial (hereafter: TRD-OLT) patients attended an on-site clinical longitudinal follow-up at 6 months. All patients ceased any use of psychiatric medication at the latest 14 days before the trial, and psychotherapeutic care was discontinued 21 days before the trial commencement date.

Of the 19 TRD-OLT patients, 15 were retained (mean age=42.75, SD=10.15, 4 female) for the present analysis after 4 were excluded due to excessive fMRI head motion. In this cohort, a 12 channel head coil was used to acquire 240 volumes of rs-fMRI in ~8 minutes: TR=2000ms, TE=31ms, 36 axial slices, flip angle=80 degrees, bandwidth=2298Hz/pixel, GRAPPA acceleration=2). T1 MPRAGE structural scans were acquired using the same parameters as the MDD-RCT cohort. Importantly, this TRD-OLT dataset only had the BDI collected as a depression outcome measure, not the primary outcome measure of the MDD-RCT, the QIDS.

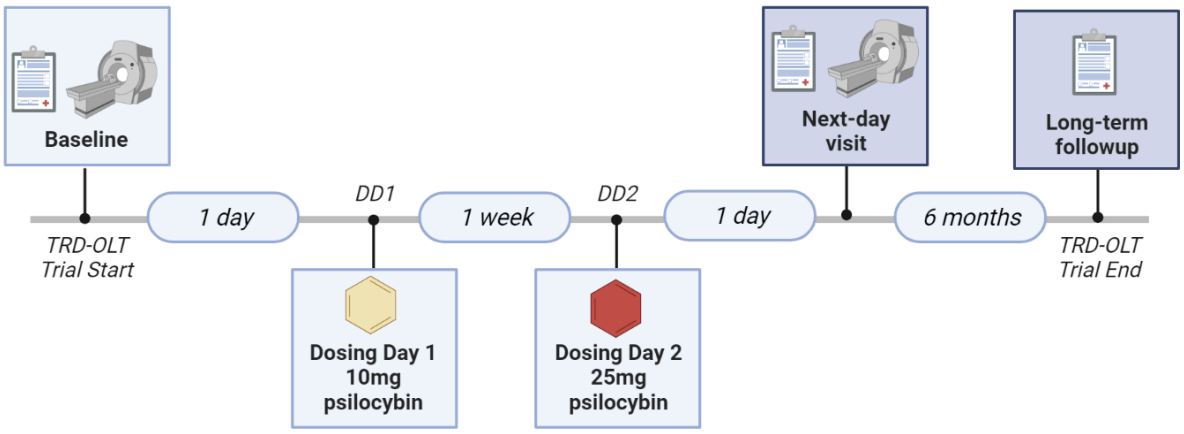


Fig. 19: Overview of the TRD-OLT trial (replication dataset) structure. Patients who were recruited to the open-label trial attended a 1-day pre-treatment baseline session during which BDI scores and an eyes-closed resting-state fMRI scan were collected. This was followed by two psilocybin therapy dosing days (DD), separated by 1 week: A low-dose of psilocybin (10mg) was orally ingested on DD1 and followed by a high-dose dose (25mg) on DD2. The follow-up fMRI and clinical assessment occurred one day after DD2. These patients were also longitudinally followed up at 6 months.

Double-Blind Randomized Controlled Trial of Psilocybin *vs* SSRI for Major Depressive Disorder (MDD-RCT)

The data in this study were originally collected for the preregistered randomized controlled trial published by Carhart-Harris et al., see Carhart-Harris et al. (2021) for detail. In brief, recruitment was carried out *via* formal (trial networks) and informal (social media and word of mouth) channels. The inclusion criteria were identical to the TRD-OLT study. Video telephone interviews were performed using the 17-item Hamilton Depression Scale (HAM-D-17) to assess whether participants met the cutoff >= 17, to be classified as moderate-to-severe major depressive disorder (MDD). A confirmed depression diagnosis and detailed medical history were obtained for each of the shortlisted patients from their respective general physician. Patients who were thereafter deemed eligible, subsequently had face-to-face physical and mental health assessments led by a trial-based psychiatrist, which were followed by their first psychological support session. Again, all patients ceased any use of psychiatric medication at the latest 14 days before the trial, and psychotherapeutic care was discontinued 21 days before the trial commencement date.

The trial was 6 weeks long, requiring each participant to attend six visits within this timeframe (see Fig.19). Their assignment to the different therapeutic arms was randomized using a number generator, implemented by Imperial College London staff who were not involved in the trial. This assigned patients to either the (i) *psilocybin* [psychedelic] arm, in which patients received two 25mg doses of psilocybin 3 weeks apart with only crystalline cellulose placebo in between, or the *escitalopram* [SSRI] arm, in which 10mg of the SSRI were administered daily for the first three weeks, with 20mg of it in the following three weeks. Importantly, in the SSRI arm two 1mg doses of psilocybin were administered as their placebo on 'dosing days', as to standardise expectations (and prevent 'unblinding') between the groups, both were truthfully told that they would be receiving psilocybin, but not at which dosage. Medications and placebos were pre-packaged in non-disclosing format for all research visits.

In this chapter, I focus on a subgroup of 42 of the originally n=59 recruited participants. Of the n=59, only n=50 underwent fMRI scans associated with the trial. One of these had to be excluded due to

using cannabis during the duration of the trial, four more because of excessive head motion during scanning, and three more due to aborting the taking of their pills, leaving a final sample of n=42 (n=20 escitalopram/SSRI arm, 6 female, mean age 38.7±11.03; n=22 psilocybin/psychedelic arm, mean age 41.9±10.96). Like the whole trial sample, all participants in this neuroimaging subsample partook in six visits (see Fig.19).

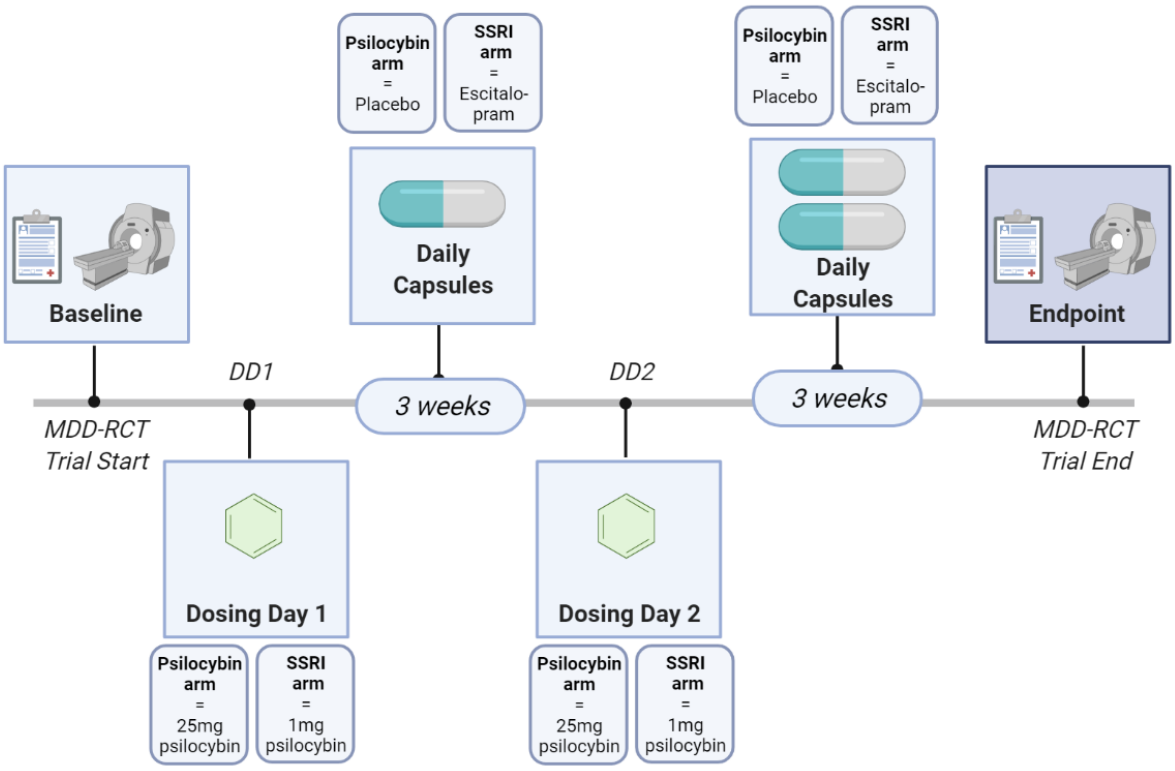


Fig. 19: MDD-RCT trial structure. At *visit 1* participants underwent a baseline fMRI scan (pre-trial), and attended an introductory therapy session with their mental health professionals. *Visit 2* (dosing day 1; DD1) occurred the following day, where both groups received their respective dosages of psilocybin (25mg for Psilocybin group, and 1mg for Escitalopram/SSRI group), under direct supervision of mental health professionals to ensure no adverse events, and with discharge criteria evaluated by the trial psychiatrists. Upon discharge, participants were provided with their pill container of capsules (containing crystalline cellulose for psilocybin, and escitalopram for the SSRI group) and were briefed to ingest one capsule each morning until the next research visit in three weeks' time. Psychological debriefing (*visit 3*) was scheduled for the following day. *Visit 4* (research visit, DD2) was scheduled for three weeks after *visit 2* (DD1), to give patients their second dose of either psilocybin or placebo. *Visit 5* occurred the following day consisting of an integrative psychological session of attentive open listening styles. Following visit 5, participants were across both treatment arms asked to take two capsules every morning, thus doubling the dose of escitalopram in the SSRI arm of the study for three weeks, with no consequence to the psilocybin arm's administration of crystalline cellulose. Finally, on *visit 6* which occurred three weeks after DD2, the same procedures as on *visit 1* were repeated, including fMRI recordings and behavioural/clinical assessments to establish the trial outcomes for each subject.

Baseline (pre-trial) and Endpoint (post 6-week trial) assessments consisted of an rs-fMRI scan and collection of the two outcome measures, namely the 16-item Quick Inventory of Depressive Symptomatology-Self Report (QIDS-SR-16; scores 0-27, greater QIDS-SR = greater depressive symptoms; responder classified as >=50% reduction of score over trial) and of Beck Depression Inventory (BDI1A; responder classified as >=50% reduction of score over trial). For other additionally collected measures, see (R. Carhart-Harris et al., 2021). On dosing days, the Emotional Breakthrough Inventory, which assessed acute subjective experiences of overcoming challenging emotions and experiences in response to drug administration was also collected (Roseman et al., 2019).

Resting-state fMRI (rs-fMRI) was collected in two (one pre-trial, one post-trial) ten minute scans with the participant's eyes closed. All imaging was performed on a 3T Siemens Magnetom TrioTim MRI scanner. Anatomical T1 scans were acquired using GRAPPA fast spoiled gradient echo scans in an axial orientation, with a field of view = 256× 240× 160 providing 1mm isotropic voxel size (TR/TE = 2.3/2.98ms; inversion time = 450ms; flip angle = 20◦). The rs-fMRI acquisitions lasted ten minutes, and used a multislice acquisition sequence gradient echo planar imaging sequence (EPI; FoV = 192mm, 64×64 acquisition matrix, parallel acceleration factor = 2, 90◦ flip angle, TE=30ms, TR=1.25ms). Forty four axial slices were acquired with interleaved slice acquisition order, at 3mm thickness with zero slice gap (3mm isotropic voxels).

Spatial and Temporal Preprocessing

To maintain comparability with the previous chapter(s), preprocessing for all scans was again performed using the CONN functional connectivity toolbox utilising statistical parametric mapping (SPM12; Wellcome Centre for Human Neuroimaging, London, UK) functions on a MATLAB (2018b, The Mathwork, Inc. Natick, Massachusetts, USA) platform. The preprocessing pipeline was identical to Chapter IV.

Serotonergic Raphe Nuclei Whole-brain Seed-to-Voxel Correlation Analyses

Functional connectivity analyses of the raphe in this chapter used an approach tailored to the clinical context of the analyses. Instead of contrasts of seed-to-voxel connectivity between groups, here I performed whole-brain seed-to-voxel correlations with outcome measures (QIDS and BDI), using the CONN toolbox correlation and GLM options. Explicitly, whole-brain temporal correlations for the serotonergic raphe seeds were again computed using General Linear Models. The functional connectivity analyses produced seed-to-voxel parameter estimate β-images, and these were entered into whole-brain correlations with primary (QIDS) and secondary (BDI) outcome scores (pre-trial; post-trial; Δ post – pre-trial) to reveal connectivity clusters of significant association with outcome scores. Significant clusters identified in this analysis setup are therefore those clusters that show a statistically significant correlation between raphe connectivity to given voxels and the respective depression measure, after the relevant corrections of thresholding at voxel level $p<0.005$ (uncorrected) and cluster level $p<0.05$ (FWE-corrected for multiple comparisons), in keeping with the previous chapters.

Binary Logistic Regressions to predict Outcomes

Whereas linear regression is suitable when dealing with continuous data, it is not appropriate to use when regression towards a binary outcome, i.e. a yes/no measure, is performed. In these cases, I used binary logistic regression, which fits a sigmoidal curve with a minimum value of 0 and a maximum value of 1. Fitting of this curve for a continuous predictor variable therefore calculates thresholds to determine whether a predictor data point is classifiable as consistent with a 'No' (i.e. 0) or 'Yes' (i.e. 1) outcome.

Percent Amplitude of Low Frequency Fluctuations (PerAF) to approximate Spontaneous Activity in the Raphe

Measurement of the amplitude of low frequency fluctuations in the BOLD signal can be used to obtain voxel-level metrics of spontaneous brain activity. Recent work has suggested that utilising percent amplitude of fluctuations is the most test-retest reliable and appropriate metric (Jia et al.,

2020) and preferable to previous approaches such as fractional amplitude of low frequency fluctuations (Egorova et al., 2017). In accordance with the processes also utilised in other software solutions, the CONN toolbox uses a Fourier transformation on the timeseries of each voxel within the ROI mask to the frequency domain to compute the power spectrum of the BOLD signal. Subsequently, the square root of each frequency in the power spectrum is calculated, with the averaged square root across 0.01–0.08 Hz being ALFF. I then converted the ALFF values into PerAF for the raphe (and other nuclei). In accordance with the protocol by Jia et al. (2020), PerAF was calculated as the percentage change of ALFF relative to mean BOLD signal intensity at each time point and averaged across the whole timeseries.

5.3 Results

5.3.1 Clinical Outcomes in the Neuroimaging Trial Subsamples

The overall results of the TRD-OLT and MDD-RCT trials have previously been published (Carhart-Harris et al., 2016 & 2021). Both found significant reductions in depression symptoms upon psilocybin (and in the case of the MDD-RCT also upon SSRI). The participants who underwent neuroimaging were subsamples of the larger trial samples. Therefore, here I re-analysed outcomes with the respective trial's primary pre-registered depression measures to ascertain that these subsamples are representative of the overarching cohorts.

In the TRD-OLT neuroimaging cohort, significant decreases ($t(14)=7.131$, $p<0.001$) of depression severity (BDI) were observed when comparing baseline BDI scores ($M=34.13\pm1.833$) to those collected at 1-week after last dosing ($M=14.6\pm3.073$; see Fig.21A.i). This significant difference ($t(14)=4.769$, $p<0.001$) was also found when comparing BDI scores from the 6-months follow-up ($M=18.6\pm3.069$) to baseline (see Fig.21A.ii). Together, these results suggest that like in the greater trial data, this imaging subsample displays fast-acting subacute and long-lasting depression symptom reduction in response to psilocybin.

In the MDD-RCT trial, the psychedelic treatment arm showed significant ($t(21)=6.355$, $p<0.001$) psilocybin-induced depression symptom reductions at 3 weeks after the last dosing ($M=6.136\pm1.024$)

compared to baseline (M=13.955±0.679) measured with the QIDS questionnaire. A significant decrease in depression severity (t(19)=5.222, p<0.001) between baseline (M=16.35±1.016) and post-trial (M=9.95±1.41) was also observed with SSRI treatment. There was no significant difference (t(40)=0.815, p=0.42) between the treatment arms in the strength of overall depression symptom reduction (psilocybin: M=-7.818±5.771; SSRI: M=-6.4±1.226, see Fig.21B.i & B.ii).

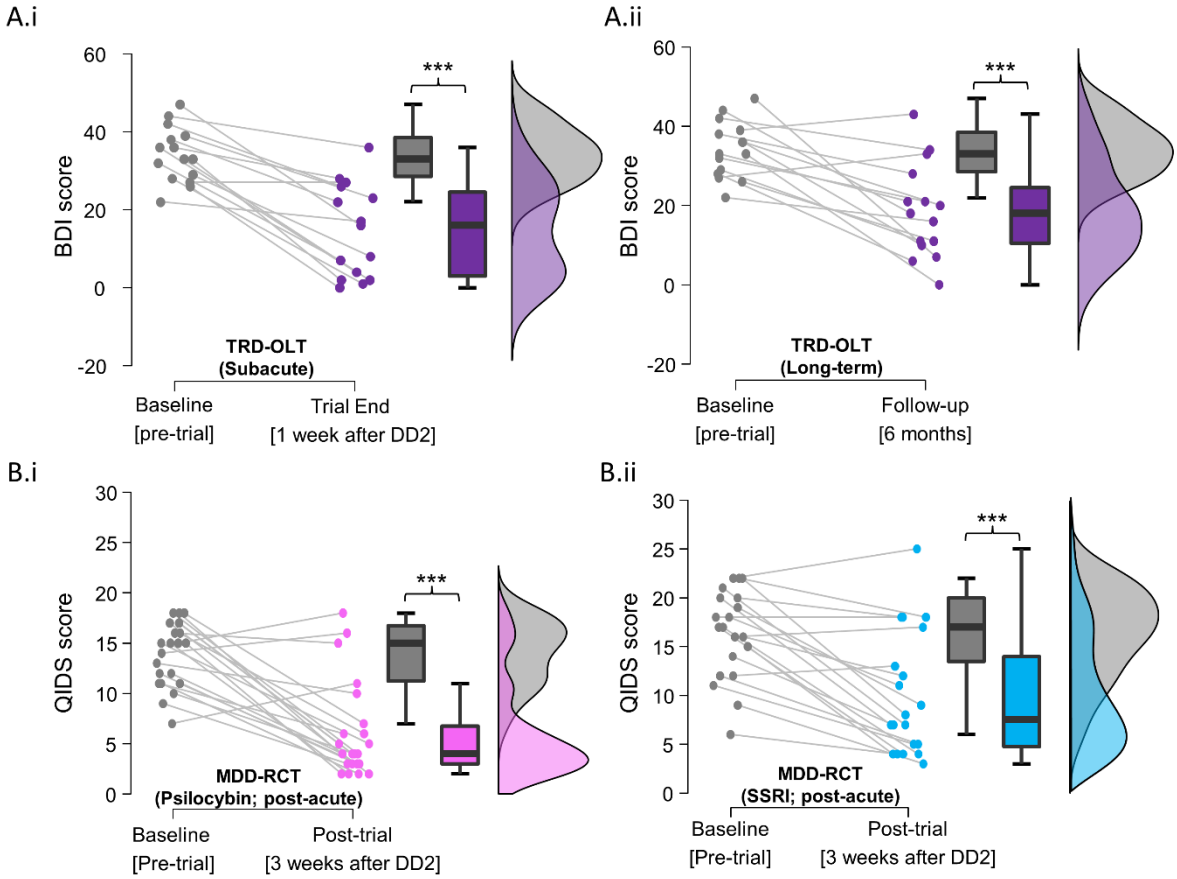


Fig. 21: Outcomes in the neuroimaging subsamples are consistent with those of the overarching trials. (A.i) In the TRD-OLT cohort, significant decreases in depressive symptomatology measured with the BDI questionnaire were found both at 1 week after dosing, and at (A.ii) 6 months after the trial was conducted – thus suggesting quick, but long-lasting depression symptom reduction. (B.i & B.ii) In the MDD-RCT cohort both the psychedelic and SSRI treatment arm showed significant decreases in depressive symptomatology measured with the QIDS questionnaire. The overall decrease in depression scores between the treatment arms was not significantly different. *** indicates p<0.001.

5.3.2 Median Raphe connectivity to the precuneus is associated with depression symptom reduction, demonstrating treatment-monitoring and potential diagnostic utility

To establish whether any sub- and/or post-acute raphe connectivity across the two trials was associated with depression symptom improvement I performed whole-brain median (MnR) and also

dorsal raphe (DR) seed-to-voxel connectivity correlations that used the respective pre-registered primary outcome measures as their covariate (i.e. BDI for TRD-OLT, and QIDS for MDD-RCT). Although I expected that raphe connectivity to the precuneus could be of relevance on the basis of the results reported in Chapter IV, these analyses were purposely not restricted to this target but rather assessed all non-MnR voxels in the brain. Despite no such pre-selection of the PCu, the data-driven seed-to-voxel correlations with depression symptom reduction strongly implicated solely connectivity of the Median Raphe (MnR) to the PCu as key.

In the TRD-OLT cohort's scans that were collected 1-day after last dosing with psilocybin, the only significant clusters of post-trial MnR seed-to-voxel connectivity associated with improvements in depression score (i.e. decreases in BDI, Δ BDI Post – pre-trial) were in the PCu ($R=-0.733$; $p<0.001$, $BF_{10}=56.374$, Fig.22.A). The greater connectivity to these clusters was after the trial (i.e in the sub-acute phase of drug effects), the greater the magnitude of depression symptom reduction was in the TRD-OLT cohort. This importance of MnR-PCu coupling for depression outcome was reinforced by the complementary analysis in the MDD-RCT cohort responders, where imaging was performed post-acutely 3 weeks after last dosing. In this sample, the only significant cluster in MnR whole-brain correlation with treatment outcome was again in the PCu with a similarly negative association with depression symptom reduction (Δ QIDS; post – pre-trial; $R=-0.385$, $p=0.024$, $BF_{10}=3.008$; Fig.22.B).

This indicates that the greater MnR connectivity to the PCu was after the dosing/trial across both cohorts, the greater the depression symptom reduction was. Supporting the specificity of this effect for the MnR, no associations for DR or other monoaminergic nuclei connectivities were found. (The correlation in the MDD-RCT was purposely agnostic to the treatment arm participants were in (i.e. pooled psychedelic and SSRI arms), even when considering the treatment arms separately, the PCu remained the largest depression-reduction associated cluster in the whole-brain MnR correlations; see see Appendix CV, Fig.CV.1). Furthermore, the potential importance of MnR-PCu interplay was additionally solidified as in the full sample of the MDD-RCT cohort (n=42) I found that pre-trial MnR whole-brain seed-to-voxel correlation with baseline depression severity was also related solely to a

cluster in the PCu (R=-0.588, p<0.001) – meaning that the lower MnR connectivity to the precuneus was before the trial, the more depressed participants were (see Appendix CV, Fig.CV.2).

Taken together, these data-driven analyses converge on a special importance of raphe-precuneus coupling for depression treatment – and suggest that raphe connectomics may have treatment-tracking and potential diagnostic utility in depressive disorders.

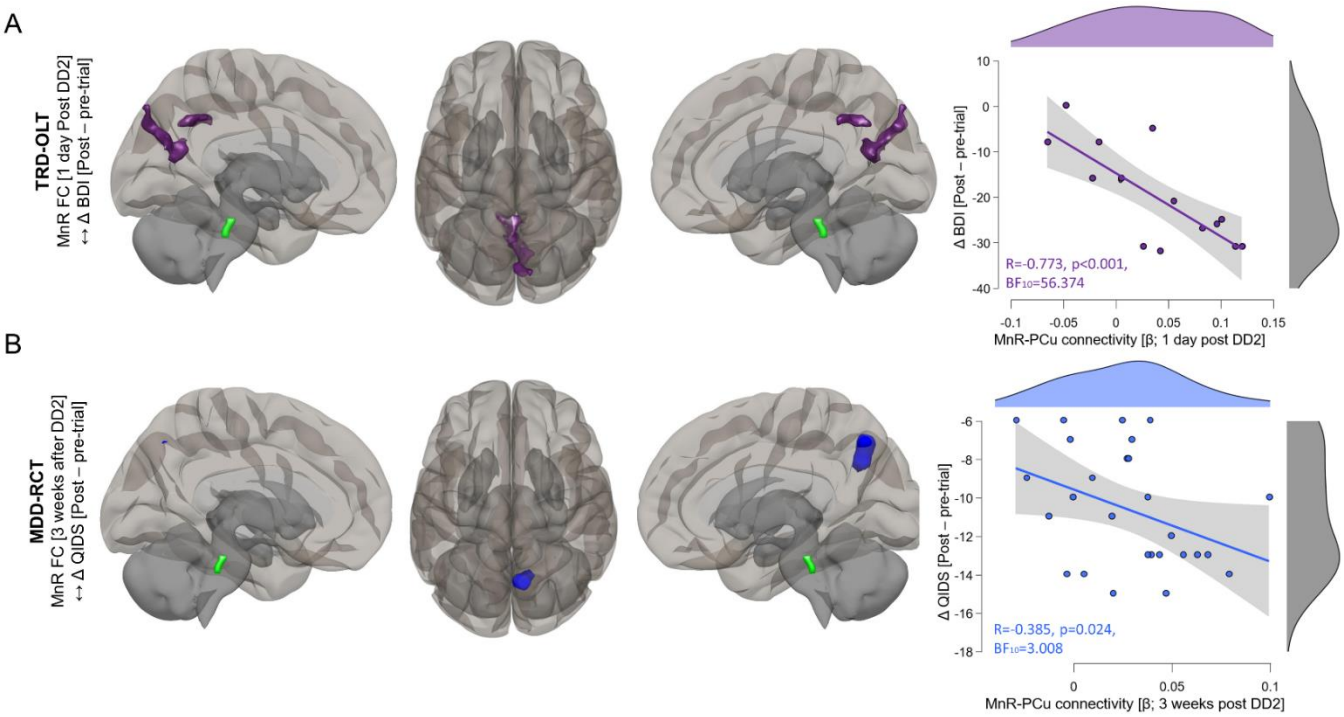


Fig. 22: MnR seed-to-voxel whole-brain correlations with depression symptom reduction converge on median raphe connectivity to precuneus as associated with outcome. (A) In the TRD-OLT cohort scans were collected 1 day after dosing with psilocybin. The only significant seed-to-voxel connectivity clusters associated with decreases in the BDI (Δ QIDS 1 day post dosing – Pre-trial) were in the precuneus. This negative association indicates that the greater the connectivity of the MnR to these clusters became, the greater the depression symptom reduction was. (B) In the MDD-RCT cohort, scans were collected 3 weeks after last dosing with psilocybin. MnR seed-to-voxel connectivity post-trial correlations with QIDS change (Δ QIDS 3 weeks post dosing – Pre-trial) in treatment responders (n=27) again revealed a significant cluster in the precuneus (blue, R=-0.489, p=0.005). This association that was agnostic to the treatment arm participants were in, was also negative – indicating that the greater this MnR-PCu connectivity was after the trial, the more reduced depression ratings were. Whole-brain seed-to-voxel correlations with depression outcome measures were performed in the CONN toolbox second-level analysis options. Statistical thresholds for seed-to-voxel correlations with outcome measures were voxel level p<0.005 (uncorrected) and cluster level p< 0.05 (FWE-corrected).

5.3.3 Sub- and post-acute psilocybin-induced increases in MnR-PCu connectivity are associated with improvements in depressive symptomatology

Motivated by the previous results that the absolute level of MnR-PCu connectivity in a patient may have potential uses for monitoring treatment-efficacy, I aimed to establish whether treatment-induced MnR connectivity *increases* to the precuneus in comparison to baseline (Δ MnR-PCu FC Post – pre-trial; cluster common to psychedelic and SSRI arm from Fig.21b) are associated with better treatment outcomes and/or proportional to improvement in depression scores.

In the TRD-OLT cohort, responders (M=0.063±0.017) showed significant increases in MnR-PCu connectivity when compared to baseline ($t_{(7)}$=-5.080, p<0.001), whereas non-responders did not (t(6)=1.219, p=0.269; M=-0.039±0.036; see Fig.23.A.i & A.ii). This finding was replicated in the psychedelic treatment arm of the MDD-RCT, where treatment responders showed a significant increase in post-trial MnR-PCu connectivity (M=0.029±0.008) compared to baseline/pre-trial (M=0.015±0.006; normality violated, Wilcoxon T=35, p=0.047; see Fig.23.B.i), whereas no significant difference was observed in non-responders to psychedelic treatment (Pre-trial M=0.037±0.008; Post-trial M=0.040±0.034; Wilcoxon T=9, p=0.844; Fig.23.B.ii). SSRI treatment responders ($t_{(10)}$=-0.413, p=0.452; Fig.23B.i) and non-responders ($t_{(8)}$=-1.196, p=0.131; Fig.23B.ii) both did not show significant differences between pre- (responders M=0.020±0.005; non-responders M=0.012±0.010) and post-trial timepoints (responders M=0.025±0.009; non-responders M=0.037±0.016; see Fig.23.C.i & C.ii). Taken together, these results are consistent with psychedelic-induced upregulation of raphe connectivity being associated with the best treatment outcomes – without such an increase occurring in SSRI treatment.

Nevertheless, treating depression outcome as a categorical variable (i.e. responders vs non-responders) might be insufficient to resolve if MnR connectivity changes exist on a spectrum along which might improvements in connectivity might in fact be proportional treatment benefit experienced by a participant. To this end, I subsequently used a correlational approach with normalised depression score changes (% change compared to baseline/pre-trial).

Psychedelic-induced change in MnR-PCu connectivity was strongly associated with relative depression symptom reduction in the TRD-OLT cohort (R=-0.756, p=0.001, BF_{10}=79.889). The stronger the increase in MnR-PCu coupling in comparison to baseline, the greater the depression symptom reduction (% BDI change post-dosing *vs* pre-trial) was. In the MDD-RCT cohort these findings replicated, as again only in the psilocybin arm was a significant relationship (R=-0.424, p=0.025; magenta; Fig.23.D.ii) between MnR-PCu connectivity and relative QIDS depression symptom reduction (% QIDS change post-trial vs pre-trial) found, whereas I found no such relationship in the SSRI arm (R=0.109, p=0.601, BF_{10}=3.157; cyan Fig.23.D.ii). This result once more replicated with relative changes in the simultaneously recorded BDI score (% change Post – pre trial), where only the psilocybin arm showed a significant relationship of MnR-PCu connectivity change with BDI reduction (R=-0.456, p=0.016, BF_{10}=4.399; magenta Fig.23A.i), whereas the SSRI arm did not (R=-0.035, p=0.442; cyan Fig.23B.i).

Altogether, these findings suggest that a psychedelic-induced increase of MnR connectivity occurs in the sub- and post-acute phases after dosing in those who respond to treatment – and that the improvement in MnR-PCu connectivity is proportional to the improvement in depressive symptomatology. This might follow the acute ‘knockout’ of raphe connectivity as observed with LSD in Chapter IV, thus possibly being consistent with the allostatic ‘reset’ model proposed in this and the previous chapter. Whether following this raphe reset or not, these results suggest that psychedelic-induced improvements in raphe functionality (that are detectable as early as subacutely 1 day after and as late as post-acutely 3 weeks after dosing) might be involved in the depression-alleviating effect of psychedelics, but not traditional SSRIs.

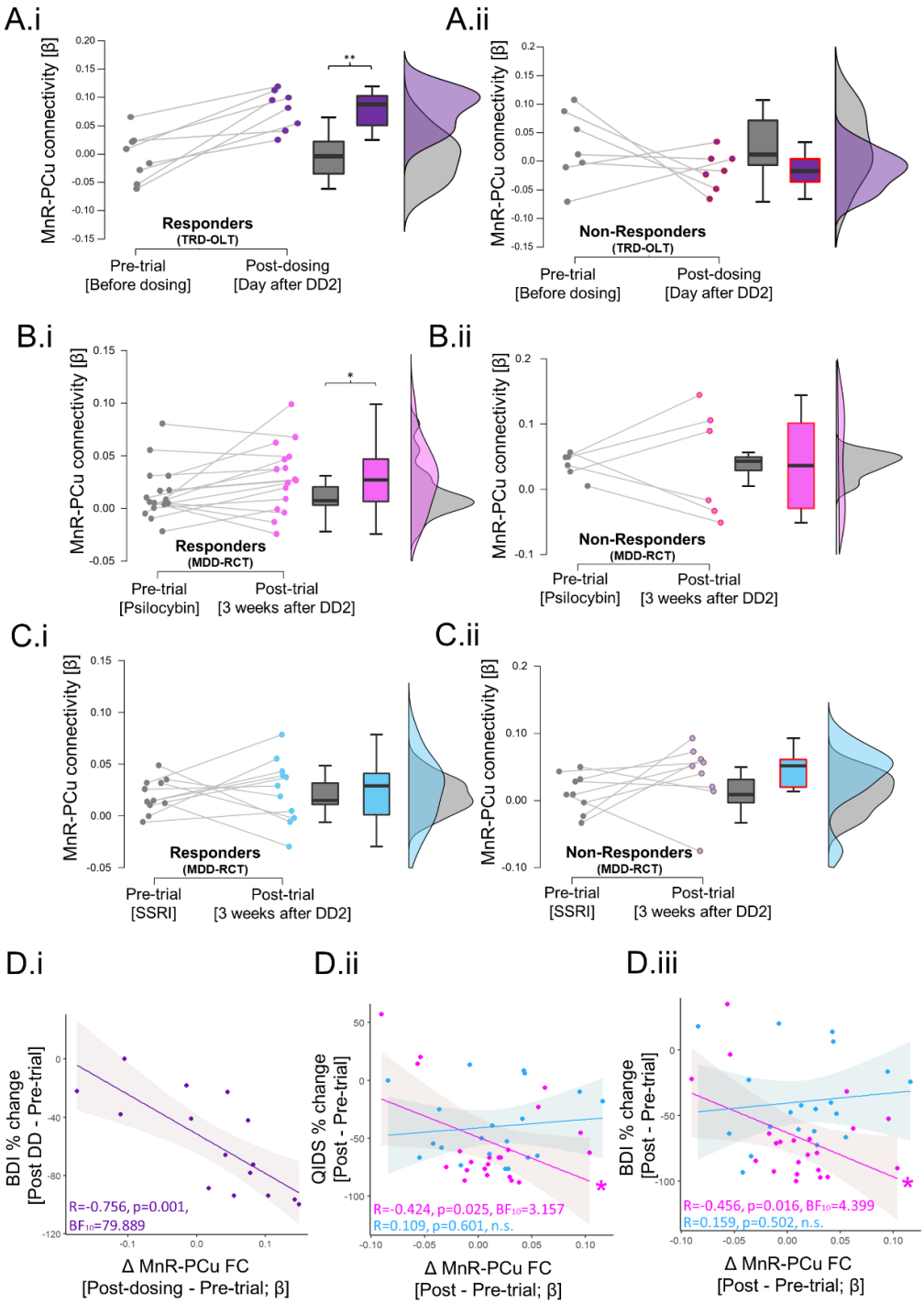


Fig. 23: MnR-PCu connectivity is significantly increased in psychedelic treatment responders across both trials, and the level of MnR-PCu connectivity increase is correlated to strength of relative depression symptom reduction. (A.i) Participants classed as treatment responders in the TRD-OLT cohort showed significant increases in MnR-PCu connectivity from before the trial (grey) to post-trial (1 day after dosing; purple). (A.ii) Non-responders to psychedelic treatment did not show a significant increase in MnR-PCu connectivity (purple with red border). (B.i) Participants in the psilocybin arm of the MDD-RCT who were classed as responders also showed a significant upregulation of MnR-PCu connectivity, whereas (B.ii) non-responders did not. (C.i & C.ii) Both responders and non-responders to classical SSRI treatment with escitalopram did not show a significant increase in MnR-PCu connectivity (cyan). (D) Treating depression symptom reduction as a normalised continuous variable (percent change of pre-registered depression measures QIDS and BDI respectively), (D.i) in TRD-OLT participants a very strong relationship was observed between connectivity increases and depression symptom reduction (purple). (D.ii & D.iii) Across the whole MDD-RCT treatment arms, only the psychedelic arm showed significant correlations of MnR-PCu connectivity increases with greater depression symptom reduction (% QIDS and BDI; magenta), whereas the SSRI arm did not (cyan)

5.3.4 Upregulated MnR-PCu connectivity one day post-dosing is predictive of significant depression reduction lasting 6 months

To further ascertain the importance of subacute MnR-PCu connectivity increases for depression treatment outcome, I additionally assessed its relationship with long-term depression reduction. In the TRD-OLT cohort, participants were followed up longitudinally at 6 months after their last dosing day. At this 6-months visit, BDI scores were taken in order to assess the longevity of treatment effects: if their BDI score was still more than 50% lowered in comparison to pre-trial, participants were classed as long-term responders.

Using this classification of long-term responder status as a binarized outcome, I calculated a logistic regression that used the MnR-PCu connectivity change (Δ MnR-PCu FC; Post dosing – pre-trial, z-transformed) 1 day after dosing as a continuous predictor, with 10000 bootstrapping simulations. It was found that MnR-PCu connectivity increase at 1-day post-dosing is a significant predictor for long-term responder-status (X^2=4.674, p=0.031; Wald test p=0.018; Odds ratio: 2.19). The outcome of a participant being a long-time responder was 2.19 times more likely for each unit increase in upregulated MnR-PCu connectivity 1 day after dosing (see Fig.24A). This lends support to the hypothesis that psychedelic-induced upregulation of MnR-PCu connectivity, consistent with a knockout-to-reset effect on the raphe, is involved in the therapeutic effect of psilocybin and its longevity.

5.3.5 Acute psychedelic effects of emotional breakthrough are predictive of improved post-acute MnR-PCu connectivity

Given the observation that *acute* effects of a psychedelic entail the strong downregulation of MnR-PCu connectivity, it would have been ideal to resolve whether this acute 'knockout' of MnR-PCu connectivity provides a stimulus that indeed causes reorganisation of raphe functionality and connectivity to an improved (and depression-alleviating) state. Regrettably, the *de facto* relationship between acute and post-acute effects could not directly be assessed in neither the MDD-RCT, nor TRD-OLT trial data, as participants were not scanned during acute administration of psilocybin. However, in the MDD-RCT the emotional breakthrough inventory (EBI), a measure that captures

aspects of the acute experience in integration with the therapeutic container[23] (Roseman et al., 2019) was collected during acute administrations on dosing days. This behavioural variable provided the best available proxy for the strength of acute substance effects at dosing, which should putatively also capture MnR-PCu disconnection. I hypothesized that greater average EBI ratings might hold predictive value for the emergence of more control-like, i.e. positive MnR-PCu connectivity at the post-trial timepoint in the MDD-RCT cohort. To test this, a logistic regression was performed in which the outcome of MnR-PCu connectivity being positive (+ve) or negative (-ve) after the trial was treated as the binarized outcome measure, and average EBI ratings across the two dosing visits were used as their continuous predictor, again with 10000 bootstrapping simulations performed. Only in the psilocybin arm (X^2=4.778, p=0.029, Wald test p=0.045; see Fig.24B.i) were EBI ratings a positive predictor of whether positive MnR-PCu connectivity was observed post-trial (Escitalopram: X^2= 1.233, p=0.267, n.s.; see Fig.24B.ii). In the psilocybin arm, the odds of positive MnR-PCu connectivity post-trial were increased by 1.6% for each one-unit increase in the EBI score (Odds ratio = 1.016). This supports the hypothesis that the strength of the acute experience (and contained therein acute disruption of MnR-PCu connectivity) is predictive of positive post-trial MnR-PCu FC.

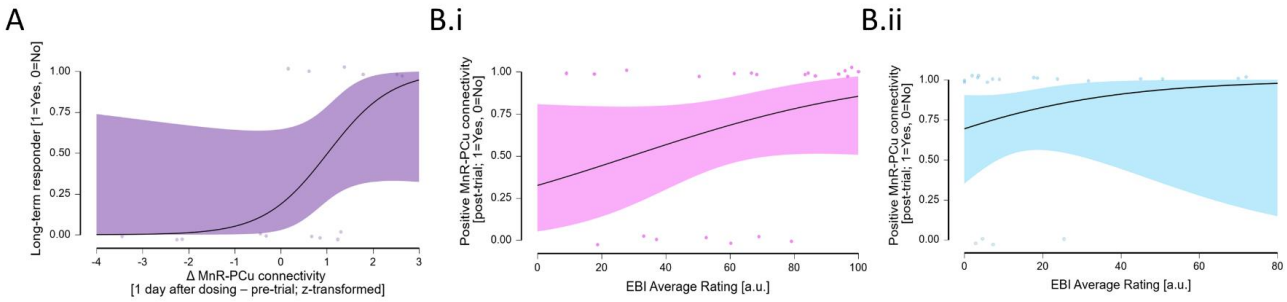


Fig. 24: Long-term responder status in the TRD-OLT is predicted by subacute MnR-PCu connectivity upregulation, while post-acute positive MnR-PCu connectivity is predicted by ratings of emotional breakthrough in the MDD-RCT psilocybin arm. (A) A Logistic regression that treated long-term responder status in the TRD-OLT cohort (i.e. 50% reduction of BDI scores lasting to six months post-trial) as its outcome variable found that subacute MnR-PCu connectivity upregulation was a positive predictor for longevity of depression symptom reduction (purple). For each 1 unit increase in (B.i) In the psilocybin treatment arm of the MDD-RCT (magenta), scores on the Emotional Breakthrough Inventory (EBI) were positive predictors for whether MnR-PCu connectivity was positive after the trial or not, with a 3.3% increase in this chance with each unit on the EBI. (B) No significant predictive power was observed for the EBI in the escitalopram subgroup (cyan). Logistic regression was performed in JASP v.14.0.0.

[23] The therapeutic 'container' is an emerging definition of inter-active and inter-dependent building of the physical, psychological and interpersonal context by caregiver and patient in which therapeutic effects best arise (Jain & Penn, 2021).

5.3.6 Psychedelic-induced sub- and post-acute MnR-PCu connectivity changes are associated with increases in precneus-DMN(+) connectivity

As reported above, in acute psychedelic administrations MnR-PCu connectivity disruption was associated with more impaired PCu-DMN(+) connectivity. Subacutely however, it has previously been reported that after psychedelic administration enhanced DMN integrity is observed, which gave rise to a DMN 'reset' hypothesis (Carhartt-Harris et al., 2018). It is plausible that psychedelic-induced modifications to the raphe (and resultant changes in serotonergic modulation of particularly the PCu) are an underpinning mechanism for such downstream large-scale connectivity changes. To test this relationship, I re-utilised the same analysis approach as in Chapter IV, assessing whether treatment-induced changes in MnR-PCu connectivity might affect how strongly the PCu is connected to those clusters that were part of its whole-brain connectivity at baseline. Across both the TRD-OLT and MDD-RCT, these whole-brain connectivity clusters of the PCu clusters' again encompassed largely DMN, but also to a lesser extent DAN aspects, and are thus also referred to as DMN(+; see Fig.25A&B) .

In the TRD-OLT cohort, the greater MnR-PCu connectivity changes were, the greater the increase in PCu-DMN(+) connectivity was ($R=0.545$, $p=0.018$, $BF_{10}=2.899$; see Fig.25A), suggesting that subacute increases in MnR connectivity may underpin DMN integrity. Similarly, in the MDD-RCT, only in the psilocybin subgroup was a significant relationship ($R=0.363$, $p=0.049$, $BF_{10}=4.157$) of treatment-induced changes in MnR-PCu connectivity (Δ MnR-PCu connectivity Post – Pre-trial) with increases in PCu-DMN(+) connectivity found, again suggesting a putative neuromodulatory influence of serotonergic connectivity on PCu-DMN(+) connectomic integrity (SSRI arm: $R=0.278$, $p=0.118$; see Fig.25B). Altogether, these results suggest that psilocybin-induced upregulations in raphe connectivity may underpin the previously observed psychedelic post-acute 'DMN reset'.

Finally, to establish whether MnR-PCu connectivity might exert its depression-alleviating influence *via* downstream effects on DMN(+) integrity, I performed average causal mediation analyses, that used changes in MnR-PCu connectivity as their independent variable/predictor, DMN(+) integrity changes and/or post-trial modularity as their mediator, and reductions in BDI and/or QIDS as their

outcome measure. I found significant total effects of MnR-PCu connectivity changes on percent decreases in depression scores across psychedelic treatment in the TRD-OLT (β = -271.629, CI=-390.66:-152.602, p<0.001) and MDD-RCT cohorts (psilocybin: β = -315.093, CI=-691.17:39.21, p=0.028; SSRI: β = 73.6, CI=-195.85:343.05, p=0.592). However, across both cohorts, there were no significant average causal mediation effects *via* DMN(+) integrity, suggesting that rather than being mediated by these downstream possible epi-phenomena, depression symptom reduction may, at least partially, be directly driven by improvements in raphe-cortex coupling.

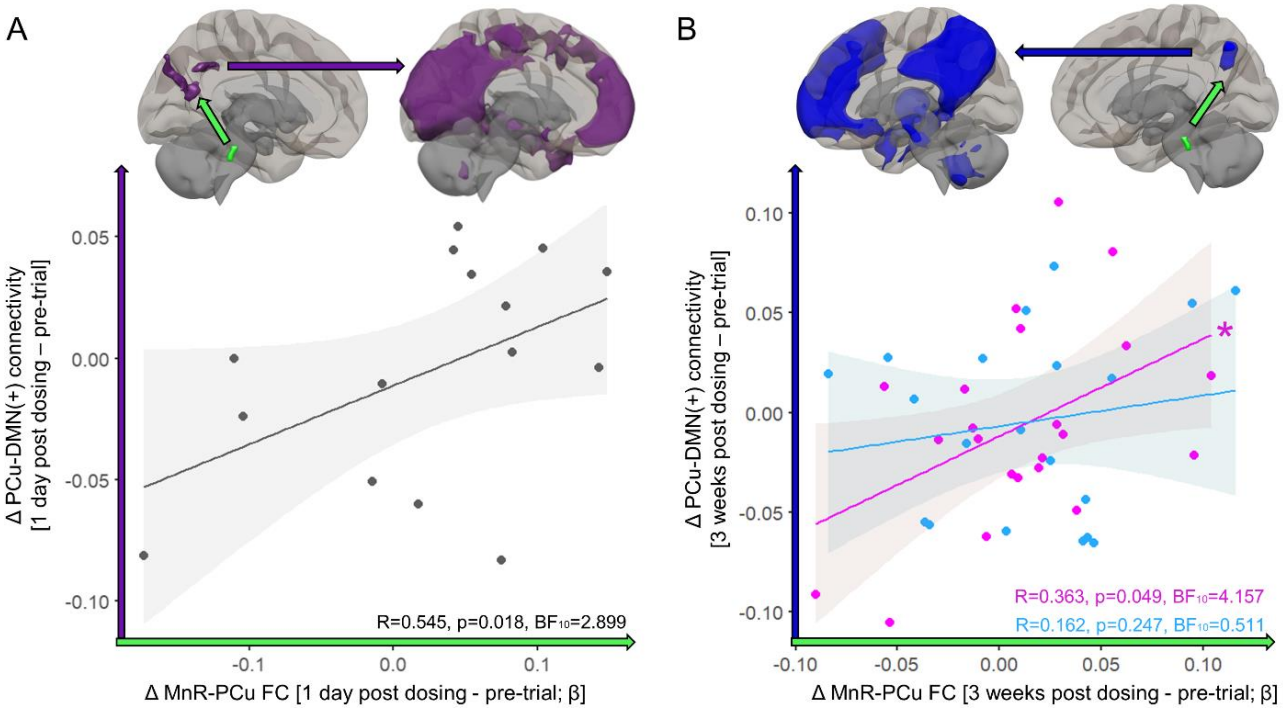


Fig. 25: Psilocybin-induced increases in DMN integrity are associated with enhanced MnR-PCu connectivity strength. (A & B) Across both the TRD-OLT (purple) and MDD-RCT (magenta and cyan) cohorts, greater Δ PCu-DMN(+) functional connectivity strength was significantly associated with MnR-PCu connectivity increases in psilocybin, but not SSRI, treatment. Correlations used Spearman's rank. Correlation coefficients and Bayes factor were extracted using JASP and RStudio.

5.3.7 Spontaneous Raphe BOLD fluctuations are increased only after psychedelic, not after SSRI treatment

The above results altogether suggest that improvements in raphe connectivity enhancements may be involved in successful treatment of MDD. However, if a psychedelic-induced 'raphe reset' is indeed occurring, this may affect not just connectivity, but also activity of the MnR. As the literature suggests that both psychedelics and SSRIs have acute inhibitory effects on raphe activity (Aghajanian et al., 1968, 1970; Aghajanian & Marek, 1999; Foote et al., 1969), I hypothesized that post-acute raphe activity should be increased in the psychedelic treatment arm, as the psychedelic and serotonin are not competing at relevant receptor sites anymore. Instead, as SSRIs induce downregulation of serotonin reuptake, a homeostatic signal to decrease raphe-derived serotonin provision and thus MnR activity might still be operant in the SSRI treatment arm.

To test this, I used percent amplitude of low frequency fluctuations (PerAF; Jia et al., 2021) of the MnR to assess spontaneous activity, and tested for both treatment arms whether there were significant differences between the pre- and post-trial timepoints. In support of the raphe reset hypothesis, in the psychedelic treatment arm there was a significant increase in PerAF (F=-1.917, df=21, p=0.034, see Fig.26A) at the post-trial timepoint compared to pre-trial. The psychedelic-induced increase in PerAF was however not found for the TRD-OLT cohort, suggesting that the activity increase might occur later than MnR-PCu connectivity increases, i.e. more than 1 day after dosing.)[24] Importantly, no significant correlations of MnR PerAF with depression outcome measures were found in either arm of the trial, suggesting that sub- and post-acute connectivity changes may be more relevant to therapeutic effects than activity changes.

[24] Across both cohorts no significant PerAF changes were found for any of the other monoaminergic brainstem nuclei (i.e. DR, VTA, LC).

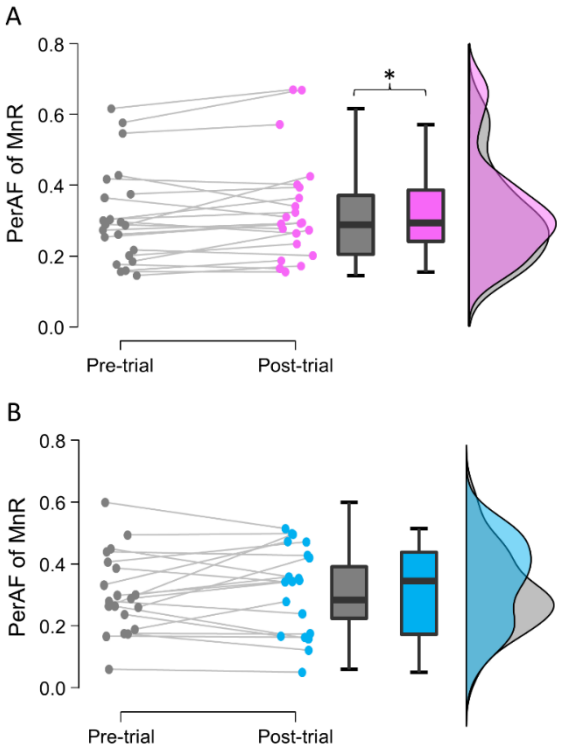


Fig. 26: Psilocybin treatment is associated with increased spontaneous BOLD activity (measured as PerAF) in the median raphe, whereas SSRI treatment is not. (A) Spontaneous BOLD activity in the median raphe nucleus was increased significantly between pre- and post-trial in the psychedelic treatment arm (magenta) of the MDD-RCT cohort. (B) The SSRI treatment arm (cyan) did not show a significant increase in MnR BOLD fluctuations. * = $p<0.05$

5.4 Discussion

This chapter provides the first *in vivo* evidence that psychedelics have long-lasting sub- and post-acute effects on a serotonergic source nucleus of the human brain. Strikingly, I found that data-driven raphe connectivity assessments might hold clinical utility for the identification of biomarkers for depression and its treatment, as analyses of psychedelic depression trial data revealed raphe-precuneus coupling at sub-/post-acute timepoints after psychedelic dosing to be strongly associated with depression symptom reduction. Upregulation of raphe-precuneus coupling occurred with the best treatment outcomes and was proportional to symptom reduction across both sub- (1 day) and post-acute (3 weeks) timepoints after dosing, and not observed in SSRI treatment. Furthermore, the strength of raphe functionality improvement was predictive of longevity of treatment effects and associated with increases in precuneus-DMN connectivity. Moreover, I demonstrate that the post-acute emergence of positive MnR-PCu connectivity was predicted by acute psychedelic experience,

thus tying the psychedelic acute 'knock-out' of MnR-PCu connectivity from **Chapter IV** to psychedelic-induced post-acute upregulation of this connectivity coupling. Taken together, these data thus provide empirical support for the working hypothesis that psychedelics (but not SSRIs) may through an allostatic process 'reset' raphe-cortex coupling and possibly serotonergic system function – constituting a mechanism that could plausibly underpin their antidepressant effects, and distinguish them from SSRI treatment.

To begin with, my data-driven analyses identified that raphe-precuneus coupling is the only connectivity pattern in sub- and post-acute rs-fMRI after both psychedelic trials that is associated with treatment outcome. Across both cohorts, the greater raphe-precuneus coupling was after the trial, the better the depression symptom reduction experienced by a participant was. This adds onto the finding that lower pre-trial MnR-PCu connectivity was diagnostically associated with greater depression severity elaborates and extends previous observations of deficits in raphe-wholebrain (Anand et al., 2019; Han et al., 2019) and raphe-PCC connectivity in depressed patients (Ikuta et al., 2017) – which sit in contrast to the otherwise positive and strong coupling of the MnR to the PCu/PCC observed in healthy populations (Bär et al., 2016; Beliveau et al., 2015; Singh et al., 2022). No other monoaminergic brainstem source nuclei showed any associations with initial or post-trial depression, suggesting that MnR-precuneus coupling might plausibly be a sufficiently specific non-invasive biomarker for the serotonergic deficit at the centre of the monoamine hypothesis of depression (Cosci & Chouinard, 2019; Delgado, 2000; Hamon & Blier, 2013). The specific association with the precuneus in particular could reflect that serotonergic modulation of this cortical integration and network node is especially relevant – which is consistent with previous findings that this cortical node's normal connectivity and activity may safeguard against MDD (Höflich et al., 2019). Indeed, as mentioned in Chapter IV, the abundance of serotonin receptors (particularly 5HT2ARs) in the precuneus suggests it may be especially susceptible to alterations in serotonergic tone (Beliveau et al., 2017). As depletion of serotonin has been demonstrated to negatively affect raphe connectivity (Bär et al., 2020; Weinstein et al., 2015), MnR-PCu hypoconnectivity could thus indicate lowered serotonergic tone provision to this cortical node, or be a marker for a global serotonin deficit.

Most importantly however, my results suggest that the sub-/post-acute consequences of psychedelic dosing on the serotonergic system may be diametrically opposed to those observed in the acute setting. Explicitly, raphe-precuneus coupling was significantly upregulated 1 day and 3 weeks after dosing in depressed patients, with the extent of enhancement of raphe-cortex coupling proportional to treatment symptom reduction. A psychedelic-induced increase in MnR-PCu connectivity might thus be a fast-occurring phenomenon apparent as early as one day and as late as three weeks after dosing. Altogether, these observations suggest that a psychedelic-induced allostatic upregulation of raphe function may underpin psychedelic therapeutic effects, which provides empirical evidence in favour of the *knockout-to-reset* hypothesis posited at the beginning of this chapter. Instead, this effect was not detectable in the SSRI trial arm, with no significant upregulation of raphe-precuneus coupling occurring. While it is possible that other raphe connectivity alterations than the MnR-PCu relationship might be involved in the response to SSRI treatment, the present analyses are indicative of a less successful or lack of upregulation of the diagnostic and treatment-tracking MnR-PCu connectivity biomarker. This raises the possibility that efficacious SSRI treatment does not entail an enhancement of serotonergic source nucleus connectivity – most likely as the acute effects, i.e. raphe knockout, are not similar between continuous SSRI and single/double dose psychedelic administration.

The post-acute upregulation of raphe functionality as a likely sequela of the acute psychedelic raphe 'knockout' is also resolved within the confines of the present data: The greater EBI scores during the dosing session were, the more likely a given participant was to have positive MnR-PCu connectivity after the MDD-RCT trial. Based on the assertion that acute psilocybin effects should be similar to LSD (given relatively similar pharmaco-dynamic profiles, observations and reports), EBI scores should thus entail the acute raphe-'knockout', which provides a preliminary linkage between acute psychedelic experience and the suggested subsequent post-acute raphe "reset" and the observed treatment effects. It is important to acknowledge however that the EBI also captures influences of the therapeutic container/setting (Roseman et al., 2018, 2019), whose contribution to the psychedelic experience and overall therapeutic outcome should not be underestimated (Thal et al., 2021).

Nevertheless, the raphe-'reset' may form a central column of a combination of factors contributing to successful psychedelic treatment.

One aspect that is important to resolve, is that the suggested raphe *knockout-to-reset* parallels previous observations of acute hypo- and post-acute hyper-connectivity of the DMN, which was coined the "DMN reset". This phenomenon has itself been suggested to underpin therapeutic effects (Carhart-Harris et al., 2012, 2017). The findings in this and the previous chapter indicate that MnR-PCu connectivity increases are a possible neuromodulatory underpinning of these effects. MnR-PCu connectivity disruption due to LSD, i.e. MnR-PCu hypo-connectivity was associated with disintegration of the DMN, whereas post-acute psychedelic-induced increases in PCu-DMN connectomic integrity are associated with proportional increases in MnR-PCu connectivity. As mentioned in Chapter IV, serotonergic signalling has been suggested to produce neuromodulatory environments that especially favour default mode network brain states over others (Conio et al., 2019), which supports that raphe function changes should have DMN-level consequences. Although SSRI treatment has also been suggested to upregulate PCu/PCC-DMN connectivity (Kraus et al., 2014), in the present analysis this effect was absent in the SSRI arm and DMN integrity in the SSRI arm appeared independent of changes in raphe functionality. This reinforces the view that SSRI effects may be mediated *via* mechanisms that do not adaptively reset serotonergic source nucleus function.

Taken together, the data across **Chapter IV** and **V** therefore are consistent with an acute psychedelic "knockout" of serotonergic function (captured in disrupted MnR-PCu connectivity) precipitating an adaptive re-emergence of raphe functionality, i.e. a "raphe reset" (captured in increased MnR-PCu connectivity and possibly also activity). This reset might move the serotonergic system away from a state of *hypo-function* towards one of normal or hyper-function – which might allow at least partial alleviation of the monoaminergic/serotonergic deficit associated with depression. The mechanistic sequence underpinning such a phenomenon might function as follows: In acute administration psychedelics outcompete endogenous serotonin at binding to particularly the 5HT2A receptor

(5HT2AR). Given the homeostatic organisation of transmitter systems, vast whole-brain binding of the psychedelic at serotonergic receptors will send inhibitory feedback to the raphe to downregulate provision of serotonin, with psychedelic signalling *via* inhibitory autoreceptors in the brainstem (5HT1AR) further inhibiting the median raphe (Behzadi et al., 1990; Kosofsky & Molliver, 1987; Vertes & Linley, 2007). This is what preclinical animal experiments suggest as well, as raphe activity effectively ceases in acute administration (Aghajanian et al., 1968, 1970; Aghajanian & Marek, 1999; Foote et al., 1969; Rogawski & Aghajanian, 1981). This process might disrupt both activity *and* connectivity of the raphe – which might already be impaired in depressed individuals (i.e. a hypo-serotonergic state). In these patients, a psychedelic-induced acute downregulation of the serotonergic system might constitute a sufficient "stressor" (as specified in biological-systems allostatic theory) that pushes the median raphe past a 'reset-point', i.e. to a level of hypo-function where fundamental neurobiological processes engage that serve to establish "new stability (i.e. homeostasis) through change" (McEwen, 2000; McEwen & Wingfield, 2003).

The drivers of this process might be various second messenger systems, and in the longer term also epigenetic changes to serotonin-related gene expression (dos Santos & Hallak, 2020; Soga et al., 2021).

The quickness and stability of this raphe upregulation is in the present samples already observable one day after psychedelic dosing (TRD-OLT), and as long as 3 weeks after dosing (MDD-RCT). A long-lived post-acute raphe hyper-function (compared to baseline) is a plausible treatment mediator for psychedelic depression treatment through alleviating serotonin deficits. Additionally, it is also well-positioned to underpin other long-lasting phenomena, such as the recently suggested post-acute hyperplastic phase (Brouwer & Carhart-Harris, 2021) and the associated post-psychedelic 'afterglow' (Psiuk et al., 2021; Sampedro et al., 2017), as many neuroplasticity mechanisms are dependent on serotonergic signalling.

Nevertheless, to establish whether serotonergic system function is indeed 'reset', future neuroimaging work will need to assess acute, post-acute and long-term follow-up in MDD psychedelic treatment,

using simultaneous PET-rsfMRI scans with serotonin-system related tracers (e.g. for both 5HT2A and SERT; Canli & Lesch, 2007; Saulin et al., 2012). This could clarify whether connectivity and activity patterns observed here do relate to sustained changes in serotonin tone. Additionally, various genetic and other factors may co-determine whether the acute downregulation of raphe functionality can induce the suggested allostatic process. Some psilocybin-treated patients who do not show anti-depressant effects in the MDD-RCT cohort may not respond to psilocybin binding to 5HT2ARs in the same way, possibly due to relatively widespread 5HT2AR polymorphisms (Schmitz et al., 2021). In support of such a different response occurring, those who experience the weakest psychedelic effects also show the weakest or full absence of anti-depressant effects. Indeed, while for most participants this double (or potentially even a single) psychedelic dose are sufficient for an upregulation of raphe connectivity, the allostatic load required in some patients might be different due to other comorbidities (e.g. through dopaminergic antagonism of serotonin; de la Cruz et al., 2021; Ishii et al., 2017; Ogawa et al., 2014). As such, future work will need to also establish whether acute drug-induced disruption of MnR-PCu connectivity may – if the 'knockout' pathologically persists once acute drug effects subside – actually be associated with prolonged anxiogenic adverse events in psychedelic depression treatment (Andrews et al., 1994).

Taken together, these findings raise a central corollary question: Could it be that psychedelics target a central rate-limiter of depression pathology in the raphe in a way that is "closer to the source" of the monoaminergic deficit – closer than SSRI broad-scale downregulation of serotonin reuptake? To answer this question, the long-term follow-ups of the MDD-RCT and other psychedelic depression trials should utilise neuroimaging to ascertain (i) whether long term benefits of psychedelics might outperform SSRI treatment, and (ii) whether lack of sustained upregulation of MnR connectivity/activity coincides with diminished or lack of long-term stable treatment effects. Should long-term effects of raphe connectivity and activity on depressive symptomatology be found, and these not be observed with traditional antidepressant treatment, then this could indicate that upregulation of postsynaptic availability might be counterproductive for self-sustaining antidepressant effects. Indeed, a very recent meta-analytic work found evidence that long-term SSRI use in fact may

reduce serotonin availability as would be expected in a homeostatic transmitter system model (Moncrieff et al., 2022). On the other hand, isolated findings suggest that raphe serotonin provision and activity can over time be subtly amplified by an SSRI in mice (Dankoski et al., 2016), suggesting that treatment effects of psychedelics and SSRIs could over time converge. Even if this should be the case, the question remains whether continuous SSRI medication is truly favourable over a single or double psychedelic stimulus from adherence, logistical, efficiency and therapeutic standpoints. As a result, it will be key to continue comparative studies of these interventions to establish the fundamental differences in their mechanisms.

I acknowledge various limitations of this work. As in the previous chapter, the serotonergic specificity of the median raphe ROI remains to be established using complementary work. The consistency across the work in the HAAN and the newly released brainstem navigator (Edlow et al., 2012; Bianiardi et al., 2022), however supports the ROI as being appropriately placed and specific. Nevertheless, it is striking that despite many previous implications of the dorsal raphe nucleus in depression in both preclinical and clinical contexts, I did not find effects centred on the DR. It is possible that the present chapter or previous studies might have inadvertently captured signals common to both DR and MnR nuclei, or alternatively over-dichotomized them. The DR is more easily delineable, but also closer to various sources of white matter noise, which might further complicate reliable detection of its signals in a heterogenous sample. Furthermore, a potentially strongly confounding factor in the current experimental setup is that participants in the SSRI treatment arm received a ‘microdose’ of psilocybin on the research visits (1mg). While this was assumed to have negligible experiential effects (Madsen et al., 2019), it is possible that the dose may have been sufficient to elicit subclinical changes in raphe functionality. While the rationale for this was to standardize expectations between the two groups, it will be important for future work to compare fully homogenous groups to establish *de facto* unique effects of SSRIs in comparison to psilocybin. Relatedly, the fact that I utilised a precuneal ROI that is common to both treatment arms of the RCT is advantageous to standardize across the cohorts for comparability. Nevertheless, this might under-estimate the treatment-specific effects of either group, as evidenced by the fact that while the PCu is

implicated in the treatment-arm-specific whole brain correlations, the clusters are slightly different between the two groups. Furthermore, once a research visit occurred participants were effectively unblinded by the occurrence of psychedelic experiences in the psilocybin group, and the absence of these in the other. This might itself have affected how participants self-rated depression severity due to disappointment/anxiety. To combat this, future work may need to consider the use of drug-dose differentiated subgroups or of cross-over designs, despite the enormous logistical burden this might require. Furthermore, the inter-relationship of the median raphe with the dorsal raphe, which I did not assess, may hold additional importance in MDD (Hornung, 2012; Jasinska et al., 2012). The remaining concerns are similar to the previous chapter, given the purposely similar processing and approach.

Taken together with the previous chapter, the present results thus provide the first *in vivo* evidence that psychedelics have marked and interrelated acute and sub-/post-acute effects on serotonergic source nucleus function in the human brain. These effects are mirror images of one another, with acute disruption putatively followed by a sub-/post-acute enhancement in raphe functionality, as captured in raphe-posterior cortex coupling. In the broader context of this thesis, this demonstrates that raphe connectomics can provide disease- and treatment-relevant insights in depression, as it emerged to have efficacy-monitoring and potential diagnostic utility – thus fulfilling central requirements set out for biomarkers that are urgently needed for the advancement of CNS pharmacological treatments (in DOC, and beyond; Edlow, Sanz et al., 2021). Correspondingly, usage of raphe-cortex coupling as a modifiable biomarker in depression trials may allow crucial sub-clinical treatment-efficacy monitoring through a process that is simple and ready to implement, relatively low-cost and non-invasive. More broadly, the observation that after the psychedelic ASC there are lasting changes in a transmitter nucleus' connectivity which are associated with depression-alleviation recapitulates that brainstem influences do not constitute mere activating arousal, but have consciousness-relevant and somatic functions (Satpute et al., 2019). Altogether, **Chapter V** thus solidifies this thesis' case that monoaminergic nuclei from the brainstem are involved in ASCs, and extends this perspective to ASCs' post-acute sequelae. Finally, the observation that powerful

pharmacological stimuli such as a psychedelic drug can precipitate re-emergence of previously impaired transmitter-system-specific nucleic connectivity and activity might be informative for treatment frameworks outside the MDD context, such as for DOC (compare **Chapter II** and **III**).

Appendix Experimental Chapter

6.1 Preface and Overview

With the advent of the COVID-19 pandemic, much of my work focus was redirected towards curating, processing and analysing a 3T rs-fMRI database of COVID-19 patients who were imaged at follow-up timepoints of 6 and 12 months. The following analyses were motivated by the consistent implication of potential brainstem involvement in COVID-19 acute symptomatology (such as breathlessness, disorders of consciousness etc), high expression of the ACE2 receptor in the brainstem and recent observations that viral protein in histopathology can be located in the pons and midbrain. While some COVID patients experience disorders of consciousness (Fischer et al., 2022), this was not the case in this cohort, and as such did not strictly sit within the scope of the assessment of ASCs as suggested in this thesis. As such, these particular analyses are presented here as an appendix chapter, as they provided the opportunity to assess whether – in the absence of transmitter-specific hypotheses – brainstem connectomics can show clinical utility to characterise potential disease and symptom contributors. Specifically, in this appendix chapter I methodologically advance on the previous experimental chapters by beginning with a gene-map-based brainstem region-of-interest to iteratively characterise potential transmitter system deficits in survivors of COVID-19 with long-term symptoms. I use a simple-to-implement connectivity metric to make individual patients' transmitter nuclei connectivities in this clinically-heterogenous sample maximally comparable to normative data – and lay out the use of partial correlations to identify unique contributions of neurotransmitter nuclei for Long-COVID symptomatology.

The data in this chapter was collected by Doris Chatfield, Anne Manktelow and various other WBIC collaborators at the University of Cambridge during the COVID-19 pandemic. I curated and co-organised the database, performed all analyses myself and wrote the chapter independently with input from Emmanuel A Stamatakis.

“Un-masking” persistent monoaminergic brainstem dysfunction in long term follow-up of COVID-19 patients

6.1.1 Summary

Generalised brainstem dysfunction has been suggested to be involved in both acute and long-lasting effects of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). While neuropathological work has demonstrated that the brainstem shows neuro-invasion by SARS-CoV-2 and shows signs of neuroinflammation in fatal cases, no empirical evidence for its persistent dysfunction in COVID-19 survivors has been found yet. Using rs-fMRI recordings of survivors of severe COVID-19 that was collected at 6 months after initial infection/hospitalisation, I tested whether brainstem nuclei connectivity may be dysregulated in COVID-19 survivors – and whether particular transmitter system connectivity deficits may relate to long-term symptomatology. To begin with, I found that the region of the brainstem that is the most enriched in the Angiotensin-converting enzyme 2 receptor (the point of cell entry of SARS-CoV-2) is strongly disrupted in comparison to healthy controls. Considering individual brainstem nuclei overlapping with this ACE2-enriched region showed that both monoaminergic and glutamatergic nuclei were disconnected. However only dopaminergic (VTA) and serotonergic (MnR and DR) disconnections survived correction for multiple comparisons. Only the dopaminergic ventral tegmental area’s connectivity impairment showed strong unique associations over and above the other nuclei with clinical symptomatology at the 6-months timepoint – with greater impairments in VTA functionality being associated with worse outcomes across multiple sub-domains of the Short Form 36 questionnaire (SF-36). Finally, in a subsample of participants who underwent an additional follow-visit at 12 months after initial infection/hospitalisation, I found that improvements (increases in comparison to first visit) in VTA functional connectivity are associated with improvements in symptomatology. Considered together with very recent preclinical evidence that SARS-CoV-2 impairs dopamine synthesis in the acute disease phase, these results suggest that monoaminergic impairments may persist long after acute phases and that related treatment strategies may hold promise for the treatment of Long-COVID.

6.1.2 Introduction

Over the past two years, the coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has affected more than 275 million people worldwide. Importantly, meta-analyses have found that up to one in three COVID-19 patients show neurological manifestations in the acute phase (Chou et al., 2021; Misra et al., 2021), with many going on to report neurological post-acute sequelae long after the initial infection as part of the collection of symptoms referred to as Long-COVID (Crook et al., 2021; Nalbandian et al., 2021). A key substrate that has been proposed as a mediator of these neurological deficits is the human brainstem – as COVID-19-induced dysfunction of this brain region is ideally positioned as a neural "bottleneck" to bring about both acute and prolonged neurological deficits (Becker, 2021; Proal & VanElzakker, 2021; Yong, 2021).

Firstly, in respect of acute neurological manifestations, the brainstem contains the respiratory centres of the human central nervous system (CNS), the disruption of which has been suggested to bring about the commonly reported breathlessness in COVID-19 (Manganelli et al., 2020). This is in line with neuropathological analyses of fatal cases, in which both viral RNA and protein have been found in the brainstem of deceased patients, confirming the neurotropism of SARS-CoV-2 – which may induce localised brainstem dysfunction (Matschke et al., 2020). Indeed, the brainstem is likely particularly vulnerable to SARS-CoV-2 neuro-invasion as the pons and medulla have the highest density of the Angiotensin-converting enzyme 2 (ACE2) receptor of the whole brain, which is the receptor *via* which the virus gains cell entry (Chen et al., 2021; Lukiw et al., 2020). This causes a local immunological response, as seen in consistently replicated findings of brainstem inflammation in these regions (Yong, 2021). Importantly however, less than 30% of severe COVID-19 cases seem to show structural abnormalities on magnetic resonance imaging (MRI), and a recent meta-analysis of MRI imaging in COVID found no structural changes in the brainstem at all (Najt et al., 2021). This highlights that COVID-induced acute and long-term deficits may fundamentally be underpinned by the impairment of brainstem *function*, which – when considered in a neurotransmitter-specific fashion

– may provide treatment targets that are especially relevant in the context of an ever growing incidence of Long-COVID.

In respect of post-acute manifestations, patients who survive COVID-19 – much like previous SARS survivors (Moldofsky & Patcai, 2011) – often face long-lasting and persistent symptoms that have equally been hypothesized to be underpinned by disruptions of central brainstem functions (Becker, 2021; Crook et al., 2021; Najt et al., 2021; Nalbandian et al., 2021; Ogier et al., 2020; Yong, 2021): such as anxiety, depression, low energy and fatigue, as well as physical impairments. Indeed, these deficits have in non-COVID contexts been strongly associated with altered function of the neuromodulatory transmitter nuclei contained within the pons and midbrain (Avery & Krichmar, 2017; Marder, 2012). As such, assessing their dysfunction provides an explicit opportunity to identify treatable deficits and generate mechanistic hypotheses in Long COVID. This has during the pandemic motivated various publications which posited the likely involvement of persistent brainstem dysfunction in the (equally persistent) neurological sequelae of COVID-19, based especially on the brainstem nuclei's arousal and autonomic functions (Al-Sarraj et al., 2021; Becker, 2021; Yong, 2021). Mapping dysfunctions of these systems to symptomatology has however not been empirically performed yet. I submit, in extension of the previous chapters, that in order to understand the brainstem as a potentially fundamental effector system of the COVID-19 disease and its sequelae, and to produce actionable treatment targets for Long-COVID, it is crucial to complement ideas of the more widely-suggested "generalised" brainstem dysfunction with a transmitter system- and nucleus-specific view.

Consequently, I sought to resolve whether persistent sequelae of COVID-19 may be associated with brainstem dysfunction, and whether a brainstem nuclei functional connectivity analysis may be suitable to disentangle related sub-effector systems as treatment targets. To this end, I assessed the connectivity of a combination of histologically- and genetically characterised brainstem regions of interest (ROI) in a sample of COVID-19 patients of varying initial disease severities at 6 months after infection (n=44) compared to healthy controls (n=42). Specifically, I aimed to resolve whether

particular transmitter nuclei connectivity profiles may be associated with specific symptomatic manifestations of Long-COVID, using partial correlational analyses. Finally, in line with the hypothesis that brainstem dysfunction may at least partially underpin the severity of symptoms but may be reversible, it was tested whether improvement in brainstem function at 12 months in a subset of participants who had repeat visits (n=12) may bring about positive changes in symptomatology.

6.2 Materials and Methods

Participants and Data Acquisition

All patients admitted to Addenbrookes Hospital with COVID-19 between 10th March 2020 and 31st July 126 2020, aged 18 years or older, who tested positive to Covid-19 as the basis of an acute hospitalisation but survived the acute illness and attended a follow up visit were eligible to take part in this cohort study. This comprised a total of 489 patients, of whom 49 provided written informed consent to participate in the study. Recruitment was carried out through the NIHR COVID-19 BioResource, which received ethical approval from the East Anglia - Cambridge Central Research Ethics Committee (REC 17/EE/0025). Age- and sex-matched controls were recruited using the same study application, and supplemented by subjects recruited through an ethically approved protocol used for magnetic resonance imaging sequence development and quality control. Clinical data used in these analyses were obtained from inpatient electronic medical records, and from cardiorespiratory and neurological assessment at follow-up clinical and research visits. An additional group of 42 age- and sex-match non-hospitalised controls was recruited for neuroimaging.

The WHO Covid-19 10-point scale was used to assess initial COVID-19 severity at time of hospitalisation (Marshall et al., 2020). Various fluid biomarkers were collected, particularly the greatest readout of haematological (platelets), inflammatory (C-reactive protein, CRP, serum ferritin), immunological (inteleukin-6, IL-6), and hepatic dysfunction (bilirubin), as well as coagulatory (D-

dimer), prothrombin time (PT) and activated partial thromboplastin time (APTT; Asakura & Ogawa, 2020; Bangash et al., 2020; Iba et al., 2020; Levi et al., 2020).

Breathing function was assessed at approximately 3 months after initial hospitalization *via* CareFusion Micro 1 Handheld Spirometer (Vyairre Medical GmbH.; Hoechenberg, Germany) to obtain measures of peak expiratory flow (PEF), forced expiratory volume in 1 second (FEV1), forced vital capacity (FVC) and FEV1/FVC ratio. Measurements were taken three times, with a minute-long intermission after each attempt. Before and after participants partook in a 6-minute walk test, pulse oximetry, monitored *via* GE Carescape V100 Dinamap Vital Signs Monitor (GE Healthcare Systems, Chicago, Illinois, USA), was utilised to obtain a measure of arterial oxygen saturation and heart rate (Bois et al., 2012; Crapo et al., 2012; PL et al., 2003).

Research visit: neurological assessment and image acquisition/pre-processing

During a scheduled research visit 6 months after initial hospitalisation/diagnosis, imaging data were acquired using a 3T Siemens Prisma*fit* System (32-channel head-coil) at the Wolfson Brain Imaging Centre at the University of Cambridge.

3D-structural scans were acquired using a T1-weighted sequence (3D Magenetisation-Prepared Rapid Gradient-Echo, 3D MPRAGE), with repetition time (TR) = 2 ms; echo time (TE) = 2.99 ms; inversion time (TI) = 880 ms; flip angle α= 9°; field of view (FOV) = 208 × 256 × 256 mm3; resolution = 1 mm isotropic; accelerated factor (in-plane acceleration iPAT) = 2; acquisition time, 5 min.

Eyes-closed Resting state Echo-Planar Imaging (EPI) was acquired as 477 volumes with 64 slices for whole brain coverage (TR = 735ms; TE = 30ms; FOV = 210mm x 210mm; resolution = 2.38 x 2.38 x 2.4mm) in sessions lasting 5 minutes and 51 seconds.

The preprocessing and denoising pipeline for this data was identical to the core experimental chapters in this thesis. However, to remain consistent with BioBank data practices due to follow-up work from this project requiring integrability and comparbility, the data were linearly detrended and filtered using a band-pass filter of [0.008 0.09] Hz, rather than the otherwise used high-pass filter. After

quality control, 44 patients had structural and functional resting state functional magnetic resonance imaging (fMRI) data of sufficient quality to be included in the brainstem-centric analyses.

Brainstem Nuclei Functional Connectivity 'Distance' analyses

To most accurately resolve brainstem nucleic connectivity changes in this heterogenous cohort of participants with Long-COVID, an alternative approach to the previous chapters was used to compute functional connectivity profiles of all transmitter-specific HAAN ROIs (glutamatergic: mesencephalic reticular formation, parabrachial nucleus, oral pons; noradrenaline: locus coeruleus; serotonergic: median and dorsal raphe; dopaminergic: ventral tegmental area). This approach was motivated by making COVID-19 survivors maximally comparable to healthy controls, seeking to quantify a 'distance' from control functional connectivity for each individual participant.

Firstly, the faithful co-registration of HAAN ROIs to the anatomical and functional scans was assessed visually. Then, seed-to-voxel connectivity analyses were performed in the control cohort, to obtain a mask of the group-level average "normative connectivity" of brainstem nuclei. For this, temporal correlations for each brainstem nucleus were computed for all other voxels in the brain using General Linear Models. The functional connectivity analyses produced parameter estimate whole-brain β-images which were thresholded at $p<0.005$ voxel-level and $p<0.05$ cluster-level (corrected for multiple comparisons) to create binarized maps of average whole-brain connectivity for each nucleus.

Thereafter functional connectivity coupling of each HAAN nucleus to its nucleus-specific map was computed in the Long-COVID cohort for each participant. β estimates were extracted from the GLM using REX. To make these estimates directly comparable to the control average connectivity strength, the obtained beta values were transformed into z-scores, meaning that the estimate was converted to a parameter that reflects how many standard deviations away from control-like connectivity an individual participant's connectivity was.

Correlations with Short Form 36 Questionnaire

Quality of life, cognition and mental health questionnaires were collected (Generalised Anxiety Disorder-7 (GAD-7), Patient Health Questionnaire-9 (PHQ-9), Patient Health Questionnaire-15 (PHQ-15), Posttraumatic Stress Disorder Checklist-5 (PCL-5)). However, as these are partially ordinal scales they were excluded from primary analyses. Instead, the scores from the Short Form-36 questionnaire (SF-36) with subscores *as per* most recent guidance (physical functioning (SF36-PF), role limitation physical (SF36-RLP), role limitation emotional (SF36-RLE), energy dimension (SF36-ED), emotional wellbeing (SF36-EW), social functioning (SF36-SF), pain (SF36-P) and general health (SF36-GH)) were used.

These measures were entered into correlations with the functional connectivity z-scores for respective nuclei, which used Spearman's rank and/or Kendall's Tau. As frequentist tests of the null hypothesis cannot conclude in favour of the H_0 but merely to its rejection, Bayesian statistics were used in a subset of analyses (using JASP v.14.0.0). Furthermore, in order to resolve domain-specific *unique* contributions of a given transmitter nucleus in Long-COVID symptomatology, when a significant primary seed-to-symptom correlation was found, I subsequently controlled for the connectivity profiles of all other brainstem nuclei and age as nuisance covariates in a partial correlation to ascertain that the initial correlation was indeed fully driven just by the nucleus identified in the primary correlation .

ACE2 receptor peak region-of-interest in the brainstem

Predicitive mRNA expression maps for the ACE2 receptor are available in MNI-152 space from the Neuroimaging Lab at the Medical University of Vienna (https://www.meduniwien.ac.at/neuroimaging/mRNA.html). These are based on the Allen Brain Atlas (https://portal.brain-map.org/). This ACE2 map in MNI-152 space, was masked with an explicit mask of the brainstem. To capture the within-brainstem peak of predictive ACE2 expression, the map was then thresholded to retain only the top 15% of predictive ACE2. This additional ROI was used to begin the analyses with a physiologically-relevant starting point, as SARS-CoV2 enters human cells *via* the ACE2 receptor.

6.3 Results

6.3.1 ACE2-rich brainstem area shows connectivity impairment in Long-COVID

Firstly, I aimed to ascertain whether a brainstem dysfunction can be captured with fMRI, and specifically functional connectivity, at 6 months after initial hospitalisation in COVID-19 survivors. To utilise a physiologically-relevant but relatively general brainstem ROI, I used an area that is the most enriched in the ACE2 receptor. This ROI captured, consistent with pre-existing literature (Chen et al., 2021; Lukiw et al., 2020), a larger pontine region overlapping in particular with monoaminergic brainstem nuclei (see Fig.AC1a & Fig.AC2f). In healthy controls, this area showed wide-ranging brain connectivity, capturing in particular Default Mode Network (DMN) and midline regions. Instead, COVID-19 survivors showed a significantly lower connectivity of this ACE2-rich ROI to this map (red, Fig.1A) when compared to controls (see Fig.AC1b). The strength of this impairment in brainstem connectivity was predicted by/associated with the severity of initial illness as measured both with the WHO severity scale (see Fig.AC1c) and the fluid biomarker level of C-reactive protein, a blood-based systemic inflammation measure (see Fig.AC1d). This suggests that COVID-19-induced brainstem impairment can be long-lasting and still detectable using neuroimaging at 6 months, and that brainstem impairment might be involved in eliciting initial disease severity in a way that is still detectable long after hospitalisation. In exploratory analyses with SF-36 subscores however, the functional connectivity impairment of this ‘high-vulnerability’ ACE2-rich brainstem region showed no correlations with any clinical subdomains that had been collected at the 6 month follow-up visits.

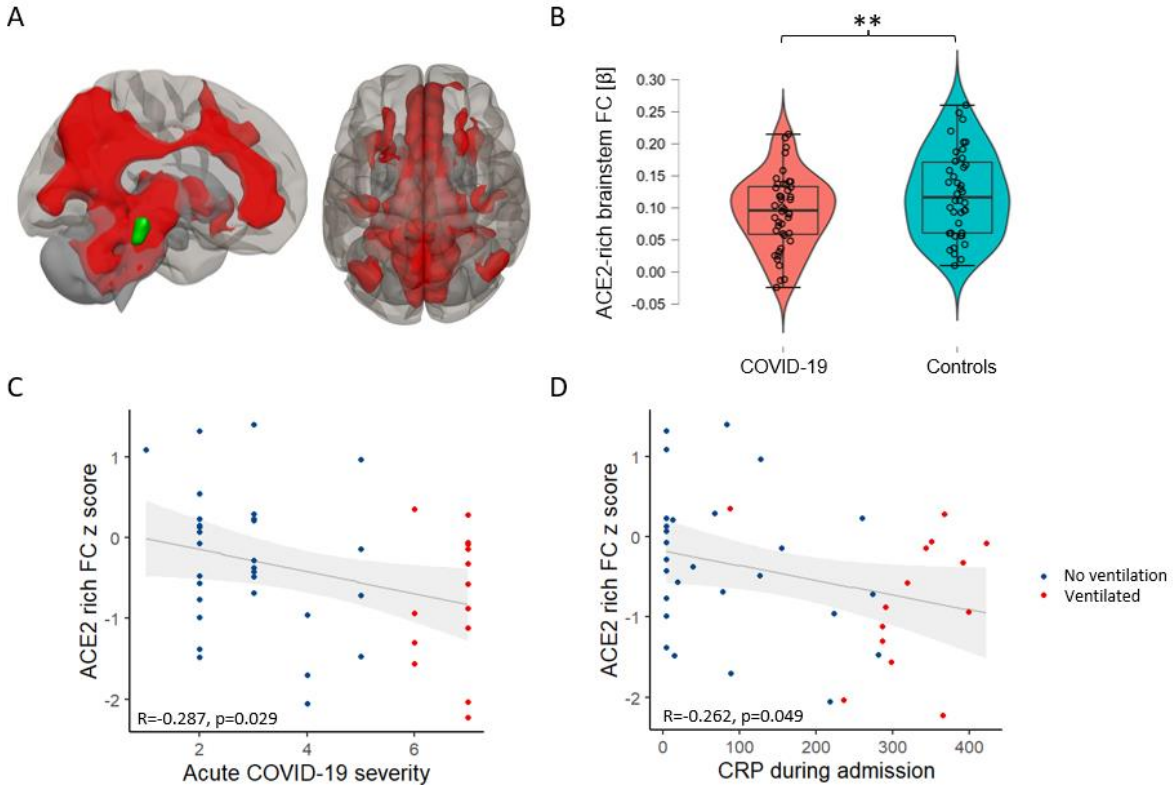


Fig. AC1: ACE2-richest brainstem region is disconnected in severe COVID-19 survivors. (A) Control connectivity map (red) of the ACE2-rich brainstem region. (B) The COVID-19 cohort showed significantly lower connectivity to the control connectivity map (red; A) than the Control cohort. (C) Acute COVID-19 severity, measured on the WHO scale was predictive of the strength of brainstem functional connectivity impairment (z score), as was (D) the acute level of C-reactive protein, an inflammation marker. FC = Functional connectivity

6.3.2 Brainstem connectivity deficit can be resolved into transmitter-specific sub-components

Given clear pre-existing associations between specific neurotransmitter systems and reported symptoms of Long-COVID, together with these nuclei's spatial overlap with the ACE2 rich brainstem region, I hypothesized that the long-term deficits observed at 6 months may be better resolved by assessing particularly neuromodulatory monoaminergic and glutamatergic arousal nuclei. These were the dopaminergic ventral tegmental area, the serotonergic median and dorsal raphe nuclei, the noradrenergic locus coeruleus (all neuromodulatory nuclei) and the glutamatergic mesencephalic area, oral portion of the pons, and parabrachial complex. We excluded the cholinergic pedunculopontine nucleus despite its availability in the HAAN as it showed no overlap with the ACE2 receptor map.

Further resolving and extending the result found for the ACE2 rich ROI, all brainstem nuclei that partially overlapped with it (with the exception of mesencephalic reticular formation and locus

coeruleus) showed significantly lower mean connectivity in the COVID patients than the controls (see Fig.2). When applying Bonferroni multiple comparisons correction, only the dopaminergic VTA, and serotonergic DR remained significant. As the violin and distributed boxplots in Fig. 2 show, a few participants also showed higher β-values/standardized z-scores (i.e. higher connectivity) than the control mean after COVID, suggesting that in some participants there may be an upregulation of connectivity.

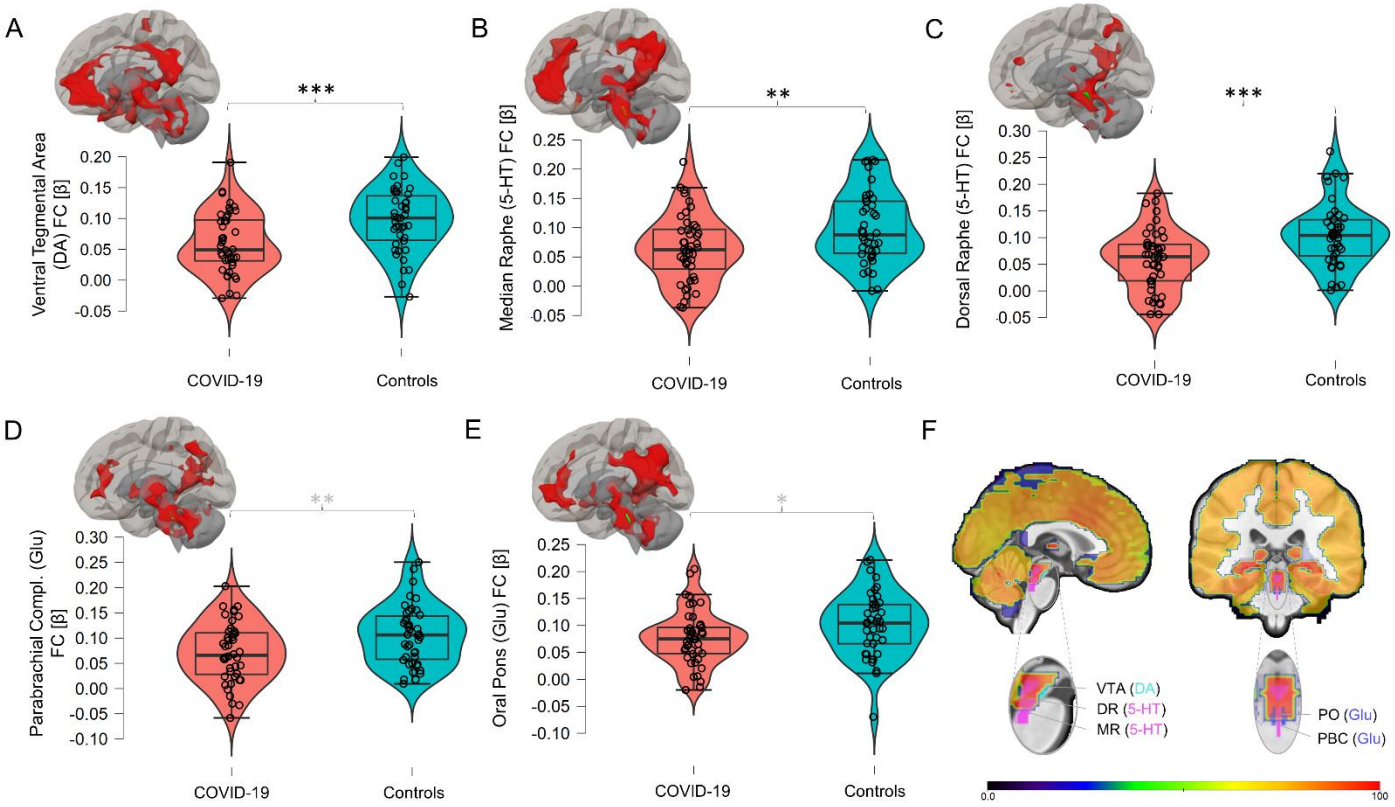


Fig. AC2: Connectivity is lowered across monoaminergic and glutamatergic nuclei post COVID-19. Red brain maps show average connectivity map of controls. The violin plots with standard bar graph elements show the individual connectivity data points of controls (azure) and COVID-19 patients (red) at 6 months after initial illness. (A-C) The connectivity of brainstem nuclei to control maps was significantly lowered across the monoaminergic ventral tegmental area, median and dorsal raphe , in a manner that remained significant after Bonferroni correction for multiple comparisons (black asterisks). (D-E) The glutamatergic parabrachial complex and oral pons also showed significantly lowered connectivity in COVID-19 survivors, but this did not survive multiple comparisons (grey asterisks). (F) Overlap of monoaminergic and glutamatergic nuclei with the whole-brain ACE2 receptor map to highlight highest density of ACE2 overlapping with pontine neuromodulatory nuclei.

6.3.3 Brainstem nucleic connectivity deficits are associated with clinical outcome at 6 months

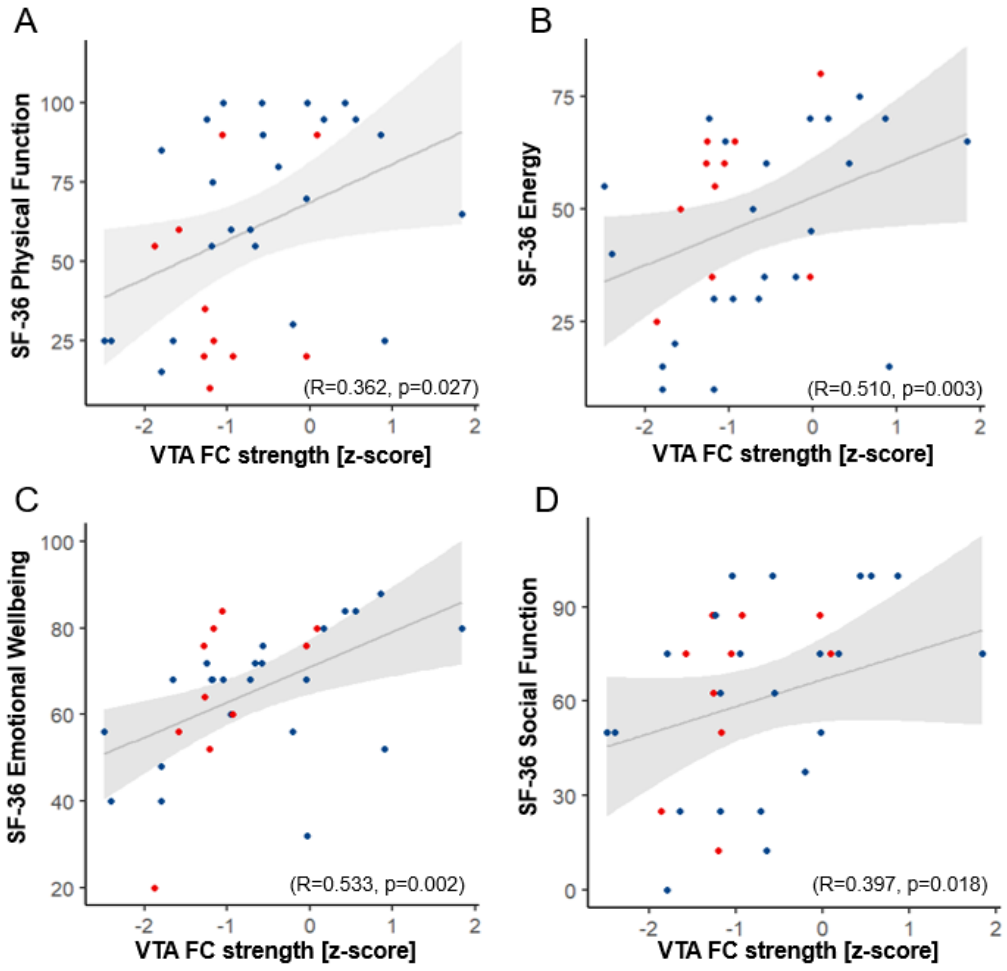
Next, I explored whether transmitter nuclei connectivity deficits might be associated with domain-specific clinical deficits at 6 months measured with the SF36 scale. Importantly however, given that disruption of brainstem connectivity was found across multiple nuclei and transmitter systems, I wanted to ascertain that *unique* contributions of different transmitter systems/nuclei were captured. As such, I performed primary exploratory correlations with the battery of clinical examinations (see Methods), and when a significant primary correlation was found, the connectivity strength of all other nuclei that showed significant differences was regressed out (nuisance covariate).

I hypothesized, given the widely-reported symptoms of depression and somatic dysfunction in the literature of both acute and post-acute COVID-19 (Becker, 2021; Crook et al., 2021; Ogier et al., 2020; Proal & VanElzakker, 2021; Yong, 2021), that the monoaminergic modulatory nuclei should be strongly implicated, whereas the roles of not purely modulatory nuclei (e.g. glutamatergic) should be less relevant here. All correlations were controlled for age.

In support of this hypothesis, no significant correlations across all clinical variables were found for the two glutamatergic nuclei (PBC and PO), whereas various significant correlations were found for the monoaminergic neuromodulatory brainstem nuclei.

In the primary, exploratory, correlation analyses, the serotonergic median raphe nucleus (MnR) was found to be correlated with the SF-36 Emotional Wellbeing score ($R=0.392$, $p=0.029$), whereas the DR only showed a trend in this direction $R=0.260$, $p=0.072$), indicating that the more serotonergic connectivity was disrupted, the more severely emotional wellbeing was impacted. Most strikingly however, the dopaminergic ventral tegmental area's (VTA) connectivity impairment was associated broadly with a variety of SF-36 functions: Emotional Wellbeing ($R=0.524$, $p=0.001$), Social Functioning ($R=0.375$, $p=0.017$), Role Emotional ($R=0.577$, $p<0.001$), as well as Energy ($R=0.498$, $p=0.002$), Physical function ($R=0.375$, $p=0.016$), and Role Physical ($R=0.361$, $p=0.021$) – thus strongly implicating dopaminergic system function across the spectrum of long-term post-COVID symptoms.

Substantiating the importance of the VTA, the secondary partial correlations revealed that out all nuclei only the associations of the VTA (with Social Function (R=0.397, p=0.018) Emotional Wellbeing (R=0.533, p=0.002), Energy (R=0.510, p=0.003), Physical Function (R=0.362, p=0.027) and Role Physical (R=0.345, p=0.036)) remained significant when controlling for all other nuclei – whereas the relationships of all other nucleic connectivities became non-significant. Further substantiating this finding, only VTA correlations had Bayesian support (Emotional Wellbeing, BF_{10}=26.595; Physical Function, BF_{10}=11.629; see Fig.AC3). Altogether, these findings thus suggest



that dopaminergic system alterations may be involved in mediating Long-COVID symptomatology.

Fig. AC3: Unique associations of brainstem nuclei with outcome measures reveal monoaminergic ventral tegmental area dysfunction as central in outcome after COVID-19. Red = ventilated, blue = unventilated during acute illness. Controlling for age, and all other nucleic connectivities, only associations of the VTA FC strength with SF-36 measures of (A) physical function, (B) Energy, (C) Emotional Wellbeing, and (D) Social Function remained significant. BF reported in-text.

6.3.4 Long-term changes in ventral tegmental area connectivity are associated with improvement in symptoms

The above observations of VTA associations with symptomatology concerned the first neuroimaging visit of the participants at 6 months after the acute illness. However, a small subsample of participants (n=12) returned at 12 months for a repeat visit. This allowed the preliminary assessment of whether improvements in brainstem connectivity at 12 months compared to the 6-months timepoint may be associated with improved symptomatology. Explicitly, I tested tested this in domains that the VTA showed any covariance with at 6 months post-COVID-19.

In concordance with the above hypothesis, VTA connectivity increase (Δ VTA FC 12 – 6 months visit) was significantly associated with better scores in physical function and role physical on the SF-36 scale (see Fig.AC4). This indicates that recovery and improvement in dopaminergic brainstem function (captured in functional connectivity), may be key to the recovery from Long-COVID, and might in turn provide important information to develop treatment strategies for easing symptoms and aiding recovery.

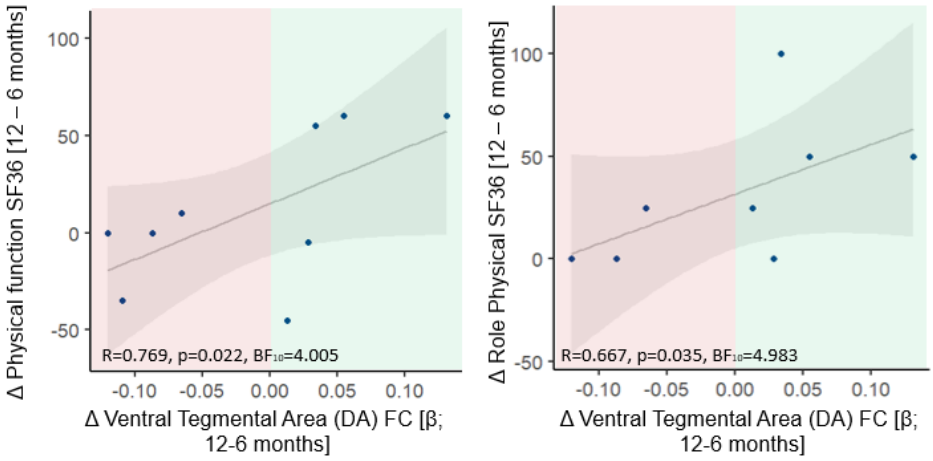


Fig. AC4: Improvements in dopaminergic ventral tegmental area functional connectivity at 12 months are associated with improvements in key symptoms. SF-36 subscore changes between research visits at 6 and 12 months for Physical Function and Role Physical were associated positively with changes in ventral tegmental area functional connectivity between these two visits. Green binding box highlights increased FC compared to previous visit, whereas red bounding box lowered connectivity.

6.4 Discussion

In this Appendix chapter, I report the first empirical evidence of both persistent generalised and transmitter-system specific brainstem functional connectivity deficits in patients who have survived COVID-19, validating and extending various pre-existing hypotheses in the emerging COVID-19 literature (Becker, 2021; Crook et al., 2021; Pereira, 2020; Yong, 2021). The extent of brainstem dysfunction is associated with long-term outcomes on the emerging spectrum of Long-COVID, with particular importance of the dopaminergic source nucleus in the VTA. Furthermore, the observation in the preliminary subset of patients that improvements over time in this connectivity are associated with clinical improvements, supports that brainstem function may be a suitable treatment target – and that brainstem connectomics might be a central tool to stratify patients to receive the right treatments, and track their efficacy. I discuss these findings below and suggest that future studies should explore the efficacy of dopaminergic drugs with good safety profiles and accessibility as a uniquely suitable experimental therapeutic route for Long-COVID.

To begin with, the brainstem area richest in the ACE2 receptors and thus most vulnerable to COVID-19 neuro-invasion showed dysfunctional connectivity profiles in the present cohort. This provides empirical support for the theoretical assertions in the literature that COVID-19 is associated with a long-lasting perturbation of brainstem signalling – of a region the virus has been reported to enter (Matschke et al., 2020; McQuaid et al., 2021; Meinhardt et al., 2020). It is plausible that the impairment of brainstem function may be brought about by direct neurotropic and localised inflammatory/immune activity effects as the pons is particularly exposed to these, as the area of high ACE2 levels lacks a blood-brain barrier (Xu & Lazartigues, 2020). Indeed, the possibly most-replicated finding in COVID-19 histopathology is that of brainstem inflammation/encephalitis in areas coincident with the present ROI (Proal & VanElzakker, 2021). As both WHO-scale measured initial COVID-19 severity and acute levels of the systemic inflammation marker C-reactive protein are predictive of the extent of brainstem connectivity impairment, brainstem dysfunction indeed may be

COVID- and localised inflammation-*induced*, rather than a non-specific sequela. Altogether, although the ACE2-rich ROI is coarse and of limited physiological and treatment-informing relevance, such a broad-scope approach might in future work be able to stratify Long-COVID sufferers into those with and without some generalized brainstem dysfunction.

Importantly the coarser ROI connectivity deficit could be resolved into transmitter-system-specific connectivity impairments. As hypothesized, the neuromodulatory nuclei of the brainstem were most strongly associated with the neurological and neuropsychiatric sequelae of COVID-19, whereas the glutamatergic nuclei did not show such associations. Indeed, it is important to highlight that some participants who had no or just light PASC (post-acute sequelae of COVID; Nalbandian et al., 2021) symptoms, showed nucleic connectivities that were heightened above the benchmark means of controls in our analysis. This highlights the possibility that, persistent 'dysfunction' of brainstem signalling in the more severe cases may capture the absence of an allostatic, compensatory process. This is mirrored in all correlational results that show that those participants with z scores above the means of controls across particularly the monoaminergic nuclei are overall scoring better (and control-like) on the SF-36 subscales.

Indeed, monoaminergic function may be especially important to the battery of post-COVID-19 symptoms assessed, as only the VTA connectivities statistically survived controlling for connectivity profiles of all other nuclei and had Bayesian support. With associations that ranged from emotional wellbeing to energy/fatigue as well as general physical function, the dopaminergic VTA is an interesting candidate system for therapeutic interventions in this population, with many dopaminergic drugs that have solid safety profiles, and good tolerability (Faraone, 2018; Federici et al., 2005). Indeed, rodent immunostaining has recently revealed the VTA to be particularly enriched in ACE2 receptors (Hernández et al., 2021), and this nucleus has been associated with chronic fatigue syndrome and myalgic encephalomyelitis which are conditions that share many long-term somatic symptoms with Long-COVID (Baraniuk, 2022).

Most centrally, VTA dopaminergic function might be particularly likely to be impaired by SARS-CoV-2 due to a specific linkage: the gene that is the most strongly co-expressed with ACE2 is Dopa Decarboxylase (DDC), which converts L-DOPA into dopamine – making it crucial for the production of dopamine (Mpekoulis et al., 2021). As previously suggested by Nataf (2020), this co-expression pattern between ACE2 and DDC indicates that there may be a functionally relevant linkage between ACE2 expression and dopamine (and serotonin) synthesis. As it is established that SARS-CoV-2 infection downregulates ACE2 receptor expression levels, this might in turn also strongly downregulate the synthesis of dopamine (and serotonin as DDC is also involved here), thereby precipitating neurological sequelae that are associated with a resultant transmitter deficit.

This assertion is supported by observations from ACE2-knockout mice, as these show heavily downregulated levels of serotonin in both blood and brain, which should generalise to dopamine (Klempin et al., 2018). Additionally, dopaminergic system (and DDC) function have been suggested to be immuno-protective against viral infection (Frakolaki et al., 2019) and runaway inflammation (Vidal & Pacheco, 2020), meaning that a disruption of dopamine synthesis pathways, and resultant dysfunction of its main source nucleus in the VTA, may form part of both acute and post-acute disease phases of COVID-19. In strong support of this hypothesis, recent findings suggest that SARS-CoV-2 viral loads in patients with acute illness are negatively associated with the level of DDC expression, indicating that acute COVID-19 may directly impair dopamine synthesis (Mpekoulis et al., 2021). This effect of dopamine-downregulation may persist until long after the initial illness, as COVID-induced effects on ACE2 expression in-concert with long-lasting ACE2 auto-antibodies could trigger a sustained proinflammatory state and consequently incur a vicious cycle of monoamine downregulation and thus lower defences against inflammation – which might resultingly manifest as Long-COVID/PASC.

The observation that in those with 12-months follow-up the improvement of physical symptoms was proportional to the improvement in VTA connectivity importantly highlights that the connectivity (and possibly transmitter deficits) may be reversible. This upregulation of connectivity may reflect a delayed allostatic process in some patients, or may alternatively be a systems-level correlate of

downregulation of systemic inflammation, which allows normal dopamine (and serotonin) synthesis. Regarding specific treatment agents, there may broadly be two differing strategies: (i) upregulation of DDC activity, which would need to target protein kinase A or G, as their phosphorylation is known to upregulate DDC activity (Federici et al., 2005) or (ii) upregulation of dopaminergic signalling with well-known dopaminergic agonists such as methylphenidate, which has previously been demonstrated to upregulate VTA connectivity (Spindler et al., 2021). It is important to acknowledge however that strategy (ii) may transiently downregulate DDC activity further (Federici et al., 2005) as less synthesis of the transmitter is required in the face of supplementation.

In the immediate context of this thesis, a pivotal function of the dopaminergic brainstem nucleus for Long-COVID-19 symptomatology is biologically plausible. A stronger disruption of VTA functionality (and possibly of other monoaminergic nuclei, such as the raphe too) might in some patients lead to the previously reported delirium, and in severe cases, even disorders of consciousness occurring upon or after COVID-19 (Edlow, 2021). This highlights once more that different levels of brainstem dysfunction exist, which might at low severity first impair various other vegetative and somatic functions before leading to an altered state of consciousness – although Plum & Posner (2003)'s definition of a disorder of consciousness would likely include many different manifestations of Long-COVID.

It is important to acknowledge various limitations of this work. Firstly, the experimental setup was not able to assess acute COVID-19 effects on the brainstem, as patients were typically heavily sedated in hospital settings (which would have affected brainstem connectivity analyses; see Chapter II**)**. As such, and by not having behavioural/clinical measures for controls, this work lacks the ability to delineate whether brainstem dysfunction is truly COVID-induced or may be representative of other pre-existing conditions or uncontrollable circumstantial factors such as lock-downs. However, the initial severity and fluid marker predictive power does suggest a linkage between initial illness and brainstem dysfunction. It also has to be acknowledged that in cleaner, non-clinical data a more sophisticated statistical approach such as structural equation modelling would have been preferable, but the collected data was by necessity not complete, nor structured enough to apply such measures

without having to defer to imputation – which is prone to biases in heterogenous cohorts (Carvalho et al., 2020). Finally, it is important to acknowledge that despite the many indications in preclinical work, dysregulation of brainstem nucleic connectivity still cannot yet conclusively be seen as synonymous with lowered transmitter provision, which blood-based biomarker and simultaneous PET/rs-fMRI will have to address further in future analyses.

In conclusion, I here provide the first evidence that brainstem nucleic connectivity dysregulation might capture the widely only theoretically suggested persistent brainstem dysfunction after COVID-19. Correspondingly, brainstem impairment might best be seen in a transmitter-specific way as again the empirical evidence points towards monoaminergic, and especially dopaminergic impairments above others. Connectivity of the dopaminergic source nucleus in the VTA is associated with particular symptomatology, and constitutes a physiologically-plausible treatment target in the ever-more common condition of Long-COVID. More generally, this Appendix chapter therefore demonstrates that stepwise, data-driven nucleic connectivity assessments can contribute to the formulation of mechanistic perspectives, even in pathologies where explicit transmitter-specific hypotheses are absent. This reinforces the potential exploratory clinical utility of brainstem nuclei connectomics.

Chapter VII

7.1 General Discussion

7.1.1 Summary of Results

In this thesis, I investigated *in vivo* monoaminergic brainstem nuclei function in humans in pathological and pharmacological consciousness perturbations. Moving beyond previous characterisations of brainstem function in ASCs, this nucleus-specific approach was motivated by strong parallel streams of preclinical and clinical evidence implicating monoaminergic transmitters in consciousness perturbation. Altogether, across its experimental chapters, this thesis provides the first *in vivo* evidence that disrupted functional connectivity of the dopaminergic and serotonergic brainstem nuclei might be a hallmark feature of chronic and transient ASCs, which could underpin established large-scale network dysfunction, as well as associated behavioural and clinical states of perturbed consciousness. Taking sum of the findings, these ASC-associated and pharmacologically-modifiable *in vivo* connectivity dysregulations have various potential translational, diagnostic and therapeutic implications – and can therein begin to close central knowledge gaps outlined in the introduction (**Chapter I**).

Specifically, DOC patients and propofol sedation provided an ideal starting point and proof-of-concept for this work, due to strong evidence in both animals and humans for a role of dopaminergic function for consciousness maintenance. Motivated by this, in **Chapter II** I demonstrated that both pathological and pharmacological consciousness perturbation are characterised by a similar connectivity deficit of the dopaminergic VTA to the PCu/PCC: which is associated with DMN large-scale network dysfunction, behaviour/outcome, and is modifiable by the catecholaminergic drug methylphenidate. Secondly, in **Chapter III**, I demonstrated that VTA connectivity to the thalamus is

preserved in responsive DOC patients, and that the level of this connectivity (together with VTA-PCu/PCC connectivity) might determine relevant network- and whole brain function as posited in the anterior-forebrain mesocircuit model. Taken together, **Chapters II** & **III** on the VTA provide *in vivo* evidence of dopaminergic system dysfunction in ASCs, in turn identifying function of this source nucleus as a potential diagnostic and therapeutically-relevant hallmark and target that can complement existing mechanistic frameworks.

Thirdly, in **Chapter IV** I found that acute effects of the serotonergic drug LSD entail a marked connectivity disruption of the raphe, also here to the PCu: with the disruption of MnR being proportional to not just DMN disintegration but also psychedelic experiential intensity, suggesting potential mechanistic involvement of this brainstem nucleus in the psychedelic ASC. Utilizing a PET-receptor map-based approach, I also characterised that relative 5HT2AR receptor density might drive this raphe disconnection in pharmacologically-plausible fashion. This suggests that even in a markedly different ASC to DOC/sedation, monoaminergic and specifically serotonergic brainstem dysregulation is a feature. Finally, whereas Chapter IV demonstrated downregulations in MnR connectivity in the *acute* psychedelic experience, in **Chapter V** I found that the *sub*/*post-acute* effects of the psychedelic ASC are characterised by an upregulation of this MnR-PCu connectivity – and that this upregulation might underpin the depression-alleviating effects of psilocybin, in distinction from a classical SSRI. The enhancement of MnR-PCu connectivity was proportional to depression symptom reduction and increases in DMN integrity, and was predictive of the longevity of treatment effects. Taken together with the observation that acute psychedelic experience predicted the post-acute MnR-PCu connectivity strength, Chapters IV & V thus suggest that psychedelic effects on the raphe are mirror images of one another between acute and sub/post-acute phases – and might *via* a knockout-to-reset architecture underpin the therapeutic potential of these drugs.

In the following I discuss these results and their overarching translational, diagnostic and therapeutic implications in the context of existing literature, highlight the limitations of the present work, and formulate potential routes for future investigations.

7.2.1 Monoaminergic Connectivity Disruption as an ASC Hallmark Provides Multidirectional Translational Bridges

This thesis provides evidence that both chronic pathological (DOC; **Chapter II** & **III**) and acute pharmacological ASCs (propofol sedation, **Chapter II**; LSD, **Chapter IV**) are characterised by disruptions of the connectivity of monoaminergic brainstem nuclei. As laid out in the respective chapters, in animals these nuclei have been extensively studied using both pharmacological and lesion approaches, consistently implicating their function and integrity in wakefulness – and their *dys*function conversely in altered wakefulness (Andrada et al., 2012; Brown et al., 2010; Brown, Purdon, & Dort, 2011; J. Li et al., 2018). While in human pathological ASCs (e.g. chronic DOC), some brainstem structural impairment in the form of lesions or loss of projecting fibres was previously demonstrated in such regions (Edlow et al., 2013a; Fischer et al., 2016; Snider et al., 2019, 2020), it was only inferred that dys*function* of specifically monoaminergic nuclei could be involved in human pathological and pharmacological ASCs (Edlow, Claassen, et al., 2020; Edlow et al., 2021). Therein, this thesis' results of dopaminergic and serotonergic brainstem connectivity alterations provide a critical step forward in resolving that brainstem function might be altered at the level of individual monoaminergic nuclei in pathological and pharmacological perturbations of consciousness – which opens up long-required multi-directional translational bridges.

Firstly, these results provide *preclinical-to-human translation* of findings from animal research. To begin with, the dysfunctional connectivity of the dopaminergic VTA common to both sedation and DOC is highly convergent with preclinical evidence of the VTA's importance for wakefulness and anaesthesia emergence in animals (Bian et al., 2021; Kenny et al., 2015; Palmiter, 2011; Solt et al., 2014; Taylor et al., 2016). Similarly, psychedelic-induced inhibitions of raphe activity established in animals also appear to have an *in vivo* correlate in the specific connectivity disruption observed here. This allows a progression beyond the thus-far assumed generalisability of transmitter systems-level accounts of e.g. anaesthetic drug effects from animal to humans, towards frameworks that can establish transmitter nuclei as similarly dysregulated (and thus conserved) in humans. Consequently, my findings tighten the translational interface between *in vivo* preclinical and *in vivo* clinical

investigations for neurotransmitter- and brainstem-related inquiries, which has been suggested as an imperative step for a long time (Avery & Krichmar, 2017; Beissner, 2015; Beissner et al., 2014; Parvizi & Damasio, 2001a; Satpute et al., 2019; Sclocco et al., 2018; Silva et al., 2010; van den Brink et al., 2019; Vogt & Laureys, 2005b; Vytlacil et al., 2014). Although future work using both liquid biomarkers (CSF and blood) and PET imaging will have to establish to what extent connectivity impairments relate to *de facto* transmitter deficits, strong alterations in the co-activation of these monoaminergic source nuclei and their targets are likely to affect transmitter provision either locally and/or globally (Pfeffer et al., 2021; van den Brink et al., 2016; Shine et al., 2019; van den Brink et al., 2019). Recent findings from a similar approach used in autism spectrum disorder corroborate that decreases in functional connectivity of brainstem nuclei relate to levels of associated transmitter metabolites measurable as liquid biomarkers (Mccarty et al., 2021). Altogether, the results in this thesis therefore support that animal transmitter systems-level insights on wakefulness might be relevantly measurable and testable *in vivo* in human ASCs – providing a key stepping stone to advance the characterisation of the necessary and sufficient neural substrates of wakefulness and consciousness.

Secondly, these results also have *cross-modality translational* implications. This is again possibly best highlighted through the parallel and strikingly similar implication of VTA connectivity in both sedation and DOC. This suggests, that – in the absence of structural brainstem damage – impairment of the dopaminergic brainstem might be universally involved in loss and impairment of consciousness – but the reversibility of this similar connectivity deficit in sedation might reveal mechanisms by which 'waking-up' effects may be achieved in DOC (Kelz et al., 2019; Kushikata & Hirota, 2014; Monti & Monti, 2007; Nir et al., 2019; N. E. Taylor et al., 2013). The understanding of the neurochemical bases of different ASCs might be greatly enhanced by cross-comparisons of different ASCs – which should identify shared brainstem-level correlates across pathological and pharmacological ASCs. Indeed, the observation that serotonergic raphe connectivity is impaired in psychedelic administrations in a strikingly similar fashion to the aforementioned VTA impairment, suggests that monoaminergic brainstem function might be universally important to consciousness

maintenance irrespective of ASC type (van den Brink et al., 2016; Shine et al. 2018, 2019) and that the functionally most relevant influences might share a common architecture *via* the posterior cortex (Vogt & Laureys, 2005; see following section). To truly resolve a common relevance of all monoaminergic nuclei to consciousness, these findings should be extended to, and complemented by, analyses of the noradrenergic locus coeruleus and the dopaminergic sub-portion of the ventrolateral periaqueductal gray across different ASCs in future work – as the aminergic 'axis' might have complementary and 'in-concert' roles for consciousness maintenance (Schwarz & Luo, 2015; Totah et al., 2018; S. Zhang et al., 2016). Given insights from research on physiological consciousness alterations such as sleep-wake transitions on these nuclei, ASC-associated momentary and acute deficits in one system might be partially compensated for by another system that has convergent functionality. Such convergent functionality and homeostatic balancing is especially plausible given the common synthesis pathway of monoamines (Goldberg et al., 2014), widely co-expressed receptors (Ishii et al., 2017) and extensive intra-brainstem excitatory and inhibitory connections (Adell et al., 2002; Hornung, 2003a; Huang et al., 2019; Kauer & Polter, 2019; Lu et al., 2006; Morales & Margolis, 2017; Ntamati et al., 2018; N. E. Taylor, Pei, Zhang, Vlasov, Davis, Taylor, Weng, van Dort, et al., 2019; Taylor et al., 2014). Although the present work did not resolve interrelationships of monoaminergic and other neurotransmitter nuclei to delineate what Satpute et al. (2018) have called the overall "dynamic recipes" of cortical modulation, the observed results suggest that monoaminergic function might be a useful target and plausible rate-limiter for conscious-specific neural processes, based on its cross-ASC implication. As such, utilising both preclinical-to-clinical and cross-modality translational and comparative perspectives might greatly facilitate formulation of mechanistic hypotheses of brainstem-cortex/brainstem-cerebrum interaction.

Most importantly, the combination of these perspectives would empower *bi-directional back-translation* aimed at causal inference. Transmitter-specific hypotheses developed from cross-modal observations in humans *via* brainstem nuclei analyses could be formally tested back in animals with novel non-invasive *in vivo* optogenetic techniques, even during high-field neuroimaging (Luppi, Cain, Spindler and Gorska et al., 2021). These techniques allow for the modulation of very specific and

deeply seated brainstem targets (Hong, 2020). Employing translational hypothesis-driven optogenetics could thus establish causal relationships between manipulation of a brainstem nucleus and effects on the animal, the broader brainstem, and on concomitantly recorded macroscopic neuroimaging markers (Vesuna et al., 2020). Therein, leveraging insights from brainstem transmitter analyses together with these technological developments can provide insights into the necessity and/or sufficiency of a given nucleus' function for normal wakefulness – thus making systems-level investigations accessible that are otherwise impossible in humans. As such, the multi-directional translational opportunities and directions potentially afforded by the present results could help to significantly refine the model of brainstem-cortical interaction required for consciousness.

7.3 A Special Relationship of the Brainstem and the Posterior Cingulate/Precuneus

The results throughout the experimental chapters in this thesis consistently point towards a special relationship of the monoaminergic nuclei with the PCu/PCC. This is a striking result, given the consistent association of this posterior transmodal hotspot with consciousness and its impairment (Alves et al., 2019; Cavanna & Trimble, 2006; Leech et al., 2012; Liu et al., 2014; Margulies et al., 2009, 2016; Raichle, 2015; Rosazza et al., 2016; Vogt & Laureys, 2005a; H. Wu et al., 2022; X. Wu et al., 2019; Xie et al., 2011; H. Zhang et al., 2017). It has long been suggested on the basis of previous observations and of associated tracing studies that the PCu/PCC might be especially reliant on brainstem signalling, and required for brainstem signalling to have consciousness-supporting function, as posited by (Vogt & Laureys, 2005). They theorised that it is both necessary and potentially sufficient for consciousness that direct (and indirect *via* anteroventral thalamus) brainstem signalling is integrated in this cortical region. The observations made in this thesis provide empirical evidence in favour of this hypothesis of a centralised position of the posterior cortex, and suggest that the widespread ASC-associated changes in the precuneus and posterior cingulate (and broader retrosplenial cortex) function might be driven by changes in the monoaminergic neuromodulation of this posterior cortical hotspot.

It is highly plausible that the precuneus/PCC might be particularly vulnerable to shifts/disruptions in modulation. Firstly, this region is projected to from both the VTA and the raphe (Edlow, 2021; Hornung, 2012; Hornung, 2003b), and has lots of metabotropic monoamine receptors *via* which their signalling molecules can alter the synaptic and electrochemical properties of PCu/PCC neurons (Beaulieu & Gainetdinov, 2011; Beliveau et al., 2017; Heiss & Herholz, 2006; Ishii et al., 2017; Lanfumey & Hamon, 2004; Nagano-Saito et al., 2017). Indeed, the posterior cortical transmitter landscape is one of the most complex and diverse in the brain (Impieri et al., 2019), which might partially explain how this region (specifically the precuneus) has experienced the greatest spatial expansion in human evolution (Bruner et al., 2017). This complex receptor-ome is central to allowing the precuneus flexible and multimodal connectivity profiles to be accomplished, which is captured in it having the greatest decoupling of its connectivity profiles from underpinning microstructure (Paquola et al., 2019) – and this uncoupling is thought to be fundamentally reliant on neuromodulatory influence (Shine et al., 2018, 2019). Conversely, this suggests that the PCu/PCC's transmodal association cortex function is, at least partially, the result of and enabled by functional brainstem-derived neuromodulation of the complex PCu/PCC physiology. In turn, and in line with Vogt & Laureys (2005) and Cavanna & Trimble (2006), even single monoaminergic system impairment might powerfully disrupt the neural landscape and cortical properties required for consciousness (Shine et al., 2018). My results that PCu/PCC connectivity to the rest of the brain is impaired in proportional fashion the more altered dopaminergic VTA or serotonergic raphe connectivity to this region is across different ASCs, provides empirical *in vivo* evidence of this theory. Indeed, recent findings that stimulation of the retrosplenial cortex can mimic ketamine effects to elicit a dissociation-like status, further corroborate the key role of the attuning of precuneal properties for consciousness (Vesuna et al., 2020). Importantly, it is often overlooked that the *in vivo* murine models used by Vesuna et al (2020) show the greatest activity disruption in a dissociative state not in the precuneus/retrosplenial cortex, but rather in the midbrain, possibly coincident with monoaminergic nuclei. Adopting experimental setups such as that of Vesuna and colleagues (2020) might allow for the causal sequence of modulatory effects to be established, namely whether effects from the precuneus/PCC flow to the brainstem or the other way around, or synergistically between them.

However, even if effects should start at the level of the cortex (such as proposed in Chapter IV for LSD's mechanism) the feedback to the brainstem and resultant perturbation of tonic modulation might still serve as a direct and rate-limiting contributor to ultimate impairment of the PCu/PCC given its inherent reliance on neuromodulation. This is supported by the work of Nagano-Saito and colleagues (2017) which reveals that posterior cortex dopamine receptor occupancy is very strongly correlated with that region's whole-brain connectivity levels to the DMN.

It is also important to highlight that the PCu/PCC is not only implicated in ASCs in the literature, but further in various neurodegenerative and psychiatric disorders. Its interplay with monoaminergic nuclei might be of primary importance to conscious state, but once this is at or past the physiologically-required consciousness-enabling level (the consciousness 'bottleneck'), alterations in tonic MnR and VTA connectivity may very plausibly influence affective states (Satpute et al., 2019), trophic support (Black & Rogers, 2020; Tovar-y-Romo et al., 2014) and other large-scale functions, consistent with the multitude of transmitter-associated functions in the literature. Indeed, the observation that the post-acute MnR-PCu/PCC connectivity increase after treatment with a psychedelic is associated with depression-alleviation, supports such a consciousness-first-affective-second view of brainstem 'arousal variety' of signals. Indeed, as Parvizi & Damasio (2001) posited, it is central to understand that the overlap and multitudinous functions of the different nuclei are understood as a homeostatic system that entails consciousness at its core, but regulates the organismal state more broadly, i.e. representing wakeful, affective and other varieties of "arousal" (Satpute et al., 2019).

Considered together, my results thus provide empirical evidence that the functional link between the brainstem and PCu/PCC is of central importance – and that its disruption may at least partially underpin ASCs and the associated large scale phenomena such as network dysfunction. This does not preclude that other modulatory partnerships, such as to the densely projected-to thalamus are not also of direct relevance to conscious state as demonstrated in Chapter II. Indeed, a comprehensive brainstem-cerebrum interaction model should consider nucleic partnerships with various important

subcortical and cortical nodes as bi-directional feed-forward and feed-back signalling partners. Nevertheless, the consistency of the brainstem-PCu/PCC connectivity alterations across ASCs provides a preliminary step towards such a model that can begin to characterise the neurobiological bases of consciousness impairment – and associated large-scale network-level phenomena.

7.4 Functional Diaschisis: Monoaminergic Nuclei Impairment Might Have Large Scale Consequences

The results that VTA and raphe connectivity to the PCu/PCC is across different ASCs associated with the integrity of the default mode network (DMN) is a critical step forward for understanding the potential neurochemical/neurobiological bases of network impairments in ASCs, and to allow the predominantly macroscopic neuroimaging accounts of human brain function associated with consciousness to move towards more biologically-informed mechanistic frameworks.

To begin with, recent work at 7T has demonstrated that the VTA and raphe show among the strongest connectivity of the whole subcortex to the DMN, as does the central lateral thalamus (Li et al., 2021). This has re-framed earlier results of monoaminergic nuclei connectivity to DMN nodes (Bär et al., 2016; Beliveau et al., 2015; Buckner & DiNicola, 2019) into an explicit participation of these nuclei in the DMN. This conceptual extension provides a new dimension to initial systems-level hypotheses for how these nuclei might contribute to network function, *via* what the authors referred to as "funnel effects" of small structures, i.e. their function having large-scale consequences *via* global projections (Parent & Hazrati, 1995), which recapitulates the case made in this thesis. Although my experimental work was largely carried out before the publication of these hypotheses, my results are directly concordant with them and provide evidence that disconnection of the monoaminergic VTA and raphe from the precuneus/PCC might be mediators of the arguably most-replicated ASC-associated finding of DMN dysfunction. Indeed, the observation made throughout the chapters and across different ASCs that disruption of PCu/PCC-DMN connectivity is proportional to how disconnected specific monoaminergic nuclei are, provides a potential *in vivo* "scale-up" for what the consequences of brainstem nucleic impairment known from animal studies might be (Avery & Krichmar, 2017; Harris-

Warrick & Johnson, 2010; Klaassens et al., 2017; Marder, 2012; Marder et al., 2014). This "scale-up" constitutes an additional translational function of the present results, in allowing a potential bridge between the pre-existing micro/mesoscopic (transmitter system-level) insights and established macroscopic (network-level) phenomena. This relevance of monoaminergic dysfunction for network integrity might be fundamentally related to the concept of *diaschisis*. Diaschisis is the principle that a lesion at a distant site can affect (many) other sites throughout the brain (Carrera & Tononi, 2014) – or in other words that large-scale dysfunction can be the consequence of focal harder-to-observe issues. Therein, not just a structural, but a functional 'lesion' in a transmitter brainstem nucleus might underpin larger scale deficits – meaning that these nuclei are correspondingly targets to reinstate network- and other cortex-level processes required for consciousness in pathological ASCs (Edlow, Barra, et al., 2020).

Some have gone so far to suggest that all cortical connectivity landscapes captured in networks are ultimately epi-signatures and epiphenomena of underlying neuromodulation. This is founded on the observation that selective monoaminergic and catecholaminergic drugs can powerfully alter the intrinsic connectivity, brain-wide network topography and interactions in the human brain (Pfeffer et al., 2021; van den Brink et al., 2016; Shine et al., 2019. These results intriguingly centre once more on the monoamines, whose source nuclei have consequently been proposed as candidate tonic drivers of network-level properties (Avery & Krichmar, 2017; Marder, 2012; Marder et al., 2014) – and that network-level dysfunction is thus likely to always reflect at least some level of (either primary or secondary) brainstem impairment across different ASCs. My results equally suggest that accounts of consciousness-relevant cortical network dysfunction might be incomplete without explicitly addressing subcortical systems that might be contributing to and/or driving them. Both direct brainstem-to-cortex and indirect brainstem-to-thalamus(-to-cortex) influences (see Chapter III) might mediate the thus-far typically cortico-centric network topography and integrity. Although this thesis' findings support this assertion, more precise pharmacological paradigms and intra-subject control conditions will be required to take a stepwise meso-to-macroscopic perspective, where modulatory brainstem system involvement can be established to span various cortico-centric phenomena.

Altogether however, the present results suggest that simple brainstem nuclei connectomics might provide a framework to empower more fundamentally biological accounts of ASC large-scale network dysfunction – potentially aiding to overcome a key limiting factor for the clinical utility of fMRI, across many indications (Buckner et al., 2013; Buckner & DiNicola, 2019; Shine et al., 2019). This potentially generalisable utility of the neurotransmitter-specific connectomics approach beyond ASCs might be reflected in the findings from psychedelic administrations. The diametric opposition of the acute and post-acute effects of a psychedelic on MnR-PCu/PCC, and simultaneously also DMN+, connectivity captures a plausible mechanism to underpin previously-established network-level effects based on an involved effector system – and can thus begin to empirically explain anti-depressant effects of psychedelics, which the otherwise biologically agnostic network approach cannot (Carhart-Harris & Nutt, 2017; Nichols, 2012, 2016). This on the one hand reinforces that the function of brainstem nuclei outstrips consciousness-maintenance alone, but most importantly once more highlights that monoaminergic brainstem nodes should form part of therapeutically-relevant network models and perspectives.

7.5 Refining the Arousal-Awareness Framework

The results in this thesis also complement the two-dimensional arousal-awareness framework (see Fig.1, Chapter I; Laureys, 2005; Laureys et al., 2004; Laureys, Boly, et al., 2009). Indeed, while the binarization of brainstem function being associated merely with setting the 'level' of consciousness and the cortex with the 'content' of consciousness might be clinically utile, the present results reinforce that these axes are not unidimensional, nor fully independent (Satpute et al., 2019).

To begin with, the psychedelic experiments revealed that monoaminergic brainstem nuclei dysregulation is not only associated with pathologically and/or sedated states of consciousness (as found for the VTA). Instead, raphe functionality changes were associated with measurably *qualitative* changes in sensory perception and experience as found in LSD and psilocybin administrations. While the covariance of these content (i.e. awareness) changes with raphe

dysfunction cannot establish causation, these findings are nevertheless strongly suggestive of the fact that brainstem influences are associated with both arousal *and* awareness. This is in direct correspondence to the concepts presented by (Parvizi & Damasio, 2001a) who suggested that brainstem signalling – as an expression of its overarching homeostatic function – should underpin both *contents* and *level* of consciousness. Even if involvement of the brainstem in the contents of consciousness might remain impractical to establish causally (due to the impossibility of obtaining spoken reports from animals), even the sheer association of brainstem function with the arousal dimension in the framework might need to be refined: The original arousal-awareness framework classified clinically-observable arousal as present and at 'normal' levels in MCS and UWS patients. However, work in this thesis has demonstrated that monoaminergic brainstem function is in fact impaired in such patients compared to controls. Therein, while some level of brainstem-derived arousal might indeed be operant, it is not the physiologically-relevant type of arousal-related brainstem signalling that is qualitatively and quantitatively sufficient to allow the emergence of functional consciousness. The corresponding right 'recipes' might need to be understood as orchestrated synergistically by different brainstem neurotransmitter nuclei influences (Satpute et al., 2019).

Therefore, it is feasible that brainstem-derived tonic neuromodulation is neither solely arousal- nor fully awareness- related, but rather sets a 'neural reference space', within which these two classical dimensions can interact to produce functional consciousness. Expressly, brainstem diffuse neuromodulatory influence may constitute a distinct process central to unifying how awareness and arousal can be integrated to enable consciousness. Thus, disturbances in the neuromodulatory preconditions of this reference space create the disentanglements observed in the classical awareness-arousal space (Olszewski & Baxter, 1982). Although this thesis could not directly assess neuromodulation – as neuroimaging remains impractical for such work – in the context of previous work, my results corroborate the possibility that at least monoaminergic neuromodulation might need to be considered as distinct from pure awareness and arousal.

As an inception point, brainstem modulatory nucleus function (captured in connectivity and activity) could be mapped as a third non-linear axis in the original awareness-arousal layout. Explicitly, arousal could be re-mapped to be relevant to reticular formation function, whereas neuromodulation as an expression of the diffuse neuromodulatory system (DNMS) operation (Olszewski & Baxter, 1982). In this adapted framework, conditions such as the locked-in syndrome would likely fall into more differentiated positions in such a X(arousal)-Y(awareness)-Z(neuromodulation) space, as evidence in the fact that lesions in LIS typically spare the DNMS, whereas lesions in other DOCs do affect it (Barbic et al., 2012.; Hocker & Wijdicks, 2015; Sciacca et al., 2019; Smith & Delargy, 2005). Differentiating these three axes could thus empower differential diagnoses, but despite enhancing cross-modality comparisons, such a multi-dimensional space can for now only be seen as a working hypothesis and starting point.

Altogether, a working hypothesis to extend the awareness-arousal framework could thus help to characterise different mechanistic contributors to ASCs – but also to unify the nomenclature surrounding ASCs. Previous descriptions of "altered states of arousal" might in fact not solely represent states of altered activating signals from the reticular formation to the cortex and rest of the brain (Baraniuk, 2022; Brown, Purdon, & van Dort, 2011; Clauss & Nel, 2006; Edlow et al., 2013b; Hindman et al., 2018; Liu et al., 2018; Schiff, 2008; Singh et al., 2022; Snider et al., 2019; Valko et al., 2016), but rather altered dynamic modulatory brainstem function. Consequently, such an extension on the basis of the present results and pre-existing hypotheses, has implications for disease models and therapy frameworks due to the resultant identifiability and drug-ability of actionable targets.

7.6 Implications for Disease Models and Therapy Development in ASCs and beyond

As mentioned previously in this chapter, the present work has various possible implications for mechanistic frameworks of disease and treatment. While the only pathologies dealt with in this dissertation are DOC and depression (and secondarily COVID-19), the related insights might still have broader applicability.

To begin with, my findings suggest that disease models for DOC might have to explicitly incorporate brainstem nuclei *function*, rather than just structural lesions, to be able to account for the symptomatology and neural underpinnings of DOC. In this way, as suggested in Chapter II and III, DOC might represent a brainstem nucleic *functional* disconnection syndrome (not just a *structural* disconnection syndrome, as previously suggested for coma (Edlow et al., 2013b). Indeed, my results that find VTA connectivity to both the PCu/PCC and the thalamus to be perturbed in a manner relevant to those nodes' whole-brain connectivity and responsiveness are consistent with a model of DOC in which the brainstem is a bottleneck/funnel for downstream brain deficits.

Explicitly, the anterior forebrain mesocircuit extension I suggested might provide both (i) potential biomarkers to monitor dopaminergic treatment efficacy, and (ii) means of identifying novel treatment targets through *in vivo* neuroimaging, which could feasibly be developed to be applicable in individual patients (see next section). Indeed, the VTA's and other nuclei's influences on posterior cortex and thalamus could be mechanistic 'missing links' to account more fully for the frontoparietal network impairment observed and suggested by Schiff and colleagues (Schiff, 2010). As my results provide a source, potential targets and consequences for the dopaminergic deficit that forms the core of experimental therapeutic mechanisms based on the mesocircuit hypothesis, inclusion of DNMS influences on various nodes in the anterior forebrain mesocircuit model might greatly enhance this model's ability to account and target the multitude of consciousness-related deficits in DOC (Fridman & Schiff, 2022). It is highly probable however that related transmitter deficits are not homogenous across patients, and that their impairment is partially also structurally driven – indicating that both the mesocircuit model and therapy development need to consider multiple targets, ranging from different nuclei and transmitter systems to pre-and post-synaptic processes (see future directions).

Equally informative, though for a different indication, are the findings on the acute and post-acute effects of psychedelics, and how the latter may serve to alleviate depression by putatively resetting raphe functionality. To the best of my knowledge, thus-far there have been no clear demonstrations of the monoaminergic deficit hypothesized to be at the heart of depression pathophysiology *in vivo* in

humans. Equally so, no biological account of the psychedelic treatment mechanisms of depression was provided, especially no account that could explain why substances such as psilocybin have very long-lasting anti-depressant effects after single or double administration (Aday et al., 2020; Andersen et al., 2021; Artin et al., 2021; Canal, 2018; Carhart-Harris et al., 2012; Chi & Gold, 2020; Muttoni et al., 2019; Palhano-Fontes et al., 2019; Roseman et al., 2018; Schenberg, 2018; Spriggs et al., 2020; Vollenweider & Kometer, 2010). My results can begin to close these relevant gaps for a systems-level account of how psychedelic acute effects might be opposed to their post-acute effect, and resultantly can underpin depression treatment. Similarly to the MnR-PCu/PCC connectivity that is disrupted during LSD administrations, an impairment in MnR-PCu/PCC connectivity was also associated with depression severity – but post-acutely, i.e. after psychedelic treatment, this same connectivity is upregulated in proportion to the treatment benefit experienced by the patient. Despite many animal studies dealing with the effects of both classical antidepressants and psychedelics on the raphe in the 1980s and 1990s, the focus moved from them towards receptor sites in the cortex in recent years (R. Carhart-Harris & Nutt, 2017). My findings return to the raphe, and demonstrate that a re-attuning of raphe function in response to psychedelics is intuitively suitable to underpin their long-lasting effects and to distinguish them from SSRIs. This complements previous disease models, as it demonstrates that *an in vivo* serotonergic deficit might indeed be occurring in humans (as postulated in the monoaminergic hypothesis), and that psychedelic treatment is putatively acting on a source nucleus related to this. Instead, SSRIs' continuous upregulation of serotonergic tone might be doing the opposite, by further downregulating raphe functionality due to an over-abundance of serotonin at post-synaptic sites. Altogether, this therefore motivates future investigations that further tease apart whether these treatments are fundamentally different in terms of their post-acute effects, or whether (as also previously suggested in animal studies; (Dankoski et al., 2016)) both modes might ultimately re-stabilise raphe functionality as a central mechanistic arbiter in depression. Importantly however, these results also demonstrate a potentially central facet of pharmacological treatment responses of the brainstem nuclei, namely that the acute and post-acute effects of a substance may be fundamentally different.

This difference in acute *versus* post-acute and chronic effects of neurotransmitter-focused pharmacological stimuli on the respective brainstem source nuclei might be highly informative back in the DOC and brain injury context too. If the aim of a given pharmacological intervention is to re-initiate the normal function of a transmitter system (such as for dopamine), exploring the possibility of pharmacologically-induced allostatic system resets might be useful: i.e. to depress certain brainstem nuclei beyond a point at which allostatic processes re-organise and re-initiate their functionality (McEwen, 2000; Mcewen & Wingfield, 2003; Ramsay & Woods, 2014). While initially a counter-intuitive concept, this possibility is fundamentally intertwined with the homeostatic organisation of transmitter systems (Baumeister et al., 2014). Furthermore, such a 'reset' principle could plausibly underpin the 'paradoxical' effects of $GABA_A$ agonists such as zolpidem in DOC patients (Noormandi et al., 2017). Expressly, their inhibitory action may depress functionality in brainstem nuclei who are already impaired, both *via* downregulating excitatory afferents and *via* direct inhibitory receptors in the nuclei themselves. The additional disruption through activity downregulation, might lead to allostatic re-organisation of transmitter system functionality. Although this consideration is purely theoretical at this point in time, it might add a critical facet to therapy development for DOC: Adjuvants (such as dopamine receptor agonists, dopamine synthesis upregulators and reuptake inhibitors) alone might be mainly symptomatic treatments. Although they might acutely upregulate dopaminergic tone and even related biomarkers (as I found for methylphenidate), these might be unable to sustainably alter root causes of dopaminergic and other brainstem nucleic hypo-function in the same way a powerful 'knockout-then-reset' framework might (McEwen, 2000; Mcewen & Wingfield, 2003; Ramsay & Woods, 2014). Consequently, it might be important to trial such approaches and monitor how the acute and post-acute changes in transmitter system function might differ, and whether sustained changes in nuclei connectivity and activity are associated with improved clinical presentation and/or even re-emergence of consciousness. Firstly however, it will be important to establish whether 'reset' effects are (i) truly selective to single transmitter systems or whether effects reverberate to other nuclei, and (ii) whether they can be brought about without causing side-effects which would be prohibitive in the intensive care setting. This would be best complemented by performing direct comparisons to alternative stimulation

techniques (ultrasound, deep-brain, etc), which should be trialled in targeting individual transmitter nuclei based on the present results.

7.7 Possibility of Non-Invasive Biomarkers of Neurotransmitter System Function

As discussed in the individual chapters and immediately above, it is possible that brainstem connectomics might provide preliminary biomarkers for specific neurotransmitter nuclei function that have translational and clinical value. By 'biomarker' I refer to its classical and basic definition as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Atkinson et al., 2001).While there is more validation of this approach and connectivity patterns required, my results meet key requirements of biomarkers: Brainstem nucleic connectomics appear to have diagnostic utility across indications, are modifiable by pharmacological agents in both promoting and demoting direction, covary with disease severity and outcome across many different domains, contexts and over time – and importantly appear sufficiently selective (i.e. specific to particular implicated transmitter systems). While this altogether suggests that *in vivo* nuclei connectivity could be a correlate for transmitter function, thus-far absent in human neuroimaging, this opportunity needs to be carefully replicated to iteratively establish actual clinical applicability. Most importantly a central question remains, namely whether connectivity dysregulation is indicative of a release deficit at the disconnection site, or of a release deficit globally – or whether connectivity shows no relationship to deficits. Although recent findings suggest that connectivity of brainstem nuclei and transmitter metabolite levels in bio-liquids are correlated (Mccarty et al., 2021), a more suitable test for their association remains to perform simultaneous PET rs-fMRI in humans, and ideally corroboration of these findings through microcialysis experiments which could quantify *de facto* transmitter levels in animals during simultaneous neuroimaging.

Although there is a lot of further work required to characterise the ultimate utility of brainstem connectomics for clinical contexts, the present findings and approach still provide an inception point to make biological systems-level insights available through mainstream neuroimaging. As such, this

approach might despite its current methodological limitations be a lot closer to a true *bio*-marker than many previously suggested neuroimaging markers that often might provide some surrogate endpoints, but not simultaneously include relevant effector systems (Lai, 2019). As such, re-assessing transmitter nuclei connectomics in existing data of sufficient quality can also *a posteriori* complement the more macroscopic, especially network-level, markers through adding potential effector systems and treatment targets across many different indications (see future directions).

7.8 General Limitations

While I discussed many of the limitations specific to each piece of experimental work in the respective chapters, there are general limitations of the present work that need to be highlighted. Broadly speaking they fall into two inter-related categories, namely the constraints of 3T fMRI neuroimaging, and assumptions about the homogeneity and location of nuclei.

To start with, the signal-to-noise ratio (SNR) of fMRI is in general low, but certainly the lowest in the brainstem, with signal being down to 5% or less when compared to cortical regions (J. Li et al., 2021). The SNR is especially limited in the particular locations of the monoaminergic nuclei, as the VTA is close to the 'circle of Willis' and the raphe close to the cerebral aqueduct – meaning that they are especially vulnerable to cardiorespiratory pulsatility artifacts (Sclocco et al., 2018). Nevertheless, I employed explicit anatomical brainstem masking prior to any smoothing and the aCompCor denoising procedure to maximally downregulate such artefacts, both of which have previously shown to control well for various noise sources. Still, the small cross-sectional area of nuclei in the millimetre order, means that the present analyses are operating at the spatial resolution limit of 3T rs-fMRI (Beissner, 2015). As in-plane resolutions of sub-5mm would be preferable, the present results will need to be replicated at ultrahigh field, which also benefits from greater SNR and better gray/white matter contrast. However, for brainstem-cortex functional connectivity relationships, there is a strong translatbility of findings from 7T to 3T, which provides confidence in the present results and their potential clinical utility – as only 3T imaging is routinely available in such settings.

Highfield imaging might also empower the identification of nuclei through magnetisation transfer images (Ye et al., 2021), though only for those nuclei rich in iron. Importantly however, the ultrahigh resolution might still require significant smoothing to allow the accommodation of inter-subject heterogeneity in the absolute location of a given nucleus.

Another methodological limitation is that the present work used both voxel-based and cluster-based correction approaches for multiple comparisons, which ultimately skews towards larger connectivity clusters in contrasts. Indeed, ideally future work should use non-parametric statistical approaches such as Bayesian statistics which do not have homogeneity of variance assumptions, as these assumptions can often be violated in brainstem analyses (Beissner et al., 2014). However, the assumptions required for the simple, readily-implementable general linear model approach in the present work were extensively controlled and established. Nevertheless, restrictively corrected frequentist p-values might – as opposed to Bayesian approaches – produce false negative results, thus under-estimating potential brainstem nucleic effects.

The second major general limitation concerns the precise localization and homogeneity of the brainstem nuclei. For a longer time, it was difficult to perform analyses such as the ones in this thesis, due to the absence of appropriate probabilistic and/or *ex vivo* atlases of the neurotransmitter nuclei in the human brainstem. In recent years however, there have been various templates published, from a variety of con- and divergent methodologies (Adil et al., 2021; Aggarwal et al., 2013; Bianciardi et al., 2018a; Bianciardi et al., 2022; Edlow et al., 2012; Tang et al., 2018). As far as can be established from the published methodology, the Harvard Ascending Arousal Network (HAAN) atlas is the only histologically-characterised atlas, which can therefore provide nuclei templates that are based on the neurotransmitter phenotype of a given brainstem area. However, it is important to acknowledge that this is only the predominant type, as indeed the brainstem areas considered in this thesis have been found to also contain not just dopaminergic and serotonergic neurons respectively, but also GABAergic subpopulations or even some smaller subpopulations of a different neuromodulatory transmitter type. (Breton et al., 2018; Browne et al., 2018; Hernández-Vázquez et al., 2018; J. P. Hornung, 2012; Huang et al., 2019; Jackson et al., 2009; Y. Li et al., 2019; Pollak Dorocic et al.,

2014; N. E. Taylor, Pei, Zhang, Vlasov, Davis, Taylor, Weng, Dort, et al., 2019; S. R. Taylor et al., 2014). This means that with the advent of higher field imaging it might be necessary to further sub-parcellate homogenous groups within certain nuclei to be able to characterise transmitter-specific imaging correlates. As such, the translatability from a given nucleus deficit to a transmitter deficit might be limited in its biological validity – although recent work suggests that such nuclei-transmitter deficit overlaps are being found (Mccarty et al., 2021). In relation to this, it is also relevant that the HAAN might only be able to capture the location of the nuclei correctly to a partial extent, as the nuclei were traced manually in a single *ex vivo* brain specimen and then transferred into MNI-152 space. However, recent parcellations (e.g. Bianciardi et al., 2022) are overall concordant with the general location of the monoaminergic nuclei, which increases confidence in the preliminary utility of using this template for this work.

A final limitation that is partially also related to field strength is the large voxel size of the rs-fMRI scans used in the present analyses throughout this thesis. The resolution of voxels is 3x3mm in-plane resolution with a slice gap of 3.75mm for the DOC and propfol study, and 3mm isotropic for the psychedelic and COVID functional scans. In the case of small regions, such as the ventral tegmental area and the raphe nuclei, this voxel size could indicate that off-target signal may be captured, as volume averaging effects could occur – meaning that multiple diffuse neuromodulatory nuclei inadvertently contribute to the signal extracted in the experiments despite considering single brainstem ROIs. At the present field strength of 3T it is difficult to delineate exact differences between nuclei, which will be instrumental in future work with larger field strengths. Nevertheless, my analyses in the COVID population (Appendix Chapter) used a partial correlation approach to identify that through controlling for different nuclei's BOLD signatures nucleus-specific contributions to e.g. correlations with clinical outcome can be obtained. Such approaches, that explicitly consider and control for brainstem-general and cross-nucleus signal may provide an inception point for a route by which at least approximately nucleus-specific information may be obtained from signals of individual ROIs. Nevertheless, future work should focus systematic efforts on the reliable and differential identification of nuclei, which should ideally become delineable in subject-space as well.

These limitations highlight that there is additional work to be done on making approaches to brainstem functional connectomics more standardized, robust, and transmitter-system specific to empower a potential clinically-useful tool for understanding ASCs and other neurotransmitter-related disorders. Nevertheless, the approach I have employed provides one of the few and only ways to address hypothesis-driven transmitter-system specific questions in humans, and the results fit intuitively at the translational interfaces this work was concerned with – meaning that the present results can take an important first step in the direction of making transmitter system inquiries non-invasively accessible in clinical neuroscience.

7.9 Future Directions

As detailed in the above sections, this thesis provides evidence of monoaminergic brainstem dysfunction in ASCs that can and should motivate various follow-up studies.

Firstly, future studies, particularly using higher field imaging, should aim to contextualise the function of the VTA and raphe with other brainstem transmitter nuclei, such as the locus coeruleus, periaqueductal gray, pedunculuopontine nucleus and other structures (Beissner, 2015; Leitch et al., 2010; Parvizi & Damasio, 2001b, 2003; Sclocco et al., 2018). This should not only assess the relationship of all these nuclei to the subcortex and cortex (to resolve the required brainstem-cerebrum interaction model), but should explicitly address how nuclei-nuclei connectivity and influences might be affected in ASCs and other pathologies. Such work will require ultrahigh field strengths, as at 3T such interrelationships are not readily assessable (Singh et al., 2022). The interrelationships of the nuclei may be able to identify whether certain nuclei might affect others more readily in ASCs, and whether certain nuclei might thus provide the most suitable stimulation or pharmacological targets (Cain et al., 2021, 2022). To establish such hierarchies, in human neuroimaging advanced methods such as Grainger causality (Seth et al., 2013) and a brainstem-adapted form of dynamic causal modelling (Stephan et al., 2010) should be used. However, for empirical causality optogenetic animal work will be required (Hong, 2020; Vesuna et al., 2020).

The general extension to other nuclei should also be embraced in the general experimental application of brainstem analyses in DOC, which has previously been suggested (Edlow, Barra, et al., 2020): namely personalised nuclei connectome mapping. Individual DOC patients may have equally individual brainstem nucleic connectivity (and thus transmitter-system) deficits. This importantly encompasses both functional and structural deficits, which might exist on a comorbid spectrum. In terms of functional connectivity, even in the present sample of DoC patients – which was curated to not contain structural brainstem abnormalities – brain-wide PCu/PCC connectivity analyses revealed disconnections from sites coincident with non-dopaminergic brainstem nuclei (overlapping with glutamatergic, cholinergic and serotonergic transmitter nuclei of the HAAN). Therein, the neuromodulatory deficits that need to be addressed by treatment strategies might be best understood through a multimodal lens that assesses both functional and structural integrity of all nuclei and their projections, to operationalise the most likely intervention to be efficacious (Edlow, Barra, et al., 2020; Mccarty et al., 2021). As such, future work should explicitly explore individual-specific functional (and structural) nuclei connectome mapping for its predictive and treatment-tracking value in individuals to provide a rational framework for precision-medical therapies in DoC. However, such an approach, focussed solely on functional connectivity and activity of nuclei could also be applicable to various other pathologies in which transmitter systems are thought to be dysfunctional, such as Parkinson's disease, schizophrenia, depression and others.

Once it is established that group-level and individual brainstem connectivity biomarkers are correlates of relevant transmitter levels and dysfunctions (see previous section), the inclusion of brainstem nuclei into *in-silico* modelling might be particularly useful to understand how behavioural and clinical phenomena associated with ASCs and their neural manifestations come about. Expressly, the analysis of different nuclei would create a wealth of data across multimodal and multivariate approaches. Moving beyond machine learning on neuroimaging which has already been used to predict prognosis in DOC and to differentiate states of consciousness (healthy consciousness from DOC or sedation), whole-brain modelling approaches such as dynamic mean field models have already begun to include receptor maps into their frameworks (Jobst et al., 2021; Kringelbach et al.,

2020; Pfeffer et al., 2021). These models' ability to account for whole-brain functional profiles, stimulus events and behavioural manifestations might be greatly enhanced through including multiple transmitter nuclei, and could produce biologically-plausible predictive models that can reduce redundancy between metrics and targets, as well as identify synergy between many identifiable neural correlates of DOC. This might provide a much-needed accelerator to distil clinically-relevant predictions from such models. Such nuclei-enriched *in silico* models might – also in other pathologies – thus allow the creation of increasingly realistic computational models of transmitter-related disease. As it has been suggested that effectively all drugs that have neuroactive properties in brain injury act directly on neurotransmitter systems, such models might therefore be especially suitable for phenotypic drug discovery by allowing potential drug effects to be *a priori* tested computationally, which might help to overcome a previously under-appreciated bottleneck for the development of neurologically efficacious therapeutics (Kawa et al., 2015; McGuire et al., 2018; Zheng & Tong, 2015).

Furthermore, a more general future direction motivated by the present results is for the concomitant usage of PET in transmitter related disease areas, such as DOC. While PET is prohibitively expensive for routine clinical usage, its ability to assess biological effectors directly *via* conjugate radioligands makes it an indispensable tool to understand transmitter deficits. Expressly, using multi-tracer PET could differentiate whether transmitter deficits are of pre-synaptic origin (i.e. at nucleic level) or related to insufficient postsynaptic receptor occupancy (i.e. not necessarily from nucleic sources) (Fridman et al., 2019; Fridman & Schiff, 2022). Such approaches, though costly, time- and resource-intensive could through usage of simultaneous PET/rs-fMRI establish the experimental and clinical utility of the simple brainstem connectomics based biomarkers suggested in this thesis. This would first need to use a proof-of-concept approach in healthy participants undergoing e.g. anaesthesia and/or sedation to establish how PET-signal and fMRI nucleus effects relate to one another, while iteratively utilising receptor and transporter ligands for respective transmitter systems. Furthermore, the usage of PET and fMRI *in vivo* should be complemented by PET-to-*post-mortem* analyses, wherein a transmitter-system ligand could be used on brainstem and other brain tissue when a patient

donates their brain after death to assess whether *in vivo* detected deficits show a neuropathological correlate.

Finally, replication of my findings through larger multicentre clinical and experimental cohorts is required to substantiate the replicability and generalizability of monoaminergic transmitter deficits detected with rs-fMRI – and to validate whether the easily-implemented analysis methods and tests presented in this thesis are appropriately sensitive and robust. To this end, international collaborations, in which multiple centre's DOC data are pooled and standardized, will greatly enhance confidence in the present results and approach.

7.10 Conclusion

Together, the studies in the present thesis indicate that monoaminergic brainstem nuclei function is dysregulated in altered states of consciousness – and might contribute to behavioural, network-level and other associated phenomena previously established in the literature. Therein, this work provides primary evidence that the role of the brainstem for different pathological and pharmacological ASCs might be best understood through the lens of specific transmitter nuclei, and that monoaminergic functional changes in posterior cortical connectivity and modulation might be especially important for conscious state. Considered in-concert, these results begin to resolve a central piece of brainstem-cerebrum interplay at a systems-level perspective, therefore also initiating the bridging of foundational micro- and mesoscopic aspects of CNS function to the typically macroscopic perspectives in neuroimaging of ASCs. As such, the present findings support the translational, diagnostic and therapeutic utility of assessing brainstem nuclei in ASCs and beyond – and reinforce that the brainstem is a fundamental piece of the puzzle of understanding not just consciousness *per se*, but brain function as a whole.

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List of CONN atlas anatomical regions with corresponding anatomical labels. This table is applicable to all chapters.

FP r	(Frontal Pole Right)	**PaCiG l**	(Paracingulate Gyrus Left)
FP l	(Frontal Pole Left)	**AC**	(Cingulate Gyrus, anterior division)
IC r	(Insular Cortex Right)	**PC**	(Cingulate Gyrus, posterior division)
IC l	(Insular Cortex Left)	**PCu/Precuneus**	(Precuneus Cortex)
SFG r	(Superior Frontal Gyrus Right)	**Cuneal r**	(Cuneal Cortex Right)
SFG l	(Superior Frontal Gyrus Left)	**Cuneal l**	(Cuneal Cortex Left)
MidFG r	(Middle Frontal Gyrus Right)	**FOrb r**	(Frontal Orbital Cortex Right)
MidFG l	(Middle Frontal Gyrus Left)	**FOrb l**	(Frontal Orbital Cortex Left)
IFG tri r	(Inferior Frontal Gyrus, pars triangularis Right)	**aPaHC r**	(Parahippocampal Gyrus, anterior division Right)
IFG tri l	(Inferior Frontal Gyrus, pars triangularis Left)	**aPaHC l**	(Parahippocampal Gyrus, anterior division Left)
IFG oper r	(Inferior Frontal Gyrus, pars opercularis Right)	**pPaHC r**	(Parahippocampal Gyrus, posterior division Right)
IFG oper l	(Inferior Frontal Gyrus, pars opercularis Left)	**pPaHC l**	(Parahippocampal Gyrus, posterior division Left)
PreCG r	(Precentral Gyrus Right)	**LG r**	(Lingual Gyrus Right)
PreCG l	(Precentral Gyrus Left)	**LG l**	(Lingual Gyrus Left)
TP r	(Temporal Pole Right)	**aTFusC r**	(Temporal Fusiform Cortex, anterior division Right)
TP l	(Temporal Pole Left)	**aTFusC l**	(Temporal Fusiform Cortex, anterior division Left)
aSTG r	(Superior Temporal Gyrus, anterior division Right)	**pTFusC r**	(Temporal Fusiform Cortex, posterior division Right)
aSTG l	(Superior Temporal Gyrus, anterior division Left)	**pTFusC l**	(Temporal Fusiform Cortex, posterior division Left)
pSTG r	(Superior Temporal Gyrus, posterior division Right)	**TOFusC r**	(Temporal Occipital Fusiform Cortex Right)
pSTG l	(Superior Temporal Gyrus, posterior division Left)	**TOFusC l**	(Temporal Occipital Fusiform Cortex Left)
aMTG r	(Middle Temporal Gyrus, anterior division Right)	**OFusG r**	(Occipital Fusiform Gyrus Right)
aMTG l	(Middle Temporal Gyrus, anterior division Left)	**OFusG l**	(Occipital Fusiform Gyrus Left)
pMTG r	(Middle Temporal Gyrus, posterior division Right)	**FO r**	(Frontal Operculum Cortex Right)

pMTG l	(Middle Temporal Gyrus, posterior division Left)	**FO l**	(Frontal Operculum Cortex Left)
toMTG r	(Middle Temporal Gyrus, temporooccipital part Right)	**CO r**	(Central Opercular Cortex Right)
toMTG l	(Middle Temporal Gyrus, temporooccipital part Left)	**CO l**	(Central Opercular Cortex Left)
aITG r	(Inferior Temporal Gyrus, anterior division Right)	**PO r**	(Parietal Operculum Cortex Right)
aITG l	(Inferior Temporal Gyrus, anterior division Left)	**PO l**	(Parietal Operculum Cortex Left)
pITG r	(Inferior Temporal Gyrus, posterior division Right)	**PP r**	(Planum Polare Right)
pITG l	(Inferior Temporal Gyrus, posterior division Left)	**PP l**	(Planum Polare Left)
toITG r	(Inferior Temporal Gyrus, temporooccipital part Right)	**HG r**	(Heschl's Gyrus Right)
toITG l	(Inferior Temporal Gyrus, temporooccipital part Left)	**HG l**	(Heschl's Gyrus Left)
PostCG r	(Postcentral Gyrus Right)	**PT r**	(Planum Temporale Right)
PostCG l	(Postcentral Gyrus Left)	**PT l**	(Planum Temporale Left)
SPL r	(Superior Parietal Lobule Right)	**SCC r**	(Supracalcarine Cortex Right)
SPL l	(Superior Parietal Lobule Left)	**SCC l**	(Supracalcarine Cortex Left)
aSMG r	(Supramarginal Gyrus, anterior division Right)	**OP r**	(Occipital Pole Right)
aSMG l	(Supramarginal Gyrus, anterior division Left)	**OP l**	(Occipital Pole Left)
pSMG r	(Supramarginal Gyrus, posterior division Right)	**Cereb l**	(Cerebellum Left)
pSMG l	(Supramarginal Gyrus, posterior division Left)	**Cereb r**	(Cerebellum Right)
AG r	(Angular Gyrus Right)	**Ver**	(Vermis)
AG l	(Angular Gyrus Left)		
sLOC r	(Lateral Occipital Cortex, superior division Right)	**iLOC l**	(Lateral Occipital Cortex, inferior division Left)
sLOC l	(Lateral Occipital Cortex, superior division Left)	**ICC r**	(Intracalcarine Cortex Right)
iLOC r	(Lateral Occipital Cortex, inferior division Right)	**ICC l**	(Intracalcarine Cortex Left)
SMA L	(Juxtapositional Lobule Cortex -formerly Supplementary Motor Cortex- Left)	**MedFC**	(Frontal Medial Cortex)
SubCalC	(Subcallosal Cortex)	**PaCiG r**	(Paracingulate Gyrus Right)

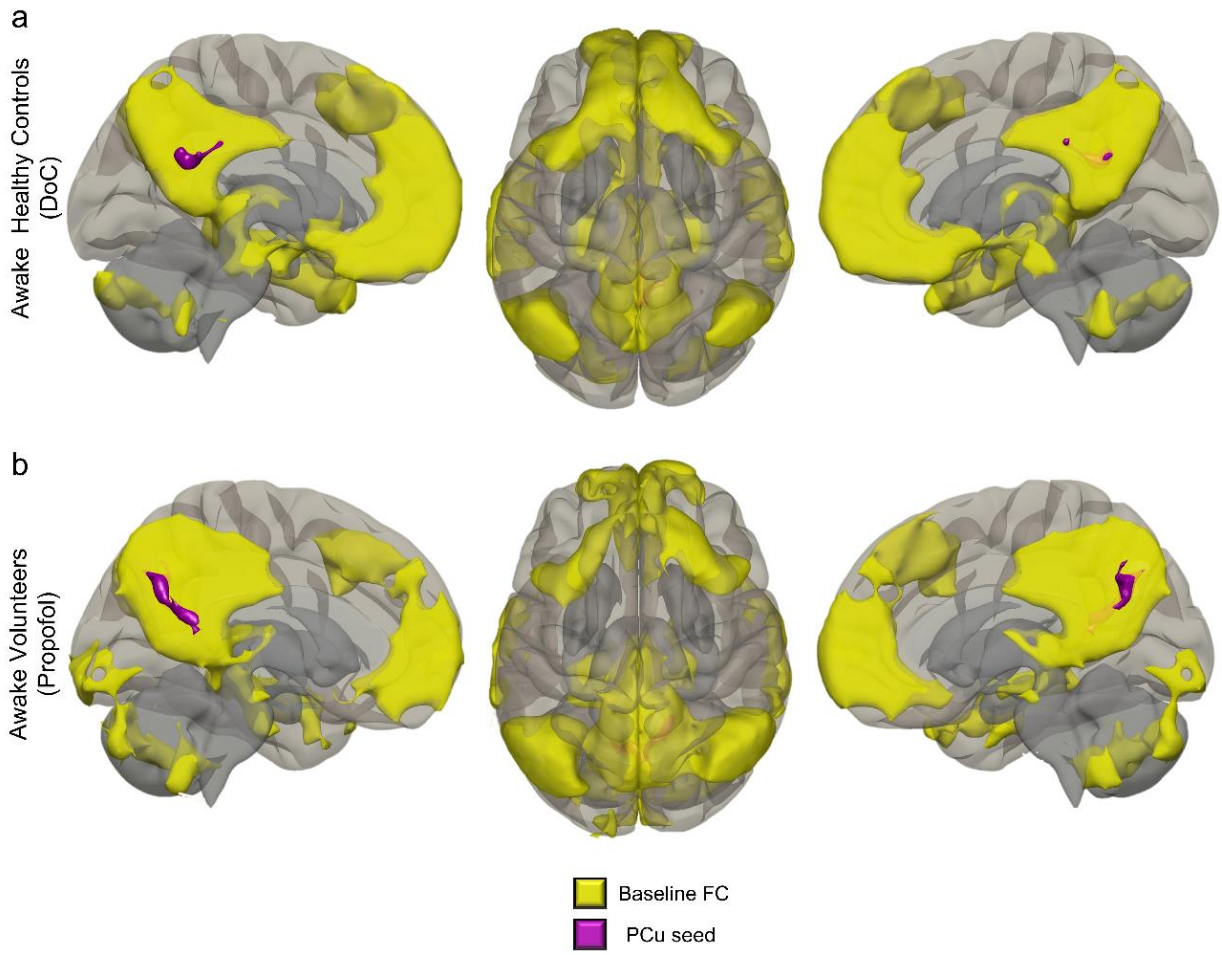


Fig. CII.1: Clusters from which VTA disconnected in both cohorts were sufficient seeds for full Default Mode Network (DMN). In new seed-to-voxel analyses in the respective control conditions. (i.e. healthy controls for DoC patients (a); awake volunteers in the propofol cohort (b), the respective PCu/PCC cluster showed baseline connectivity patterns to all canonically identified DMN regions (Buckner & DiNicola, 2019).

Effort to Compress – Additional information on Algorithm

ETC works as follows: For a given input sequence, the algorithm iterates over the sequence, and replaces the subsequence of a given length that occurs most frequently with a new symbol. For instance, given the binary input string ‘11010010’ and length 2, the first iteration will identify the pair ‘10’ as the most frequent subsequence (as it occurs more often in the original sequence than either ‘00’, ‘01’ or ‘11’). This pair will thus be replaced with a new symbol, such as ‘2’, resulting in output string ‘12202’. The algorithm is then applied again to the new sequence produced by the previous iteration, yielding the new sequence ‘3202’, and iterating until the output sequence becomes constant or its length is reduced to unity: 11010010 ↦ 12202 ↦ 3202 ↦ 402 ↦ 52 ↦ 6; the total number of iterations required for the algorithm to terminate is thus 5 in the present example.

The number of iterations required to transform the input sequence to a constant sequence by iterating the substitution procedure corresponds to the value of Effort-To-Compress. Since this number is at most L - 1 (with L being the length of the original input sequence), the measure can be normalised as:

$$ETC_{norm} = \frac{ETC}{L-1}$$

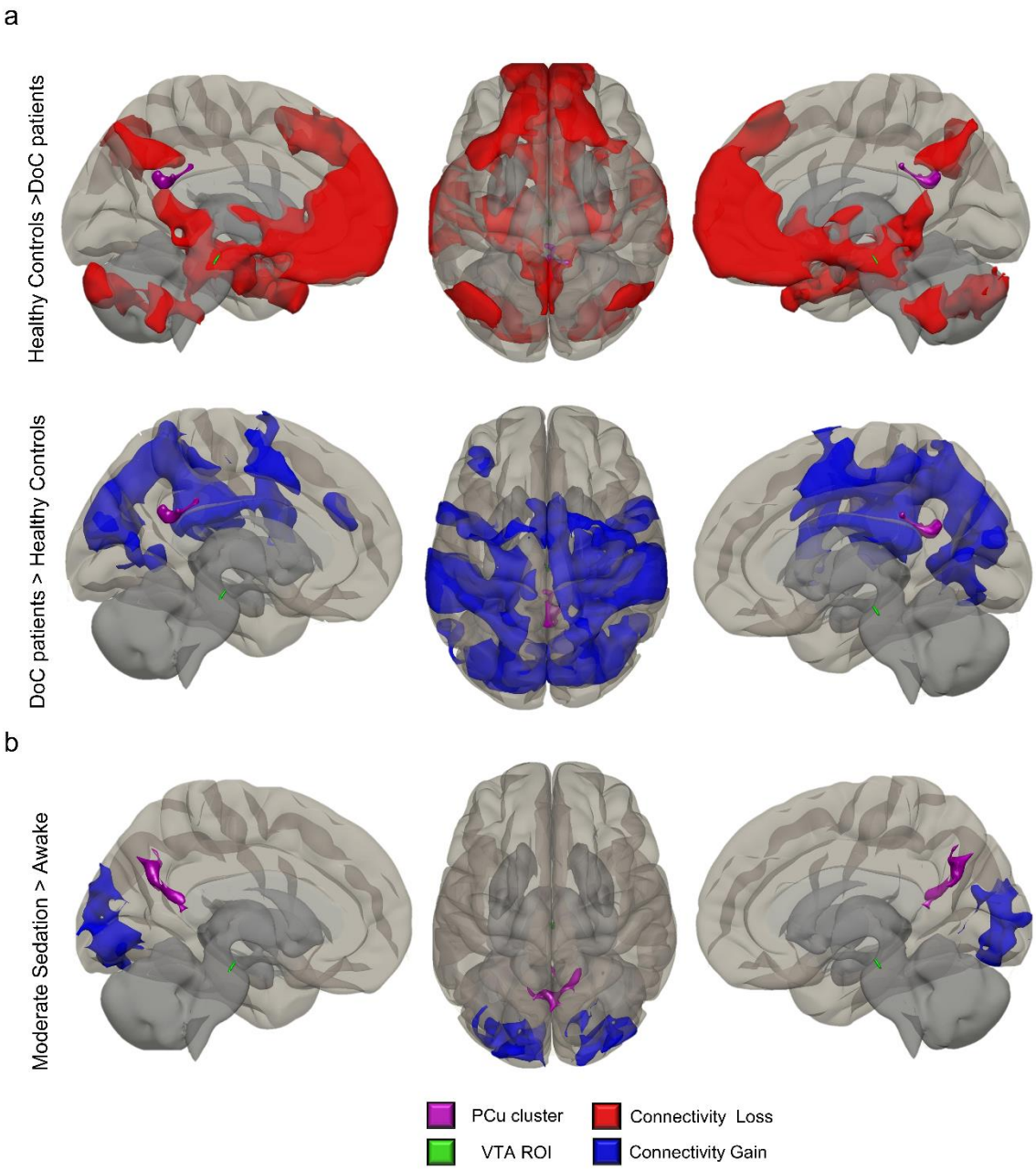


Figure CII.2: 'Downstream' connectivity changes of PCu/PCC clusters in perturbed states of consciousness. (a) In a contrast of Healthy controls *versus* DoC patients, the PCu/PCC cluster the VTA lost connectivity with in DoC patients, showed 'downstream' losses of connectivity (red) with DMN regions, and increases in connectivity with areas not commonly considered part of the DMN (blue). (b) In a contrast of moderate sedation against awake controls, the PCu/PCC cluster the VTA lost connectivity with in Propofol sedation showed 'downstream' occipital connectivity gains (blue). Renderings were made using the CONN toolbox' 3D template. Left is left.

***Table CII: Significant* clusters of PCu/PCC 'downstream' connectivity changes in contrasts with respective control conditions.** CONN atlas labels, MNI coordinates, cluster extent and FWE-corrected p-values are reported. DMN regions in **bold**. Results were thresholded at p<0.005 voxel-level (uncorrected) and p<0.05 cluster-level (FWE-corrected).For clusters over 10000 voxels, only labels for regions which occupied over 250 voxels within those clusters are reported.

			FP l+r, **MedFC**, **MidFG** l+r, **PC**, **Precuneus**, Brainstem, TP l+r, **AC**, PaCiG l+r, toMTG l+r, pMTG l+r, Hippocampus l+r, SFG l+r, Thalamus l+r, aPaHC l+r, pPaHC l+r, aMTG l+r, Caudate l+r, Amygdala l+r, FOrb l+r, Accumbens l+r, IC l+r, Putamen l+r, SubCalC, pTFusC, aITG l+r. pITG l+r, aSTG r, LG , Cereb1,3,45,6,8,9,10 l+r, Ver3, 45,8,9	-04 +46 -04	32652	0.000
Disorders of Consciousness	**Healthy > DoC Patients**	↓Loss	Cereb1,2,7,8 r	+38 -70 -36	1680	0.000
			Precuneus, PC	+00 -60 +38	987	0.000
			sLOC l, **AG** l	-44 -72 +48	974	0.000
			sLOC r, **AG** r	+48 -64 +48	681	0.000
	DoC Patients > Healthy	↑Gain	sLOC l+r, PreCG l+r, PostCG l+r, SPL l+r , SMG l+r, SMA l+r , AC, PO l+r, iLOC r, IFG oper r, Cuneal l+r, LG r, PT r, MTG, ICC, OP	-58 -36 +30	28189	0.000
			FP l, MidFG l	-34 +32 +32	390	0.008
			toMTG l, iLOC l	-56 -54 +00	348	0.016
Propofol Sedation	**Moderate Sedation > Awake**	↑Gain	OP l, LG l, iLOC l, OFusG, sLOC l, ICC l	-12 -78 -08	1995	0.000
			OP r, iLOC r, OFusG r, sLOC r, ICC r, Cereb 1 r	+40 -80 -06	1192	1192

Table CII.M: Significant clusters of baseline connectivity revealed in seed-to-voxel analyses using the PCu clusters as seeds in new seed-to-voxel connectivity analyses. Associated CONN, labels, MNI coordinates cluster extent and FWE-corrected p-value are denoted. Results were thresholded at p<0.001 voxel-level (uncorrected) and p<0.005 voxel-level (uncorrected) and p<0.05 cluster level (FWE-corrected). For clusters over 10000 voxels, only labels for regions which occupied over 250 voxels within those clusters are reported.

Condition	*Significant positive FC at rest (CONN-label)*	*Peak MNI Coordinates*	*Cluster size*	*p-FWE corrected*
Awake (Healthy controls DoC dataset)	**Precuneus**, **FP l+r. PC,** sLOC l+r, pMTG l, SFG l+r, **MidFG l+r**, **MedFC, AC,** PaCiG l+r, SFG l+r, TP l+r, pMTG l+r, SubCalC, **AG l+r**, Cereb2 l, Hippocampus l+r, Brainstem, Thalamus l+r, aMTG l+r, Cereb9 l+r, pPaHC l+r, Forb l+r, pITG l+r, aPaHC l+r, Amygdala l+r, Accumbens l+r, LG l+r, Cereb45 l+r, aSTG l+r, IC l+r, pTFusC Cereb8 l+r,	+00 -40 +30	28218	0.000
	pMTG l, TP l, aMTG l, FOrb l, pITG l, aSTG l, aITGl, IC l, toMTG l	-66 -18 -18	3513	0.000
	pMTG r, TP r, aMTG r, FOrb r, pITG r, toMTG r, aSTG r, FP r, aITG r, Amygdala r, pSTG r, IC r	+28 +18 -24	3049	0.000
	sLOC l, **AG l**	-40 -64 +38	2622	0.000
	sLOC r, **AG r**	+48 -62 +36	2238	0.000
	Cereb 2 l, Cereb 1 l	-34 -72 -38	989	0.000
	Cereb 2 r, Cereb 1 r, Cereb 7 r, Cereb 8 r	+20 -86 -34	840	0.000
	Cereb 9 l+r, Ver9, Brainstem, Cereb 8 r	-06 -54 -42	762	0.000
	Precuneus, PC, sLOC r, **AG** r, Cuneal l+r, Hippocampus r, Ver45, LG l+r, pPaHC l+r, Thalamus l+r,	-02 -62 +32	14619	0.000
	ICC l+r, Brainstem, Cereb45 l, AC, PreCG l, SCC l+r, sLOC l, iLOC r, aPaHC l, pTFusC l, SPL r, pSMG r, Ver4, PreCG r	+02 +64 +22	3522	0.000
Awake (Volunteers Propofol dataset)	**FP** r, **MedFC**, PaCiG r, **FP** l, PaCiG l, **AC**, SubCalC, SFG l+r	-40 -72 +38	3476	0.000
	sLOC l, **AG** l, SPL l, pSMG l, iLOC l	+22 +38 +42	1681	0.000
	MidFG r, SFG r, FP r	+08 -82 -40	1522	0.000
	Cereb2 l+r, Cereb1 l+r, Cereb7 l+r	-28 +22 +48	1089	0.000
	MidFG l, SFG l, FP l	+66 -30 -10	786	0.000
	pMTG r, aMTG r, pITG r, toMTG r	-60 -04 -22	706	0.000
	pMTG l, aMTG l, TP l	-04 -54 -50	515	0.000
	Cereb9 l+r	+22 -28 -10	275	0.000
	Hippocampus r, pPaHC r, Thalamus r, Brainstem, Amygdala	+06 -96 -12	160	0.002
	OP r, LG r, OP l	+00 +50 +44	120	0.010
	SFG l+r, PaCiG r	+48 +18 -34	115	0.013

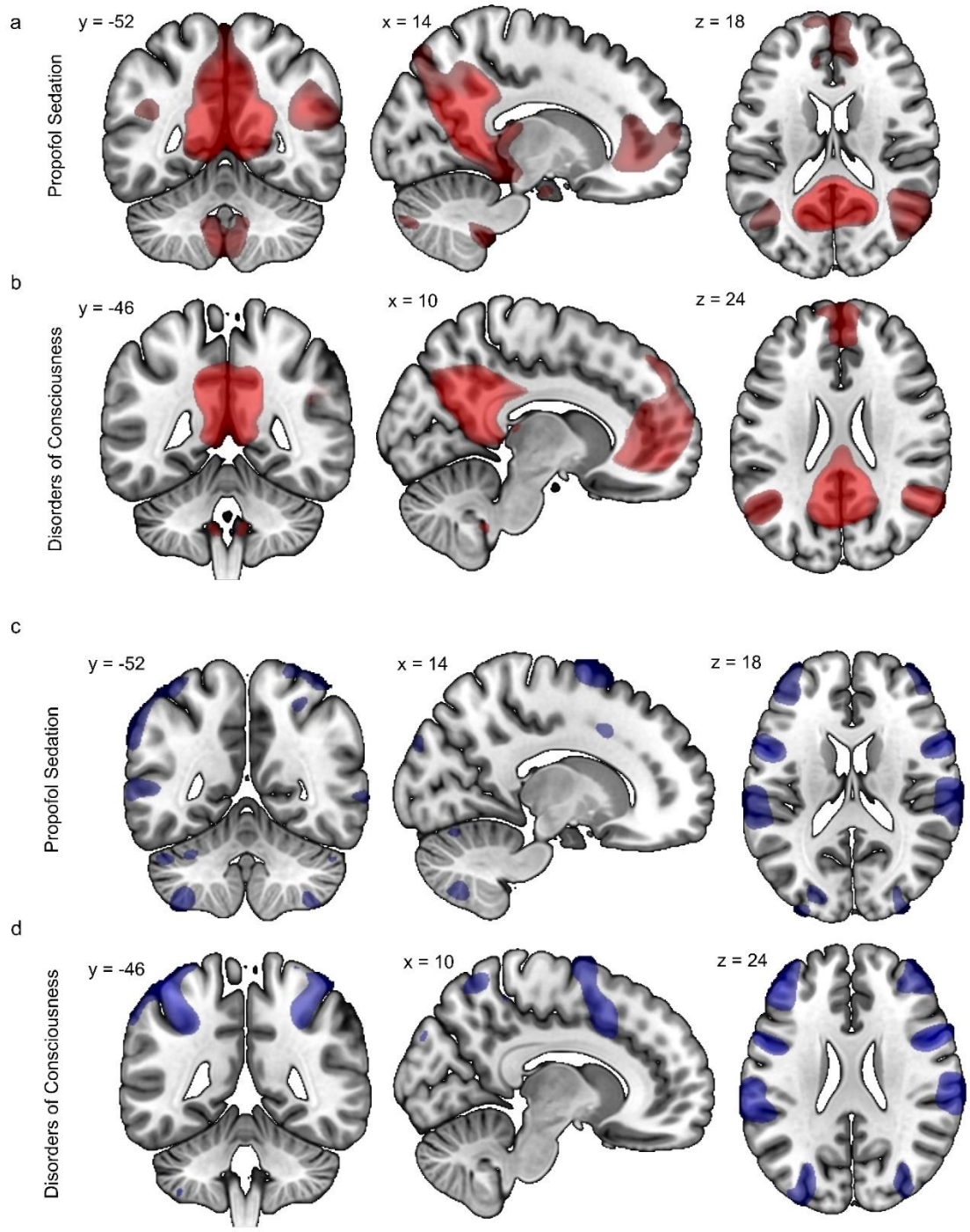


Fig. CII.3: Neurosynth functional connectivity maps generated with and centred on the peak MNI coordinates for the respective precuneus cluster for each cohort. Across both populations, the respective PCu/PCC clusters showed similar positive (a & b) and negative (c & d) functional connectivity patterns.

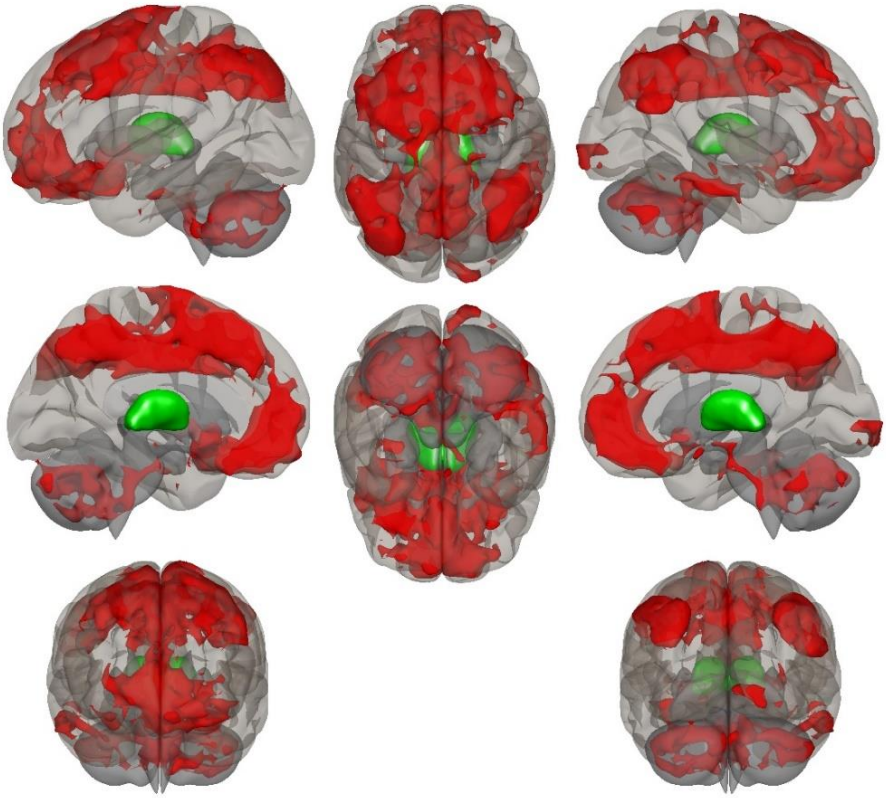


Fig. CIII.1: Seed-to-voxel disconnections of thalamus in DOC patients (contrast of Controls > DOC patients). The disconnections of the thalamus that were observed spanned from the largest cluster centred on the Precuneus, via effectively all regions of the default mode and frontotemporal networks (see Table below.)

Table 1SM: Seed-to-voxel connectivity contrasts using the thalamus as a seed. Associated CONN, labels, MNI coordinates cluster extent and FWE-corrected p-value are denoted. Results were thresholded at p<0.001 voxel-level (uncorrected) and p<0.005 voxel-level (uncorrected) and p<0.05 cluster level (FWE-corrected). For clusters over 10000 voxels, only labels for regions which occupied over 250 voxels within those clusters are reported.

Contrast	***Significant positive FC at rest (CONN-label)***	***Peak MNI Coordinates***	***Cluster size***	***p-FWE corrected***
Controls > DOC	Precuneus, PC, SFG l+r, MidFG l+r, AC, FP l+r, PreCG l+r, PaCiG l+r, MedFC, Forb l+r, SMA l+r, SPL l+r, Putamen l+r, , LG l+r, pPaHC l+r, AG l+r,	-04 +10 +54	28142	0.000
	ICC l+r, Brainstem, Cereb45 l, AC, PreCG l, SCC l+r, sLOC l, iLOC r, aPaHC l, pTFusC l, SPL r, pSMG r, Ver4, PreCG r	+00 -34 -36	6801	0.000
	sLOC r, AG r, SPL r, pSMG r, PostCG r	+36 -46 +40	3185	0.000
	OP r, LG r iLOC r, ICC r,	+16 -104 -02	391	0.000
	PreCG r, PostCG r, SPL r	+20 -26 +70	1522	0.000
	aPaHC l, Hippocampus l, aTFusC l,	-34 -16 -26	169	0.000

Appendix Chapter IV (Appendix CIV)

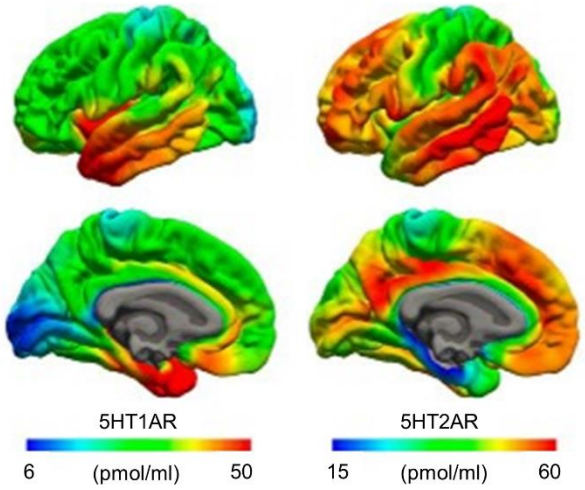
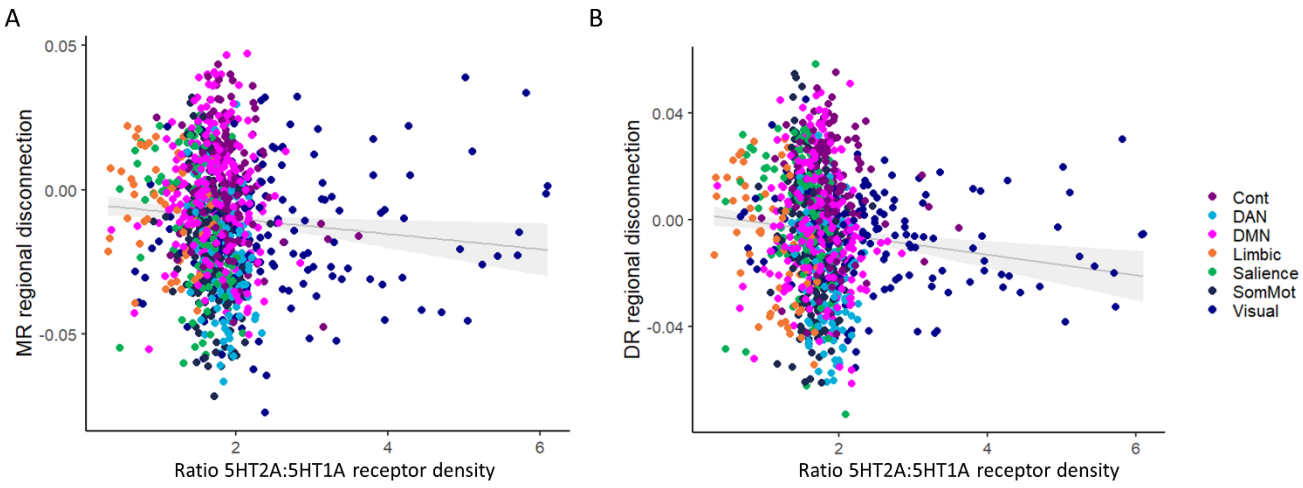


Fig. CIV.1: Average density (B_{max} of PET signal) maps for the two 5-HT targets considered in Chapter IV. Plotted on the common FreeSurfer surface (left hemisphere; lateral view, upper and medial view, lower). These plots highlight that 5HT2AR is much more widely expressed, but that there are pockets of co-expression with 5HT1AR to heterogenous extents throughout the brain. The areas from which I observed disconnections in Chapter IV, overlap in particular with the co-expressions at posterior cortical and subcortical/thalamic level, as well as cerebellum.

Fig. CIV.2: Strength of LSD-induced raphe disconnection is associated with relative regional 5HT2A receptor density also when visual network regions are included. For both of the serotonergic source nuclei, the average level of regional disconnection observed in our sample (Δ connectivity change LSD-Placebo) was significantly associated with the relative density of 5-HT2AR (over and above 5HT1AR) in the region in question, meaning that higher relative regional 5-HT2AR levels were a predictor for stronger raphe disconnection from that region. Colours in graphs correspond to established labels of canonical resting state networks (Yeo et al., 2011; DMN = Default Mode Network; DAN = Dorsal Attention Network; ECN = Executive Control Network; LN = Limbic Network; SN = Salience Network; SMN = Somatomotor Network; as per the Schaefer 1000 parcellation. Grey line is overall regression line with 95% confidence interval. Correlation rendering was made in RStudio, with both frequentist and Bayesian regression strengths, significances and support extracted using JASP v14.0.0.



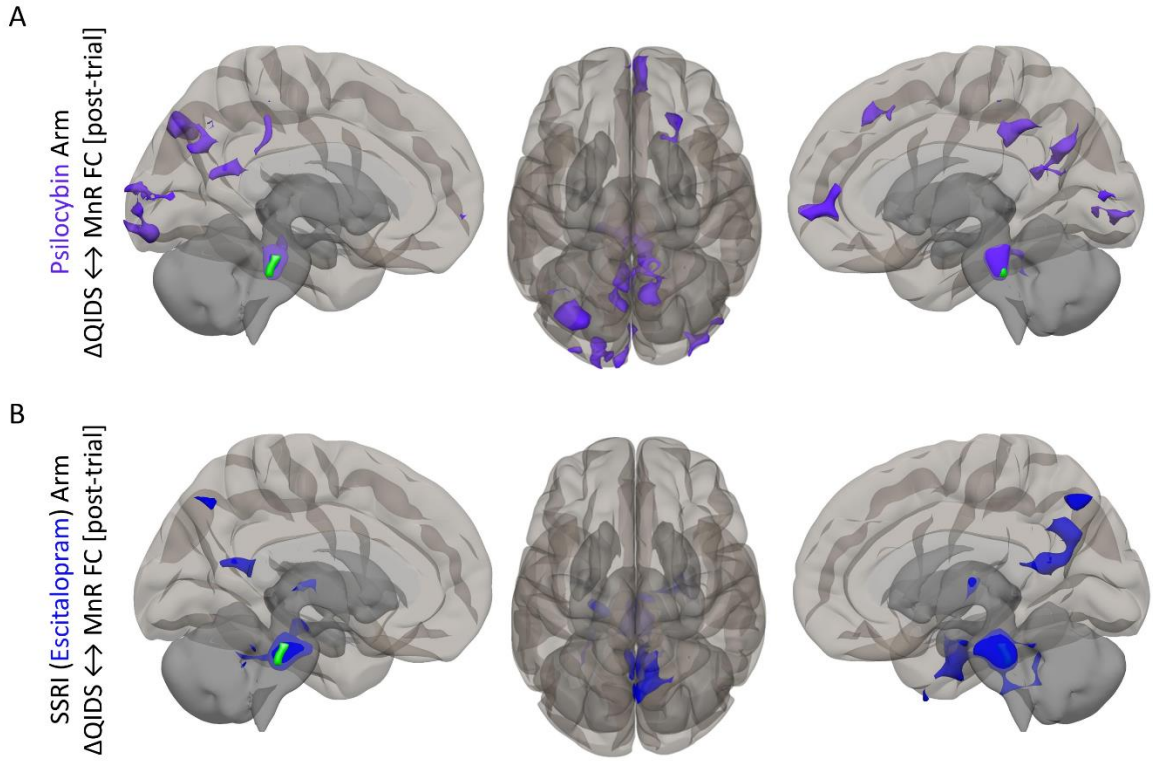


Fig. CV.1: Whole-brain correlations of post-trial MnR seed-to-voxel connectivity with QIDS depression reduction reveal similar posterior cortex clusters across both treatment arms in the MDD-RCT trial. (A and B) both reveal connectivity changes to the precuneus and posterior cingulate, where in both treatment arms, the clusters are the largest.

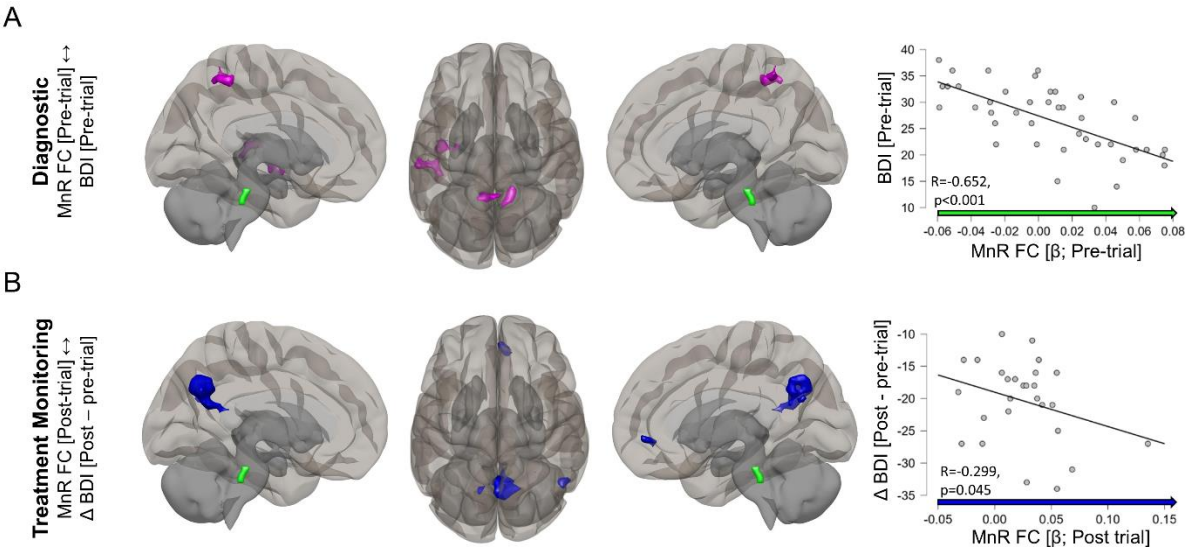


Fig. CV.2: Whole brain correlations of MnR seed-to-voxel connectivity with the Beck Depression Inventory (BDI) as the outcome measure replicate the findings made with the QIDS. (A) Also when using the BDI, the only cluster that remained significant in the seed-to-voxel correlations of pre-trial MnR connectivity and pre-trial depression severity was in the precuneus. (B) Equally, the largest cluster of association of MnR post-trial connectivity with reduction in BDI (Δ BDI post – pre-trial) was again in the precuneus. Additional small clusters coincided with other DMN regions.

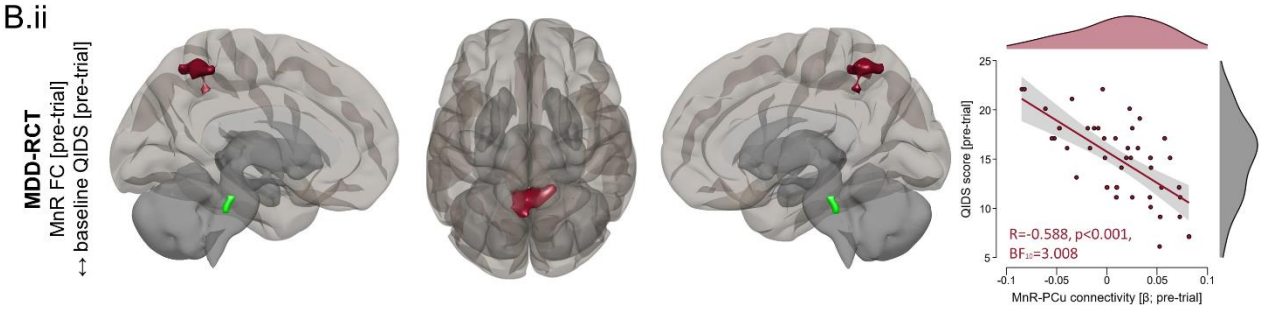


Fig. CV.3: In MDD-RCT cohort whole brain correlations of MnR pre-trial seed-to-voxel connectivity are associated with depression severity at baseline. (B.ii) The more impaired connectivity between the median raphe and the precuneus (dark red) was before the trial, the more depressed participants of both treatment arms scored on the QIDS.

8.3 List of Figure Legends

Ithaka gave you the marvelous journey. | Without her you wouldn't have set out.

She has nothing left to give you now.

And if you find her poor, Ithaka won't have fooled you. | Wise as you will have become, so full of experience,

you'll have understood by then what these Ithakas mean.