Human translational studies investigating the neurocognitive effects of 5-HT_4 receptor agonism

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“All the most acute, most powerful, and most deadly diseases, and those which are most difficult to be understood by the inexperienced, fall upon the brain.”

Hippocrates

Abstract

Major depression is common, costly, and a global cause of significant morbidity. First-line antidepressant medication has a clinically problematic delay before therapeutic efficacy and is ineffective for a subset of those affected. In addition, many patients with depression, as well as other mental illnesses, find that cognitive impairment has a substantial impact on their quality of life and functioning. Cognitive impairment is poorly treated with medication such as antidepressants, and can persist after remission. Based on animal behavioural studies, as well as molecular and neurochemical evidence, 5-HT_4 receptor agonism has been suggested to have putative antidepressant effects and to improve cognition. However, there is relatively little evidence in humans demonstrating such effects.

The aim of this thesis is to investigate whether it is possible to translate the pre-clinical neurocognitive effects of 5-HT_4 agonism into humans. This would help to elucidate whether 5-HT_4 receptor agonism may have therapeutic potential within psychiatry as an antidepressant and / or a pro-cognitive treatment.

A series of experimental medicine fMRI studies were conducted using the 5-HT_4 receptor agonist, prucalopride, in healthy volunteers. Low dose prucalopride led to improved behavioural cognition on a memory encoding and implicit faces task, and increased neural activation in memory processing regions including the hippocampus. Resting-state fMRI provided further mechanistic support for prucalopride as a pro-cognitive agent. The latter findings were partially confirmed in a clinical population of unmedicated depressed patients treated with a novel 5-HT_4 agonist, PF-04995274. Here, 5-HT_4 agonism with PF-04995274 increased activation in another memory processing region and modulated activity within the hippocampus; however, the behavioural pro-cognitive behavioural effect seen with prucalopride was absent. Although there was no evidence of an antidepressant-like effect in experimental medicine studies, a pharmaco-epidemiological study suggested that one year of standard dose prucalopride for the treatment of gastrointestinal disorders was associated with reduced risk of depression.

Overall, this thesis identifies that 5-HT_4 receptor agonism has neurocognitive effects consistent with other pro-cognitive medications in healthy participants and depressed patients. Data using electronic health records indicates that it may also reduce incidence of future depressive episodes. Future studies should consider confirming the correct dose of individual agonists to ensure optimised central receptor binding in clinical populations, using larger sample sizes to allow for variability inherent in clinical populations, and / or to assess the effect of medication over a longer time period.

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Contents

Long Abstract

More than 300 million people around the world are suffering from depression at any one time. However, first-line antidepressants take several weeks before therapeutic effect, and for one third of patients clinical response is negligible. Therefore, there is a need to discover new antidepressant treatments that target novel mechanisms to produce a faster onset of action. Impairments in cognitive functioning also occur across many psychiatric disorders, including depression. These deficits negatively affect quality of life and functioning but are poorly targeted by current treatments for the disorder (such as antidepressants in depression). Thus, another therapeutic priority is to explore the neural underpinnings of these cognitive impairments to enable the development of more effective treatments. The serotonin 4 (5-HT_4) receptor subtype has recently emerged as one such possible target – suggested as both a rapid antidepressant and cognitive enhancer – based on experimental work in animal studies.

In this thesis, I investigate whether we can translate the neurocognitive effects of 5-HT_4 receptor agonism seen in animals to human neuropsychological models. Chapter One presents the research background to this topic, and Chapters Two to Six present experimental data.

Chapter One establishes the current therapeutic challenge for depression and cognition more broadly across mental disorder, and provides a review of how agonism at the 5-HT_4 receptor may link to depression and cognition. In this, I discuss the existing findings of 5-HT_4 receptor agonism in terms of neurocognitive effects in animals and how we can investigate the translation of these effects into humans.

Chapters Two, Three and Four describe results from an experimental medicine fMRI study investigating the effects of six days' treatment with the 5-HT_4 receptor agonist, prucalopride, in healthy human volunteers.

Chapter Two examines the effect of prucalopride on behavioural and neural memory processing. It was found that participants who had received prucalopride performed significantly better that a placebo treated group on a memory recall task. Prucalopride also increased hippocampal activity bilaterally as well as increasing activity in the right angular gyrus (a linked memory processing region). This suggests that 5-HT_4 receptor activation may potentially have a pro-cognitive effect in humans, associated with activation of regions involved in hippocampal-associated memory processing.

Chapter Three examines the effect of prucalopride on the neural processing of emotional faces. Participants receiving prucalopride were more accurate at identifying the gender of emotional faces. Prucalopride was also associated with reduced activation in a network of regions corresponding to the default mode network (DMN). However, there was no evidence that prucalopride treatment produced a positive bias in the neural processing of emotional faces. This chapter provides further support for a pro-cognitive effect of 5-HT_4 receptor agonism in humans. Although these neurocognitive investigations did not suggest an antidepressant-like profile of prucalopride in humans, possible explanations for this apparent lack of effect are discussed.

Chapter Four investigates the effect of prucalopride on resting-state functional connectivity (rsFC) in the healthy human brain. Network analyses identified that participants in the prucalopride group had enhanced resting-state functional connectivity in the central executive network (CEN)

and the posterior / anterior cingulate cortex (PCC / ACC). Seed analyses also showed greater rsFC between the left and right rostral ACC and the left lateral occipital cortex ACC, and reduced rsFC between the hippocampus and other regions of the DMN. This indicates that prucalopride may enhance rsFC between regions involved in cognitive networks and reduce rsFC within the DMN, suggesting a neural network mechanism for the pro-cognitive effects seen in Chapters Two and Three.

Evidence from Chapters Two to Four suggests possible pro-cognitive effects of 5-HT_4 agonism in humans, with little evidence for an antidepressant-like profile of action. Therefore, two further studies were undertaken to evaluate this. Chapter Five assessed the effects of 5-HT_4 receptor agonism on a memory task in a group of participants with un-medicated depression, assessing whether it is possible to translate the pro-cognitive effects seen in healthy participants into a clinical population. For this study, a different 5-HT_4 agonist (PF-04995274) was used. Chapter Six details a study using a large dataset of live electronic health records, to see if longer-term treatment with a 5-HT_4 agonist (prucalopride) is associated with positive changes in longitudinal mental health outcomes.

Results in Chapter Five indicated that, like prucalopride, PF-04995274, relative to placebo was able to modulate activity within the hippocampus in depressed patients. In addition, PF-04995274 also increased activation in the lateral occipital cortex. Unlike prucalopride, PF-04995274 did not produce improved performance in a behavioural memory task. This suggests that 5-HT_4 receptor agonism broadly affects hippocampal activity within humans in both healthy and depressed participants, although the notable differences between the studies are discussed.

In Chapter Six, the TriNetX electronic health records network was used to perform an emulated target trial, comparing the incidence of mood, anxiety and cognitive disorders over a two-year period following prucalopride initiation versus alternative anti-constipation agents. Compared to alternatives, one year of prucalopride was associated with reduced risk of several mental illnesses, mostly notably depression. Negative control outcome and supplementary analyses indicated these findings were specific and robust. Possible mechanisms for this are discussed including generalised pro-cognitive effects or modifications of the gut-brain axis. This study suggests that prucalopride may provide additional mental health benefits for patients receiving it as an anti-constipation agent.

Finally, Chapter Seven summarises results from this thesis and discusses the implications for these within the context of the current research base. I also discuss methodological factors that should be considered when interpreting the findings from each chapter. In particular, I discuss strengths and limitations with the different study designs and techniques used. I finish the thesis with a discussion of the possible directions for future research into 5-HT_4 receptor agonism and its potential clinical niche in psychiatry.

Abbreviations

5-HT = serotonin

5-HT_4 = serotonin receptor number four

ACC = anterior cingulate cortex

BDNF = brain derived neurotrophic factor

BDI = Beck Depression Inventory

BET = Brain Extraction Tool

BIDS = Brain Imaging Data Structure

BMI = body mass index

BOLD imaging = blood oxygen level dependent imaging

CEN = (central) executive network

95% CI = 95% confidence interval

CREB = cyclic-AMP response element binding protein

CSF = cerebral spinal fluid

DICOM = Digital Imaging and Communications in Medicine

DMN = default mode network

DRN = dorsal raphe nucleus

DSM = Diagnostic and Statistical Manual of mental disorders

EHI = Edinburgh Handedness Inventory

EPI = echo-planar imaging

ERK = extracellular signal-regulated kinase

EPQ = Eysenck Personality Questionnaire

FEAT = fMRI expert imaging analysis tool

fMRI = functional Magnetic Resonance Imaging

FSL = FMRIB's Software Library

FWE = family-wise error

GABA = gamma-aminobutyric acid

GM = grey matter

GP = General Practitioner

HAM-D = Hamilton Rating Scale for Depression

HR = hazard ratio

IBS = Irritable Bowel Syndrome

IQ = Intelligence Quotient

mRNA = messenger ribonucleic acid

ms = milliseconds

NART = National Adult Reading Test

NCO = negative control outcome

OR = odds ratio

PANAS = Positive and Negative Affect Scale

PCC = posterior cingulate cortex

PET imaging = positron emission tomography imaging

RESTAND = Effect of PF-04995274 on emotional processing in un-medicated depressed patients

rsFC = resting-state functional connectivity

ROI = region of interest

RSN = resting-state network

RT = reaction time

s = seconds

SCID = Structured Clinical Interview for DSM-5

SD = standard deviation

SEM = standard error of the mean

SHAPS = Snaith-Hamilton Pleasure Scale

SN = salience network

SSRI = selective serotonin reuptake inhibitor

STAI-S = Spielberger State-Trait Anxiety Inventory, State Version

STAI-T = Spielberger State-Trait Anxiety Inventory, Trait Version

VAS = Visual Analogue Scale

WM = white matter

Declaration & publications stemming from this work

The chapters in this thesis represent my own work, under the supervision of Catherine Harmer, Philip Cowen and Susannah Murphy. Other support received is detailed in Acknowledgements.

Chapter 1: Draws on elements of (i) Murphy SE, de Cates AN, Gillespie AL, Godlewska BR, Scaife JC, Wright LC, Cowen PJ, Harmer CJ (2021). Translating the promise of 5HT4 receptor agonists for the treatment of depression. Psychological Medicine 51, 1111–1120; (ii) de Cates AN, Wright LC, Martens MAG, Gibson D, Tuerkmen C, Filippini N, Cowen PJ, Harmer CJ, Murphy SE (2021). Déjà vu? Neural and behavioural effects of the 5-HT_4 receptor agonist, prucalopride, in a hippocampal-dependent memory task. Translational Psychiatry 11, 497; (iii) de Cates AN, Martens MAG, Wright LC, Gibson D, Gould van Praag CD, Capitao LP, Cowen PJ, Harmer CJ, Murphy SE (2022). The effect of the 5-HT_4 agonist, prucalopride, on an fMRI faces task in the healthy human brain. Frontiers in Psychiatry 13, 859123.

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Chapter 3: Based on work published as de Cates AN, Martens MAG, Wright LC, Gibson D, Gould van Praag CD, Capitao LP, Cowen PJ, Harmer CJ, Murphy SE (2022). The effect of the 5-HT_4 agonist, prucalopride, on an fMRI faces task in the healthy human brain. Frontiers in Psychiatry 13, 859123.

Chapter 4: Based on work under review as de Cates AN, Martens MAG, Wright LC, Gibson D, Spitz G, Gould van Praag CD, Suri S, Cowen PJ, Murphy SE, Harmer CJ. 5-HT_4 receptor agonist effects on functional connectivity in the human brain; Implications for pro-cognitive action. Biological Psychiatry: Cognitive Neuroscience and Neuroimaging.

CHAPTER 1

Research Background

1.1 General introduction

Globally, depression is ranked as one of the largest contributors to morbidity with more than 300 million people affected at any one time (WHO 2018; 2022). One in five people experience at least one lifetime episode (Malhi and Mann 2018), approximately two thirds of which respond to first-line antidepressant treatments (De Carlo et al. 2016). Yet, up to one third of patients do not achieve remission following an adequate trial of two antidepressants, and instead may be considered to be resistant to treatment with significant consequences for their occupational and social functioning (Malhi and Mann 2018; Rush et al. 2009). Furthermore, patients receiving the most commonly-used first-line antidepressants (SSRIs, selective serotonin reuptake inhibitors) usually experience a delay before therapeutic benefit (Duman 2007). Within this context, there is a pressing need to develop new treatments for depression that target novel mechanisms and have a faster onset of therapeutic action.

Impairments in cognitive functioning occur across many psychiatric disorders, including affective, psychotic and neurodevelopmental conditions (Etkin et al. 2013; Millan et al. 2012). However, these cognitive deficits seem separable from primary symptoms of the mental disorder. That is, medications targeting the disorder are not effective at improving cognition (Millan et al. 2012) and cognitive problems persist after remission from the other symptoms (Rock et al. 2014). This may in part relate to their breadth: cognitive dysfunction covers many impairments including learning and memory, attention and processing speed (Millan et al. 2012). Given the significant impact such

impairments can have on quality of life, there is also a critical need to understand the neural underpinnings to enable the development of effective treatments.

The 5-HT_4 receptor has recently emerged as one such potential target (Murphy et al. 2021). Studies using animal models suggest that agonism at this receptor might improve mood as well as enhance learning and memory, which if realised, would be clinically relevant for a spectrum of mental disorders.

Therefore, the aim of this thesis is to investigate whether we can translate the neurocognitive effects with 5-HT_4 receptor agonism seen in animals to human neurocognitive models. In this introductory chapter, I provide a broad overview of the current problems in terms of medication for depression and cognition, and of the potential of the 5-HT_4 receptor as a novel treatment target. I will then review the likely mechanisms of action of the 5-HT_4 receptor in terms of neural functioning, the neurocognitive findings of 5-HT_4 receptor agonism in animals, and how we might be able to investigate the translation of these effects into humans.

1.2 Pathophysiology of depression and its association with cognitive dysfunction

1.2.1 Overview of the problem

Despite a significant body of research spanning several decades, we still do not fully understand major depressive disorder and its causes. This likely relates to complexity within the disorder itself: the same clinical syndrome may be due to various biological aetiologies i.e. neurotransmitter function (excitatory and inhibitory neurotransmission), neuroinflammation, neurotrophins, oxidative stress, chronic stress, endogenous opioids, and endocannabinoids and

endovanilloids (Malhi and Mann 2018; Pitsillou et al. 2020). Although, these biological factors may interconnect via a broader neuroplasticity hypothesis of depression (Price and Duman 2020), this vast array of potential causes may explain why at least a third of patients do not respond to first-line antidepressants (Rush et al. 2009). Social and psychological factors (such as childhood traumatic experiences and poverty) are also known to be relevant in the aetiology of depression (Malhi and Mann 2018) but these will not be discussed in detail here due to the predominant biological focus of this thesis.

Patients taking first-line antidepressants, such as SSRIs, also usually experience a delay before any therapeutic benefit (Duman 2007). This at least partly relates to the generalised increase in extracellular serotonin that occurs with the onset of SSRI administration, which leads to broad agonistic activity including at the 5-HT_{1A} autoreceptor (Artigas 2013). This initially reduces release of serotonin into the synaptic cleft that only resolves with desensitisation of the 5-HT_{1A} autoreceptor over time.

Thus, a "one size" treatment for depression does not fit all, and our existing treatments are unacceptably slow. Nonetheless, despite this complexity regarding aetiology and difficulties with response to treatment, people with depression show consistency in many symptoms and MRI findings such as reduced hippocampal volume. These include behavioural deficits (i.e. cognitive impairment), and abnormalities within the brain relating both to structure (i.e. reduced hippocampal volume) and function (i.e. abnormal hippocampal functioning and functional connectivity more generally).

1.2.2 Cognitive deficits as part of depression

Poor cognition is an important facet of the diagnosis of depression for all ages, affecting two thirds of individuals (Halahakoon et al. 2019). It can be divided into “cold” cognition, which describes problems spanning attention and concentration, learning and memory, executive function, processing speed, reward, and language (Etkin et al. 2013; Millan et al. 2012; Rock et al. 2014), and “hot” cognition, which relates to emotional processing and motivational states (Godlewska and Harmer 2020). In this thesis, when I use the term cognition, I am generally referring to “cold” cognition, unless stated otherwise.

Cognitive deficits are often particularly troubling to patients, second only to low mood in their impact according to a re-analysis of STAR*D data (Fried and Nesse 2014). Importantly however, cognitive deficits seem to be separable mechanistically from low mood (Halahakoon et al. 2019). First, antidepressants that successfully target mood do not consistently improve cognition. For example, the longitudinal iSPOT-D trial found that cognitive impairments did not improve with any SSRI including escitalopram, sertraline, or modified-release venlafaxine (Shilyansky et al. 2016). Second, in systematic reviews of remitted depression, multiple domains of cognitive impairments persist for up to 50% of people even after low mood has resolved (Rock et al. 2014; Semkovska et al. 2019), with the cognitive domains affected generally consistent across studies (i.e. attention, response inhibition, memory, processing speed, information processing). The antidepressant drug, vortioxetine is licensed in the United States specifically for cognitive impairment in depression due to the evidential promise of early studies (Frampton 2016; Mahableshwarkar et al. 2015; McIntyre et al. 2017; McIntyre et al. 2014), although more recent data has not consistently replicated statistical superiority in comparison to SSRIs (Nierenberg et al. 2019; Vieta et al. 2018).

Although often not formally assessed, episodic memory problems are a particular concern (Darcet et al. 2016). These memories pertaining to previous life experiences are often recalled too generally (Van Vreeswijk and De Wilde 2004), which causes reduced ability to solve problems and imagine the normal breadth of future events. As a consequence, cognitive flexibility and the ability to plan for the future is impaired (Price and Duman 2020). For this and other reasons, poor cognition objectively affects quality of life and functioning, with adverse outcomes such as a higher risk of unemployment (Baune et al. 2010; Knight and Baune 2018; Knight et al. 2020; McIntyre et al. 2013).

Two recent studies from the same group examined baseline cognitive deficits and their relation to severity of symptoms and to treatment response in almost 100 patients with depression and a similar number of healthy controls. In the first study, in unmedicated depression, there were no correlations between individual symptoms of poor cognition and depression severity (Dam et al. 2021). In the second study, following initiation of standard antidepressant treatment, most cognitive domains improved but these were unrelated to changes in mood symptoms, and no individual baseline cognitive measure was related to antidepressant response (Dam et al. 2022). Interestingly, the same study also found that the most severe cognitive deficits at baseline were associated with poorer clinical response to antidepressants at 8 weeks but not 12 (Dam et al. 2022). Taken together, these new findings support previous evidence that cognition is separable from other symptoms in depression, although severity of cognitive impairment may be linked to delayed treatment response.

Therefore, understanding the neural correlates of cognitive impairments within depression is important, as this may help guide us towards treatments that are more effective. As might be

expected, these correlates are far from completely clear, but our present understanding is that structures such as the hippocampus and functional connectivity within the brain are involved.

1.2.3 The role of the hippocampus within depression

The hippocampus sits deep within the medial temporal lobe and is integral for episodic memory in animal and non-human primates (Roux et al. 2021). Synaptic plasticity within this brain region is an important and necessary mechanism for memory encoding and storage (Martin et al. 2000), which occurs here via appropriate activity-dependent remodelling of the hippocampus and its connections (Roux et al. 2021).

The hippocampus is a key region within the context of depression (Liu et al. 2017; Price and Duman 2020). There may be several reasons for this (Tartt et al. 2022): (i) its role in stress responses and the hypothalamic-pituitary-adrenal (HPA) axis (Sheline et al. 2019); (ii) its involvement in the limbic system and with regions associated with emotional processing and memory (such as the amygdala and the anterior cingulate cortex (ACC)) (Richardson et al. 2004); and (iii) its unusual capacity for neurogenesis into adulthood (unlike most other brain regions) (Eriksson et al. 1998). Thus, structural and functional changes within the hippocampus are suggested to play a major role in impaired cognition both within the context of depressive disorder and beyond (Roux et al. 2021)

Reduced hippocampal volume in depression has been a consistent finding (Santos et al. 2018; Schmaal et al. 2016; Sheline et al. 2019). It is associated with markers of disease progression and severity, such as the number of episodes (i.e. worse with increasing number), duration of illness (i.e. worse with early onset), and treatment resistance (Belleau et al. 2019; Schmaal et al. 2016).

Whether hippocampal volume depletion precedes or follows depression, and the exact role of chronic stress within this, is debated (Belleau et al. 2019). However, there is general agreement that reduced hippocampal volume probably results from reduced levels of neurotrophic factors (such as brain derived neurotrophic factor (BDNF). This reduces neurogenesis within the hippocampus leading to loss of synapses and their normal plasticity (Price and Duman 2020; Roux et al. 2021); this impairs the ability of neuronal connections to remodel and adapt in line with contextual environments (Price and Duman 2020).

In this way, depression also affects hippocampal functioning i.e. its connections with other brain regions and involvement in brain processes such as mood and cognition (Tartt et al. 2022). In untreated depression, when compared with healthy controls, the hippocampus appears to have impaired connections with both the amygdala (affecting mood) (Cullen et al. 2014; Hamilton and Gotlib 2008) and prefrontal cortex (affecting cognition) (Cao et al. 2012; Hao et al. 2020). Moreover, these connective abnormalities appear to correlate with depression severity (Cullen et al. 2014), indicating a potentially causal relationship.

1.2.4 The role of functional connectivity changes in depression

People with current or previous symptoms of depression also show changes within resting-state neural networks related to cognition compared with healthy volunteers. These include connections between individual regions, as well as between and within larger networks. For example, a large meta-analysis of resting-state fMRI data in depression identified that there was decreased functional connectivity within and between the prefrontal cortex and limbic regions, including the hippocampus (Kaiser et al. 2015). Abnormalities in functional connectivity also can be seen at the level of larger canonical networks, namely the default mode network (DMN, or

medial frontoparietal), central executive network (CEN, or lateral frontoparietal), and salience network (SN, or midcingulo-insula) (Bhaumik et al. 2017; Dong et al. 2019; Neufeld et al. 2018; Wang et al. 2020). Interconnections are frequent between these three functional networks and thus they are known as the "triple network"(Menon 2011). In depression, alterations in functional connectivity within the triple network appear to occur in both directions depending on the context (i.e. decreased resting-state functional connectivity (rsFC) between the DMN and CEN, and increased rsFC between the SN and the CEN (Dong et al. 2019)).

These neural network abnormalities seen in depression are suggested to impair cognitive control and flexibility, information processing for both hot and cold cognition, and engender negative biases of self, others and the environment (Price and Duman 2020). For example, large human datasets show that appropriate connections between these specific networks are directly associated with successful cognitive performance (Shen et al. 2018), and both the type / severity of behavioural cognitive dysfunction and abnormal neural functional connectivity have been suggested as vulnerability markers for future episodes of depression (Langenecker et al. 2018; Marchetti et al. 2012). This highlights the interconnected nature of these deficits in terms of clinical outcomes. Interestingly, using resting-state fMRI in a large dataset, serotonin receptor neural networks specifically linked to the 5-HT_4 receptor were found to have greater functional connectivity in the parietal cortex and operculum associated with depression diagnoses, symptoms of low mood, anxiety and panic, and slower behavioural responsiveness to reward (Salvan et al. 2022). This also suggests a role for specific receptor types in functional connectivity within mental illnesses such as depression, including the 5-HT_4 receptor.

1.2.5 Summary

The cause of depression is poorly understood. It is probable that many aetiologies exist across the population, although there seem to be consistent findings at the group level (such as suboptimal synaptic plasticity and hippocampal abnormalities). The hippocampus is thought to be structurally and functionally different in individuals who have depression compared to healthy controls. More broadly, functional connectivity in the brain has been shown to be affected in depression. Neural abnormalities, in particular those relating to the hippocampus and functional connectivity, are associated with cognitive impairment, a significant problem for many patients with depression affecting their ability to function in daily life.

1.3 Cognitive deficits exist within mental illness more broadly

Cognitive deficits also occur across other mental disorders outside of depression. Some are more fluctuating in nature, and some appear to follow a more persistent or even show a progressive course (Etkin et al. 2013).

Schizophrenia has been associated with problems in cognition since its first description as "dementia praecox", with episodic memory deficits being central to the disorder (Guo et al. 2019a). A recent longitudinal cohort study determined that cognitive decline in schizophrenia begins over a decade before the first episode of psychosis, indicating that there may be a role for medication to address cognitive impairment even before antipsychotics are usually prescribed (Jonas et al. 2022). Although longitudinal data is limited, individuals with other psychotic disorders also demonstrate similar (although less extreme) cognitive deficits (Sheffield et al. 2018). In bipolar disorder, for example, it seems that patients have normal IQ in childhood (Zammit et al. 2004), but that cognitive impairments also predate the start of illness as deficits

are already discernible when patients present with their first psychotic symptoms (Bora and Pantelis 2015).

Cognitive deficits also occur within the neurodegenerative disorders, Alzheimer's disease (Joe and Ringman 2019) and Parkinson's disease (Das et al. 2019). Impairments within both of these disorders are generally progressive in nature, namely dementia, with episodic memory impairments featuring early and increasing with time as broader cognitive deficits occur.

Therefore, any medication with potential pro-cognitive benefits may also be of interest outside of depression in terms of therapeutic potential (Millan et al. 2012). However, whether a single pro-cognitive agent may target deficits across all disorders is uncertain (Colwell et al. 2022; Roux et al. 2021), and it is possible that progressive neurodegeneration such as that seen in the dementias may require agents with different properties.

1.4 A role for the 5-HT_4 receptor in mood and cognition

Agonism at the serotonin$_4$ receptor subtype (5-HT_4) receptor has recently been proposed as a potential target to improve mood and cognition within humans (Murphy et al. 2021). Studies suggest that 5-HT_4 agonism leads to behavioural improvements in animal models tapping learning and memory, and low mood / anxiety, alongside compatible molecular and neurochemical changes.

1.5 Characteristics of the 5-HT_4 receptor

1.5.1 Location

Since its first description almost 35 years ago (Dumuis et al. 1988), the 5-HT_4 receptor has been discovered both in the brain as well as in various peripheral locations including the heart (Kaumann 1990), gut (Craig and Clarke 1990), adrenal glands (Idres et al. 1991) and bladder (Tonini and Candura 1996). In the mammalian brain, 5-HT_4 receptors are located postsynaptically, and expressed in areas involved in the processes of emotion and cognition, including the basal ganglia, hypothalamus, hippocampus, amygdala, and neocortex (Cai et al. 2002; Compan et al. 1996; Roychowdhury et al. 1994; Vilaró et al. 2005). 5-HT_4 receptors are mainly situated on gamma-aminobutyric acid (GABA) neurones in the nucleus accumbens and striatum (Compan et al. 1996), glutamate pyramidal cells in the medial prefrontal cortex and hippocampal CA regions (Cai et al. 2002; Vilaró et al. 2005) and hippocampal granule cells in the dentate gyrus (Vilaró et al. 2005). 5-HT_4 receptors have also been found in the rat dorsal raphe nucleus (DRN) in autoradiographic studies (Varnäs et al. 2003; Vilaró et al. 2005). However, as mRNA encoding 5-HT_4 receptors does not seem to be present here, it is likely that they are located on DRN afferent neurons rather than DRN serotonin cells *per se* (Vilaró et al. 2005).

Human PET studies confirm that 5-HT_4 receptors have a high density in the caudate, putamen, and nucleus accumbens, with lower expression across cortical and limbic areas, including the hippocampus and amygdala (Beliveau et al. 2017). Low levels of 5-HT_4 binding are also reported in the thalamus and raphe nuclei (Beliveau et al. 2017). Similarly, the highest levels of human 5-HT_4 receptor mRNA have been found in the caudate and putamen, with lower levels in the amygdala and hippocampus (Medhurst et al. 2001). Post-mortem human studies support these findings with 5-HT_4 receptors in the basal ganglia (caudate nucleus, putamen, nucleus accumbens, globus pallidus and substantia nigra), as well as the hippocampus (particularly in the CA1 region), and an

intermediate density in the neocortex and amygdala (Bonaventure et al. 2000; Varnäs et al. 2003). Similar studies confirm that these receptors are lost in significant numbers in neurodegenerative cognitive diseases, especially in the hippocampus (Reynolds et al. 1995), indicating the clinical relevance of these receptors.

1.5.2 Molecular effects

5-HT_4 receptors are transmembrane-spanning excitatory G protein-coupled receptors. Their activation results in increased neuronal excitation mediated via adenylyl cyclase and an increase in cyclic AMP (cAMP) (Bockaert et al. 2008; Dumuis et al. 1988). However, interestingly, 5-HT_4 receptor activation also initiates several important G protein-independent pathways, including the activation of tyrosine kinase Src and, in turn, neuronal ERK (extracellular signal-regulated kinase) (Barthet et al. 2007). These latter mechanisms are known to be important particularly for synaptic plasticity in the hippocampus via long-term potentiation (Norman et al. 2000).

5-HT_4 receptors in the prefrontal cortex (PFC) control the firing rate of serotonergic cells in the midbrain. For example, 5-HT_4 receptor knockout mice have half the basal firing rate of DRN 5-HT cells compared to wildtype mice (Conductier et al. 2006). Furthermore, drug-induced 5-HT_4 receptor activation of medial PFC cells excites 5-HT cells in the DRN, which in turn produces a rapid and sustained increase in basal firing here (Faye et al. 2019; Lucas et al. 2005; Lucas and Debonnel 2002). Surprisingly, this increase in firing can be seen within 30 minutes of the administration of the highly-selective 5-HT_4 receptor agonists, prucalopride and RS67333, suggesting that agonism might have the potential to have rapid clinical effects (Lucas et al. 2005; Lucas and Debonnel 2002). This excitation of raphe cells contrasts sharply with the decrease in

raphe cell firing seen with SSRIs after initial administration, a molecular mechanism probably associated with their delayed onset of action (Blier et al. 1998).

1.5.3 General neurochemical effects

5-HT_4 receptors also indirectly affect neuronal function through their modulation of neurotransmitter release and neuroplasticity proteins. Focussing firstly on neurotransmitters, this includes increased release of acetylcholine (Consolo et al. 1994; Johnson et al. 2012; Siniscalchi et al. 1999), as well as GABA (Bijak and Misgeld 1997), dopamine (Bockaert et al. 2004; 2008) and histamine (Johnson et al. 2012).

The acetylcholine release stimulated by 5-HT_4 receptor agonism may be particularly important as a mechanism of improved cognition (Hagena and Manahan-Vaughan 2017; King et al. 2008; Rebholz et al. 2018). 5-HT_4 receptor activation in animals enhances the release of acetylcholine in the frontal cortex and hippocampus (Consolo et al. 1994; Siniscalchi et al. 1999). Initially this was suggested to relate to increased activity in the nucleus basalis, the hub of cholinergic innervation to the cortex and hippocampus (Orsetti et al. 2003). However, a dual label in-situ hybridisation study found that 5-HT_4 mRNA was not detected in basal forebrain cholinergic cells, indicating that 5-HT_4 receptors are not synthesised by cholinergic cells *per se*. Instead, 5-HT_4 receptors do appear to be synthesised by GABA and glutamate neurons in and around the basal forebrain (Penas-Cazorla and Vilaro 2015). This has subsequently been supported by findings that the 5-HT_4 receptor agonist, prucalopride, similar to ketamine, alters synaptic transmission via the APMA glutamate receptor (Chen et al. 2020). However, the 5-HT_4 receptor does seem important for acetylcholine release in the frontal cortex and hippocampus as these effects are blocked by 5-HT_4 receptor antagonists (Murphy et al. 2021). Therefore, 5-HT_4 receptors are an important and

necessary part of the cholinergic neural system, although probably acting indirectly through other neurotransmitters, such as glutamate and GABA.

For the last twenty years, we have understood that the clinical effects of antidepressants may be mediated through increased neurogenesis and synaptic plasticity, via an increased production of neuroplasticity proteins (D'Sa and Duman 2002; Duman 2002). A few weeks of SSRI administration to animals typically leads to increased expression of the neuroplasticity proteins, BDNF and CREB, as well as hippocampal cell proliferation (Malberg et al. 2000; Warner-Schmidt and Duman 2006). 5-HT_4 receptor agonists can have similar effects, but importantly much more quickly (Ishizuka et al. 2014; Lucas et al. 2007; Pascual-Brazo et al. 2012). For example, after seven days of 5-HT_4 agonism these effects are equivalent to that seen with 14 to 21 days of SSRI administration (Malberg et al. 2000; Pascual-Brazo et al. 2012; Samuels et al. 2016). In fact, the first neuroplastic changes are identifiable after only three days administration of a 5-HT_4 agonist (Lucas et al. 2007; Pascual-Brazo et al. 2012). Thus, 5-HT_4 receptor agonists may modulate neural plasticity in a similar way to SSRIs; that they can do so more quickly than SSRIs suggests a potential niche as rapidly-acting medication.

1.5.4 5-HT_4 receptor action specifically within the hippocampus

5-HT_4 receptors have been identified on hippocampal glutamatergic neurones (King et al. 2008), which is the largest cellular population within the hippocampus. From a neurochemical point of view, 5-HT_4 receptors are involved here in synaptic plasticity including the cellular processes of memory: long-term potentiation (LTP) and its inverse, long-term depression (LTD) (Murphy et al. 2021). Overall, 5-HT_4 receptor activation seems to promote information encoding preferentially through LTP in the dentate gyrus and CA1 (the area associated with “place cells” that activate

when stimulated by specific locations (O'Keefe and Recce 1993)), and does so over any encoding via LTD. In fact, 5-HT_4 receptor activation seems to prevent persistent LTD in all hippocampal subfields (Kemp and Manahan-Vaughan 2005; Marchetti-Gauthier et al. 1997). However, the exact process that occurs within each hippocampal region appears to vary across different subunits due to the differential expression of 5-HT_4 receptor isoforms (a,c,n) (Agrawal et al. 2020; Hagena and Manahan-Vaughan 2017).

Furthermore, electrophysiological recordings have identified that prucalopride administration mediates synaptic plasticity in CA3 via modulation of glutamate AMPA receptors (Chen et al. 2020), and that RS67333 administration does this in CA1 via modulation of GABA pathways (Lecouflet et al. 2020). In this way, 5-HT_4 receptors appear to support the nuanced encoding of different information within the hippocampus due to the various processes by which this can be conducted (Hagena and Manahan-Vaughan 2017; Marchetti et al. 2004; Twarkowski et al. 2016), and to ensure a “reset” of plasticity to a baseline level when required (Roux et al. 2021). Therefore, neurochemically, functions in the hippocampus are complex, but relate to different processes of learning and memory, and also modulation of glutamate and GABA neurotransmission.

5-HT_4 receptors have other effects within the hippocampus that are important for cognition. For example, as described earlier, 5-HT_4 receptors have the capacity to stimulate G-protein independent processes (Barthet et al. 2007) that play a role in synaptic plasticity by potentiating hippocampal spine growth (which in turn is known to influence learning and memory (Kozono et al. 2017; Restivo et al. 2008; Schill et al. 2020)). This occurs from embryonic development onwards (Agrawal et al. 2019). In addition, theta oscillations affect cellular excitability and are paced by acetylcholine inputs (Roux et al. 2021). 5-HT_4 receptor agonists also are known to

increase theta oscillations in the hippocampus (Johnson et al. 2012; Wang et al. 2022), and also to decrease apoptosis of hippocampal neurones (Hashemi-Firouzi et al. 2021), both of which have been linked to improved cognitive performance.

Finally, 5-HT_4 receptors may also influence inflammatory processes in the hippocampus. In an early-onset mouse model of Alzheimer's disease, chronic 5-HT_4 receptor activation reduced levels of two important pro-inflammatory mediators (IL-1β and MCP-1) in the entorhinal cortex, which normally promote astrogliosis and neuronal loss (Baranger et al. 2017). 5-HT_4 receptor activation may therefore prevent the early destruction of glial cells and thus support an optimal immune environment in the brain.

1.6 Animal studies examining the role of 5-HT_4 receptors in learning and memory

1.6.1 Overview and breadth of pro-cognitive effects

5-HT_4 partial agonists have shown promising behavioural results on learning and memory tasks in animals. In particular, rodents have shown improved performance during learning and memory tasks (Hagena and Manahan-Vaughan 2017; King et al. 2008), with effects obvious after only a few doses (Lamirault and Simon 2001; Lucas et al. 2007). This appears consistent for hippocampal-dependent learning and memory (Hagena and Manahan-Vaughan 2017), including object recognition (Freret et al. 2012; Lamirault and Simon 2001; Levallet et al. 2009), spatial learning (Fontana et al. 1997; Lelong et al. 2001), and place recognition (Lamirault and Simon 2001; Orsetti et al. 2003), as well as olfactory-associated learning (Marchetti et al. 2004; Marchetti et al. 2011), social memory (Letty et al. 1997) and attention (using the five choice serial reaction time task) (Hille et al. 2008). There have also been positive effects in animals with

advanced age (Lamirault and Simon 2001) and in animal models of Alzheimer's and Parkinson's disease (Baranger et al. 2017; Giannoni et al. 2013; Guo et al. 2019b; Wang et al. 2022). There is evidence that these benefits persist with chronic agonism (14 to 30 days) (Hashemi-Firouzi et al. 2021; Quiedeville et al. 2015), and pre-administration of 5-HT_4 agonists prior to training seems to show the most consistent results (Lamirault and Simon 2001; Lo et al. 2014; Meneses and Hong 1997; Orsetti et al. 2003).

1.6.2 5-HT_4 receptor agonism and cognition in animal studies, and the relationship with acetylcholine

Benefits of 5-HT_4 receptor agonists in terms of performance on learning and memory tasks have been particularly linked with acetylcholine release, although this may be mediated by non-cholinergic cell populations (Penas-Cazorla and Vilaro 2015). There are two main bodies of evidence for this: (i) reversal of anticholinergic-induced cognitive impairments with 5-HT_4 agonists (Cachard-Chastel et al. 2008; Fontana et al. 1997; Lo et al. 2014; Marchetti-Gauthier et al. 1997; Moser et al. 2002); and (ii) enhancement of the effects of 5-HT_4 agonists on behavioural cognition with acetylcholinesterase inhibitors (Cachard-Chastel et al. 2007; Freret et al. 2012; Lamirault et al. 2003; Mohler et al. 2007; Moser et al. 2002). For example, Lo and colleagues demonstrated that pre-administration of the muscarinic antagonist, scopolamine, impaired rodent performance on tasks of spatial learning and contextual fear. However, these impairments lessened when scopolamine was given alongside the selective 5-HT_4 agonist, SSP-002392 (Lo et al. 2014). Moreover, this occurred in a dose-dependent manner with the highest dose of SSP-002392 completely preventing any impairment, and SSP-002392+scopolamine-treated animals showing improved spatial retention memory compared to those receiving only scopolamine for at least the following three days (Lo et al. 2014). Furthermore, scopolamine-induced deficits in spatial learning and memory were reversed with even low doses of the 5-HT_4 agonist, prucalopride,

when it was given alongside the acetylcholinesterase inhibitor, donepezil (Cachard-Chastel et al. 2007). Interestingly 5-HT_4 receptor agonist action may also promote secretion of a neurotrophic protein that adversely affects the neurotoxic amyloid-β peptide, part of the pathophysiology of Alzheimer's disease (Lecoutey et al. 2014). For these reasons, donecopride has been developed with both cholinesterase inhibitor and 5-HT_4 agonist properties potentially for Alzheimer's disease.

Interestingly, 5-HT_4 receptor knock-out mice do not appear to demonstrate learning and memory behavioural deficits as might be expected (Segu et al. 2010). However, low-dose scopolamine seems to reveal otherwise hidden impairments in the long-term spatial memory of knock out rodents (Segu et al. 2010). Therefore, it is possible these animals undergo early developmental adaptations to minimise downstream impacts on the cholinergic system but they may retain some vulnerability to cognitive deficits when exposed to a minor challenge.

1.6.3 5-HT_4 receptor agonism and cognition in studies relating to sleep

Sleep impairments are known to adversely affect behavioural cognition, likely related to impacts on neural processing during memory acquisition and consolidation (Dag et al. 2019). In a total sleep deprivation condition in rodents, intra-hippocampal (CA1) injection of the 5-HT_4 agonist, RS67333, administered pre-training, reversed the otherwise deleterious effects on memory acquisition (Eydipour et al. 2020).

1.6.4 5-HT_4 receptor activation and cognition in animal models of low mood or anxiety

5-HT_4 receptor activation also appears to be able to improve cognition in animal models of low mood or anxiety. Chronic administration of the 5-HT_4 receptor agonist, RS67333, improved

various learning and memory deficits occurring secondary to corticosterone, whereas chronic fluoxetine led to only partial improvement (Darcet et al. 2016). Furthermore, the 5-HT_4 receptor agonist, RS67333, improved emotional memory performance in a passive avoidance test in the Flinders Sensitive Line rat model of depression (Eriksson et al. 2012).

1.6.5 5-HT_4 receptor antagonism in animal models of cognition

There are far fewer studies assessing 5-HT_4 receptor antagonism and cognition. More recent studies suggest, intriguingly, that antagonism alone may have similar effects to agonism. The 5-HT_4 receptor antagonist, RS23597, was able to reverse the deleterious effects of total sleep deprivation on memory acquisition; a 5-HT_4 agonist had the same effect in this study (Eydipour et al. 2020). The 5-HT_4 antagonist, GR113808, as well as the 5-HT_4 agonist, BIMU-8, both improved working memory in substantia nigra pars compacta-lesioned rats (analogous to Parkinson's disease) (Guo et al. 2019b). This finding was replicated in rodents with lesions in the medial forebrain bundle (another model of Parkinson's disease) using the same 5-HT_4 receptor agonist and antagonist (BIMU-8 and GR113808) (Wang et al. 2022). However, earlier studies found that 5-HT_4 agonist pro-cognitive effects were abolished when they were co-administered with 5-HT_4 receptor antagonists (Fontana et al. 1997; Lamirault and Simon 2001; Mohler et al. 2007; Moser et al. 2002). This suggests some complexity relating to whether agonists and antagonists are administered at the same time or separately in terms of the resulting behavioural impact.

1.6.6 Summary

5-HT_4 receptor agonists show consistent behavioural benefits in animal models of learning and memory, and may have particular clinical potential for ameliorating the cognitive deficits that occur with low mood or poor sleep. This is closely linked to their capacity to release acetylcholine

in brain regions linked to cognition, and to have specific effects on the hippocampus (such as promoting cell proliferation and spiny growth, potentiating theta oscillations, and inhibiting apoptosis). Findings with receptor antagonists are mixed but this may relate to timing of administration.

1.7 Animal studies examining the role of 5-HT_4 receptors in mood and anxiety

1.7.1 Overview and breadth of effects on mood and anxiety

Further to their specific role in learning and memory, animal behavioural studies have also demonstrated that 5-HT_4 receptor agonists are rapidly active in models designed to capture anhedonic behaviour. For example, selective 5-HT_4 receptor agonism reduced immobility in the forced swim test with a single dose of prucalopride (Lucas et al. 2007) and a few days of RS67333 (Gomez-Lazaro et al. 2012; Pascual-Brazo et al. 2012). In addition, three days of RS67333 increased sucrose consumption in the chronic mild stress model (Lucas et al. 2007) and in the corticosterone model (Pascual-Brazo et al. 2012). Three days of RS67333 also was sufficient to reduce excess activity in the olfactory bulbectomy model (Lucas et al. 2007). With a week of RS67333, a complete reversal of the effects of chronic corticosterone on sucrose consumption was seen, similar to the effect of three weeks of fluoxetine (Pascual-Brazo et al 2012). Therefore, 5-HT_4 receptor agonists consistently ameliorate anhedonia-like behaviours in rodents produced by chronic mild stress and corticosteroids, and similar models.

1.7.2 5-HT_4 receptor agonism and speed in animal models of low mood / anxiety

An important facet of these results is the rapidity of action with which 5-HT_4 receptor agonists produce effects compared to SSRIs (Gomez-Lazaro et al. 2012; Lucas et al. 2007; Pascual-Brazo et

al. 2012). These rapid behavioural results are supported by similar findings neurochemically. Three days of 5-HT_4 receptor agonism resulted in desensitisation of 5-HT_{1A} autoreceptors, increased CREB phosphorylation and increased hippocampal cell proliferation (Lucas et al. 2007), effects typically seen after two or three weeks of SSRI use.

1.7.3 5-HT_4 receptor agonism is involved with SSRI action

5-HT_4 receptors also contribute to both the behavioural and neurochemical action of classic antidepressants (SSRIs) in animal models, although there is some inconsistency across studies (Cryan and Lucki 2000). First, 5-HT_4 receptor agonists are able to modify the effects of SSRIs. For instance, the 5-HT_4 receptor agonist, RS67333, potentiated the impact of acute paroxetine on serotonin tone in the rat hippocampus (Licht et al. 2010). Second, impairing 5-HT_4 receptor function reduces the antidepressant effect of SSRIs. For example, in a chronic steroid model of depression / anxiety, GR125487, a 5-HT_4 receptor antagonist, blocked the behavioural and neurochemical effects of fluoxetine (Mendez-David et al. 2014). This is further supported by 5-HT_4 knock out mice studies where fluoxetine was unable to reverse the negative effects of olfactory bulbectomy c.f. wildtype mice (Amigo et al. 2016) and where hippocampal cell proliferation in the dentate gyrus did not occur c.f. wildtype mice (Imoto et al. 2015). Third, chronic administration of monoaminergic antidepressants involving serotonin, such as paroxetine, fluoxetine, and venlafaxine, decreases 5-HT_4 receptor density in the rodent basal ganglia and hippocampus (Licht et al. 2009; Vidal et al. 2010; Vidal et al. 2009). Thus, 5-HT_4 receptor activation seems necessary for SSRIs to have an antidepressant / anxiolytic effect (Murphy et al. 2021).

Intriguingly, administering 5-HT_4 receptor agonists with SSRIs prevents the typical reduction in raphe firing that usually results on SSRI initiation (Lucas et al. 2010). A recent study using

transcriptomic-neuroimaging mapping to study the effects of fluoxetine during optogenetic activation of the DRN (designed specifically to release serotonin) suggests a possible mechanism for this: fluoxetine upregulates DRN activation of serotonin receptor neural networks specifically related to the 5-HT_4 receptor (and downregulates activation of those related to the 5-HT_{1A} autoreceptor) (Salvan et al. 2022). That is, SSRI administration may alter interactions between brain regions expressing 5-HT_4 receptors, and potentially increase target response for a 5-HT_4 receptor agonist. This indicates that 5-HT_4 receptor agonists may also have clinical potential as an antidepressant augmentation strategy.

1.7.4 5-HT_4 receptor agonism and anxiolysis

5-HT_4 receptor agonists have also been shown to lead to rapid anxiolysis in rodents (Chen et al. 2020; Faye et al. 2019; Mendez-David et al. 2014; Pascual-Brazo et al. 2012). Although it is challenging to model anxiety separately from low mood in rodent models, anxiety is typically assessed by measuring feeding or pleasurable activities in novel, open or exposed environments. A week of RS67333 reduced latency to feed in the novelty suppressed feeding (NSF) paradigm in the same manner as two weeks of fluoxetine (Pascual-Brazo et al. 2012). Interestingly, replications of this study have been mixed. Mendez-David and colleagues did not replicate this finding although they did find reduced anxiety-related behaviour in the open field and elevated plus maze with seven days of RS67333, and an even broader effect at 28 days administration (Mendez-David et al. 2014). However, more recently, using a single dose of RS67333, Faye and colleagues did replicate anxiolysis according to these same three assessments (elevated plus maze, the open field and NSF) and Charousaei and colleagues replicated anxiolysis using the elevated plus maze (Charousaei et al. 2021; Faye et al. 2019). A single dose of RS67333 also resulted in reduced anxiety in a model of chronic cannabinoid exposure (Abboussi et al. 2016).

Interestingly, anxiolysis with 5-HT_4 agonism seems rapid and independent of hippocampal neurogenesis (unlike SSRIs) (Mendez-David et al. 2014). Therefore it may relate to direct activation of medial prefrontal cortex 5-HT_4 receptors (Faye et al. 2019) and involve AMPA or NMDA receptor modulation (Charousaei et al. 2021; Chen et al. 2020). However, the findings in animal models of anxiety are difficult to separate from antidepressant potential due to limitations in translating human symptoms into distinct rodent models of psychopathology.

1.7.5 5-HT_4 receptor antagonism in animal models of depression and anxiety

Studies examining the effect of 5-HT_4 receptor antagonism in animal models of depression or anxiety have been sparse and mixed. Two studies found reduced anxiety on the elevated plus maze with a single dose of the 5-HT_4 receptor antagonists, SB204070, GR113808 (Silvestre et al. 1996) or SB207266A (Kennett et al. 1997; Silvestre et al. 1996). However, a single dose of similar agents (SDZ205-557, GR113808, or SB204070) found no effect on rodent behaviour in the light / dark choice test (Costall and Naylor 1997). Interestingly, and analogous to the cognitive data with antagonism, Bell and colleagues noted that both a 5-HT_4 agonist *and* antagonist separately led to reduced anxiety on the elevated plus maze when compared with diazepam control (Bell et al. 2014). However, this latter finding has not been replicated.

1.7.6 Summary

5-HT_4 receptor agonists consistently ameliorate anhedonia-like behaviours and anxiety-like behaviours in rodents, across various models and with different agonists. They are also associated with release of neurotrophic proteins needed for neurogenesis. These effects occur more quickly than that seen with SSRIs, and 5-HT_4 agonism seems a necessary part of the mechanism by which

SSRIs are clinically effective. Similar to effects on cognition, findings with 5-HT_4 receptor antagonists are mixed.

1.8 A potential role for 5-HT_4 receptors within other mental disorders

1.8.1 Overview

In addition to cognitive and mood / anxiety disorders, 5-HT_4 receptor agonists have been suggested to have a potential therapeutic role in other psychiatric disorders, namely psychosis and eating disorders and eating disorders / addiction.

1.8.2 Psychosis and schizophrenia

Serotonin has trophic effects, and thus abnormal serotonin signalling related to impaired 5-HT_4 receptor activity has been suggested to decrease neuronal survival (Cho and Hu 2007). This may be particularly affected in schizophrenia (Eggers 2013). Glutamate signalling is also disrupted in psychotic disorder, and 5-HT_4 receptors are linked to glutamate receptors in various ways. For example, 5-HT_4 receptor stimulation modulates glutamate signalling via AMPA receptors, and gene expression correlations exist between 5-HT_4 receptors and both AMPA and NMDA receptors (Chen et al. 2020; Park et al. 2021). Therefore, 5-HT_4 receptor dysfunction may affect both serotonin and glutamate pathways suggested to be relevant for psychosis.

In human studies, the severity of reduced hippocampal volume in first episode psychosis correlates with the density of 5-HT_4 hippocampal receptors (Park et al. 2021). Furthermore, as cognitive impairments are common in psychosis, and appear to link to both development and

progression of the disorder (Jonas et al. 2022; Karcher et al. 2022), the pro-cognitive potential of 5-HT_4 agonism may be of particular interest.

1.8.3 Addiction and eating disorders

There is also biological plausibility for 5HT_4 agonism to be involved in the overlap between addiction and anorexia. Motivation to self-restrict in anorexia despite the need for energy is thought to involve dysfunction in the nucleus accumbens (Bockaert et al. 2011; Schwartz et al. 2000), a vital region for reward processing and addiction. The stimulant drug, MDMA, acts via 5-HT_4 receptor activation within the nucleus accumbens to reduce feeding (Jean et al. 2007). Thus, dysfunction of eating due to 5-HT_4 receptor modulation in the nucleus accumbens may be part of the mechanism by which anorexic animals become "dependent" on diet restriction (Jean et al. 2012). However, detailed studies on 5-HT_4 receptor activity within the wider context of substance misuse and other eating disorders remain pending.

1.9 5-HT_4 receptors and the gut-brain axis

1.9.1 Overview: the gut-brain axis may be another mechanism for the effects of 5-HT_4 receptor agonism

5-HT_4 receptors may also exert behavioural and neural effects via the gut-brain axis. The nervous systems of the brain and the gut (the latter known as the enteric nervous system (ENS)) are closely connected via several mechanisms. For example, via the autonomic nervous system, limbic areas have descending projections to the ENS (Furness 2012), and the adrenal cortex and medulla release various neurotransmitters (including serotonin) to control gut functioning via the

ENS (Smith and Vale 2022). 5-HT_4 receptors are highly expressed on enterochromaffin cells and in ENS neurones, which when activated modulate gut activity (via the release of acetylcholine affecting smooth muscle functioning) (Agrawal et al. 2020). Focus has centred on two potential and possibly overlapping mechanisms for a specific gut-brain connection involving 5-HT_4 receptors: the microbiome and its interaction with the immune system (Margolis et al. 2021) and modulation of serotonin itself (Shine et al. 2022).

1.9.2 The microbiome and a link with mental disorder

The microbiome (the billions of commensal micro-organisms that line our gut) has recently received attention for its role in facilitating communication between the gut and the brain (Margolis et al. 2021). Of particular relevance, this impact is known to extend throughout the lifespan (Margolis et al. 2021) and to involve both cognition (Gareau 2014) and mood (Cryan and Dinan 2012). Cross-sectional human studies suggest that changes in the diversity and amounts of microbiota and microbial metabolites are associated with many different brain disorders, including depression and disorders of cognition (e.g. Parkinson's disease, and Alzheimer's disease) (Bastiaanssen et al. 2019). However, these studies have struggled to provide consistent results that demonstrate causality in the relationship; a common issue with cross-sectional studies (Long-Smith et al. 2020). This makes it difficult to determine how much weight to place on the role of the microbiome in cognition and depression at this time.

1.9.3 The microbiome and one potential mechanism for an interaction with mental health: immune modulation

The microbiome and gut-signalling are also affected by immune mechanisms. For example, gut bacteria may release lipopolysaccharide (LPS, an immune agonist), which then accesses the brain

via the systemic circulation. LPS typically causes “sickness behaviour” and is linked with the development of depressive and anxious behaviours in rodents and humans (Lasselin et al. 2020). A growing number of studies indicate that people with depression have differences in their gut microbiome compared to healthy individuals, although the exact manner of the differences vary between studies (Kelly et al. 2016; Valles-Colomer et al. 2019; Zheng et al. 2016). This inconsistency in study outcomes may be due to the different technologies used for assessment, and other influences that affect individual composition and function of gut microbiota (e.g. age, type of diet, use of antibiotics) (Margolis et al. 2021). Nonetheless, intriguingly, transplanting the microbiome of depressed individuals into a healthy rodent leads to altered neurocognitive outcomes and depressive-like behaviours in the animal, indicating that microbiota abnormalities may be strongly implicated in the aetiology of abnormal neural functioning (Kelly et al. 2016; Zheng et al. 2016)

1.9.4 The gut-brain axis and the role of serotonin

Furthermore, serotonin levels in the brain are intimately connected with, and possibly an extension of, those found in the gut. 5-HT neurones appear in the gut in embryonic development and modulate the development of other neurotransmitter neurones (Takaki et al. 2014). In adulthood, variations in serotonergic tone may facilitate different levels of cognitive functioning with higher gut serotonin levels enabling recruitment of networks to enhance cognitive flexibility (Shine et al. 2022). Moreover, 5-HT_4 receptors appear to be activated by normal gut micro-organisms to help induce maturation of the adult enteric nervous system. For instance, mice kept in a germ-free environment have slower gut transit time and an abnormally high degree of nestin-expressing neuronal stem cells. Both of these normalise following bacterial colonisation, which is dependent on signalling via 5-HT_4 receptors (de Vadder and Mithieux 2018).

In addition, many bacteria express tryptophan decarboxylase enabling them to turn dietary tryptophan (the precursor of serotonin) into tryptamine. Microbially-derived tryptamine is then able to affect gut motility via 5-HT_4 receptors (Bhattarai et al. 2018). However, it is not yet clear whether bacterial-derived tryptamine is able to reach the brain in order to influence neural processing directly (Margolis et al. 2021). Serotonin functioning may also be affected in the gut by abnormalities within the HPA system: gut functioning is affected via 5-HT_4 receptors due to an increased release of steroid hormones due to stress (Agrawal et al. 2020) and chronic stress is intrinsically linked with depression (Pitsillou et al. 2020; Sheline et al. 2019).

1.9.5 Summary

Varied systems connecting the gut and the brain (both of which involve 5-HT_4 receptors) provide an alternative, but not mutually exclusive, mechanism for any potential clinical impact of agonists at this receptor.

1.10 Preliminary human work to translate these findings

1.10.1 Evidence from 5-HT_4 receptor binding studies

PET studies have highlighted how 5-HT_4 receptor binding varies in both cognition and depression. There appears to be an inverse correlation between 5-HT_4 receptor binding and verbal memory scores, both in the hippocampus (Haahr et al. 2013) and more generally (Stenbæk et al. 2017). When serotonin tone was increased with several weeks of SSRI use in healthy males, it led to reduced 5-HT_4 receptor binding (Haahr et al. 2014), and so binding at this receptor possibly represents a real-time serotonin tone biomarker. That is, 5-HT_4 receptor availability depends on central serotonin availability, and there is an inverse relationship between endogenous serotonin

and 5-HT_4 receptor binding in healthy individuals. Therefore, healthy participants with lower memory scores may have increased 5-HT_4 receptor binding associated with a low central serotonin tone.

This is consistent with findings in early Alzheimer's disease that demonstrate increased 5-HT_4 receptor binding here correlating positively with beta-amyloid load (and negatively with cognitive test scores (MMSE)) (Madsen et al. 2011). Thus, low levels of central serotonin (which may lead to a compensatory increased 5-HT_4 receptor binding) could also explain cognitive deficits within this disorder. This is particularly interesting, as this compensatory increase in 5-HT_4 receptor expression may also help to counteract the effects of accumulating beta-amyloid in Alzheimer's; agonism at this receptor can also help increase alpha-secretase activity and speed up degradation of amyloid precursor protein (Cachard-Chastel et al. 2007; Knudsen and Hasselbalch 2021).

However, the picture is complicated in the presence of depression, and we are still working towards a full understanding. Patients with depression who were medication free had lower 5-HT_4 receptor binding than healthy controls (Köhler-Forsberg et al. 2021). Interestingly, this may support the findings of an early post-mortem case-control study, where a higher density of 5-HT_4 receptors in the caudate and frontal cortex was seen in depressed violent suicide victims compared to healthy controls (Rosel et al. 2004). However, lower 5-HT_4 binding at baseline within an unmedicated depression population was also associated with a higher concurrent anxiety at this time point (Köhler-Forsberg et al. 2022). This may indicate that higher levels of serotonin are related to anxiety symptoms with depression, and also provide a possible explanation for the temporary increase in anxiety common with the onset of SSRIs treatment (when exogenous serotonin increases)(Köhler-Forsberg et al. 2022). Similar to healthy participants, SSRI medication reduced 5-HT_4 receptor binding for all patients with depression, although the subset who

responded to SSRI medication had significantly lower 5-HT_4 receptor binding than controls (whereas those who did not respond did not differ significantly from controls) (Köhler-Forsberg et al. 2021). However, in those with increased genetic risk due to depression in a first-degree relative, lower striatal 5-HT_4 receptor binding was a risk factor for future development of depression (Madsen et al. 2014).

1.10.2 5-HT_4 receptor genetic studies

There is limited evidence that genetic polymorphisms of the 5-HT_4 receptor may be relevant for both depression and response to antidepressant medication. A case-control study reported associations between unipolar depression and polymorphisms in the splice variant region of the 5-HT_4 receptor gene (Ohtsuki et al. 2002). A GWAS (genome-wide association study) involving 706 patients from the European GENDEP project (genome-based therapeutic drugs for depression) assessed 5-HT_4 receptor genetic polymorphisms and response to antidepressants (either 12 weeks of escitalopram (n = 394) or nortriptyline (n = 312)). For the nortriptyline group, the intronic 5-HT_4 receptor genetic polymorphism, rs1345697, was found to be the strongest associated marker within the 5-HT_4 receptor gene relating to treatment response (Uher et al. 2010). However, this result did not reach statistical significance and was not assessed following longer-term administration. These findings were explored using the METADAP cohort, where homozygotes for the GG variant of this 5-HT_4 receptor genetic polymorphism, rs1345697, were less likely to improve clinically with antidepressants when compared to those with the A allele (Poinsignon et al. 2022). Although these results were prospectively assessed they used observational data, were conducted post-hoc, and there was a large attrition rate over the course of the study (58% of 492 by six months; 207 patients available for analysis at this point). Furthermore, study authors acknowledged a lack of clarity as to the exact effect of the polymorphism on 5-HT_4 receptor function. Future research with randomised data is needed as

well as a specific focus on examining the functional impact of this genetic polymorphism on receptor activity, and on assessing cognition as well as antidepressant response.

1.10.2 Potential 5-HT$_4$ receptor agonists for human translational studies

Detailed examination of 5-HT$_4$ receptor agonism in humans was previously limited due to cardiovascular side-effects with early agents. Prucalopride seems a particularly interesting candidate for further work investigating 5-HT$_4$ receptor agonism: it has good brain penetration in rodents (Johnson et al. 2012), demonstrates pro-cognitive effects in animals (Cachard-Chastel et al. 2008; Lucas et al. 2007) and has recent evidence of the ability to modulate glutamatergic transmission (Chen et al. 2019). Moreover, as a selective high-affinity 5-HT$_4$ partial agonist, prucalopride does not affect the hERG K^+ channel (c.f. cisapride) and thus it has no adverse cardiovascular action (De Maeyer et al. 2008). Initial cardiovascular concerns with the 5-HT$_4$ receptor agonist, tegaserod, due to its co-action at 5-HT$_{2B}$ receptors have not materialised in cohort data, and its license was reinstated in the United States in 2019.

After prucalopride received a license for constipation in the United Kingdom in 2010, our laboratory undertook some preliminary work assessing 5-HT$_4$ receptor agonism in humans using an experimental medicine model (Murphy et al. 2020). Here participants receiving a single low-dose of prucalopride compared to placebo demonstrated increased word recall in a verbal learning task and increased accuracy of recall and recognition of words in an emotional memory task . In this study, prucalopride also improved selection of a symbol associated with a higher reward in a probabilistic learning task.

1.10.3 The evidence gap in human translation of 5-HT$_4$ agonism

To our knowledge, the cognitive effects of prucalopride, or any 5-HT_4 agonist, have not been assessed in humans for longer than a single dose period, and its effects have not been examined neurally using functional magnetic resonance imaging (fMRI). fMRI allows us to study a proxy of activation within the brain in real-time and to correlate regional differences with specific task or rest conditions. Therefore, this allows us to investigate links between particular cognitive processes and activation within specific regions of the brain. It also allows us to examine how these are modulated by the effect of medication.

1.11 Investigating putative new medications for mental illness using experimental medicine

Conventional antidepressants work rapidly both behaviourally and neurally to decrease processing of negative information, and increase responses to positive stimuli (Browning et al. 2007; Godlewska et al. 2012; Harmer et al. 2009; Harmer et al. 2003; Harmer et al. 2004). SSRIs have been shown to decrease amygdala responses to fearful faces in healthy participants (Harmer et al. 2006; Murphy et al. 2009b), and also in people with depression (Godlewska et al. 2012). Experimental medicine studies based on this model can provide a rapid and reliable detection of drugs with clinical antidepressant activity (Arnone et al. 2009; Pringle et al. 2011; Rawlings et al. 2010), and therefore provide a useful translational approach to screen novel compounds in humans for potential antidepressant activity. These principles also apply when assessing cognition more broadly and examining potential pro-cognitive agents (Horder et al. 2009; Smith et al. 2018).

1.12 Synthesis and aims of the current thesis

5-HT_4 receptors are located in regions in the brain involved with emotional processing and cognition. Agonism at this receptor has been found to improve behavioural deficits in learning and memory including within depression / anxiety models in animals.

Likely mechanisms specifically relating to mood / anxiety effects may include increased release of neurotransmitters including dopamine and immediate firing from the dorsal raphe. Likely mechanisms specifically relating to cognition may include the enhanced release of acetylcholine, the fine tuning of memory processes such as LTP, and other direct effects on the hippocampus such as cellular proliferation, reduction in apoptosis, enhanced theta oscillations, and the promotion of learning-induced spine growth. Factors that may underpin effects on both mood and cognition include the increased production of neurotrophic proteins such as BDNF and CREB, which are thought to induce neuroplasticity, and indirect action on AMPA receptors thereby modulating glutamate functioning.

Action at the 5-HT_4 receptor is specifically involved in these behavioural outcomes because 5-HT_4 receptor agonist induced pro-cognitive effects can be blocked by the simultaneous administration of 5-HT_4 receptor antagonists. Furthermore, anticholinergic-induced impairments can be prevented or reversed by the administration of 5-HT_4 agonists.

If it is possible to translate the beneficial effects of 5-HT_4 receptor agonism on animal cognition and mood into humans, this may suggest a possible role for these drugs to ameliorate symptoms of mental illness. However, translating the effects of agents that appear promising in animals into humans is fraught with difficulty – many have failed at the clinical trial stage (i.e. NK1 antagonists

(Pringle et al. 2011)). However, pilot work examining the role of the 5-HT_4 receptor in humans appears encouraging. Therefore, this seemed a timely opportunity to undertake a translation of these effects examining neurocognitive outcomes in particular.

The aim of this thesis is to investigate whether it is possible to translate the neurocognitive effects seen in animals with 5-HT_4 receptor agonism into humans. In this thesis, I will assess the translational potential of 5-HT_4 receptor agonists in the human brain in terms of cognition (Chapter Two and Five), emotional processing (Chapter Three) and functional connectivity (Chapter Four), and then more broadly on mental health outcomes using real-world data (Chapter Six).

In summary, Chapters Two, Three and Four describe an experiment in which the effect of seven days administration of the 5-HT_4 receptor agonist, prucalopride, was investigated in healthy volunteers using fMRI. Chapter Two investigates the effect of prucalopride on hippocampal function and presents the data from an fMRI memory encoding paradigm. Chapter Three investigates the effect of prucalopride on neural responses to emotional faces and presents the data from an fMRI implicit emotional faces task. Chapter Four investigates the effect of prucalopride on resting-state networks and presents an analysis of resting-state fMRI data. In Chapter Five, the effect of the unlicensed 5-HT_4 receptor agonist PF-04995274 in un-medicated depressed volunteers was investigated; this chapter presents an analysis of the effects of this agonist on the same fMRI memory task as presented in Chapter Two. Finally, Chapter Six is an emulated target study, where we assess the impact of 12 months' prescription of prucalopride on longitudinal mental health outcomes compared to alternative anti-constipation medication.

CHAPTER 2

Neural and behavioural effects of the 5-HT_4 receptor agonist, prucalopride, in a hippocampal-dependent memory task in healthy volunteers

Based on work published as de Cates AN, Wright LC, Martens MAG, Gibson D, Tuerkmen C, Filippini N, Cowen PJ, Harmer CJ, Murphy SE (2021). Déjà vu? Neural and behavioural effects of the 5-HT_4 receptor agonist, prucalopride, in a hippocampal-dependent memory task. Translational Psychiatry 11, 497

2.1 Introduction

As detailed in Chapter One, a broad range of cognitive impairments are present in many different neuropsychiatric conditions (Etkin et al. 2013; Millan et al. 2012). Although these are often disabling for patients, they are poorly treated by current medications for the primary disorder (Millan et al. 2012).

The 5-HT_4 receptor is a promising treatment target for cognitive impairment. These receptors are expressed in brain regions associated with cognition (Beliveau et al. 2017; Cai et al. 2002; Compan et al. 1996; Roychowdhury et al. 1994; Vilaró et al. 2005) and agonism leads to rapidly improved learning and memory task performance, particularly for hippocampal-dependent memory (Hagena and Manahan-Vaughan 2017; King et al. 2008; Lamirault and Simon 2001). This may be explained by several hippocampal-focussed downstream effects including increased proliferation of cells and

growth of cellular spines (Lucas et al. 2007; Pascual-Brazo et al. 2012; Restivo et al. 2008), induction of long-term potentiation (LTP) (Marchetti et al. 2004), increased release of neurotransmitters such as acetylcholine (Hagena and Manahan-Vaughan 2017; Siniscalchi et al. 1999), and increased expression of neuroplasticity proteins (Hagena and Manahan-Vaughan 2017; Lucas et al. 2007; Pascual-Brazo et al. 2012). The importance for cognition of 5-HT_4 receptor agonism and the consequent modulation in neurotransmitter function is further demonstrated by two key preclinical findings. First, 5-HT_4 agonist-induced pro-cognitive effects can be blocked by the co-administration of 5-HT_4 receptor antagonists (Fontana et al. 1997; Lamirault and Simon 2001; Mohler et al. 2007; Moser et al. 2002). Second, anticholinergic-induced cognitive impairments can be reversed by the administration of 5-HT_4 receptor agonists (Cachard-Chastel et al. 2008; Fontana et al. 1997; Lo et al. 2014; Marchetti-Gauthier et al. 1997; Moser et al. 2002).

Given the evidence from animal models, 5-HT_4 receptor agonism was initially considered as a potential therapeutic option for primary dementias (Baranger et al. 2017; Giannoni et al. 2013; Spencer et al. 2004). However, investigating this mechanism in humans was initially limited due to the side-effect profile of early agents. Prucalopride, a selective high-affinity 5-HT_4 partial agonist with good brain penetration (Johnson et al. 2012), received a medical license for constipation in the United Kingdom in 2010. This has allowed investigation of 5-HT_4 receptor agonism using an experimental medicine model to assess potential enhancement of cognition in healthy humans. It was previously demonstrated by our laboratory that a single 1mg dose of prucalopride has pro-cognitive effects across three different tasks of learning and memory (the Rey Auditory Verbal Learning Task (RAVLT), the Probabilistic Instrumental Learning Task (PILT), and emotional memory as part of the Emotional Test Battery (ETB)) (Murphy et al. 2020). This chapter examines the effect of a longer period of prucalopride administration (six days) on behavioural and neural learning / memory processes, including an fMRI memory paradigm known to be a reliable probe of

hippocampal function and related circuitry (Filippini et al. 2009; Filippini et al. 2012). It was hypothesised that prucalopride would improve episodic memory and increase the activation of the hippocampus and related neural circuitry during memory retrieval.

2.2 Methods

2.2.1 Participants

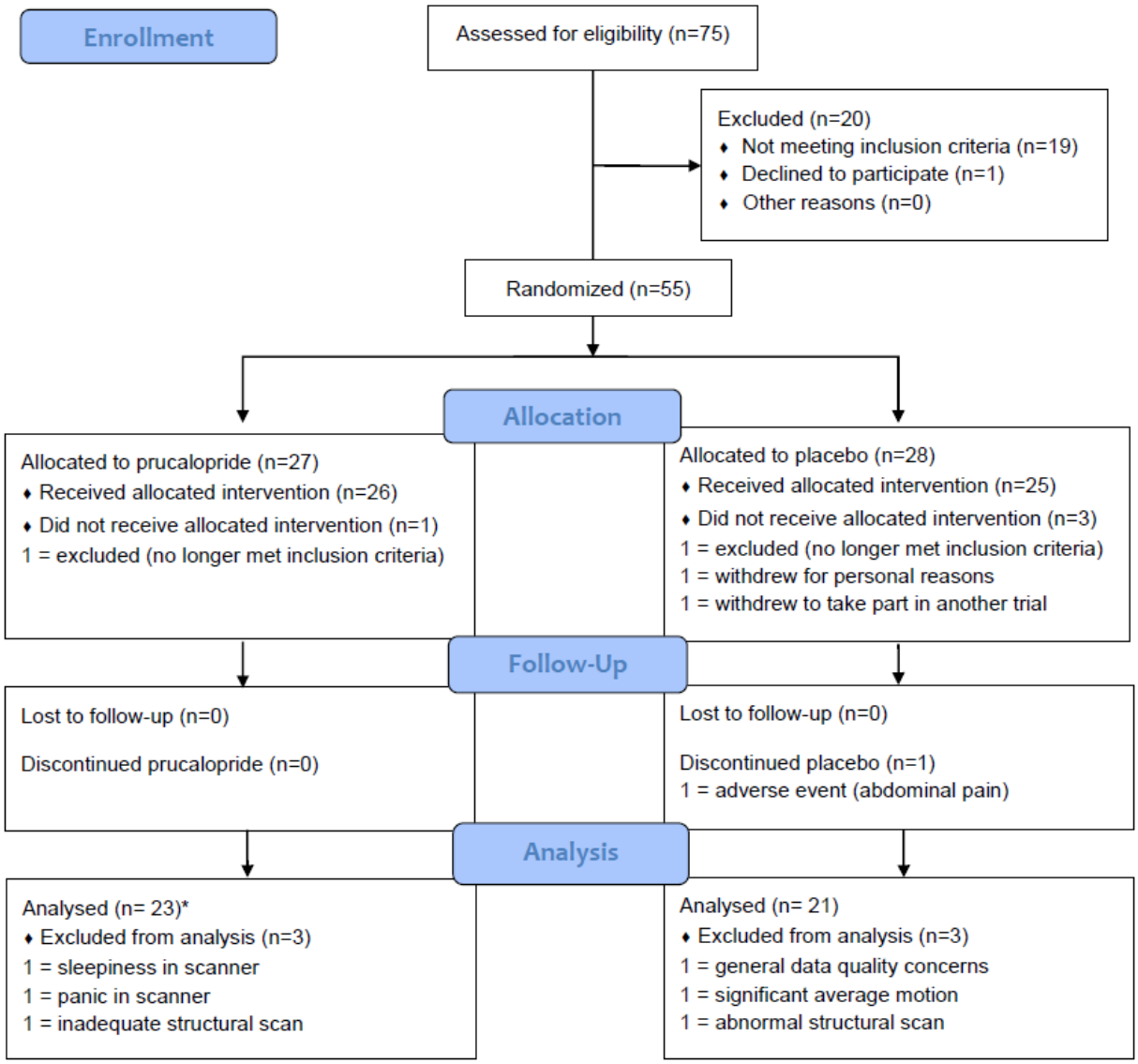
44 right-handed healthy participants (21:23 = placebo : prucalopride), between 18 and 36 years, were administered either prucalopride (six days x 1mg) or placebo, in a double-blind, randomised design. Inclusion criteria were that participants were sufficiently fluent in English to be able to understand the tasks, able to give informed consent for the study, and were screened for contraindications to prucalopride. In addition, participants were young adults of either sex (aged 18 to 40 inclusive), healthy, right-handed, not pregnant or breast feeding, and with a BMI between 18 and 30. Those potentially vulnerable to side effects of prucalopride were excluded. Exclusion criteria are included in detail in Table 1.

The study was approved by the University of Oxford Central University Research Ethics Committee (MSD-IDREC reference R57219/RE001) and a protocol including outcomes was pre-registered with clinicaltrials.gov (NCT03572790). The fMRI results detailed here (and in Chapters Three & Four) relate to pre-defined secondary outcomes. No changes to methods occurred after start of the study and flow of participants is outline in Figure 1. Participants gave written informed consent.

Table 1: Exclusion criteria for the prucalopride study

Any past or current Axis 1 DSM-V psychiatric disorder
Current usage of psychoactive medication (except the contraceptive pill, the Depo-Provera injection or the progesterone implant)
Current usage of any medication that will influence the MRI scan
Current or past history of drug or alcohol dependency
Currently pregnant or breastfeeding
Study visits due to take place during the pre-menstrual week (female participants will be asked details of their menstrual cycle to schedule the study outside this week)
Not right-handed
Body Mass Index outside the range of 18-30
History of cardiac, thyroid, or liver problems
An autoimmune disorder
Current, or a history of, gastro-intestinal disorder or irritable bowel syndrome
Epilepsy
Known lactate deficiency or any other problem absorbing lactose, galactose, or glucose
Participation in a study which uses the same computer tasks as those used in the present study
Participation in a study that involves the use of a medication within the last three months
Smoker > 5 cigarettes per day
Typically drinks > 6 caffeinated drinks per day
Any contraindication to MRI scanning (e.g. metal objects in your body, pacemakers, significant claustrophobia)

Figure 1: CONSORT diagram of flow of participants for prucalopride study fMRI scan [1]



2.2.2 Design and randomisation

The study had a between-subject, double-blind, placebo-controlled design. Participants were randomly assigned to 6 days of prucalopride 1mg (Resolor) or placebo (lactose tablets, Rayonex

[1] *= 1 additional participant excluded for Faces fMRI tasks only (due to lack of engagement for this task in scanner)(see Chapter Three)

Biomedical) in a 1:1 allocation. Randomisation was blocked in design (block size = 4) and performed using an online tool (sealedenvelope.com on 1st June 2018). Drug allocation was stratified for sex. Allocation was concealed from participants, investigators and assessors using sequential numbered containers, and the encapsulation process ensured that both capsules appeared identical. All capsules were consumed by participants in their own home.

Previous studies examining the effect of prucalopride and other serotonergic drugs on neurocognitive outcomes, including learning / memory and emotional processing, indicate that an effect size of 0.8-0.9 may be expected (Harmer et al. 2004; Murphy et al. 2020). However, given how little is known about the effects of 5-HT_4 receptors on cognition in humans, we took a conservative approach and calculated the samples size needed on the basis of a smaller estimated effect size (0.5-0.7), indicating that 17 participants per group are needed to give 90% power to detect a significant difference between the two groups with an α of 5%. It was therefore aimed to recruit 50 (25 in each group) as part of oversampling and to ensure adequate power for fMRI analyses.

By day six when imaging was carried out, prucalopride would be expected to be at a steady state (terminal half-life approximately 24h) (Frampton 2009). Testing occurred at the Department of Psychiatry and the Oxford Centre for Human Brain Activity (OHBA), part of the Wellcome Integrative Neuroimaging Centre (WIN). In female participants, testing did not take place during the premenstrual week.

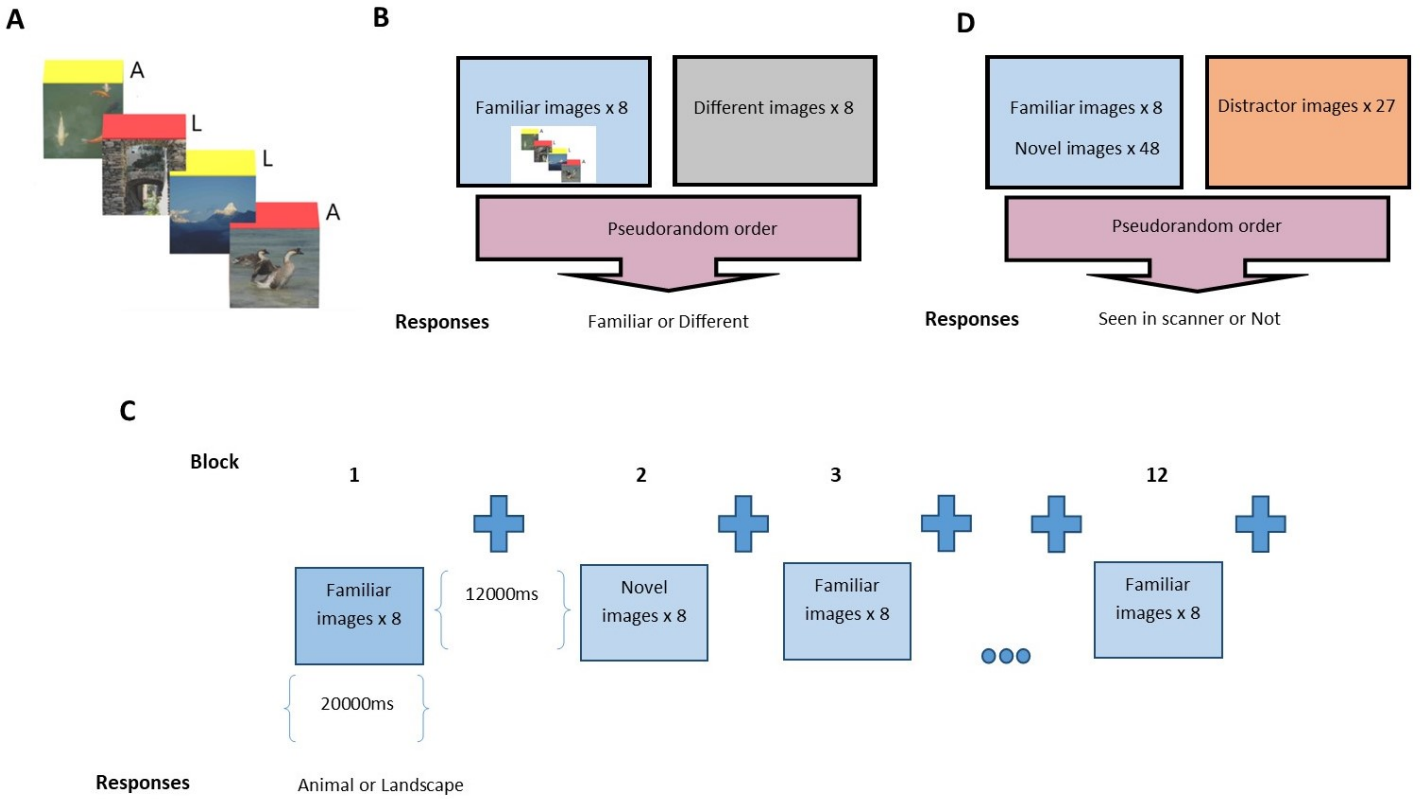
2.2.3 Questionnaire measures

Participants completed the following self-report questionnaires to obtain baseline measures of mood, anxiety, and personality: Beck Depression Inventory-II (Beck et al. 1996), Snaith-Hamilton Pleasure Scale (SHAPS) (Snaith et al. 1995), Spielberger State-Trait Anxiety Inventory, Trait Version (STAI-T) (Spielberger et al. 1983), and Eysenck Personality Questionnaire (Eysenck and Eysenck 1975). Affect and anxiety were measured at three times (baseline, pre-imaging (day 6) and post-imaging (day 6)): affect using the Positive and Negative Affect Scale (PANAS) (Watson et al. 1988) and the visual analogue scale (VAS) (Bond and Lader 1974), and for anxiety the Spielberger State-Trait Anxiety Inventory, State Version (STAI-S) (Spielberger et al. 1983). Side effects were measured at the same three time-points using a scale where participants rated the extent to which they were experiencing each of the most commonly reported side effects of prucalopride (Sajid et al. 2016). After the end of the study (day seven), participants guessed their drug allocation with a forced-choice question.

2.2.4 Memory Encoding fMRI Task

On the sixth day of prucalopride administration, participants underwent a 3T scan including an fMRI memory task designed to induce hippocampal activation. In this task (adapted from (Filippini et al. 2009; Filippini et al. 2012)), participants were presented with coloured emotionally-neutral images of animals or landscapes similar in complexity and brightness. Before the scan, randomly pre-selected pictures (four animals, four landscapes) were presented eight times in a pseudorandom order on a screen, called the “familiar” images (see Figure 2(A)). Participants were asked to identify these as animal / non-animal using a two-button response. To ensure that participants had satisfactorily encoded these images before the scan, a pre-scan memory task was performed (eight “familiar” plus eight different images involving the same number of animals and landscapes presented in pseudorandom order, where subjects were asked to identify these as familiar or different using a two-button response (see Figure 2(B)).

Figure 2: The memory encoding task design, including fMRI memory encoding training of familiar images for all participants



(A) *Before/outside the scan, 8 randomly-selected "familiar" pictures (4 animals(A), 4 landscapes(L) – 4 examples shown) were presented 8 times in a pseudorandom order on a computer screen [image presentation 3250 ms, inter-stimulus interval 500 ms, between-block interval (fixation cross) 5000 ms]. Participants identified these as animal/non-animal on a 2-button response.*
(B) *Immediately before the scan, participants underwent a pre-scan recall test where they were asked to identify which images were familiar from (A) and which were different.*
(C) *During the scan, familiar and novel images were presented as follows: image presentation 2000 ms, inter-stimulus interval 500 ms, block duration 20000 ms. There were 6 familiar and 6 novel image blocks. Between each block of images there was 12000 ms of rest, during which subjects passively viewed a fixation cross (a total of 12 rest blocks). Subjects were instructed to select from a 2-button response according to whether the images were animal / non-animal. Responses were monitored by the scanner operators to ensure compliance and accuracy and were registered by the software in a text file.*
(D) *After the scan, participants took part in a post-scan recall test where they were asked to identify which images had been seen in the scanning task (C) and which were distractors.*

During the scan, images were displayed in a pseudorandom order in a blocked design with 12 task blocks which were interleaved with rest blocks (during which participants were required to fixate on a cross on the screen) (see Figure 2(C)). In each task block, eight images were presented in a pseudorandom order: either eight "familiar" images (the images presented pre-scan) or eight "novel" images (previously unseen images). Familiar images were presented in a different order in each block. Novel images were taken from a pool of 48 images (24 animals and 24 landscapes) and each was only presented once during the task. Participants were required to indicate via a two-button response if the image contained an animal or not. Participants were also instructed to try to remember the images for a subsequent memory task. After the scan, 83 images were presented on a PC screen for 4000ms each (inter-stimulus interval 1000 ms): the eight "familiar" images (seen pre-scan and during scan), the 48 "novel" images (seen during scan only), and 27 ''distractors'' (images never seen before: 13 animals and 14 landscapes) were displayed in pseudorandom order (see Figure 2(D)). Participants were required to indicate via a button press whether the images had been seen inside the scanner or not. The task was programmed in Presentation (Neurobehavioral Systems; https://www.neurobs.com).

This task was designed to stimulate implicit visual memory recognition in the scanner for images that participants had seen before (familiar images), compared to images that were new to participants in the scanner (novel images) where implicit encoding should occur.

2.2.5 Demographic and behavioural data analysis

Demographic characteristics and baseline clinical measures were analysed using independent sample *t*-tests (continuous variables), Fisher's exact tests (sex and native language) and logistic regression (education). A repeated measures analysis of variance (ANOVA) was used to analyse

group differences in self-report measures and behavioural performance in the fMRI experimental task. Levene's test (*t*- tests) and the Greenhouse-Geisser procedure (ANOVAs) were used where appropriate. A *p*-value lower than 0.05 was used to denote statistical significance. Partial eta squared are reported as a measure of effect size. Behavioural data were analysed in SPSS (version 25, IBM). Graphs were produced using GraphPad Prism (version 9). Data were checked to ensure that tests and procedures were valid as appropriate.

2.2.6 MRI data acquisition and analysis

Blood-oxygenation-level-dependent (BOLD) fMRI and T1-weighted anatomical images were acquired using a 3-Tesla Siemens Prisma scanner, equipped with a 32-channel head matrix coil (Siemens, Erlangen, Germany). Whilst in the scanner, participants also completed an emotional faces task and resting-state scan (reported in Chapters Three and Four respectively), and an arterial spin labelling (ASL) scan, which is reported in brief here. Foam padding and a head restraint were used to control head movement. The full acquisition protocol, along with radiographic procedure is available from the Open WIN MR Protocols database here: https://open.win.ox.ac.uk/protocols/stable/1ef4ba0f-0d50-4933-b619-f9a3c6ae233a.

2.2.6.1 MRI data acquisition

fMRI acquisition: Encoding memory was assessed from a single run of 60 T2-weighted echoplanar imaging (EPI) slices covering the whole brain [repetition time (TR) 800 ms, echo time (TE) 30 ms, flip angle 52°, field of view 216 mm, slice thickness 2.4mm, voxel dimension 2.4mm isotropic, acquisition time 6min 48s]. Images were distortion corrected by an acquired fieldmap (echos at 4.92 and 7.38 ms, TR=590ms, flip angle = 46°).

Structural MRI acquisition: Additional high-resolution T1-weighted structural scans were acquired using a gradient echo sequence (TR 1900ms, TE 3.97ms, flip angle 8°, field of view 192mm, voxel dimension 1 mm isotropic, acquisition time 5min 31s) to allow later registration of the fMRI data into standard space.

ASL acquisition: Each participant also had a resting-state pCASL perfusion-weighted scan with a 2D gradient spin echo readout and a PICORE Q2T labelling scheme. ASL data were collected as tag-control pairs with a TI of 1.8 seconds and a bolus duration of 0.7 seconds. ASL imaging parameters were: repetition time: 4100ms; minimum echo time: 14.0ms; FOV read: 220mm; FOV phase: 100%; voxel size: 3.4x3.4x4.5mm; 24 slices with 4.5mm thickness; echo spacing: 0.56mm; EPI factor: 64; post-labelling delays at: 250ms, 500ms, 750ms, 1000ms, 1250ms, and 1500ms; number of dynamics/repeats: 97 (1 volume was control); acquisition time = 6min 39s; fat saturation = on. A calibration image was acquired without labelling (TR = 6000ms).

Labelling plane was set with a time of flight neck scan (TR = 21ms, TE = 3.43ms, flip angle = 30°, field of view = 200mm, voxel dimension = 0.3 x 0.3 x 1.3 mm, acquisition time = 42s). Images were distortion corrected by an acquired fieldmap (echos at 4.92 and 7.38ms, TR=482ms, flip angle = 46°).

2.2.6.2 MRI task analysis

Imaging data were analysed with FSL (www.fmrib.ox.ac.uk/fsl).

fMRI data were pre-processed and analysed using FEAT (fMRI Expert Analysis Tool), version 6.0.4, part of FSL (FMRIB's Software Library; www.fmrib.ox.ac.uk/fsl). DICOM (Digital Imaging and

Communications in Medicine) files were downloaded from the server, checked for completeness, excessive movement and visual anomalies, and converted to BIDS (Brain Imaging Data Structure)-standardised format nifti files using heudiconv (v.0.5.4 (https://github.com/nipy/heudiconv)) before pre-processing. The structural anatomical scans were brain extracted using the Brain Extraction Tool (BET)(Smith 2002). Formal MRI quality assessment was undertaken using the MRIQC package (https://mriqc.readthedocs.io/en/stable/index.html), with data considered for rejection if it fell outside the normal range of values in the derived image quality metrics. Pre-processing code is available at (de Cates et al. 2022).

Pre-processing involved: motion correction using FMRIB's Linear Image Registration Tool (FLIRT(Jenkinson et al. 2002)); deletion of non-brain tissue using BET (Smith 2002); spatial smoothing with a Gaussian kernel of 5 mm full-width-half-maximum; grand-mean intensity normalisation of the entire 4D dataset by a single multiplicative factor; high pass temporal filtering (Gaussian-weighted least-squares straight line fitting, with sigma of 90s) and B0 unwarping using fieldmap phase and magnitude images for distortion correction. No slice timing correction was applied. In addition, registration to high-resolution image and to a standard template [Montreal Neurological Institute (MNI)] was implemented using FNIRT nonlinear registration (Andersson et al. 2007).

In the first-level analysis, individual activation maps were computed using the general linear model with local autocorrelation correction. Two explanatory variables were modelled: "novel" and "familiar" images. Temporal derivatives were included in the model. Variables were modelled by convolving each block with a haemodynamic response function, using a variant of a gamma function (standard deviation 3s, mean lag 6s). No included participant demonstrated significant movement: absolute displacements were less than 1 voxel and relative displacements less than ½

voxel. At the whole-brain level, familiar images were contrasted with novel, resulting in the following model: 1) novel vs. baseline; 2) familiar vs. baseline; 3) novel > familiar; 4) novel < familiar.

In the second-level analysis, whole-brain individual data were combined at a group level (participants on placebo vs. prucalopride) using a mixed-effects analysis, and cerebral blood flow and grey matter maps as covariates of no interest. Groups were contrasted with each other, resulting in the following comparisons: 1) placebo > prucalopride; 2) prucalopride > placebo; 3) placebo mean; 4) prucalopride mean; 5) mean of all participants. Brain activations showing significant group differences were identified at the whole-brain level using cluster-based thresholding (Z>3.1, family-wise error (FWE) p<0.05 corrected). Significant interactions from whole-brain analyses were further explored by extracting percentage BOLD signal change for each type of contrast. As the hippocampus was a particular focus, it was a pre-specified region of interest (ROI). A functional ROI mask was created for the left and right hippocampus by multiplying mean activation for each contrast of interest (on whole-brain data already corrected for multiple comparisons (FWE) and Z>3.1 as described above) for all participants by the Harvard-Oxford subcortical atlas anatomical mask at a 50% threshold. Percentage BOLD signal change for each contrast in each hemisphere was extracted in order to identify the profile of drug effect. All activations are reported using MNI co-ordinates.

FSLVBM, a voxel-based morphometry style analysis (Douaud et al. 2007), was carried out to investigate potential grey matter differences between the two study-groups, underlying and potentially influencing group-related BOLD differences. Brain-extracted images (automatically created for each individual using FSLanat) were tissue-type segmented. Grey matter partial volume images were aligned to standard space using FLIRT and FNIRT registration tools. The

resulting images were averaged, modulated and smoothed with an isotropic Gaussian kernel of 2mm to create a study-specific-template. A voxel-wise general linear model was then applied using permutation non-parametric testing (5000 permutations). FSLFIRST (Patenaude et al. 2011) was used to segment the left and right hippocampus for each participant, and the volume of individual hippocampi were determined using vertex analysis. This was then normalised for each individual's brain volume and the resulting values for each hippocampus compared across groups using *t*-tests.

Regional and global blood flow was calculated for each individual. Distortion and motion corrected resting perfusion maps in units of mL/100g/min were calculated using Oxford_ASL (part of the Bayesian Inference for Arterial Spin Labelling (BASIL) tool, *https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/BASIL;* (Chappell et al. 2011; Chappell et al. 2009b) for each participant, which performs label-control subtraction, inference of voxelwise perfusion, and voxelwise calibration to obtain absolute perfusion maps, and controls for partial volume effects at the single subject level. FSL's Anatomical Processing Script (FSL_Anat, *https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/fsl_anat*) was used to pre-process each participant's high resolution T1 structural image (includes bias-field correction, brain extraction and registration to standard space via FMRIB's Linear Image Registration Tool (FLIRT) and FMRIB's Non-linear Image Registration Tool (FNIRT). The processed perfusion images were non-linearly aligned with standard space via an initial linear transformation T1 structural space (using FLIRT), followed by application of the non-linear warp from fsl_anat. A Gaussian smoothing kernel of 2.12mm was applied to all the normalised images (to match functional data). Data were interrogated using voxel-wise generalized linear model permutation nonparametric testing (5,000 permutations) with randomise (FSL's tool for nonparametric permutation inference on neuroimaging data), correcting for multiple comparisons across space (cluster-based thresholding using TFCE and a

family-wise error (FWE)-corrected cluster significance threshold of $p<0.05$ applied to the suprathreshold clusters). This results in spatial maps characterising the between-subject/group differences.

As post-hoc analyses, whole brain group feat results were also run using cerebral blood flow and grey matter maps as covariates of no interest, and sex as a covariate of interest. Hippocampal perfusion between groups was compared using fslmeants: parameter estimates of perfusion were extracted from resting perfusion maps (previously computed using Oxford_ASL in ml/100g/min) using anatomical Harvard-Oxford masks of the left and right hippocampus at 50% threshold.

2.3 Results

2.3.1 Participants

50 (100% of target) participants were recruited between 11th June 2018 and 17th May 2019. One participant was excluded from all analyses for data quality concerns raised at the time of data collection. Two other participants were excluded from fMRI analyses at the time of scanning for persistent sleepiness and acute anxiety. A further three participants were excluded from fMRI analyses for (i) a structural brain variant that affected registration to standard space, (ii) a poor quality structural scan according to MRIQC assessment, and (iii) significant motion during the scan.

Table 2: Sample demographics, baseline mood & personality

Variable (measure)	Measurement		Placebo (N=21)	Prucalopride (N=23)	P value	Odds ratio (95% CI)
Sex	*Number*	*Female*	13	13	Ref	Ref
		Male	8	10	0.77	1.25 (0.37-4.18) [2]
Age	*Mean (SD)*		24.6 (4.0)	24.7 (5.1)	1.00	
Education level	*Number*	*Postgraduate*	8	10	Ref	Ref
		Undergraduate	4	4	0.79	0.8 (0.15-4.25) [3]
		6th form / equivalent	9	9	0.74	0.8 (0.22-3.00) [4]
First language	*Number*	*English*	11	20	Ref	Ref
		Not English	10	3	0.02***	0.17 (0.04-0.73) [5]
IQ (NART)	*Mean (SD)*		112.4 (6.9)	113.4 (5.3)	0.60	
Handedness (EHI)	*Mean % right (SD)*		84.7 (17.8)	86.5 (22.7)	0.77	
Alcohol	*Mean units/week (SD)*		2.9 (2.6)	3.8 (3.6)	0.37	
Caffeine	*Mean drinks/day (SD)*		1.7 (1.1)	1.5 (1.3)	0.63	
Body mass index	*Mean (SD)*		23.1 (2.8)	23.0 (2.4)	0.85	
Mood (BDI-II)	*Mean (SD)*		0.9 (1.2)	1.0 (1.2)	0.70	
Anhedonia(SHAPS)	*Mean (SD)*		0.8 (1.7)	0.6 (1.6)	0.62	
Anxiety (STAI-T)	*Mean (SD)*		31.6 (7.5)	29.5 (5.4)	0.28	
Personality (EPQ)	*Mean (SD)*	*Neuroticism/Stability*	4.6 (3.8)	4.4 (3.1)	0.83	
		Psychotism/Socialisation	2.2 (1.9)	2.1 (1.8)	0.85	
		Extroversion/Introversion	14.9 (3.8)	14.0 (4.3)	0.47	
		Lie/Social desirability	10.6 (3.9)	9.9 (4.3)	0.57	

NART=National Adult Reading Test; EHI=Edinburgh Handedness Inventory; BDI=Beck Depression Inventory; SHAPS=Snaith-Hamilton Pleasure Scale; STAI-T=Spielberger Anxiety Inventory-Trait; EPQ=Eysenck Personality Questionnaire

[2] OR of being male in prucalopride group, compared to females
[3] OR of being undergraduate in prucalopride group, compared to postgraduate
[4] OR of being 6th form in prucalopride group, compared to postgraduate
[5] OR of being non-English in prucalopride group, compared to English

Analysis occurred in originally assigned groups. The final groups (N=44, 21:23 = placebo : prucalopride, aged 18-36) were well matched for age, BMI, level of education, use of substances, and NART scores (see Table 2). However, compared to those with English as a first language, for non-native English speakers the odds of being in the prucalopride group were 0.17 (95% CI 0.04-0.73, *p*=0.017). Randomisation guesses[6] suggested that participants were better than chance at guessing group allocation, particularly in the placebo group (correct guess: placebo 75.0%, prucalopride 60.9%). No adverse events occurred in the prucalopride group; one person discontinued placebo due to abdominal discomfort.

2.3.2 Questionnaire results

There were no significant differences in state anxiety or affect between the prucalopride and placebo group (see Table 3), and no significant differences in reported side effects (all *p*s=> 0.5).

[6] Randomisation guesses data missing for one participant

Table 3: Questionnaire measures across visits

	Placebo mean (SD) (N=21)	Prucalopride mean (SD) (N=23)[7]	P value (2dp)
Spielberger State Anxiety Inventory (STAI-S)			
Baseline	27.8 (8.1)	25.3 (4.7)	0.59
Pre-Testing	27.3 (6.0)	29.0 (5.6)	
Post-Testing	30.5 (8.4)	28.7 (6.8)	
Positive and Negative Affect Scale (PANAS) – Positive			
Baseline	32.6 (5.1)	33.9 (7.0)	0.66
Pre-Testing	33.3 (5.6)	32.3 (6.9)	
Post-Testing	31.9 (7.6)	31.4 (8.1)	
Positive and Negative Affect Scale (PANAS) – Negative			
Baseline	11.6 (2.3)	10.8 (1.3)	Included in ANOVA above
Pre-Testing	12.2 (3.1)	11.6 (2.5)	
Post-Testing	12.6 (3.4)	12.1 (3.0)	
Visual Analogue Scale (VAS) – Happy			
Baseline	70.7 (12.4)	72.3 (12.8)	0.80
Pre-Testing	72.2 (13.3)	74.6 (13.2)	
Post-Testing	73.9 (14.9)	76.6 (14.2)	
Visual Analogue Scale (VAS) – Sad			
Baseline	10.4 (13.2)	6.7 (12.8)	Included in ANOVA above
Pre-Testing	11.8 (16.7)	9.1 (15.4)	
Post-Testing	11.8 (16.5)	5.9 (10.0)	
Visual Analogue Scale (VAS) – Hostile			
Baseline	4.7 (14.3)	4.2 (10.6)	Included in ANOVA above
Pre-Testing	8.4 (17.3)	3.9 (10.5)	
Post-Testing	7.6 (18.2)	4.3 (9.9)	
Visual Analogue Scale (VAS) – Alert			
Baseline	62.1 (20.3)	70.8 (22.1)	Included in ANOVA above
Pre-Testing	62.8 (21.2)	70.0 (14.7)	
Post-Testing	57.1 (25.1)	62.2 (24.2)	
Visual Analogue Scale (VAS) – Anxious			
Baseline	14.9 (19.3)	11.0 (9.6)	Included in ANOVA above
Pre-Testing	13.6 (18.4)	14.6 (18.4)	
Post-Testing	10.7 (12.3)	8.3 (12.6)	
Visual Analogue Scale (VAS) – Calm			
Baseline	78.0 (14.5)	76.8 (12.0)	Included in ANOVA above
Pre-Testing	79.8 (13.8)	75.9 (13.8)	
Post-Testing	78.3 (17.5)	74.8 (19.1)	

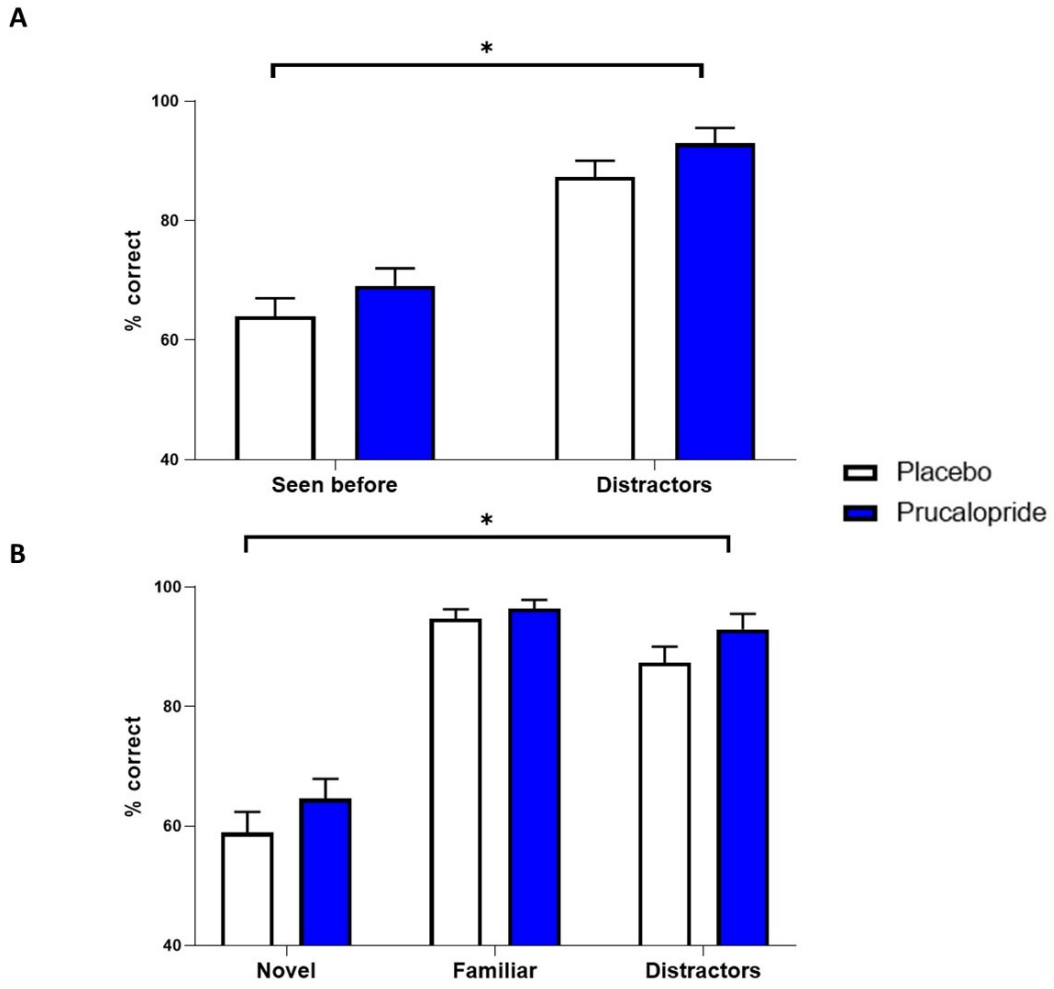
2.3.3 Behavioural results of fMRI memory task

Both groups had a high level of accuracy on the pre-scan task and were able to correctly identify the images as familiar or different with over 95% accuracy and no significant differences between

[7] results missing in prucalopride group for 1 participant for VAS only

the two groups (t(1,42)=-1.323, p=0.193; placebo mean=95.72, SD=13.02; prucalopride mean=99.43, SD=1.84; data missing for 3 participants (1 from prucalopride, 2 from placebo = 41 participants included)). This suggests that both groups had adequately encoded the pre-scan images as required.

Figure 3: Post-scan recall task results (percentage total correct at identifying image type) by group



*White = placebo, blue = prucalopride. * = p<0.05, signifying a main effect of group on ANOVA both when comparing placebo vs prucalopride for **(A)** seen before (novel + familiar) vs. distractors; **(B)** novel vs. familiar vs. distractors.*

Administration of prucalopride led to improved memory performance in the post-scan recall task: the prucalopride group were more accurate than the placebo group in selecting those seen before (novel + familiar) versus distractors ($F(1,38)=5.18$, $p=0.029$; prucalopride (mean=81.04, SEM=1.63), placebo (mean=75.67, SEM=1.70)) (see Figure 3(A)) and also distinguishing each category of image (novel/familiar/distractor) ($F(1,38)=4.806$, $p=0.035$; prucalopride (mean=84.65, SEM=1.36), placebo (mean=80.32, SEM=1.43)) (see Figure 3(B)) (data missing for 4 participants (2 from each group = 40 participants included)).

2.3.4 Analyses controlling for potential confounds

There were no group-related differences in grey matter between groups (FSLVBM: placebo > prucalopride, $p=0.91$; prucalopride > placebo, $p=0.44$) or in the size of hippocampi compared after individually normalising for total brain volume (FSL FIRST vertex: $p=0.55$ (right hippocampus), $p=0.07$ (left hippocampus)). There was also no difference between groups in either global blood flow (Oxford_ASL: $p=0.73$ (grey matter), $p=0.65$ (white matter)) or regional blood flow (placebo > prucalopride, $p=0.70$; prucalopride > placebo, $p=0.36$). Whole brain and hippocampal ROI results were similar with and without correction for cerebral perfusion and grey matter maps, and sex. Left and right hippocampal perfusion did not differ between groups: smoothed data $p=0.79$ (left hippocampus), $p=0.47$ (right hippocampus) (see Table 4). Native language and post-scan recall accuracy were not correlated using linear regression ($R^2=0.007$).

Table 4: Left and right hippocampal mean (SD) perfusion (ml/100g/min)

		Left hippocampus	**Right hippocampus**
Smoothed (2.12)	***Placebo***	52.50 (7.46)	53.84 (8.28)
	Prucalopride	51.92 (6.86)	52.17 (6.66)
Unsmoothed	***Placebo***	53.72 (7.48)	55.60 (8.48)
	Prucalopride	53.23 (7.02)	53.94 (6.87)

2.3.5 Memory Encoding Task fMRI

2.3.5.1 Main effect of task

For all participants, similar to previously described (Filippini et al. 2009; Filippini et al. 2012) the novel versus familiar contrast revealed increased BOLD fMRI signal intensity bilaterally in the hippocampus, parahippocampal gyrus, and temporal fusiform cortex. There was also increased activity to novel versus familiar pictures in association areas including the thalamus, and regions in the frontal (precentral gyrus, cingulate / paracingulate gyrus, superior frontal gyrus, frontal pole) and occipital lobes, basal ganglia (caudate, putamen) and amygdala (see Table 5).

2.3.5.2 Effect of treatment – region of interest analysis

In the left and right hippocampus, there was increased activity in the prucalopride group compared with the placebo group in response to both novel and familiar images (see Figure 4). There was a significant effect of group [$F(1,42)=5.19$, $p=0.028$, $\eta\rho^2=0.11$], although there was no significant condition*hemisphere*group interaction present. To understand this main effect of group, we explored each condition separately. When I explored the role of condition, there were significant effects of group for both novel [$F(1,42)=3.94$, $p=0.036$, $\eta\rho^2=0.10$] and familiar images [$F(1,42)=3.09$, $p=0.027$, $\eta\rho^2=0.11$].

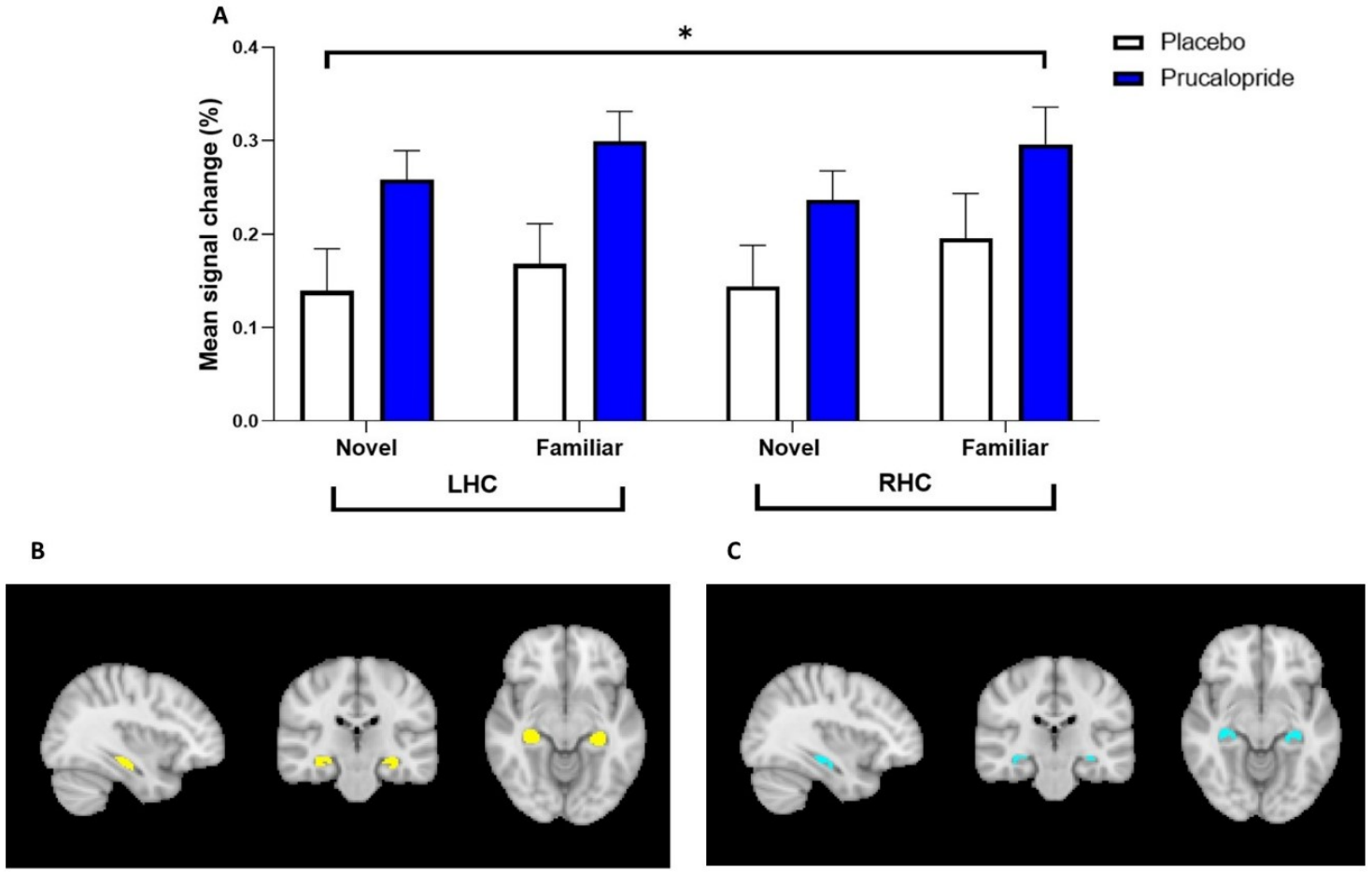
2.3.5.3 Effect of treatment – whole brain analysis

There was increased activity in the prucalopride group compared with the placebo group in response to familiar images in the right angular gyrus [prucalopride > placebo, $Z=4.1$, $p<0.005$, peak voxel location: x=42, y=-60, z=60, cluster size=168 voxels] (see Figure 5(A)). Figure 5(B) represents the group-level extracted BOLD signal change for this activation cluster.

Table 5: Cortical co-ordinates of maximal activation for clusters on the main effect of task (novel > familiar, mean of all participants)

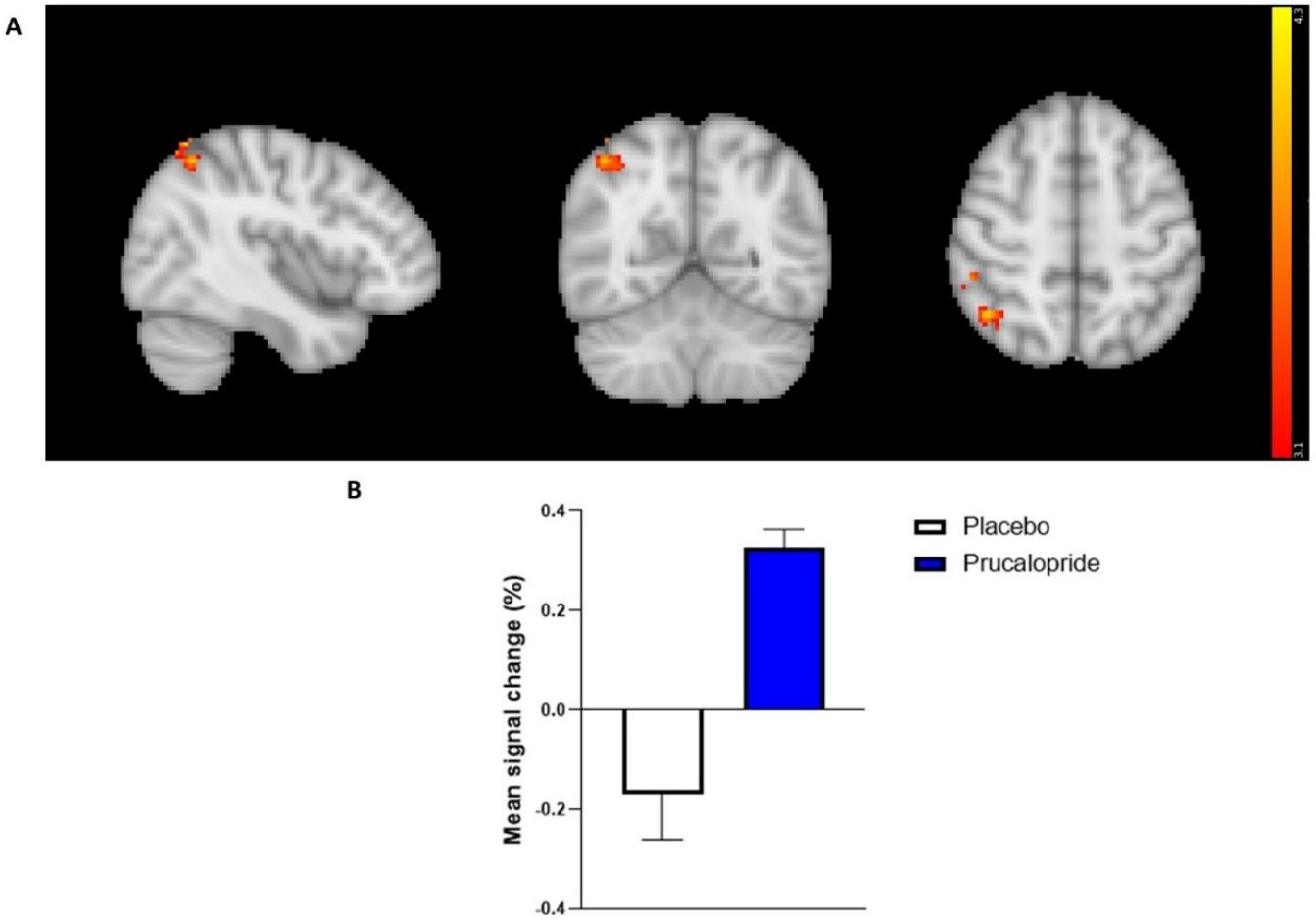
Cluster number	Size (voxels)	Z-max	p-value	Z-max location (MNI)	Regions included in cluster
1	51129	9.83	<0.001	-40,-80,-12	Bilateral occipital cortex and pole, fusiform cortex & gyrus, temporal gyrus, thalamus, hippocampus, parahippocampal gyrus, amygdala, putamen, caudate,
2	1430	7.06	<0.001	42,10,30	Right superior, middle & inferior frontal gyrus, precentral gyrus
3	332	4.92	<0.001	0,42,-24	Bilateral frontal medial cortex, cingulate / paracingulate gyrus
4	321	6.15	<0.001	20,-40,-44	Right cerebellum
5	197	4.31	0.0027	38,34,-10	Right frontal pole, frontal orbital cortex

Figure 4: Group mean of BOLD percentage signal change extracted from left (LHC) and right (RHC) hippocampi in response to novel and familiar images



(A) *Error bars show standard error of the mean. * = p<0.05, signifying a main effect of group on ANOVA.*
A functional ROI mask was created for the left and right hippocampus for each contrast of interest by multiplying mean activation for all participants by the anatomical mask at a 50% threshold: ***(B)*** *LHC and RHC mask in sagittal, coronal and axial views created for novel mean (yellow);* ***(C)*** *LHC and RHC mask in sagittal, coronal and axial views created for familiar mean (light blue).*

Figure 5: Whole-brain activation in response to familiar mean in prucalopride vs. placebo group



(A) *Sagittal, coronal and axial images depicting significantly increased activation in the prucalopride group for the familiar mean contrast in a right angular gyrus cluster (peak voxel location x=42, y=-60, z=60, Z=4.1, cluster size=168 voxels). Images thresholded at z> 3.1, p<0.05 corrected. Red to yellow colours identify increases in brain activation.*

(B) *Group mean of BOLD percentage signal change extracted from the right angular gyrus cluster (familiar mean). Error bars show standard error of the mean. White = placebo, blue = prucalopride.*

2.4 Discussion

The main findings of this chapter are that six days' prucalopride administration in young healthy participants improved recall and increased neural activation in the hippocampus and functionally related areas relative to placebo.

Specifically, in the post-scan recall task, participants on prucalopride were better able to distinguish each type of image, as well as demarcate those seen before and / or during the scan from the new distractor images. Preclinical studies have demonstrated that pre-administration of 5-HT_4 agonists before memory encoding improves learning and memory in animals (Lamirault and Simon 2001; Meneses and Hong 1997; Orsetti et al. 2003), with evidence of effects enduring with repeated administration (Quiedeville et al. 2015). Previous work in our laboratory provided an initial translation of these results into humans, where healthy volunteers randomised to a single dose of prucalopride showed better recall and recognition of words in a verbal learning and emotional memory task (Murphy et al. 2020). This chapter replicates and extends the evidence in humans of improved memory recall following prucalopride administration.

Consistent with the improved memory recall seen at a behavioural level, this chapter also demonstrated an increase in hippocampal activation for both forms of encoded image with prucalopride. Although the function of memory is not specific to one brain region, the hippocampus is known to play a central role especially when rich mental imagery is involved (Bird and Burgess 2008), and it is closely connected to memory association areas, including the angular gyrus (Seghier 2013; van der Linden et al. 2017). In addition to direct effects on the serotonergic system, prucalopride appears indirectly to reduce bursts of AMPA receptor-mediated currents in the hippocampus, altering glutamatergic transmission (Chen et al. 2019) and thus making it a

particularly interesting candidate for cognitive enhancement. GABAergic and glutamatergic neurones are also thought to mediate the increased release of acetylcholine that occurs with 5-HT_4 receptor agonism (as 5-HT_4 receptors are not present on cholinergic basal forebrain neurons) (Peñas-Cazorla and Vilaró 2015), which occurs particularly in the hippocampus and is blocked by 5-HT_4 receptor antagonists (Hagena and Manahan-Vaughan 2017; Siniscalchi et al. 1999). This study confirms that 5-HT_4 receptor agonism with prucalopride appears to lead to hippocampal activation in response to a memory stimulus, which is likely associated with behavioural effects related to cognitive performance.

Interestingly, studies using PET imaging, have found an inverse correlation between verbal memory scores and 5-HT_4 receptor binding in the hippocampus (Haahr et al. 2013) and more globally (Stenbæk et al. 2017). At first sight, this seems counter to the notion that 5-HT_4 receptors facilitate hippocampal-dependent memory. However, the authors suggested that this seemingly paradoxical finding might be explained by dependence of 5-HT_4 receptor availability on endogenous serotonin release. That is, increased 5-HT_4 receptor binding might be a marker of a lesser degree of intrinsic stimulation of 5-HT_4 receptors.

Prucalopride also significantly increased activation in a region associated with memory retrieval (the right angular gyrus) during a task where participants had to recall recently encoded information. The right angular gyrus (Brodmann area 39) has potential as a neuroimaging marker of early cognitive impairment (Lau et al. 2016), related to its close connections with the hippocampus (Seghier 2013) and as a "core hub" within the default mode network (DMN) (Vatansever et al. 2017). As activity in the angular gyrus at encoding appears to directly correlate with memory retention ability (van der Linden et al. 2017), one suggested function of this region

during memory retrieval is to distinguish objects that were or were not embedded as part of a schema during encoding.

Clinically, angular gyri lesions cause language dysfunction, low mood and poor memory (Nagaratnam et al. 2002) and can mimic dementia or pseudodementia (Benson and Cummings 1982; Nagaratnam et al. 2002). Similarly, in individuals with mild cognitive impairment (MCI), the right angular gyrus shows significantly decreased activity during resting-state fMRI compared to healthy controls (Jin et al. 2012; Lau et al. 2016). Therefore, the increased activity seen in the right angular gyrus following prucalopride administration in our study is consistent with the pro-cognitive behavioural effects observed here, and is in keeping with previous evidence of neural representations of cognition and human cognitive impairment. In support of this suggestion, Jin and colleagues found that activity within the right angular gyrus was positively correlated with behavioural memory scores for both delayed recall and learning efficiency (Jin et al. 2012).

Furthermore, abnormalities in these areas and networks do not appear to be limited to impairments within primary cognitive disorders, but also extend to other psychiatric conditions (Pauly et al. 2008). For example, grey matter volumes of the right angular gyrus are reduced in those at genetic risk of psychosis who also have currently impaired cognition compared to healthy controls (Bhojraj et al. 2011), and in first episode psychosis volumetric reduction in hippocampal subfields appears to correlate significantly with 5-HT_4 receptor density (Park et al. 2021). Importantly for clinical practice, functional connectivity impairments in the regions highlighted in this chapter appear to be amenable to improvement. For example, in patients with MCI, resting-state functional connectivity between the hippocampus and right angular gyrus increased following a six-month mind-body exercise-based intervention versus no intervention, alongside improved cognitive performance scores in the intervention group (Tao et al. 2019).

5-HT_4 receptor agonism may not facilitate all forms of learning and memory: in the pilot study of acute prucalopride administration in humans there was no effect on working memory or implicit contextual learning (Murphy et al. 2020). However, these processes involve circuitry external to the hippocampus (Nee and D'Esposito 2018; Preston and Gabrieli 2008). The dose of prucalopride (1mg) used in both our previous single and current sub-acute dosing studies was chosen following early pilot work where there were difficulties tolerating the 2mg dose, which is the standard dose used in the treatment of constipation. However, it is possible that 1mg of prucalopride may be suboptimal from the point of view of cognitive enhancement. Data is currently lacking that could indicate the dose of prucalopride required to produce the most clinically-effective activation of 5-HT_4 receptors in the brain. Use of this low dose may have decreased power in this study, and may explain, for example, why hippocampal activation to the stimulus contrast did not meet significance at the whole-brain level. Due to chance, most participants whose first language was not English were randomised to placebo. However, the memory task used here was not verbal, and there was no evidence that first language was correlated with post-scan recall task performance.

The hippocampal memory task used in this study is a validated probe of the hippocampus (Filippini et al. 2009; Filippini et al. 2012), and therefore appropriate for use with an experimental medicine approach to assess therapeutic potential for treatment-related cognitive improvement (Miskowiak and Petersen 2019). However, although the region of interest analyses involving the hippocampus demonstrate a difference between the prucalopride and placebo groups in their strength of activation, this difference did not survive correction for multiple fMRI comparisons in our whole brain analysis. Power to detect any difference at a statistical level may be improved by an increased prucalopride dose or a larger study. Unfortunately, three participants had to be

excluded from analyses due to fMRI-analysis concerns in addition to an earlier three excluded at the time of data collection for technical reasons.

This study holds promise for the future use of 5-HT_4 receptor agonists in terms of ameliorating some of the cognitive impairments associated with psychiatric disorders. It also provides evidence that the pro-cognitive effects of 5-HT_4 receptor agonism are still apparent following subacute administration; this is consistent with animal studies. Future work could include optimising the dose of prucalopride to ensure maximal effect at the 5-HT_4 receptor whilst minimising side effects. This may involve higher doses alongside peripheral 5-HT_4 receptor blockade to prevent side effects, or using a novel pharmacological agent where PET studies have indicated the dose required for optimised brain 5-HT_4 receptor occupancy (see Chapter Five). It also would be beneficial to delineate further which specific cognitive domains may benefit from 5-HT_4 receptor agonism to enable precise targeting of deficits as part of a personalised approach to treatment.

CHAPTER 3

The effect of prucalopride on a fMRI emotional faces task in healthy volunteers

Based on work published as de Cates AN, Martens MAG, Wright LC, Gibson D, Gould van Praag CD, Capitao LP, Cowen PJ, Harmer CJ, Murphy SE (2022). The effect of the 5-HT$_4$ agonist, prucalopride, on an fMRI faces task in the healthy human brain. Frontiers in Psychiatry 13, 859123

3.1: Introduction

As discussed in Chapter One, depression is a common and global disorder resulting in significant disability (Malhi and Mann 2018). However, first line antidepressants are known to have a delay of a few weeks before clinical impact, and at least one third of patients do not achieve remission with detrimental consequences on outcomes (Malhi and Mann 2018). Therefore, new and faster treatments for depression are sorely needed.

Evidence from animal studies suggests that 5-HT$_4$ receptor agonists, such as prucalopride, may represent a promising new mechanism for rapid acting antidepressant action (Duman 2007). In contrast to the delayed action of conventional antidepressants, 5-HT$_4$ receptor agonists acutely increase the firing rate of midbrain serotonergic cells, via their action on medial prefrontal cortical pyramidal cells (Faye et al. 2019; Lucas and Debonnel 2002; Moha ou Maati et al. 2016). In addition, 5-HT$_4$ receptor agonism rapidly induces the production of neurotrophic proteins

required for neuroplasticity, such as brain derived neurotrophic factor, an outcome typically only seen with chronic antidepressant treatment (Mendez-David et al. 2014).

Consistent with this, animal behavioural studies have demonstrated that 5-HT_4 receptor agonists are rapidly active in animal models of depression, ameliorating the anhedonia-like behaviours produced by chronic mild stress and corticosteroids (Alexander et al. 2010; Lucas et al. 2007; Mendez-David et al. 2014). In addition, 5-HT_4 receptor agonists have been shown to have pro-cognitive effects, and in particular facilitate hippocampal dependent learning and memory processes (Lamirault and Simon 2001; Meneses and Hong 1997; Orsetti et al. 2003), which may indicate clinical uses across a variety of mental illness diagnoses, including anxiety and schizophrenia. Consistent with this, 5-HT_4 receptor agonists have been shown to lead to rapid anxiolysis in rodents (Faye et al. 2019; Mendez-David et al. 2014; Pascual-Brazo et al. 2012), which may relate to direct activation of medial prefrontal cortex 5-HT_4 receptors (Faye et al. 2019). They also have been suggested as potential neuroprotective agents in schizophrenia as subfield volumetric reduction in the hippocampus in patients with first episode psychosis appears to correlate with 5-HT_4 receptor density (Park et al. 2021).

It has previously been shown that prucalopride (1mg, for one and six days) has a pro-cognitive effect on memory tasks in healthy volunteers ((Murphy et al. 2020) and see Chapter Two), and increases hippocampal and angular gyrus function during memory processing (see Chapter Two). However, it is currently not known whether prucalopride also has an antidepressant-like profile in humans.

Depression is associated with negative emotional biases in information processing. Serotonergic antidepressants lead to rapid shift in these informational processing biases (i.e. decrease responses to negative and increase responses to positive information) both in behavioural and fMRI studies (Browning et al. 2007; Godlewska et al. 2012; Harmer et al. 2009; Harmer et al. 2003; Harmer et al. 2004). Moreover, these shifts are detectable in both healthy (Harmer et al. 2006; Murphy et al. 2009b) and depressed participants (Godlewska et al. 2012), and they are associated with later mood change in those with low mood. Using this methodology, experimental medicine can quickly assess if a novel medication may have potential as an antidepressant in humans (Arnone et al. 2009; Pringle et al. 2011; Rawlings et al. 2010), providing a useful approach to screen putative antidepressants in humans following promising preclinical results.

This chapter examines whether short-term (6 days) administration of prucalopride affects neural and behavioural emotional processing in healthy human volunteers. This is a separate part of the prucalopride study as reported in Chapter Two and involves the same participants completing a separate fMRI task. It was hypothesised that prucalopride would decrease the neural response to negative emotional information similar to that previously observed with serotonergic antidepressants i.e. decreased response to negative vs. positive emotional stimuli in a network including the medial prefrontal cortex (including the anterior cingulate cortex), orbitofrontal cortex, amygdala, and hippocampus in an fMRI paradigm.

3.2 Methods

3.2.1 Participants

As described in Chapter Two, right-handed healthy participants were recruited and randomised to either prucalopride (6 days x 1mg) or placebo, in a double-blind randomised design (see Chapter Two for details of inclusion and exclusion criteria). No changes to methods occurred after start of the study. The flow of participants is outlined as per Figure 1 (see Chapter Two): as specified one additional participant was excluded for this chapter compared to analyses used in Chapters Two and Four, as this participant did not engage with the faces task alone (determined by behavioural responses). Participants gave written informed consent.

3.2.2 Design and randomisation

The study had a between-subject, double-blind, placebo-controlled design. Participants were assigned in a random manner to 6 days of prucalopride (Resolor) 1mg daily or placebo (lactose tablets, Rayonex Biomedical) in a 1:1 allocation. Randomisation was blocked in design (block size = 4), stratified for sex and undertaken with an online tool (sealedenvelope.com on 1st June 2018). Group allocation was concealed from participants, investigators and assessors using sequential numbered containers, and the encapsulation process ensured that both capsules appeared identical.

Participants underwent a 3T scan including fMRI tasks on day 6 as described in Chapter Two: a structural T1 scan, a faces emotion recognition test task (reported here), a memory encoding task and arterial spin labelling (ASL) scan (see Chapter Two), and a resting-state scan (see Chapter Four). By day 6 when imaging was carried out, prucalopride would be expected to be at a steady state (terminal half-life approximately 24h) (Frampton 2009). For female participants testing / scanning did not occur during the premenstrual week.

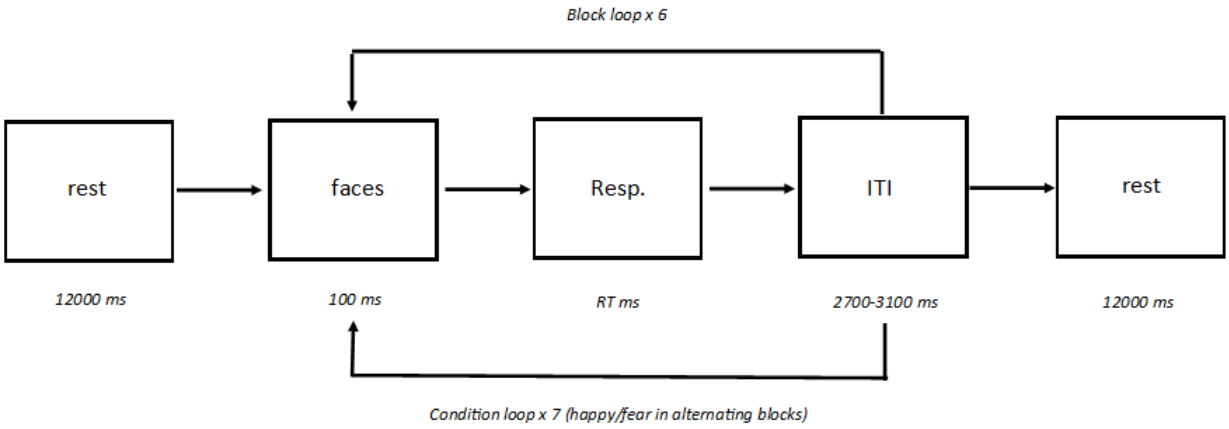
3.2.3 Questionnaire measures

As detailed in Chapter Two, to obtain baseline measures of mood, anxiety, and personality, self-report questionnaires were completed: Beck Depression Inventory-II (Beck et al. 1996); Snaith-Hamilton Pleasure Scale (SHAPS) (Snaith et al. 1995); Spielberger State-Trait Anxiety Inventory, Trait Version (STAI-T) (Spielberger et al. 1983); and Eysenck Personality Questionnaire (Eysenck and Eysenck 1975). Affect and anxiety were measured at three times (baseline (screening), pre-imaging (day 6) and post-imaging (day 6)) using: the Positive and Negative Affect Scale (PANAS) (Watson et al. 1988); visual analogue scales (VAS) (Bond and Lader 1974); and the Spielberger State-Trait Anxiety Inventory, State Version (STAI-S) (Spielberger et al. 1983). Commonly-reported side effects were also measured at these three time-points (Sajid et al. 2016). At the end of the study, participants were asked to guess their group allocation with a forced-choice question.

3.2.4 Emotional Faces fMRI Task

In the fMRI emotional processing task (Gould van Praag et al. 2021) (see Figure 6), participants were told that the aim of the task was to correctly identify the gender of each face shown in the images (male or female), with no reference made to the face emotion. This task was modified from previous versions used within our laboratory (Godlewska et al. 2012; Rawlings et al. 2010) with block lengths and orders optimised for fMRI (see (Martens et al. 2021)).

Figure 6: fMRI Faces (emotional processing) Task



Resp. = response; ITI = inter-trial interval (routine duration controlled by the component code_jitter, which generates a random number between 2.7 and 3.1s and returns this as the variable). RT = Reaction Time. ms = milliseconds.

Faces were presented rapidly for 100ms in isolation and participants had to respond to indicate the gender of the face as quickly as possible using a button press. The task followed an A-B-Rest design, where a block of condition A (fearful faces, 18 s) was followed by a block of condition B (happy faces, 18 s) then a block of rest (12 s). There was an additional rest block at the start of the experimental run. There were seven repeats of each block, generating a total 126 s of each emotion condition and 96 s of rest. Each block consisted of six trials: three male and three female images presented in a randomised order. Each image was shown once over the length of the experiment. Previous versions of this task have been shown to be sensitive to the acute effects of antidepressants on neural processing (Rawlings et al. 2010). Face images for the task were modified from the Karolinska Directed Emotional Faces (KDEF) set (http://kdef.se/index.html). The task software was written using Psychopy version 1.84.2.

3.2.5 Demographic and behavioural data analysis

Analysis of these data occurred as previously described in Chapter Two. Demographic characteristics and baseline clinical measures were analysed using independent sample *t*-tests (continuous variables), Fisher's exact tests (native language and sex) and logistic regression (education). To assess changes in subjective mood and anxiety before and after prucalopride / placebo administration, repeated measure analyses of variance (ANOVAs) were performed with time as a within-subject factor. Repeated measures analyses of variance (ANOVAs) were used to analyse fMRI behavioural task data with a between-subjects factor of the treatment group (prucalopride or placebo) and within-subjects factor of gender accuracy with face emotion (fear or happy). Post hoc analyses using independent samples t tests were performed to follow-up interactions observed. Levene's test (*t*-tests) and the Greenhouse-Geisser procedure (ANOVAs) were used as appropriate, correcting degrees of freedom where equal variances between groups could not be assumed. A *p*-value less than 0.05 was used to denote statistical significance. Partial eta squared are reported as a measure of effect size. Behavioural data were analysed in SPSS (version 25, IBM). Graphs were produced using GraphPad Prism (version 9.3.1).

3.2.6 MRI data acquisition and analysis

3.2.6.1 MRI data acquisition

Blood-oxygenation-level-dependent (BOLD) fMRI and T1-weighted anatomical images were acquired as per Chapter Two using a 3-Tesla Siemens Prisma scanner, equipped with a 32-channel head matrix coil (Siemens, Erlangen, Germany). Here we report results from the faces task. Participants also completed an encoding memory task and an arterial spin labelling (ASL) during the scan, results of which have been detailed in Chapter Two, although cerebral blood flow data is also included here as a covariate of no interest. Foam padding and a head restraint were used to control head movement.

fMRI acquisition: Facial processing was assessed from a single run of 60 T2-weighted echoplanar imaging (EPI) slices covering the whole brain [repetition time (TR) 1200 ms, echo time (TE) 30 ms, flip angle 65°, field of view 216 mm, slice thickness 2 mm, voxel dimension 2mm isotropic, acquisition time 6min 28s]. Images were distortion corrected by an acquired fieldmap (echos at 4.92 and 7.38 ms, TR=590ms, flip angle = 46°).

Structural MRI and ASL acquisition have been detailed in Chapter Two.

3.2.6.2 MRI task analysis

Faces imaging data were analysed with FSL (www.fmrib.ox.ac.uk/fsl). fMRI data were pre-processed and analysed using FEAT (FMRI Expert Analysis Tool), version 6.0.4, part of FSL (FMRIB's Software Library; www.fmrib.ox.ac.uk/fsl). DICOM (Digital Imaging and Communications in Medicine) files were downloaded from the server, checked for completeness, excessive movement and visual anomalies, and converted to a BIDS (Brain Imaging Data Structure)-standardised format nifti files using heudiconv (Halchenko 2018) (heudiconv 0.5.4 (https://github.com/nipy/heudiconv)) before pre-processing. The structural anatomical scans were brain extracted using the Brain Extraction Tool (BET) (Smith 2002). Formal MRI quality assessment was undertaken using the MRIQC package (Esteban et al. 2017) (https://mriqc.readthedocs.io/en/stable/index.html), with data considered for rejection if it fell outside the normal range of values in the derived image quality metrics. Pre-processing code is available at (de Cates et al. 2022).

Pre-processing involved various steps designed to reduce noise-related variability in the data and to improve the validity of the statistical analysis. Each participant's imaging data underwent the

following steps: (1) Removal of non-brain structures using BET (Smith 2002), (2) motion correction using FMRIB's Linear Image Registration Tool (FLIRT) (Jenkinson et al. 2002), (3) spatial smoothing using a Gaussian kernel of full-width-half-maximum 5 mm, (4) grand-mean intensity normalisation of the entire 4D dataset by a single multiplicative factor and high-pass temporal filtering cut-off = 90s (Gaussian-weighted least-squares straight-line fitting, with sigma = 45s), and (5) B0 unwarping using fieldmap rads and magnitude images for distortion correction. No slice timing correction was applied. In addition, registration to high-resolution image and to a standard template [Montreal Neurological Institute (MNI)] was implemented using FNIRT nonlinear registration (Andersson et al. 2007).

In the first-level analysis, individual activation maps were computed using the general linear model with local autocorrelation correction. Two explanatory variables were modelled: "happy" and "fear" images. Temporal derivatives were included in the model. Variables were modelled by convolving each block with a haemodynamic response function, using a variant of a gamma function (standard deviation 3s, mean lag 6s). No included participant demonstrated significant movement. At the whole-brain level, fearful images were contrasted with happy and fixation cross (baseline), resulting in the following model: 1) fear > fixation; 2) fear < fixation; 3) happy > fixation; 4) happy < fixation; 5) fear > happy; 6) happy < fear; 7) mean > fixation; 8) mean < fixation.

In the second-level analysis, whole-brain individual data were combined at a group level (participants on placebo vs. prucalopride) using a mixed-effects analysis, and cerebral blood flow and grey matter maps as covariates of no interest. Groups were contrasted with each other, resulting in the following comparisons: 1) placebo > prucalopride; 2) prucalopride > placebo; 3) placebo mean; 4) prucalopride mean; 5) mean of all participants. Brain activations showing

significant group differences were identified at the whole-brain level using cluster-based thresholding (Z>3.1, family-wise error (FWE) p<0.05 corrected). Significant interactions from whole-brain analyses were further explored by extracting percentage BOLD signal change for each type of contrast. As the anterior cingulate cortex, medial frontal cortex, orbitofrontal cortex, and left or right amygdala and hippocampus were a particular focus, they were pre-specified as regions of interest (ROI). A functional ROI mask was created for each region by multiplying mean activation for each contrast of interest (on whole-brain data already corrected for multiple comparisons (FWE) and Z>3.1 as described above) for all participants by the Harvard-Oxford atlas anatomical mask at a 50% threshold. Percentage BOLD signal change for each contrast (in each hemisphere were relevant) was extracted in order to identify the profile of drug effect. All activations are reported using MNI co-ordinates.

As post-hoc analyses, whole brain group feat results were also run with and without using cerebral blood flow and grey matter maps as covariates of no interest, and sex as a covariate of interest. FSLVBM and Oxford ASL was conducted as detailed in Chapter Two. ACC and amygdala perfusion between groups was compared using fslmeants: parameter estimates of perfusion were extracted from resting perfusion maps (previously computed using Oxford_ASL in units of ml/100 g/min) using anatomical Harvard–Oxford masks of the ACC and the left and right amygdala (amygdala at a 50% threshold).

3.3 Results

3.3.1 Participants

The sample was identical to that included in Chapter Two except that one further participant was excluded from fMRI analyses for lack of engagement with the faces task (determined from behavioural responses).

Analysis occurred in originally assigned groups. The final groups for behavioural analysis (N=43, 21:22 = placebo : prucalopride, aged 18-36) were very similar to Chapter Two in terms of baseline sample demographics, and mood and personality scores, as well as randomisation guesses and adverse events.

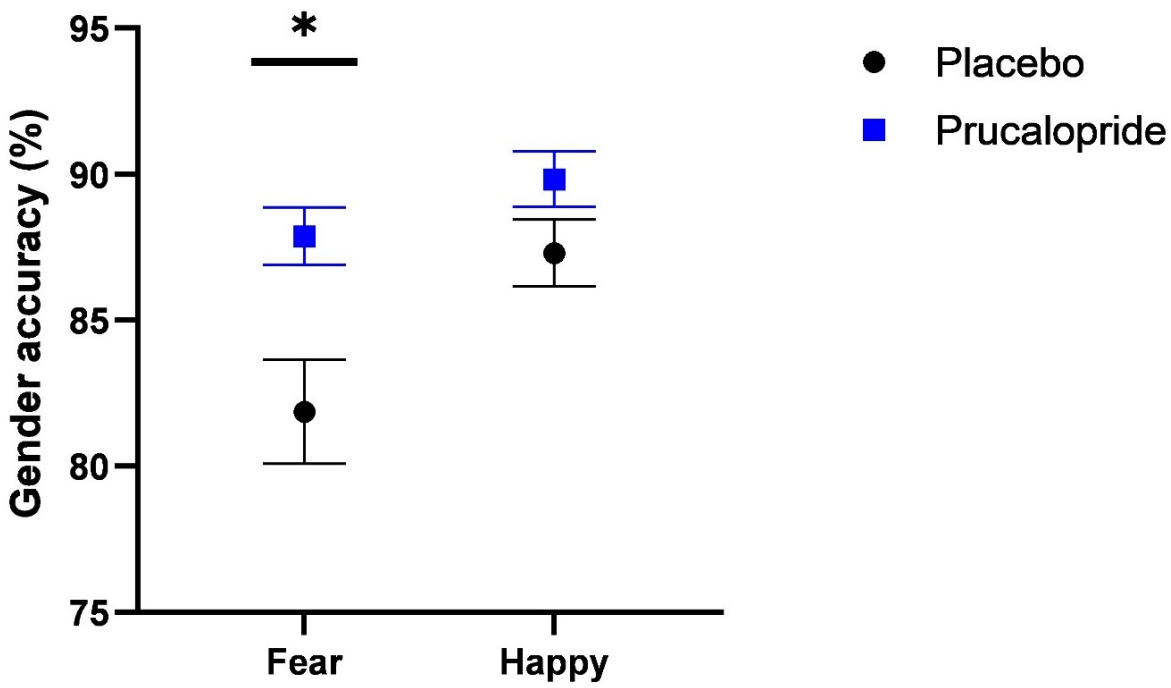
3.3.2 Questionnaire results

Similar to Chapter Two, there were no significant differences in state anxiety or affect between the prucalopride and placebo groups (all *p*s>0.5).

3.3.3 Behavioural results of fMRI Faces Task

There was a significant main effect of group (F(1,41)=7.47, p=0.009, np^2=0.15), reflecting improved accuracy in the prucalopride group to specify face gender compared with the placebo group [accuracy (%): prucalopride mean=88.85, SEM=0.80; placebo mean=84.58, SEM=1.36]. There was also a significant emotion*group interaction (F(1,41)=4.48, p=0.04, ηp^2=0.10). This interaction was driven by significantly increased accuracy for fearful faces in the prucalopride group compared with the placebo group [t(1,31.41)=-2.955, p=0.006] but no significant group differences in the accuracy for happy faces [t(1,41)=-1.702, p=0.096], see Figure 7.

Figure 7: Behavioural results of fMRI Faces Task



*Accuracy (%; unadjusted) to gender discrimination task as part of fMRI faces paradigm in placebo vs. prucalopride group. Error bars show standard error of the mean. Black circle = placebo; blue square = prucalopride. *=p<0.05 (T test; N=43; 21:22 = placebo : prucalopride)*

These results were unchanged by adjusting for non-responses (main effect of group: F(1,41)=8.91, p=0.005, ηp^2=0.18; emotion*group interaction: F(1,41)=6.91, p=0.012, ηp^2=0.14). There was no difference in average reaction speed between the groups (p=0.39).

3.3.4 Analyses controlling for potential confounds

Similar to previously reported in Chapter Two, there were no group-related differences in grey matter (FSLVBM: placebo > prucalopride, p=0.91; prucalopride > placebo, p=0.44), global blood flow (Oxford_ASL: p=0.64 (grey matter), p=0.60 (white matter)) or regional blood flow (placebo > prucalopride p=0.71; prucalopride > placebo p=0.38)). Whole brain and ROI results were similar with and without correction for cerebral perfusion and grey matter maps, and sex. Left and right

amygdala and ACC perfusion did not differ between groups: L&R amygdala smoothed data *p*=0.65 (L), p=0.87 (R); ACC smoothed data *p*=0.68 (see Table 6). Native language and overall accuracy to gender discrimination during the scan were not correlated using linear regression (R^2=0.012).

Table 6: Left and right amygdala and anterior cingulate cortex (ACC) perfusion (ml/100g/min)

		Left amygdala	**Right amygdala**	**Anterior cingulate cortex**
Smoothed (2.12)	***Placebo***	41.7	42.1	51.0
	Prucalopride	42.6	41.8	51.9
Unsmoothed	***Placebo***	41.7	42.0	52.0
	Prucalopride	42.7	41.8	52.9

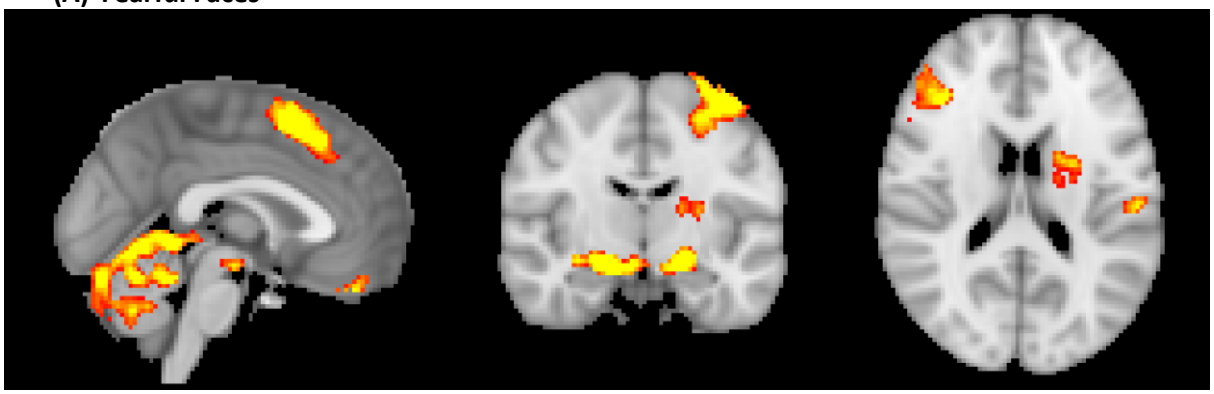
3.3.5 Emotional Faces Task fMRI

3.3.5.1 Main effect of task:

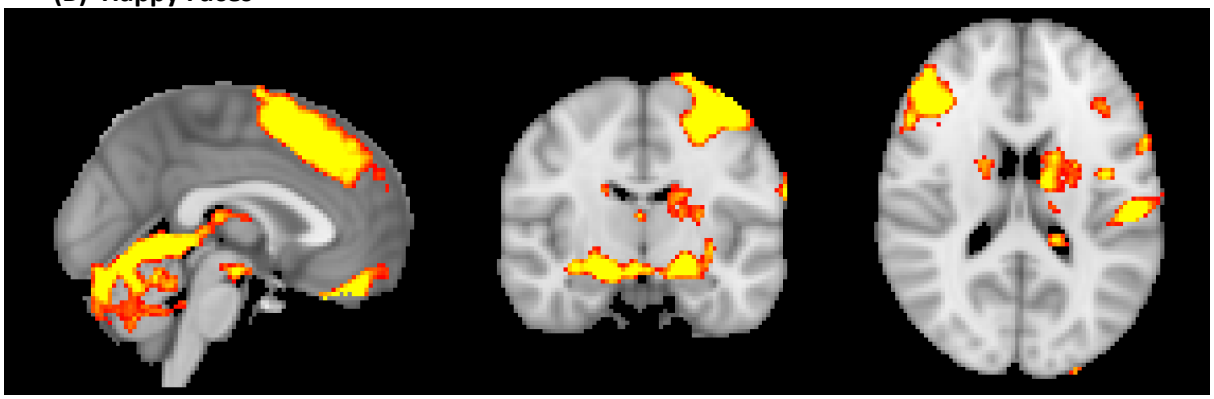
In order to determine if the functional MRI (fMRI) task engaged brain regions previously associated with fearful and happy facial stimuli, blood-oxygen-level-dependent (BOLD) activation in response to fearful faces and happy faces and the mean of both valences was compared to the baseline (i.e. fixation cross) across both groups (see Figure 8). Consistent with previous studies using implicit emotional face paradigms, significant brain activations were observed in a network of regions (Capitao et al. 2019; Fusar-Poli et al. 2009; Vytal and Hamann 2010), including the superior, middle and inferior frontal gyrus, paracingulate gyrus, frontal pole, precentral gyrus, left and right putamen, left insular cortex and left thalamus. These findings confirm that the task engaged brain areas implicated in the processing of both fear and happiness, similar to previous work with this version of the task (Martens et al. 2021).

Figure 8: Neural activity elicited by the fMRI Faces Task for all participants

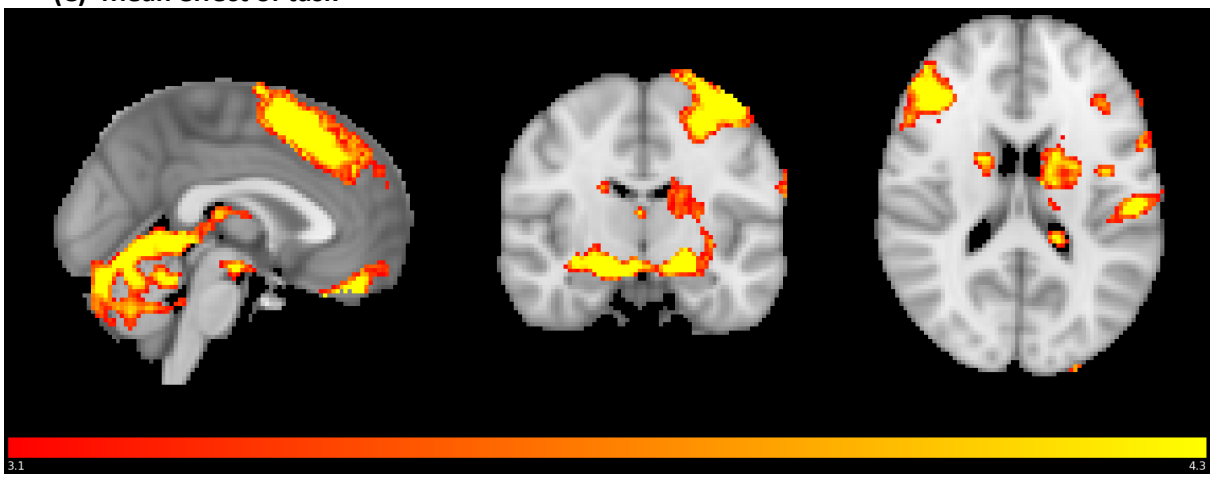
(A) Fearful Faces



(B) Happy Faces

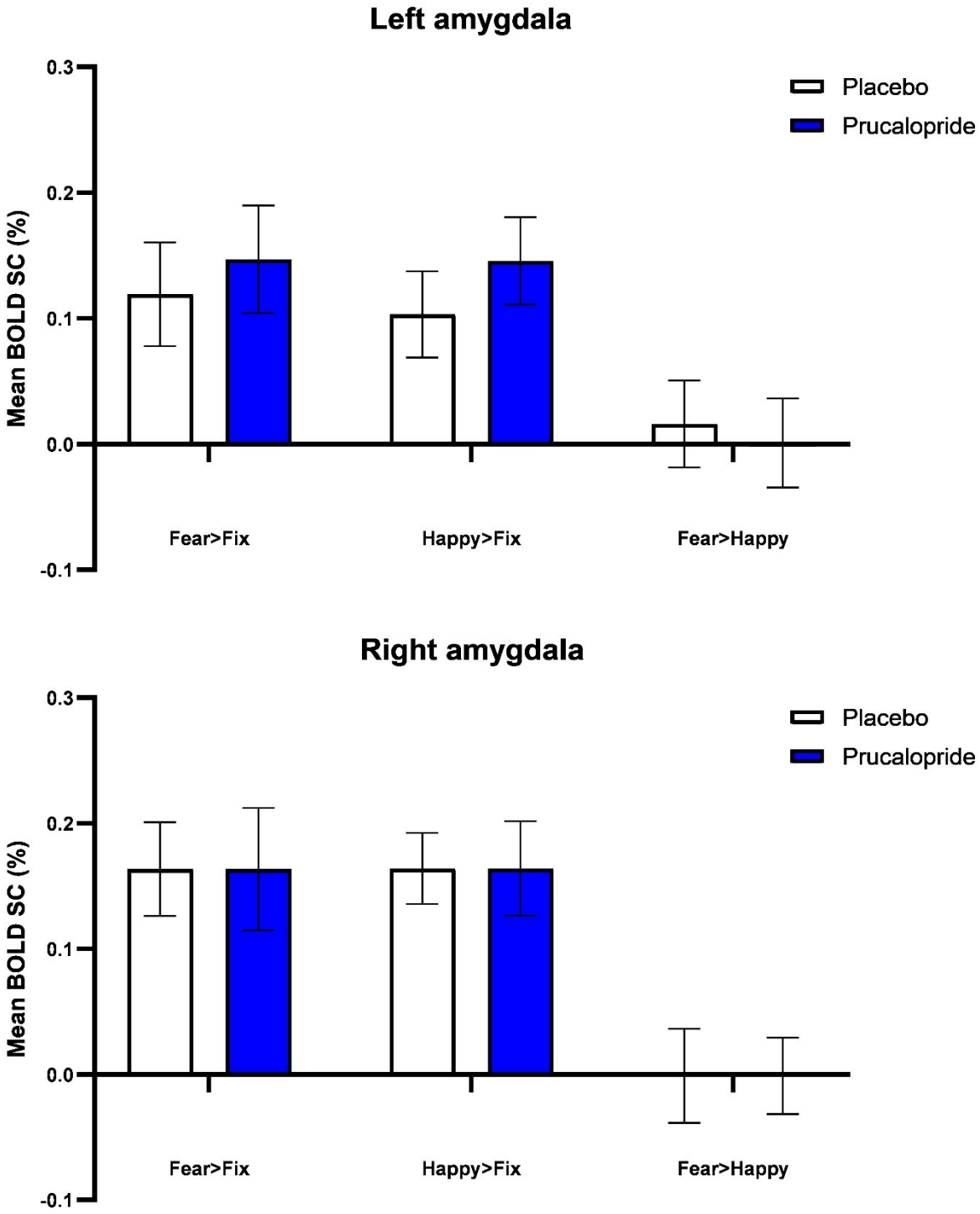


(C) Mean effect of task



*Activation of brain areas during the fMRI faces task. The sagittal, coronal, and axial images identify neural activation (red) in response to **(A)** fearful faces versus fixation cross, **(B)** happy faces versus fixation cross, and **(C)** mean of both valences, across both treatment groups. Results are shown at the 3.1 cluster threshold significance level, corrected with FWE.*

Figure 9: BOLD percentage signal change (SC) extracted from left amygdala and right amygdala



Group mean of BOLD percentage signal change extracted from L amygdala and R amygdala in response to fearful and happy images (fear>fixation; happy>fixation; fear>happy). Error bars show standard error of the mean. The anatomical mask used was the Harvard-Oxford atlas at 90% threshold.

Unexpectedly, I did not see differential whole-brain activation related to face emotion (i.e. no significant clusters were seen in the fear > happy or happy > fear contrast), and there was a similar level of amygdala activation to both fearful and happy faces bilaterally (see Figure 9). For this reason, I present the mean effect of task (mean (of fear + happy) > fixation) as our primary analysis, with reference to results relating to emotional valence as appropriate.

3.3.5.2 Effect of treatment – whole brain analysis:

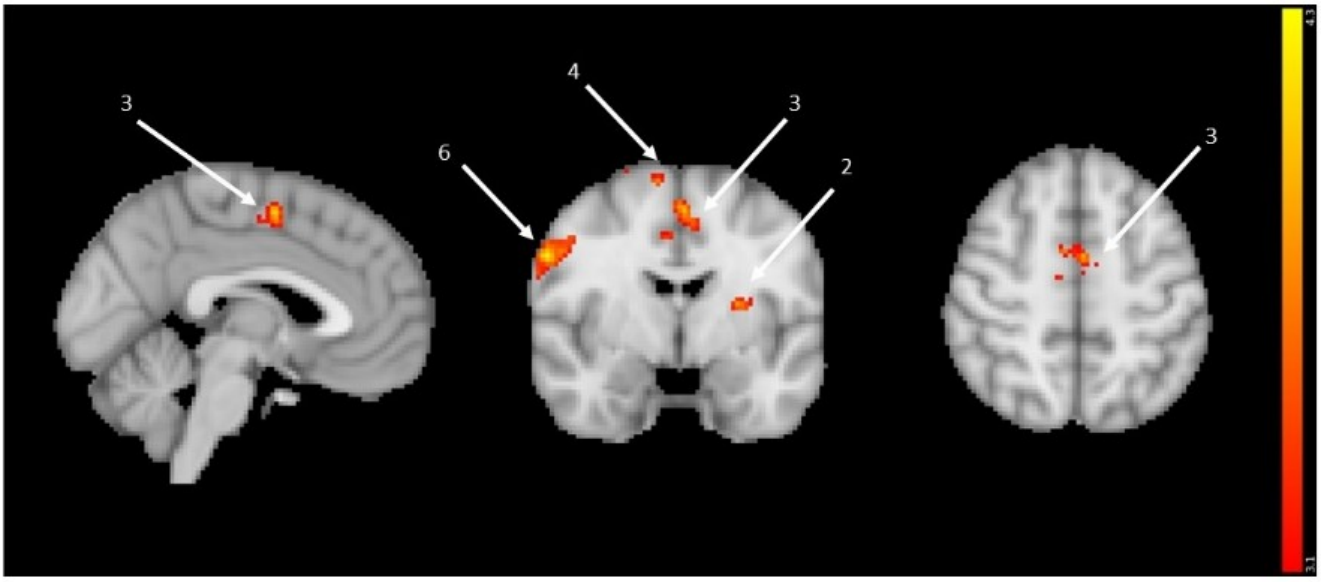
When averaging the two emotions (vs. baseline), there was altered activation in the prucalopride group in 6 clusters (i) right precentral / postcentral gyrus, (ii) left supramarginal / postcentral gyrus, (iii) right superior frontal gyrus, (iv) anterior cingulate cortex (ACC), (v) left putamen / insula, (vi) left precentral / postcentral gyrus (see Table 7 and Figure 10(A) for details of clusters). Figure 10(B) represents the group-level extracted BOLD signal change for these clusters. Across all clusters, the prucalopride group showed reduced activation in this network of areas.

Table 7: Details of activation clusters (placebo > prucalopride) from whole brain analysis for the mean effect of task

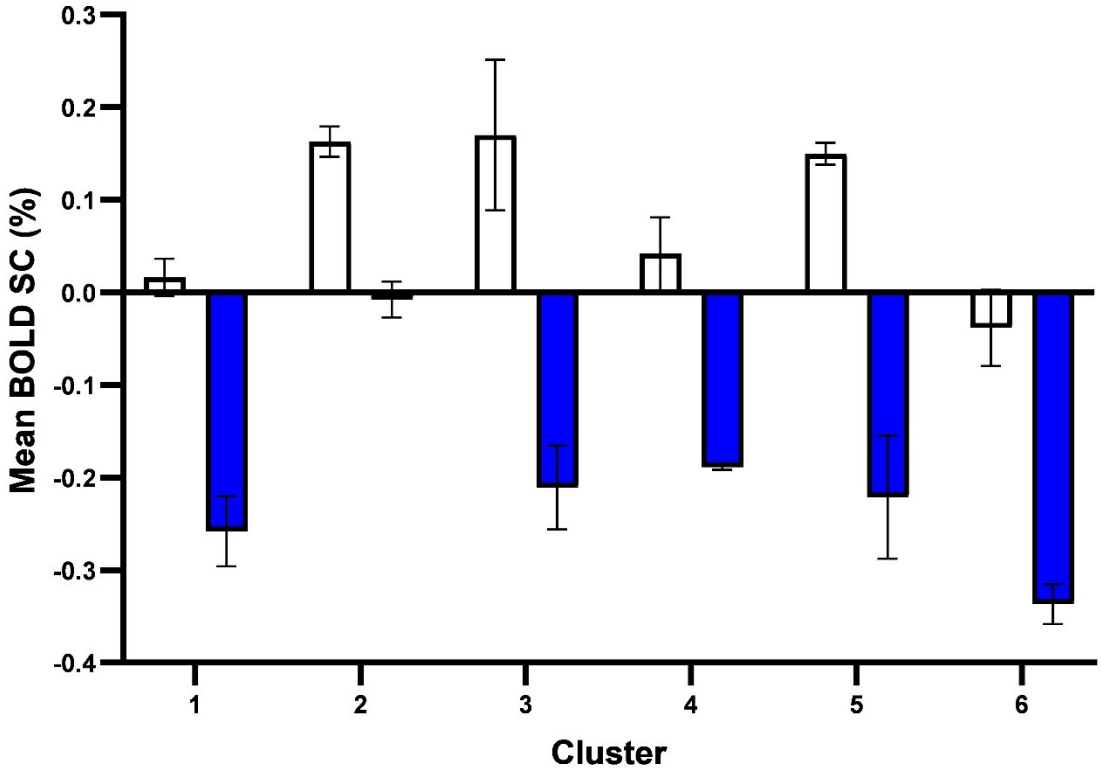
Cluster number	**Size (voxels)**	**Z-max**	**p-value**	**Z-max location (MNI)**	**Main regions involved in cluster**
6	408	4.56	<0.001	62,-2,32	R precentral / postcentral gyrus
5	299	4.6	<0.001	-62,-30,38	L supramarginal / postcentral gyrus
4	242	3.65	<0.001	12,2,68	R superior frontal gyrus
3	178	2.71	0.002	6,0,50	Anterior cingulate cortex
2	116	1.68	0.021	-28,-6,12	L putamen / insula
1	106	1.5	0.032	-48,-18,42	L postcentral / precentral gyrus

Figure 10: Whole Brain fMRI Faces Task results

A



B



(A) *Whole-brain activation in response to mean effect of task (mean > fixation) in placebo vs. prucalopride group. Sagittal, coronal and axial images (shown at MNI location 45,61,62) depicting significantly increased activation in the placebo group for the mean contrast in significant clusters (Clusters: 1 = L postcentral / precentral gyrus; 2 = L putamen / insula; 3 = anterior cingulate cortex; 4 = R superior frontal gyrus; 5 = L supramarginal / postcentral gyrus; 6 = R precentral / postcentral gyrus); cluster 1 and 5 not visible in figure. Images thresholded at z> 3.1 p<0.05 corrected. Red to yellow colours identify increases in brain activation (scale Z=3.1 to 4.3).*

(B) *Group mean of BOLD percentage signal change (SC) extracted from the 6 clusters (mean > fixation). Error bars show standard error of the mean. White = placebo; blue = prucalopride. Clusters: 1 = L postcentral / precentral gyrus; 2 = L putamen / insula; 3 = anterior cingulate cortex; 4 = R superior frontal gyrus; 5 = L supramarginal / postcentral gyrus; 6 = R precentral / postcentral gyrus*

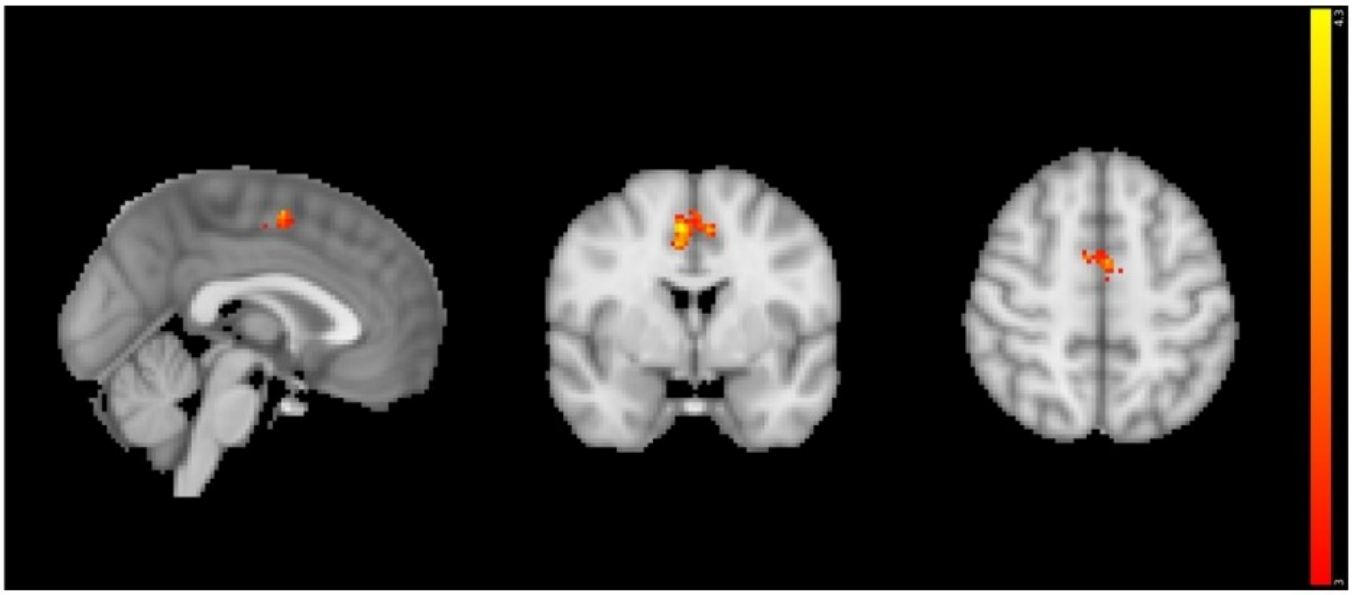
More specifically, as shown in Figure 10(B), the left and right precentral / postcentral gyrus and right superior frontal gyrus clusters showed deactivation in the prucalopride group versus no or little activation in the placebo group; the left putamen / insula cluster showed activation in the placebo group versus no activation in the prucalopride group; the ACC and left supramarginal cluster showed activation in the placebo group versus deactivation in the prucalopride group.

3.3.5.3 Effect of treatment – region of interest analysis:

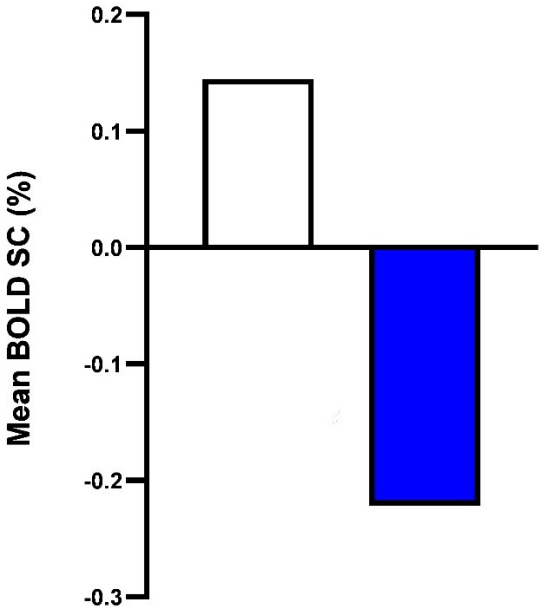
There were no significant group differences in response to fear vs. happy facial expressions, or the mean effect of task, in the medial frontal cortex, orbitofrontal cortex, or left or right amygdala. Examining the effect of prucalopride versus placebo on the left and right amygdala to fearful faces demonstrated that prucalopride did not reduce activation in the amygdala as is typically seen with serotonergic antidepressants (Harmer et al. 2006); instead there was no difference in amygdala activation between the placebo and prucalopride groups (see Figure 9).

Figure 11: ACC Region of Interest fMRI Faces Task results

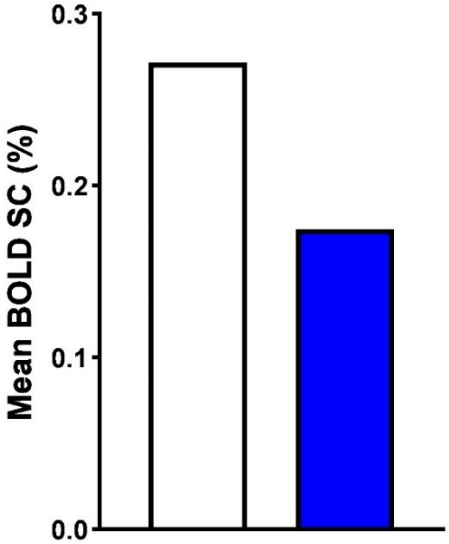
A



B



C



(A) *Region of interest activation for the anterior cingulate cortex (ACC) in response to mean effect of task in placebo vs. prucalopride group. Sagittal, coronal and axial images (shown at MNI location 45,63,62) depicting significantly increased activation in the placebo group for the mean > fixation contrast [placebo>prucalopride, Z=4.39, p<0.0002, peak voxel location: x=6, y=0, z=50, cluster size=164 voxels]. Images thresholded at z> 3.1, p<0.05 corrected. Red to yellow colours identify increases in brain activation (scale Z=3.1 to 4.3). ACC Mask = Harvard Oxford atlas*

(B) *Group mean of BOLD percentage signal change (SC) extracted from the cluster in 3(A). White = placebo, blue = prucalopride.*

(C) *Group mean of BOLD percentage signal change (SC) extracted from functional anterior cingulate cortex (ACC) mask in response to mean effect of task. A functional ROI (SVC) mask was created for the ACC for mean > fixation by multiplying mean activation for all participants by the anatomical mask. White = placebo, blue = prucalopride.*

However, in the ACC, consistent with whole-brain results, the prucalopride group showed deactivation compared with little activation in the placebo group in response to the mean effect of task (mean faces) [placebo > prucalopride, Z=4.39, p<0.0002, peak voxel location: x=6, y=0, z=50, cluster size=164 voxels] (see Figure 11(A) for cluster and (B) for group-level extracted BOLD signal change).

This pattern of reduced activation in the prucalopride group compared to the placebo group was unchanged when a region of interest small volume correction analysis using a functional mask was performed (using the same method as Chapter Two (Figure 11(C)).

3.4 Discussion

The main finding of this chapter is that after six days of prucalopride treatment, healthy young adults were more accurate at identifying the gender of faces in a rapidly presented faces task compared to the placebo group, particularly in the context of fearful faces. In addition to this improved behavioural performance, a reduction in brain activity was seen in the prucalopride group, relative to the placebo group, in response to fearful and happy faces in a network of regions, including the medial prefrontal cortex (mPFC) (anterior cingulate cortex (ACC), superior frontal gyrus), and inferior parietal lobule (supramarginal gyrus). This reduction in activation in the ACC during the task was seen in both the whole-brain and region of interest analyses.

The improvement in accuracy of gender discrimination seen in the prucalopride group is consistent with previous work demonstrating a pro-cognitive effect of acute and 6 day prucalopride administration in healthy volunteers ((Murphy et al. 2020), Chapter Two). Whilst

previously it has been reported that prucalopride acts to increase learning and memory in recall and recognition tasks, here this prucalopride-related improved performance extends to a simple perceptual discrimination task. Interestingly, this improved accuracy in the prucalopride group specifically related to fearful faces rather than happy faces, suggesting that prucalopride may have allowed participants not only to focus better on the task but also be less distracted by fearful faces compared to participants in the placebo group. It is also worth noting that participants found gender discrimination harder for fearful faces, and so this condition may have been more sensitive to changes in cognitive performance.

The rapid, specific, and potentially chronic improvements seen in learning and memory with 5-HT_4 receptor agonists in rodents (Hagena and Manahan-Vaughan 2017; King et al. 2008; Lamirault and Simon 2001; Lucas et al. 2007) have been suggested to occur secondary to an increase in neurotransmitters and neurotrophic proteins, perhaps particularly acetylcholine release (Consolo et al. 1994; Siniscalchi et al. 1999), which results in downstream effects including neuroplasticity (Hagena and Manahan-Vaughan 2017; Pascual-Brazo et al. 2012). A recent study has also shown that prucalopride reduces bursts of AMPA receptor-mediated currents in the hippocampus, altering glutamatergic transmission (Chen et al. 2019) and making it a particularly interesting candidate for enhancing cognition. However, there is less understanding from the animal literature in terms of the impact of 5-HT_4 receptor agonism on cognitive processes involved in the discrimination task used here, which could include effects on attention, amongst others.

This improved behavioural performance was accompanied by reduced activation in a network of regions, including the mPFC and inferior parietal lobule. Interestingly, this network overlaps with regions that form the default mode network (DMN). The default mode network (DMN), also known as the medial frontoparietal network (M-FPN) is a connected network of brain regions that

experience greater activity at rest than during performance of externally-orientated active cognitive tasks (Uddin et al. 2009). Abnormalities of the DMN are present in cognitive impairment – both in Alzheimer's (Greicius et al. 2004) and those at high risk of dementia (i.e. mild cognitive impairment (MCI) (Jin et al. 2012)) – but also in depression, where abnormalities may pose a cognitive risk factor for recurrent illness (Marchetti et al. 2012). Abnormal activity within the DMN is classically linked to increased rumination, but is also associated with impaired control of attention (Marchetti et al. 2012). Failing to reduce activation within the DMN whilst actively undertaking a cognitive task appears to directly relate to poor task performance, whereas appropriate "switching off" of the DMN during tasks is linked with improved cognitive performance (Petersen and Miskowiak 2021). In this way, improving appropriate deactivation of the DMN during cognitive tasks has been proposed as a transdiagnostic marker to assess for pro-cognitive effects of interventions (Petersen and Miskowiak 2021). Therefore, the decreased activation of DMN-related regions during task performance in this fMRI faces task may reflect enhanced attention and cognitive control, which is consistent with the improvement in performance accuracy seen behaviourally.

Contrary to our hypothesis, a clear antidepressant-like profile of prucalopride was not evident on the emotional processing task. In humans, in both healthy participants and depressed patients, antidepressants work rapidly to decrease brain regional activity involved in processing of negative information, and increase activity arising from positive stimuli (Browning et al. 2007; Harmer et al. 2009; Harmer et al. 2003; Harmer et al. 2004). These effects occur prior to any significant mood change in patients with depression. This emotional processing model has been shown to provide a rapid and reliable detection of drugs with clinical antidepressant activity (Arnone et al. 2009; Pringle et al. 2011), and thus provides a feasible method to screen $5HT_4$ agonists in humans for potential antidepressant activity. Results from this chapter are not consistent with

prucalopride demonstrating an antidepressant effect. That is, a general decrease in activity in response to faces rather than an emotion specific effect was seen i.e. there was no reduction in amygdala activation to fearful faces with prucalopride as typically observed with SSRIs (Harmer et al. 2006; Murphy et al. 2009a). This is a similar finding to an earlier behavioural study in healthy participants in our laboratory, which reported no evidence of an antidepressant profile of prucalopride on behavioural tasks of emotional processing following a single 1mg dose (Murphy et al. 2020).

Given the consistency of evidence from animal studies demonstrating an antidepressant-like profile of 5-HT_4 receptor agonism on a number of models of depression, it is important to consider why this effect has not yet translated to human models. Although animal models are vital in drug discovery, they are frequently poorly predictive of clinical efficacy in humans (McGonigle and Ruggeri 2014). However, we also need to consider possible other explanations for the lack of antidepressant-like profile seen in the current study. Importantly, the version of the fMRI faces task used in this study did not elicit differential activity in the amygdala in response to “happy” and “fearful” faces in this sample, unlike previous versions of the task. It is possible this relates to enhanced standardisation of the face images between emotion conditions, which was intended to minimise the confounding differences such as hair position, face size and image luminance, and provide a more constrained contrast between the emotions. In this sample, it may have led to a perception that happy faces were more threatening, and it also appears to have made the task more cognitive challenging (participants had generally reduced accuracy than seen with previous versions of this task).

Within this context, it is necessary to be cautious in interpreting the apparent lack of an antidepressant effect, a finding which needs to be replicated using a task that is more successful

in probing emotion-specific responses to face stimuli. Furthermore, a low dose of prucalopride (1mg is half the licensed dose of 2mg) was used in the current study to minimise gastrointestinal side effects. It is possible that this dose is insufficient to produce positive effects on emotional processing, and we currently lack information about the appropriate dose of prucalopride needed for optimal occupancy of brain 5-HT_4 receptors. The sample size needed (17 per group to give 90% power with $\alpha = 5\%$) was calculated on the basis of a conservative estimated effect size of 0.5-0.7, but oversampling of 25 per group was undertaken to account for fMRI analyses. Nonetheless, the study may still have been underpowered if the effect of 1mg prucalopride on amygdala activity was smaller than this effect size.

Unfortunately, three participants were excluded prior to fMRI analysis for technical reasons, and a further four were excluded during analysis for fMRI-related concerns or a lack of engagement with the task (determined from behavioural responses), which likely reduced the statistical power of the study. Due to chance, most participants whose first language was not English received placebo, but the task was not verbal in nature, and there was no evidence that first language and task performance were correlated. These findings also relate to prucalopride specifically, and there may be subtle variations in the effects of other 5-HT_4 receptor agonists (Bonaventure et al. 2000). However, there is no evidence that there are marked variations between different 5-HT_4 receptor agonists from the animal literature.

In conclusion, the behavioural results from this fMRI faces task provide further evidence that prucalopride can enhance cognition in healthy participants. This is consistent with, and extends, our previous findings of pro-cognitive effects of prucalopride on learning and memory tasks. In addition, the findings from this chapter suggest that prucalopride may reduce activation in the default mode network during task performance, which may be one mechanism by which

prucalopride improves cognition. Future work should include a more detailed exploration of the breadth of cognitive changes produced by prucalopride and other 5-HT_4 agonists, as well as of the duration of any effect considering the potential for desensitisation of 5-HT_4 receptors. Delineating the effect of prucalopride on parts of the default mode network deserves further exploration under resting-state conditions (see Chapter Four). The effect of prucalopride on emotional processing in humans also requires further exploration with a higher dose to determine if the promising findings from animal studies may indeed translate into human investigations; alternatively prucalopride's impact on longitudinal mood may be able to be explored directly using electronic health records (see Chapter Six).

CHAPTER 4

The effect of prucalopride on functional connectivity in the healthy human brain

Based on work under review as de Cates AN, Martens MAG, Wright LC, Gibson D, Spitz G, Gould van Praag CD, Suri S, Cowen PJ, Murphy SE, Harmer CJ. 5-HT$_4$ receptor agonist effects on functional connectivity in the human brain; Implications for pro-cognitive action. Biological Psychiatry: Cognitive Neuroscience and Neuroimaging.

4.1: Introduction

Preclinical data suggests that stimulating 5-HT$_4$ receptors rapidly improves learning and memory in rodent models (King et al. 2008). As discussed in Chapter One, this finding has been demonstrated using different 5-HT$_4$ receptor agonists, and across a range of cognitive paradigms (Hagena and Manahan-Vaughan 2017; Lamirault and Simon 2001; Lucas et al. 2007; Meneses and Hong 1997; Orsetti et al. 2003), and effects are maintained for up to 14-30 days (Hashemi-Firouzi et al. 2021; Quiedeville et al. 2015). Based on one pilot healthy volunteer study and the data presented in Chapters Two and Three, translation of this effect has been extended to healthy humans. A single dose of the highly-selective 5-HT$_4$ receptor agonist prucalopride increased cognitive function across three different learning and memory tasks (Murphy et al. 2020). After repeated dosing for six days, prucalopride increased neural activation in memory-associated regions during a memory task with simultaneous improvement in task performance (Chapter Two), and improved gender discrimination in an implicit emotional faces task (Chapter Three). During task activity, prucalopride also appeared to appropriately supress regions analogous to the

default mode network (DMN) (Chapter Three). Thus, Chapters Two and Three provide evidence that prucalopride may have pro-cognitive activity in humans, with suggestions for potential neural mechanisms at play.

Large human datasets show that appropriate connections between specific resting-state networks are directly associated with successful cognitive performance (Shen et al. 2018). Depression is also associated with functional changes within similar resting-state neural networks related to cognition compared with healthy volunteers (see Chapter One). These changes involve the triple network (Menon 2011) in particular, including the default mode (DMN, or medial frontoparietal), central executive (CEN, or lateral frontoparietal), and salience networks (SN, or midcingulo-insula) (Bhaumik et al. 2017; Dong et al. 2019; Neufeld et al. 2018). Therefore, the suboptimal functioning of and between components of the triple network seems to be involved in both cognitive deficits and depression.

As discussed in Chapter One, cognitive deficits as part of depression are common and disabling, are present in at least two thirds or greater of those with a diagnosis (Conradi et al. 2011; Halahakoon et al. 2019) and are rated by patients as their greatest concern after low mood (Fried and Nesse 2014; Lam et al. 2014). Despite the clear unmet need, there are almost no therapeutic agents that successfully target impaired cognition specifically as part of mental illnesses such as mood or psychotic disorder (Colwell et al. 2022). In healthy volunteers, pro-cognitive medication appears to lead to both increased and decreased connectivity including generally *reduced* resting-state functional connectivity (rsFC) between regions of the DMN (Becker et al. 2022) and *increased* rsFC in regions involved in cognition outside of the DMN, particularly parts of the prefrontal cortex (Miskowiak and Petersen 2019; Petersen and Miskowiak 2021). Consistent with this, the findings presented in Chapters Two and Three suggest that prucalopride reduces activity

in areas within the DMN in the context of a task (Chapter Three) and leads to increased activity in memory processing regions (see Chapter Two) whilst performing directed cognitive tasks with improved accuracy. The regions that I have found previously modulated by prucalopride in the context of cognitive task performance include the anterior cingulate cortex (ACC), the posterior cingulate cortex (PCC), the hippocampus, and the angular gyrus.

The aim of this chapter was to explore if subacute (six days) prucalopride in healthy humans would influence resting-state functional connectivity (rsFC) of regions involved in cognitive processing (the triple network, and seeds involving the ACC, PCC, hippocampus and angular gyrus). This used additional data from the prucalopride study. Based on existing evidence and previous results in Chapters Two and Three, it was hypothesised that prucalopride would reduce rsFC of the DMN and may have effects on other cognitive networks. Due to its size, functional heterogeneity (the ACC is thought to be linked with both emotional networks and cognitive networks), and importance in the whole brain and previous analyses of data from the prucalopride study (i.e. Chapter Three), *a priori,* we divided the ACC into two structural regions for seed placement: (i) a rostral portion thought to be more involved with prefrontal regions and affective networks (including the DMN); (ii) and a caudal portion linked with sensorimotor regions and frontoparietal networks (Margulies et al. 2007; Stevens et al. 2011).

4.2 Methods

4.2.1 Participants

Details of the study participants have been described in Chapters Two and Three.

4.2.2 Design and randomisation

Study design, randomisation and imaging protocol have been outlined in Chapters Two and Three. Resting-state data acquisition was performed with participants being asked to relax but keep their eyes open for the duration of the period (approximately six minutes).

4.2.3 Questionnaire measures and behavioural analysis

Mood, anxiety, personality, and side effects were examined with self-report questionnaires at baseline (screening), pre-imaging and post-imaging (day six). Participants were asked to guess group allocation at the end of the study. Analysis of questionnaire data was described in Chapter Two.

4.2.4 MRI data acquisition and analysis

4.2.4.1 MRI data acquisition

General scanning details have been outlined in Chapters Two and Three. This chapter reports results from the resting-state sequence of the imaging protocol.

Resting-state fMRI acquisition: A total of 220 volumes of multiecho multiband (multiband factor = 3) resting-state fMRI were acquired with a voxel resolution of 2.5x2.5x2.5mm, -30 angulation, repetition time (TR) 1600ms, echo time (TE): echo 1=15ms, echo 2=36ms, echo 3=57ms, flip angle of 70 degrees, parallel imaging PAT mode = GRAPPA 2. Scan duration was 6 minutes and 5 seconds. T1 weighted images were acquired with a voxel resolution of 1x1x1mm, TR / TE were 1900ms / 3.97ms respectively. Gradient echo phase and magnitude field maps were acquired with voxel resolution of 2.5x2.5x2.5mm to allow for distortion correction.

See Chapter Two for details of structural MRI acquisition.

4.2.4.2 Resting-state fMRI analysis

Pre-processing:

T1-weighted structural MRI and multi-echo resting-state fMRI images were pre-processed using fMRIprep 20.2.0 (Esteban et al. 2019) and a denoised optimially-combined timeseries file for each participant produced with Tedana 0.0.9 (DuPre et al. 2021; Kundu et al. 2013; Kundu et al. 2012; The Tedana Community et al. 2022). Group-level network analysis was conducted with the FMRIB toolbox. Pre-processing code is available at (de Cates et al. 2022). Pre-processing was conducted differently in this chapter compared to Chapters Two and Three to optimise the pipeline for the multi-echo resting-state data. Detailed methods are reported in the Appendix using wording as suggested by the fMRIprep and Tedana communities for reproducibility reasons, but are presented summarised here.

The T1-weighted (T1w) image was corrected for intensity non-uniformity, skull-stripped and segmented into cerebrospinal fluid (CSF), white matter (WM) and grey matter (GM), and registered to standard space using fslfast. For each subject, the BOLD images were skull stripped, and the first echo was used as the reference where necessary for later processing. Head-motion parameters with respect to the BOLD reference (transformation matrices, and six corresponding rotation and translation parameters) were estimated using MCFLIRT (Jenkinson et al. 2002).

Resting-state fMRI data were unwarped using acquired B0 field maps, co-registered to the reference run and the T1w reference using boundary-based registration. Following this, resting-

state fMRI data were registered to standard space using linear and non-linear tools. Principal components were estimated after high-pass filtering the preprocessed BOLD time-series (using a discrete cosine filter with 128s cut-off) for the two CompCor variants: temporal (tCompCor) and anatomical (aCompCor). Volumetric resamplings were performed, configured with Lanczos interpolation to minimize the smoothing effects of other kernels.

Tedana version 0.0.9 (The Tedana Community et al. 2022) produced a denoised optimally-combined time-series file for each participant, using input data from fMRIprep. A mask using the first echo for each participant was applied to the data, and then multi-echo data were optimally combined using the T2* combination method (Posse et al. 1999). This involved a series of TE-dependence metrics being calculated for each component, including Kappa, Rho, and variance explained, and component selection performed to identify BOLD (TE-dependent), non-BOLD (TE-independent), and uncertain (low-variance) components using the Kundu decision tree (v2.5;(Kundu et al. 2013).

Network analysis:

The pre-processed cleaned functional data were temporally concatenated across subjects and decomposed into independent components (ICs) using FSL MELODIC (Multivariate Exploratory Linear Optimised Decomposition into Independent Components) (Beckmann et al. 2005). Dimensionality estimation for group maps was set to 20 IC maps. They were identified as either being analogous to the most frequently reported major resting-state networks (RSNs) (Smith et al. 2009), or reflecting noise (physiological, scanner, movement). The components were first visually inspected, independently by AdeC and a colleague (MM) and subsequently by consultation with another colleague (SS) as appropriate, and then additionally inspected using

Pearson spatial cross-correlation (PCCs) to compare quantitatively to previously published maps (Smith et al., 2009). PCCs relevant to later analyses showed good correlation (see Table 8).

Table 8: Pearson spatial cross-correlation (PCC) quantitative comparison of Resting-State Networks (RSNs) found in the study to previously published maps (Smith et al., 2009).

Canonical RSN	**Agreement with Smith (PCC)**
Visual	0.7
DMN	0.6
DMN (Posterior)	0.4
Sensorimotor	0.4
Auditory	0.4
Executive control (CEN)	0.6
Right Frontoparietal	0.3
Left Frontoparietal	0.6

Dual regression was used to generate subject-specific versions of spatial maps and the associated time-series from group-average spatial maps (Beckmann et al. 2005; Filippini et al. 2009). First, for each participant, the group-average set of spatial maps was regressed (as spatial regressors in a multiple regression) into each partitipant's 4D space–time dataset. This gave a set of subject-specific timeseries, one per group-level spatial map. Then, these timeseries were regressed (as temporal regressors, again in a multiple regression) into the same 4D dataset, resulting in a set of subject-specific spatial maps, one per group-level spatial map. Subsequently, I tested for statistically significant differences between the groups across all identified networks using FSL's randomize permutation-testing tool (5000 permutations). The RSNs of interest for this analysis were from the triple network model, comprising of the default mode network (DMN), salience network and the central executive network (CEN). Other RSNs (i.e. visual, auditory) were analysed as control regions where I did not expect to see changes with prucalopride. Statistics were assessed using the Threshold-Free-Cluster-Enhancement (TFCE) approach and a family-wise-error corrected cluster significance threshold of $p<0.05$ applied to the suprathreshold clusters to

correct for multiple comparisons at the voxel level (Smith and Nichols 2009). The general linear model included the groups of interest comparison, placebo > prucalopride and prucalopride > placebo. To further visualise the results, individual Parameter Estimates (PE) values were extracted from their custom maps, using significant clusters as binary masks.

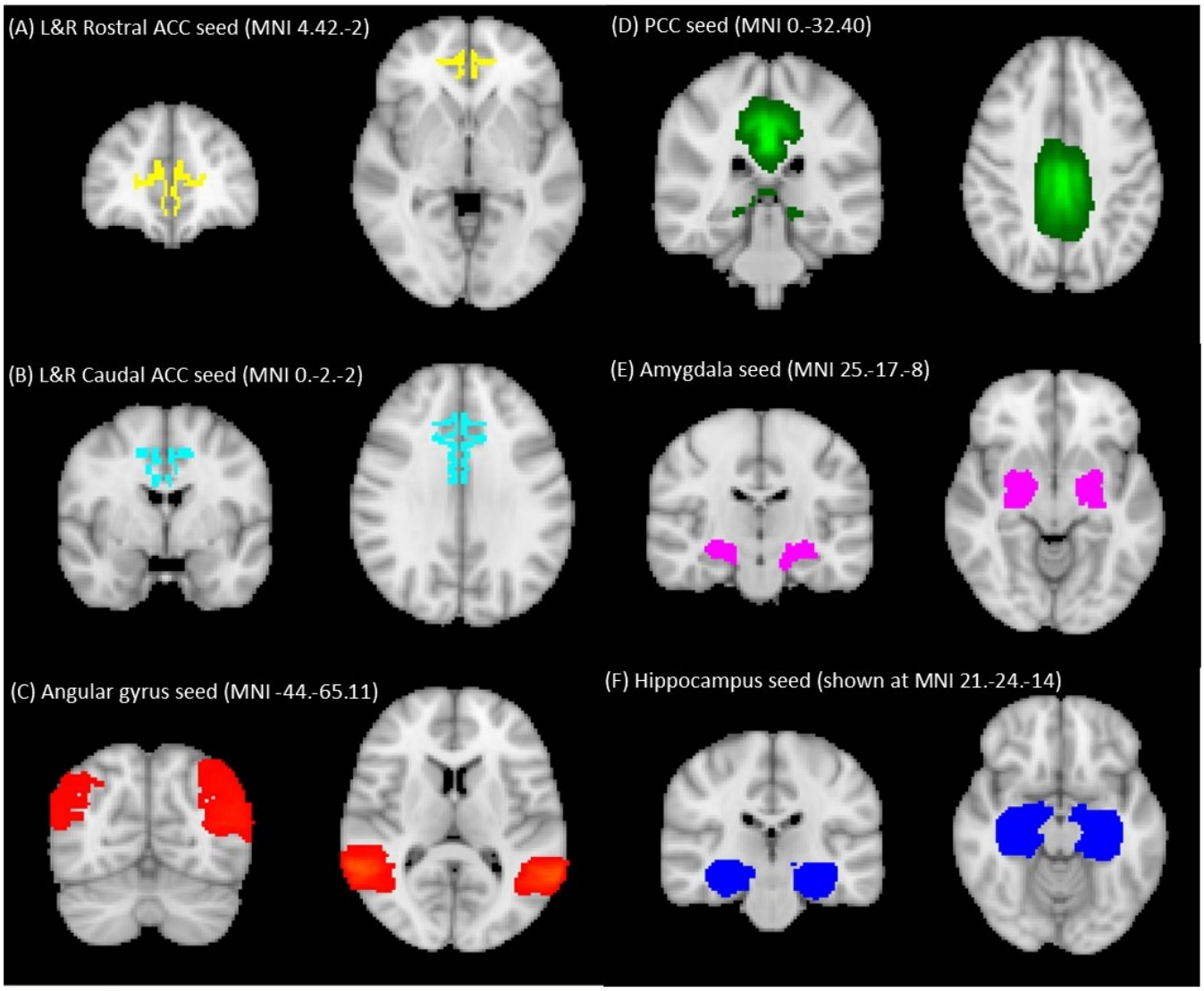
Seed analysis:

For the seed analysis, pre-determined region-specific masks were chosen: anterior cingulate cortex (ACC), posterior cingulate cortex (PCC), hippocampus, amygdala, angular gyrus. Masks were typically based on the FSL Harvard-Oxford atlas. As the ACC is a large structure with multiple roles (often summarised as affective versus cognitive), this region was subdivided into different seeds to reflect this distinction along functional lines (Vogt, Uddin) as per the FSL Talairach atlas: a rostral "affective" ACC (Brodmann areas rostral 24(a), 25, 33, rostral 32(a)) and a caudal "cognitive" ACC (Brodmann areas caudal 24(b), caudal 32(b+c)). The Talairach atlas was used to subdivide the ACC due to its increased specificity for this purpose. Masks were binarized and thresholded at 50% before creating a standard to high resolution matrix, which was applied to each mask for each participant in turn to register the mask into each individual's functional (EPI) space (see Figure 12).

I then extracted the time series for each mask for each participant. First-level connectivity was calculated as the correlation (both positive and negative) of time series of the seed with all other voxels in the brain using FSL FEAT. A white matter and cerebral spinal fluid (CSF) mask were created in standard space and then registered into the individual's functional (EPI) space before being included as covariates of no interest. FSL FEAT was then used to perform group-level analysis with two explanatory variables (prucalopride versus placebo) testing for contrasts placebo > prucalopride and prucalopride > placebo. Cluster-based thresholding (Z>3.1) was used

to identify significant clusters for each seed analysis. To harmonise this process with FSL, I used files created with the data by FSL's MELODIC ICA as well as Tedana. To further visualise results, individual Parameter Estimates (PE) values were extracted from their custom maps, using significant clusters as binary masks. All results are reported using MNI coordinates, and Bonferroni correction was applied to correct for multiple comparisons considering the six seeds used for analysis.

Figure 12: Seed maps used for seed analysis



Seed maps shown in axial, coronal, and sagittal views. Left and right-sided seeds shown together. ACC = anterior cingulate cortex; PCC = posterior cingulate cortex

Grey matter and perfusion analyses:

Distortion- and motion-corrected resting perfusion maps in units of mL/100g/min were calculated using Oxford_ASL (part of the Bayesian Inference for Arterial Spin Labelling (BASIL) tool) for each participant, which performs label-control subtraction, inference of voxel-wise perfusion, and voxel-wise calibration to obtain absolute perfusion maps, and controls for partial volume effects at the single-subject level (Chappell et al. 2011; Chappell et al. 2009a). These were non-linearly aligned with standard space via an initial linear transformation T1 structural space, followed by the application of the non-linear warp. Normalised images were merged and smoothed with a Gaussian smoothing kernel of 2.35 mm (to match resting-state data) to enable use as a voxel-dependent explanatory variable of no interest in the analysis. Grey matter maps were also generated (using feat_gm_prepare). As per Chapter Two, both of these analyses were conducted to enable use as voxel-dependent explanatory variables of no interest in the analysis.

4.3 Results

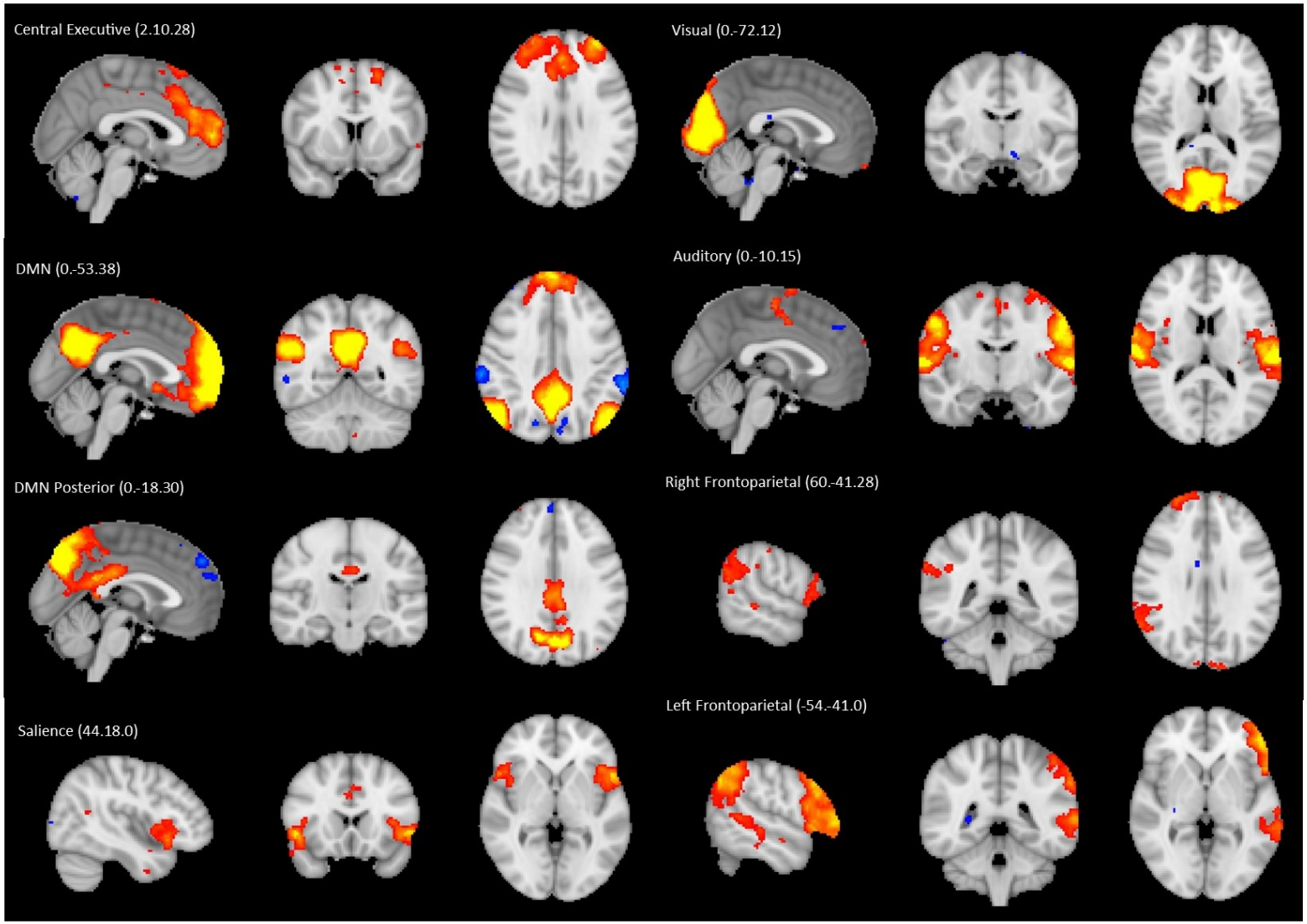
4.3.1 Participants

Chapter Two details characteristics of participants recruited and excluded within the prucalopride study (in this chapter the same participants were included as per Chapter Two). As previously reported in Chapter Two, baseline and follow up (day 6 pre and post scan) mood, anxiety and side effects showed no difference between the prucalopride and placebo group (all *p*s = >0.5).

4.3.2 Resting-state fMRI

4.3.2.1 Network analysis

Figure 13: Resting-State Networks (RSNs) identified in the study

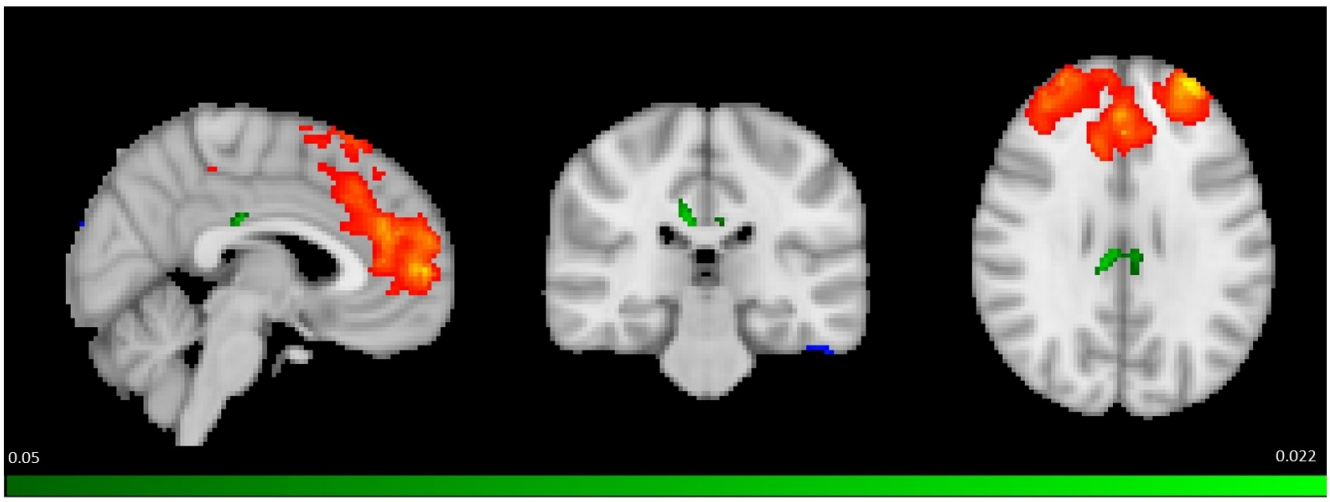


Resting-state networks shown in axial, sagittal and coronal views overlaid onto standard MNI brain. All maps thresholded at 3.1. MNI co-ordinates for each RSN location detailed and marked with crosshairs. Red signifies positive correlation, blue signifies negative correlation

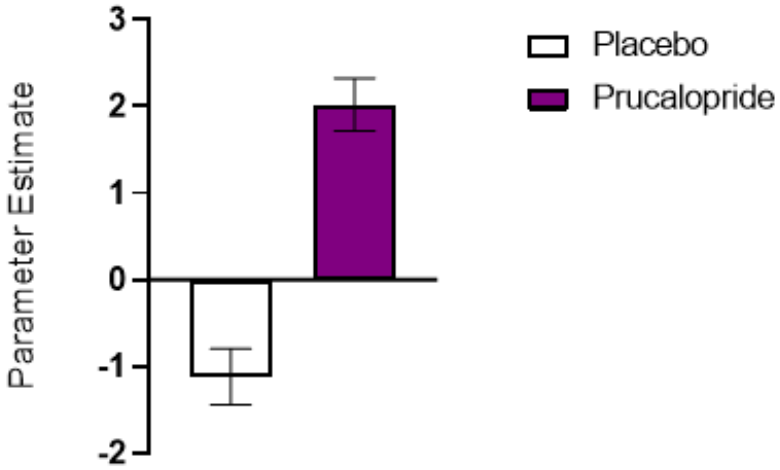
Out of the 20 ICs, eight were clearly identified as resting-state networks (RSNs), with the remainder being at least partially noise, due to physiological factors, head motion and artefacts from the scanner. All identified RSNs are displayed in Figure 13.

Figure 14: Network analysis showing greater rsFC between posterior and anterior cingulate cortices (PCC / ACC) cluster and central executive network (CEN)

A



B



(A) *Network maps. CEN shown in red/blue. Significant PCC / ACC cluster in green (prucalopride > placebo, tmax=4.8, p=0.032, MNI peak voxel x=5, y=-28, z=28, cluster size=94 voxels, colour bar indicates p (significance <0.05), shown at MNI co-ordinates 3.-23.28*

(B) *Graph showing mean Parameter Estimate (PE) in prucalopride (purple) versus placebo (white) groups representing change in rsFC in whole brain PCC/ACC cluster relative to CEN. Error bars indicate SEM.*

The prucalopride group, when compared to the placebo group, showed significantly greater resting-state functional connectivity (rsFC) between the central executive network (CEN) and a cluster predominately involving the posterior cingulate cortex (PCC) extending into the anterior cingulate cortex (ACC) (prucalopride>placebo, tmax=4.8, *p*=0.032, MNI peak voxel: x=8,y=-28,z=28, cluster size=94 voxels) (see Figure 14(A) and (B)).

4.3.2.2 Seed analysis

Changes in connectivity between seed regions and other brain regions are summarised in Figure 15 and detailed in Table 9.

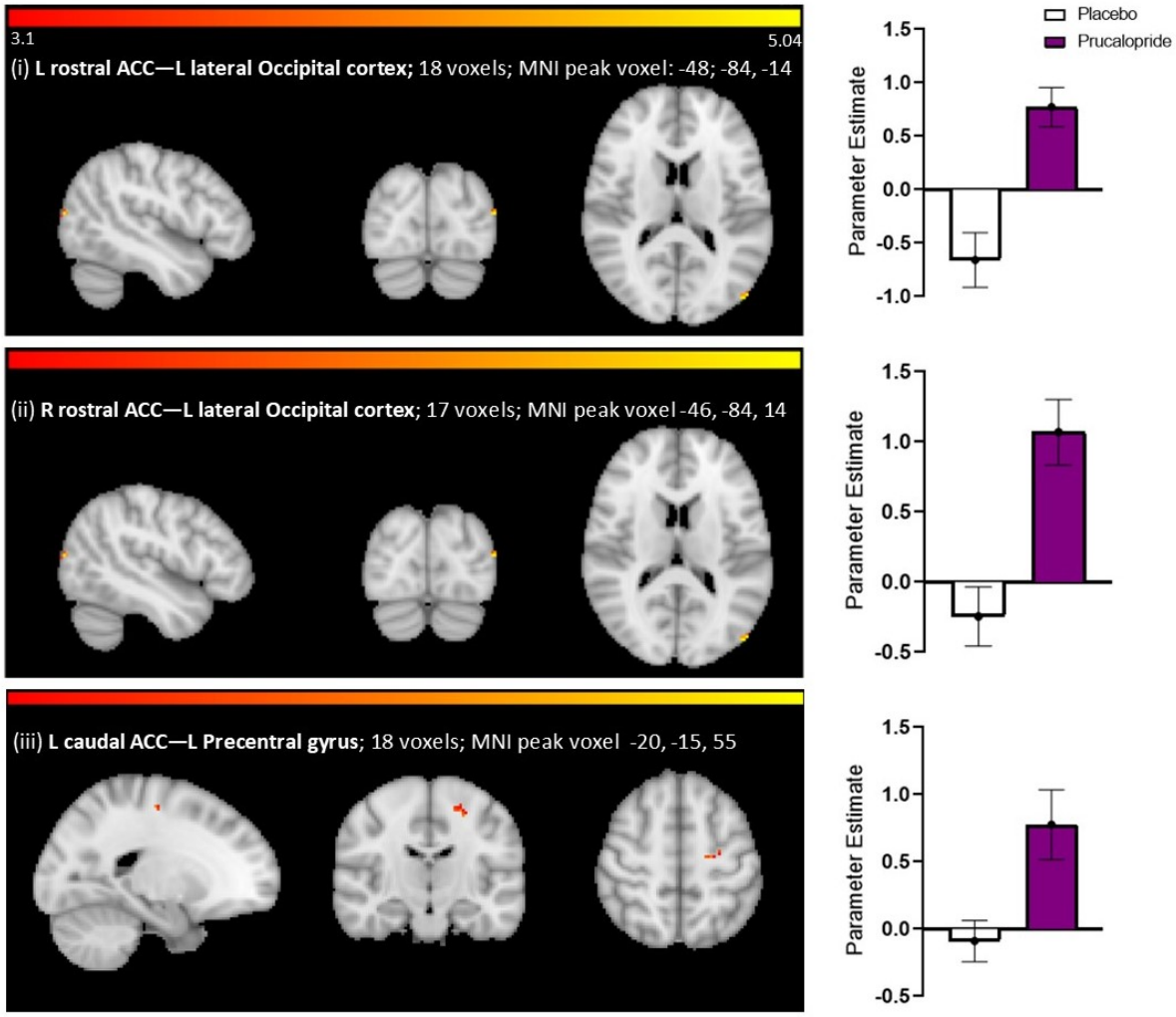
For the anterior cingulate cortex (ACC), when compared to participants taking placebo, prucalopride participants showed greater connectivity between ACC subdivisions and other attentional regions: the left and right rostral / affective ACC and the left lateral occipital cortex (LRostralACC: prucalopride>placebo, Z=4.99, corrected *p*<0.03, MNI peak voxel: x=-48,y=-84,z=14, cluster size=18 voxels; RRostralACC: prucalopride>placebo, Z=4.34, corrected *p*<0.03, MNI peak voxel: x=-46,y=-84,z=14, cluster size 17 voxels), and the left caudal / cognitive ACC and the left precentral gyrus (LCaudalACC: prucalopride>placebo, Z=4.17, corrected *p*<0.002, MNI peak voxel: x=-20, y=-18, z=56, cluster size=18 voxels) (see Figure 15 and Table 9). The right caudal / cognitive ACC was not significantly related to another region.

When compared to the placebo group, the prucalopride group also showed reduced connectivity between the left and right hippocampus and (i) the supramarginal gyrus / angular gyrus and (ii) the precentral gyrus / inferior frontal gyrus; a set of regions analogous to the DMN. This pattern

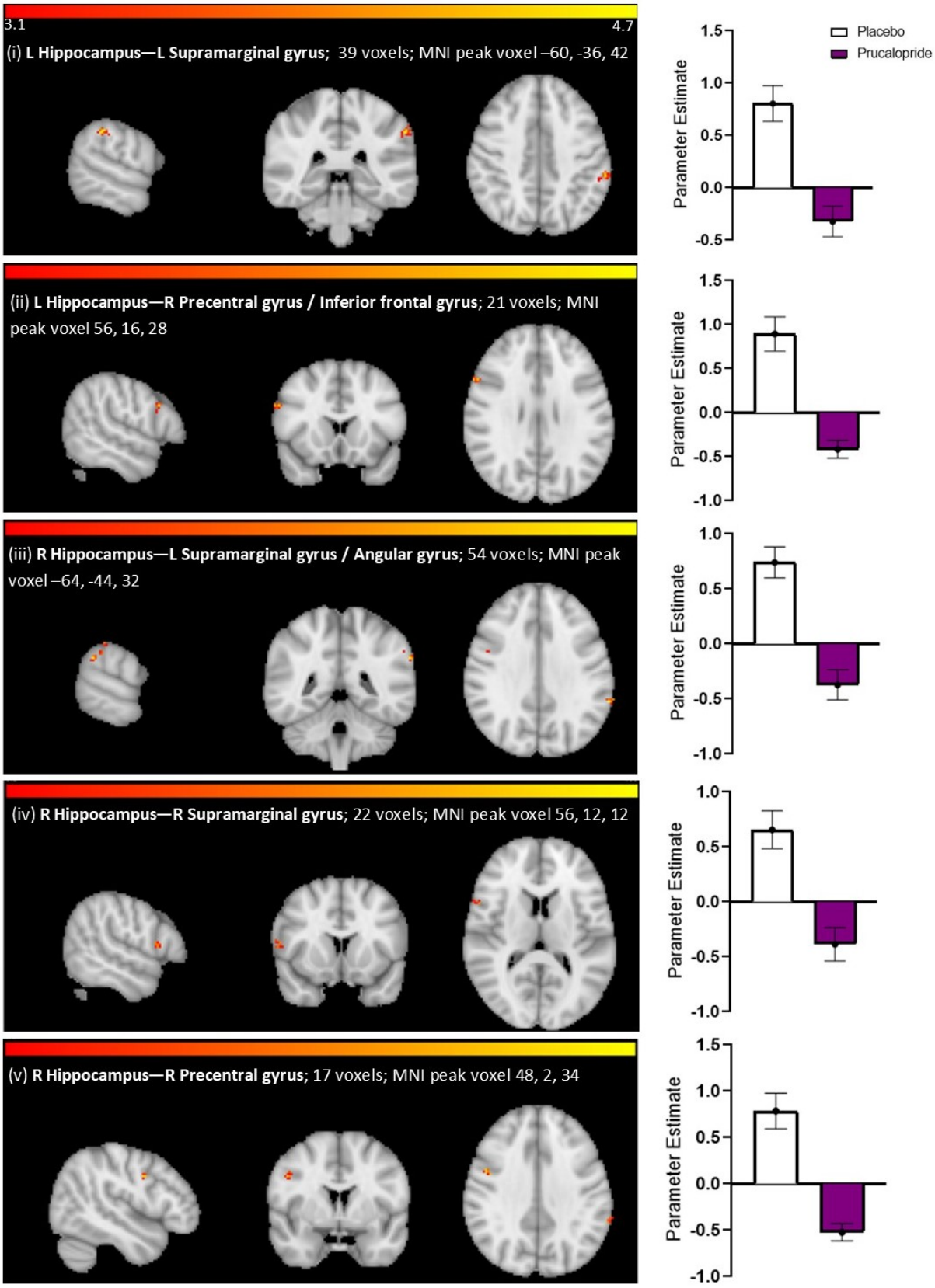
of reduced connectivity within the DMN was supported by additional findings of reduced connectivity involving seeds placed in the angular gyrus and PCC and their connections (to other regions with the DMN and visuolanguage processing regions). However, although these survived correction for multiple comparisons at the voxel level they did not survive correction for multiple comparisons at the seed level (see Figure 15 and Table 9). There were no significant findings involving the amygdala seed.

Figure 15: Network maps and corresponding parameter estimates for each seed result with significant resting-state functional connectivity (rsFC) changes with prucalopride (A): increased connectivity; (B) decreased connectivity

A



B


3.1
4.7
(i) L Hippocampus—L Supramarginal gyrus; 39 voxels; MNI peak voxel –60, -36, 42
(ii) L Hippocampus—R Precentral gyrus / Inferior frontal gyrus; 21 voxels; MNI peak voxel 56, 16, 28
(iii) R Hippocampus—L Supramarginal gyrus / Angular gyrus; 54 voxels; MNI peak voxel –64, -44, 32
(iv) R Hippocampus—R Supramarginal gyrus; 22 voxels; MNI peak voxel 56, 12, 12
(v) R Hippocampus—R Precentral gyrus; 17 voxels; MNI peak voxel 48, 2, 34
Placebo
Prucalopride
Parameter Estimate
1.5
1.0
0.5
0.0
-0.5
-1.0

Brain maps show axial / sagittal / coronal slices of Z stat images thresholded to 3.1 with cluster shown at peak voxel location. Colour bar indicates Z value. Size and MNI peak voxels for clusters shown within figure. P values shown as corrected to 3 decimal places. Parameter estimate of rsFC cluster: placebo = white, prucalopride = purple, error bars = SEM.

- ***(A)*** *(i) Seed: L rostral ACC, Connectivity result: L lateral Occipital cortex, Prucalopride > placebo, Z=4.99, corr p=0.023; (ii) Seed: R rostral ACC, Connectivity result: L lateral Occipital Cortex, Prucalopride > placebo, Z=4.34, corr p=0.028; (iii) Seed: L caudal ACC, Connectivity result: L Precentral gyrus, Prucalopride > placebo, Z=4.17,corr p=0.002*
- ***(B)*** *(i) Seed: L Hippocampus, Connectivity result: L Supramarginal gyrus, Placebo > prucalopride, Z=4.65, corr p<0.001; (ii) Seed: L Hippocampus, Connectivity result: R inferior Frontal gyrus / Precentral gyrus, Placebo > prucalopride, Z=4.35, corr p=0.005; (iii) Seed: R hippocampus, Connectivity result: L Supramarginal gyrus / Angular gyrus, Placebo > prucalopride, Z=4.61, corr <0.001; (iv) Seed: R hippocampus, Connectivity result: R Supramarginal gyrus, Placebo > prucalopride, Z=3.89, corr p<0.004; (v) Seed: R hippocampus, Connectivity result: R inferior Frontal gyrus / Precentral gyrus, Placebo > prucalopride, Z=4.38, corr p=0.03;*

4.3.2.3 Sensitivity analyses

Results were similar when grey matter and perfusion maps were included as voxel-dependent explanatory variables of no-interest for whole network and seed analyses (see Table 9).

Table 9: Details of seed results with significant resting-state functional connectivity (rsFC) changes with prucalopride (significant corrected *p* values in BOLD)

Seed region	Connectivity result	Cope	Z score	P value	Corrected P value (Bonferroni)	MNI peak voxel (x)	(y)	(z)	Cluster size (voxels)	Cluster size (voxels) with adjustment for (i) GM (ii) ASL+GM
L rostral ACC	L lateral Occipital cortex	Prucalopride > placebo	4.99	0.0038	**0.023**	-48	-84	14	18	15 19
R rostral ACC	L lateral Occipital cortex	Prucalopride > placebo	4.34	0.0048	**0.029**	-46	-84	14	17	18 18
L caudal ACC	L Precentral gyrus	Prucalopride > placebo	4.17	0.0032	**0.0019**	-20	-18	56	18	18 13
L Hippocampus	L Supramarginal gyrus	Placebo > prucalopride	4.65	<0.000001	**<0.00001**	-60	-36	42	39	39 43
	R Inferior frontal gyrus / Precentral gyrus	Placebo > prucalopride	4.35	0.00077	**0.0046**	56	16	28	21	22 25
R Hippocampus	L Supramarginal gyrus / Angular gyrus	Placebo > prucalopride	4.61	<0.000001	**<0.00001**	-64	-44	32	54	48 51
	R Supramarginal gyrus	Placebo > prucalopride	3.89	0.00067	**0.0040**	56	12	12	22	14 13
	R Inferior frontal gyrus / Precentral gyrus	Placebo > prucalopride	4.38	0.0053	**0.032**	48	2	34	17	16 16
Angular gyrus	L Inferior temporal gyrus / Occipital fusiform cortex	Placebo > prucalopride	4.23	0.018	0.11	50	-54	-22	15	20 13
	L Inferior frontal gyrus / Precentral gyrus	Placebo > prucalopride	3.6	0.028	0.17	-50	10	16	14	23 22
PCC	L Supramarginal gyrus	Placebo > prucalopride	3.75	0.010	0.062	-60	-32	42	16	14 18

4.4. Discussion

In healthy volunteers at rest, this chapter found that six days of prucalopride administration was associated with greater resting-state functional connectivity (rsFC) between the posterior cingulate cortex (PCC) / anterior cingulate cortex (ACC) and the central executive network. Additional seed analysis also identified two important findings in the prucalopride group compared to the placebo group: (i) greater rsFC between both the left and right rostral ACC and a visual attentional region, the lateral occipital cortex; and (ii) reduced rsFC as expected within the default mode network (DMN). In seed analyses, the precentral gyrus showed greater rsFC with the caudal ACC and reduced rsFC with DMN regions.

In support of the hypothesis, seed analysis demonstrated that prucalopride was associated with reduced rsFC between regions of the DMN, including the hippocampus, inferior parietal lobule (angular gyrus, supramarginal gyrus), and inferior frontal gyrus. The DMN is a set of regions that classically show intrinsic activity at rest, and relatively reduced activity (i.e. deactivate) when focus is required for external cognitive tasks and internal demands (such as autobiographical memory retrieval) (Uddin et al. 2009). A failure of the DMN to deactivate appropriately when required is associated with attentional deficits (Marchetti et al. 2012) and reduced cognitive performance in health and disease (Greicius et al. 2004; Petersen and Miskowiak 2021). Thus the finding of reduced DMN rsFC is consistent with previous work from our laboratory and in this thesis showing that acute and sub-acute prucalopride improves task-related cognition (Murphy et al. 2020) (Chapters Two and Three) and reduces activity in regions of the DMN during the performance of a memory task (Chapter Three).

The finding that six days' prucalopride administration was associated with greater rsFC between the CEN and the PCC / ACC builds on existing knowledge of the role of these regions in cognition. The PCC and ACC are both important regions in information processing and the regulation of information within the brain; the ACC has a variety of cognitive, affective and sensorimotor roles and connections (Margulies et al. 2007; Stevens et al. 2011), whereas the PCC is known for its roles in internally directed cognition, arousal and attention (Leech and Sharp 2014). The PCC (Brodmann areas 23 and 31 (Vogt et al. 2006)) seems to be particularly active during rest, and when participants retrieve autobiographical memories, plan for the future, and decide where to focus attention (Leech and Sharp 2014).

In particular, the PCC and ACC are both strongly implicated in the "triple network" model (Menon 2011) i.e. they are both connected to the executive network (CEN, or lateral fronto-parietal control network), and the salience network (SN), and are key hubs within the default mode network (DMN). Compared to healthy volunteers, those with remitted and current depression show functional changes and altered reciprocal connectivity within and between the individual networks of the triple model (Bhaumik et al. 2017; Dong et al. 2019; Kaiser et al. 2015; Neufeld et al. 2018; Wang et al. 2020). The function of these networks in relation to each other is thought to be important; dysfunction in one network may affect the others in the model, and appropriate integration of information across the model is required for optimal cognitive function (Shen et al. 2018).

The ACC also has an important role in attention, potentially mediating the effects of emotional interference, with the rostral ACC assessing the relevant information involved and the caudal ACC undertaking the control aspect (Etkin et al. 2011; Stevens et al. 2011) as a node of the salience

network (Margulies et al. 2007). This is consistent with the findings of this chapter, where greater connectivity was found in participants who had received prucalopride between the rostral ACC and lateral occipital cortex (a region that aids attention for objects that matter (Murray and Wojciulik 2004)), and the caudal ACC and the precentral gyrus (a sensorimotor region that has been implicated in visuospatial attention especially for unpredictable situations (Hahn et al. 2006)). These findings also appear to be a reproduction of those by Esposito *et al*., who similarly identified increased ACC – occipital cortex rsFC with the cognitive enhancing drug, modafinil (Esposito et al. 2013).

An interesting facet of these results is that prucalopride was associated with both increased rsFC between regions of cognition networks, and reduced rsFC within regions of the DMN. Existing literature supports this, despite some understandable variability depending on the agent involved and the population studied. Focussing on findings in healthy adults, Esposito *et al.* found a similar pattern of change following an acute dose of 100mg modafinil. Modafinal increased rsFC between the ACC and the CEN, and the lateral occipital cortex and dorsal attention network, as well as improving scores on a progressive matrices test (Esposito et al. 2013). Another example using various pro-cognitive medications found an increased rsFC between cognitive networks but reduced rsFC between the PCC and the DMN. Further analysis revealed that greater reductions in DMN rsFC were associated with better cognitive performance (Becker et al. 2022). Mueller and colleagues suggested that, in the healthy adult brain, methylphenidate both increases and decreases rsFC between cognitive and sensorimotor networks, including the ACC, PCC, precentral gyrus, and occipital cortices (Mueller et al. 2014), all regions where rsFC was affected by prucalopride in the current study. These results are similar, although with some differences, to the effects of pro-cognitive treatments within clinical populations ((Schmaal et al. 2013) [alcohol dependence]; (Péran et al. 2021) [Alzheimer's disease]; (Visser et al. 2019) [stroke]). I am not

aware of studies examining the effect of pro-cognitive medication on rsFC in depressed patients with cognitive impairment.

In summary, consistent with findings using other pro-cognitive agents, the previous (task-based: Chapters Two and Three) and current (rsFC) fMRI results with prucalopride suggest that medications with cognition enhancing properties appear to produce (i) increased connectivity within cognitive networks (such as the CEN), and (ii) reduced connectivity within the DMN and its connections (Kelly et al. 2008; Leech and Sharp 2014). Moreover, it appears that greater negative correlation between these two systems (cognitive networks versus the DMN) may be associated with better behavioural performance (Kelly et al. 2008). In this way, successful performance of cognitive demands appears dependent on the ability to dynamically modulate the DMN as well as the other cognitive networks within the triple model; and furthermore, effects within this network may be an important means by which pro-cognitive medications act.

Results from this chapter are consistent with the PCC and the ACC playing a key role in cognitive enhancement and perhaps acting as a pivot between the dual functions of these areas in attention and cognition (via the DMN and CEN in particular). That is, the PCC and ACC may help to control the balance between internally-directed attention and externally-directed focus (Leech and Sharp 2014), explaining why administration of a pro-cognitive agent such as prucalopride may both simultaneously (i) increase rsFC within cognitive networks (as per these network analyses) and (ii) reduce task-based activation (as per Chapter Three) and reduce rsFC within regions of the DMN (as per these seed analyses).

Subdividing the ACC along anatomical lines as a proxy of function allowed us to take account of some of the complexity of the ACC in its actions within resting-state networks with the use of a pro-cognitive agent. However, the transitional area between affective and cognitive functions is likely to be more complicated and gradual than the sharp distinctions necessary for analytic processes, and may be subject to inter-individual variation (Margulies et al. 2007). Furthermore, as this was an fMRI resting-state scan, I have assumed that the changes in rsFC seen here involving the hippocampus and angular gyrus relate to their role as part of the DMN as opposed to their memory processing functions. This was supported by other DMN regions interacting in a similar pattern in this study.

In terms of limitations, the results described here include some small clusters, although there is evidence of consistency within these. For example, the left and right rostral ACC both showed increased connectivity with prucalopride for the same region (i.e. the left lateral occipital cortex). In this study, we used a low dose of prucalopride (1 mg as opposed to the licenced dose of 2 mg) to reduce the risk of side effects. As noted previously, we currently lack information about the optimal dose of prucalopride needed for occupancy of brain 5-HT_4 receptors, although data from previous chapters in this thesis indicate that 1mg is sufficient to demonstrate pro-cognitive potential. The sample size required (17 per group to give 90% power with $\alpha = 5\%$) was calculated based on a conservative estimated effect size of 0.5–0.7 with behavioural analyses; in view of the less clear power calculations for fMRI data each group was oversampled. Unfortunately, power may have been affected in this chapter due to pre-analysis (3) and intra-analysis (3) exclusions. As noted previously, most participants whose first language was not English received placebo, but as there was no verbal task involved in this resting-state analysis this is unlikely to be a major concern. It should also be remembered that these findings may not generalise to other 5-HT_4 receptor agonists.

In conclusion, the results from this analysis of resting-state fMRI data following six days' of 1mg prucalopride support our previous translational work and further suggest that prucalopride may affect cognition via effects on attentional neural networks: reducing connectivity within the DMN and between the DMN and its connections whilst also enhancing connectivity within other networks involved in cognition.

CHAPTER 5

Neural and behavioural effects of the 5-HT_4 receptor agonist, PF-04995274, in a hippocampal-dependent memory task in un-medicated depression

5.1: Introduction

The work presented in Chapters Two to Four of this thesis demonstrates that the 5-HT_4 receptor agonist prucalopride has a pro-cognitive profile in healthy young adult volunteers. Using healthy participants for early mechanistic work in humans has a number of advantages; in particular, it allows the characterisation of neurocognitive effects without confounding due to symptom change. However, it is not yet known the extent to which these effects of 5-HT_4 receptor agonism generalise to a clinical population. As healthy individuals do not have baseline cognitive deficits, testing following administration of a pro-cognitive agent may be subject to ceiling effects. Alternatively, it may be more challenging to change cognition in a clinical population where cognitive deficits may be less malleable than improving cognition in healthy volunteers. To my knowledge, there have been no previous studies examining the neuropsychological effects of 5-HT_4 receptor agonists in individuals with psychiatric illness, a necessary step if we wish to assess the possible therapeutic potential of these agents.

One limitation of using prucalopride as a pharmacological probe of central 5-HT_4 receptor function is that we do not currently have PET data on prucalopride to guide the selection of the correct dose in humans for optimal receptor occupancy in the brain. As an alternative, in the

current study PF-04995274 was used, a highly selective 5-HT_4 receptor partial agonist that was made available for the current study via Pfizer through the UK Medical Research Council Industry Asset Sharing Initiative. PF-04995274 was initially developed for its putative pro-cognitive actions (with a clinical consideration for Alzheimer's Disease) (Pfizer 2011a). The dosing of PF-04995274 used here (15 mg for 7-9 days) is within the previously studied dosing and duration of administration carried out by Pfizer. Preliminary results in 48 healthy adults treated with up to 14 days' medication suggest that PF-04995274 is safe and generally well tolerated (Pfizer 2011b): mild side effects included headaches, nausea, abdominal pain, diarrhoea, flatulence and insomnia, similar to other 5-HT_4 receptor agonists. PF-04995274 is estimated to have a half-life ($t_{1/2}$) of around 30 hours, so that steady state should be achieved within about 7 days (Pfizer 2011a). PET studies have shown that 5mg of PF-04995274 produces greater than 80% receptor occupancy of brain 5-HT_4 receptors four hours after the first dose in healthy volunteers (Pfizer 2011a), indicating that 15mg should lead to sufficient receptor occupancy for clinical applications.

In this study, individuals were recruited with a current episode of major depression and who were not taking antidepressant medication or undergoing psychological therapy (to avoid any issues with polypharmacy / concurrent treatment). Apart from altering the study population and medication, the study design was as per Chapters Two to Four (the prucalopride study in healthy volunteers) to allow comparison between the effects of these two 5-HT_4 receptor agonists. Here results are reported from the same study task as Chapter Two (a memory task that directly probes the hippocampus). The aim of this chapter was to assess the effects of 5-HT_4 receptor agonism on cognition (behavioural and neural processing of memory) in this clinical population using sub-acute administration of PF-04995274. It was hypothesised, similar to the results presented in Chapter Two, that PF-04995274 would increase activation of the hippocampus with concurrent improved behavioural scores on memory testing.

5.2 Methods

5.2.1 Participants

The RESTAND (Effect of PF-04995274 on emotional processing in un-medicated depressed patients) study recruited 90 right-handed participants between 18 and 64 years old. Participants were of either sex, fluent in English and were screened for contraindications to serotonergic medication. All participants currently met DSM-5 criteria for major depressive disorder, as determined by a psychiatrist using the Structured Clinical interview for DSM-5 (SCID). Other criteria for inclusion were being willing and able to give consent for the study; being registered with a General Practitioner (GP) and consenting to the GP being informed of participation; not receiving drug or face-to-face psychological treatment for the current episode of depression; and where relevant using a highly effective method of contraception from the Screening Visit until 30 days after receiving study treatment. Exclusion criteria are detailed in Table 10.

The study was approved by the South Central Research Ethics Committee (18/SC/0076) and a protocol including outcomes was pre-registered with clinicaltrials.gov (NCT03516604). Participants gave written informed consent.

Table 10: Exclusion criteria for RESTAND

History of or current DSM-5 bipolar disorder, schizophrenia or eating disorders, or clinically significant risk of suicide
First-degree relative with a diagnosis of Bipolar Disorder type 1
Current usage of psychotropic medication
Failure to respond to antidepressant medication in current episode
Electroconvulsive therapy for the treatment of the current episode of depression
Participants undergoing any form of face-to-face structured psychological treatment during the study
Clinically significant abnormal values for screening blood tests or ECG
History of stimulant abuse (lifetime; e.g. amphetamine, cocaine), or of alcohol abuse within one year or of alcohol dependence within the lifetime
History of, or current medical conditions which may interfere with the safety of the participant or the scientific integrity of the study, including epilepsy/seizures, brain injury, severe hepatic or renal disease, severe gastro-intestinal problems, severe neurological problems
Medical conditions that may alter the hemodynamic parameters underlying the BOLD signal (e.g., inadequately treated hypertension, diabetes mellitus). Any contraindication to MRI scanning (e.g. metal objects in body, pacemakers, significant claustrophobia, pregnancy)
Pregnancy, planning a pregnancy, or breastfeeding
Participants with Body Mass Index (BMI) outside the 18 to 36 kg/m2 range at the Screening Visit
Night-shift working or recent travel involving significant change of time zones, or excessive caffeine consumption
Participation in a psychological or medical study involving the use of medication within the last 3 months

5.2.2 Design and randomisation

5.2.2.1 Overview of design and randomisation

Participants were randomised to one of three groups: PF-04995274 (7 days x 15mg), citalopram (7 days x 20mg) or placebo, in a double-blind, randomised, between-groups design. Participants were stratified by gender. Oxford Health Foundation Trust Pharmacy (Clinical Pharmacy Support Unit (CPSU), Kennington) was responsible for drawing up and holding the randomisation list (using an online 116andomization tool (https://www.sealedenvelope.com/simple-randomiser/v1/lists)), and storing and dispending study medication. The PF-04995274 and placebo tablets were provided by Pfizer and identically matched. Citalopram and placebo capsules were sourced from and encapsulated by Cardiff and Vale University Health Board, St. Mary's Pharmaceutical Unit (SMPU), to ensure that they appeared identical to other medication. Participants were instructed to take medication in the morning.

A sample size calculation was performed exactly as in Chapter Two, and thus it was aimed to recruit at least 25 individuals per group with oversampling to ensure adequate power for fMRI analyses. Based on the hippocampal region of interest results from Chapter Two, this sample size should be sufficient to reliably detect an effect size of the same magnitude (G*Power, previous effect size (f) = 0.37, with an α=0.05, to give 90% power (1-β)).

The analyses detailed in this chapter relate to the subset of recruited participants with fMRI data for the hippocampal memory task, and who received either placebo or the 5-HT_4 agonist PF-04995274 (for results purposes known as the "5-HT_4" group). This is to enable a more precise comparison with analyses conducted in Chapter Two.

5.2.2.2 Study visits

Study visits took place at the Clinical Research Facility, Warneford Hospital, Oxford and the Neurosciences Building, Department of Psychiatry, Warneford Hospital (screening, first dose and research visits) and at the Oxford Centre for Human Brain Activity (OHBA) part of the Wellcome Integrative Neuroimaging Centre (WIN) (MRI scans).

During screening, all participants underwent a medical evaluation including a review of medical history, current and past medication, weight, height and BMI measurement, vital signs assessment, 12-lead ECG, clinical laboratory blood tests for standard screening (liver function tests, urea and electrolytes), urine drug screening, and a pregnancy test where relevant. All participants completed an MRI screening safety form and handedness test to check for MRI eligibility. Following screening, the participant's GP was informed of their participation in the study and had the opportunity to communicate with the study team with any concerns.

As the study drug (PF-04995274) was unlicensed, the first study dose was administered under medical supervision. Participants meeting all study criteria returned to the Clinical Research Facility within four weeks of screening to receive the first study dose with vital signs monitored for three hours afterwards. Participants took the remaining six days' of medication at home. During the week of drug / placebo administration, participants were advised not to drink alcohol and not to carry out activities requiring full alertness if they were aware of any impairment. A researcher also contacted them by phone on day two and day four to check that there were no concerns, and participants received a daily text message reminding them to take the study medication. On day six, all participants were invited for an MRI scan. Spare medication was given

to all participants to enable completion of study visits up to and including day nine to allow for scheduling difficulties or illness.

5.2.3 Questionnaire measures

Questionnaire measures were carried out similarly to Chapter Two although questionnaires assessing state anxiety and affect (VAS, STAI-S and PANAS) were only conducted before and after research visits. As the RESTAND study involved participants with depression, they were also assessed on an observer rated Hamilton Rating Scale for Depression (HAM-D) at baseline, at the first dose visit, and at the end of the study (day 7 to 9).

5.2.4 Memory Encoding fMRI task

This was conducted exactly as detailed in Chapter Two. In brief, outside and before the scan, participants were shown eight randomly-selected "familiar" pictures (four animals and four landscapes). Participants were asked to identify these as animal / non-animal. During the scanner, these "familiar" images were shown in blocks of eight images alternating with blocks of "novel" images (six familiar and six novel blocks) interleaved with rest blocks. During the scan, participants again had to identify these images as animal / non-animal and were informed there would be a memory test afterwards. After the scan, these eight "familiar" and 48 "novel" images were shown alongside 27 "distractor" images, and participants were asked if they had seen the image in the scanner or not.

5.2.5 Demographic and behavioural data analysis

Behavioural data and questionnaires were analysed as detailed for this task in Chapter Two using SPSS (version 29, IBM). Graphs were produced using GraphPad Prism (version 9). A repeated-measures analysis of variance (ANOVA) was used to analyse group differences in self-report measures and behavioural performance in the fMRI experimental task. Levene's test (t-tests) and the Greenhouse–Geisser procedure (ANOVAs) were used where appropriate. A p-value less than 0.05 was used to denote statistical significance. Partial eta squared is reported as a measure of effect size.

5.2.6 MRI data acquisition and analysis

5.2.6.1 MRI data acquisition

MRI data was acquired as detailed in Chapter Two. The full acquisition protocol, along with radiography procedure, is available from the Open WIN MR Protocols database here: 10.5281/zenodo.6107724.

5.2.6.2 MRI task analysis

MRI analysis occurred as detailed in Chapter Two. Imaging data were analysed with FSL (www.fmrib.ox.ac.uk/fsl). fMRI data were pre-processed and analysed using FEAT (FMRI Expert Analysis Tool), version 6.0.4, part of FSL (FMRIB's SoftwareLibrary; www.fmrib.ox.ac.uk/fsl).

In summary, in the first-level analysis, individual activation maps were computed using the general linear model with local autocorrelation correction. Two explanatory variables were modelled: "novel" and "familiar" images. Temporal derivatives were included in the model. Variables were modelled by convolving each block with a haemodynamic response function with a standard deviation of 3s and meanlag of 6s. At the whole-brain level, the following model was

constructed: (1) novel vs. baseline; (2) familiar vs. baseline; (3) novel > familiar; (4) novel < familiar. In the second-level analysis, whole-brain individual data were combined at a group level (placebo participants vs. 5-HT_4 participants) using a mixed-effect analysis. Cerebral blood flow and grey matter maps were included as covariates of no interest using the same method as Chapter Two. Groups were compared in the following manner: (1) placebo > 5-HT_4, (2) 5-HT_4 > placebo, (3) placebo mean, (4) 5-HT_4 mean, and (5) mean of all participants. Brain activations showing significant group differences were identified at the whole-brain level using cluster-based thresholding (Z > 3.1, familywise error (FWE) p<0.05 corrected). Significant interactions from whole-brain analyses were further explored by extracting percentage BOLD signal change for each type of contrast. As the hippocampus was a particular focus, it was pre-specified as a region of interest (ROI). A functional ROI mask was created for the left and right hippocampus by multiplying mean activation for each contrast of interest (on already corrected whole-brain data as above) for all participants by the structural Harvard-Oxford subcortical atlas mask at 50% threshold. Percentage BOLD signal change was subsequently extracted for each contrast in each hemisphere.

As the RESTAND population were a clinical population from across adulthood (c.f. young healthy adults in the prucalopride study), for behavioural task data age and baseline HAM-D score (two possible modifiers of cognitive function) were considered as potential confounds. As with the prucalopride study (see Chapter Two), it was pre-specified to report fMRI results with grey matter and perfusion correction, as well as assessing the influence of sex as a potential confound. Similar to RESTAND behavioural data, the role of baseline HAM-D score and age as potential confounds in fMRI analyses were also considered.

5.3 Results

5.3.1 Participants

The analyses presented here focus on comparisons between the placebo and 5-HT_4 groups (for simplicity, the PF-04995274 group will be referred to as the "5-HT4" group in this chapter). In total, 53 participants were recruited to these two groups between 1st August 2018 and 19th July 2022. Analysis occurred in originally assigned groups (N=53, 26:27 = placebo: $5HT_4$). One participant (5-HT_4 group) was excluded from fMRI analyses prior to unblinding for significant movement. For the remaining 52 participants (26:26; placebo:5-HT_4), no individual participant had absolute movement greater than one voxel or relative movement more than ½ voxel, and there were no significant differences between groups in terms of movement (absolute: p=0.07; relative: p=0.71).

At baseline, the groups were well matched in terms of demographics (i.e. age, sex, BMI, first language, years of education, handedness, and substance use; see Table 11 and footnotes for details including missing data), objective severity of depression (HAM-D) and personality questionnaires. Mean HAM-D score indicated that most participants did not have severe depression, and the range of scores was similar across groups.

Table 12 and Table 13 highlight the medical and medication history across placebo and 5-HT_4 groups. Participants are listed for each separate medication and disorder they endorsed. Both groups contained a similar number of participants with ongoing medication, and there were no serious medical illnesses in any participant.

Table 11: Sample baseline demographics, mood and personality for the RESTAND study (placebo: 5-HT_4)

Variable (measure)	Measurement		Placebo N=26	5-HT_4 N=26	P value	Odds ratio
Sex	*Number*	*Male*	11	10		Ref
		Female	15	16	1.00	1.17[8] (0.39-3.56)
Age	*Mean in years (SD; range)*		31.8 (10.1; 18.9-55.4)	37.0 (13.2; 18.9-60.7)	0.11	
Years of education*	*Mean (SD)*		17.2 (2.71)	16.7 (2.50)	0.64	
First language	*Number*	*English*	22	16		Ref
		Not English	4	10	0.12	3.44 (0.91-13.0)[9]
Handedness (EHI)	*Mean % right (SD)*		81.6 (2.66)	81.1 (1.86)	0.43	
Smoking**[10]	*Mean cigarettes / day (SD)*		0.06 (0.30)	0.04 (0.20)	0.76	
Caffeine*	*Mean cups / day (SD)*		2.80 (1.72)	2.40 (1.64)	0.40	
Body mass* index	*Mean (SD)*		25.4 (4.21)	24.9 (4.64)	0.69	
HAM-D	*Mean (SD; range)*		16.9 (3.02; 13-25)	15.9 (2.32; 12-21)	0.17	
Anhedonia (SHAPS)	*Mean (SD)*		4.08 (3.12)	4.19 (2.68)	0.89	
Anxiety (STAI-T)	*Mean (SD)*		59.8 (7.22)	58.8 (9.10)	0.68	
Personality (EPQ)	*Mean (SD)*	*Neuroticism/Stability*	18.0 (4.06)	17.4 (4.55)	0.65	
		Psychoticism/Socialisation	3.38 (2.68)	4.62 (2.90)	0.12	
		Extroversion/Introversion	9.12 (5.56)	8.31 (4.92)	0.58	
		Lie/Social desirability	8.23 (5.05)	7.46 (3.71)	0.53	

[8] OR of being a female in the 5-HT_4 group, compared to males

[9] OR of being non-native English speaker in the 5-HT_4 group, compared to English speaker

[10] *= missing data (1 missing from placebo group for substances; 1 missing from placebo and 2 from 5-HT_4 groups for BMI; 7 missing from each of placebo and 5-HT_4 groups for education)

Table 12: Medical history of included participants

	Placebo	**5HT$_4$**
Eye disorders	1 (managed cataract)	0
Musculoskeletal symptoms	1	2
Previous mild concussion	1	1
Autoimmune diseases	1 (hypothyroidism (corrected))	
Mild asthma	0	2
History of kidney stones	0	2
Hayfever	0	2
Previous headaches / migraines	0	2
Dyspepsia	0	3
Gynaecological disorders	1 (endometriosis)	

Table 13: Current medication history of included participants

	Placebo	**5-HT$_4$**
Analgesics (paracetamol / ibuprofen / aspirin / codeine)	5	4
Proton pump inhibitors (omeprazole / lansoprazole)	2	1
Levothyroxine	1	0
Antihistamines	0	2
Statins	0	1
Inhalers	0	2

5.3.2 Questionnaire results: the effect of PF-04995274 during the scan day in terms of subjective affect and anxiety

There were no significant differences in state anxiety or affect between the 5-HT$_4$ and placebo group during the scan visit (all *p*s > 0.4; see Table 14).

Table 14: State anxiety and affect questionnaire results during the scan visit (placebo: 5-HT_4)

	Placebo mean (SD) N=26	**5-HT_4 mean (SD) N=24*[11]**	**P value (2dp)**
Spielberger State Anxiety Inventory (STAI-S)			0.35
Pre-Testing	44.8 (9.54)	43.3 (11.0)	
Post-Testing	44.2 (10.1)	40.4 (10.6)	
Positive and Negative Affect Scale – Positive (PANAS-P)			0.48
Pre-Testing	22.3 (6.44)	22.1 (8.56)	
Post-Testing	22.5 (8.48)	23.1 (7.74)	
Positive and Negative Affect Scale – Negative (PANAS-N)			Included in ANOVA above
Pre-Testing	17.2 (6.23)	15.1 (5.38)	
Post-Testing	14.9 (5.44)	14.1 (4.73)	
Visual Analogue Scale (VAS) – Happy			0.37
Pre-Testing	42.3 (17.3)	44.7 (22.5)	
Post-Testing	45.5 (22.6)	50.5 (20.6)	
Visual Analogue Scale (VAS) – Sad			Included in ANOVA above
Pre-Testing	38.9 (19.9)	32.3 (28.8)	
Post-Testing	31.2 (28.9)	26.8 (24.6)	
Visual Analogue Scale (VAS) – Hostile			Included in ANOVA above
Pre-Testing	13.2 (14.3)	13.1 (18.0)	
Post-Testing	13.2 (18.4)	12.2 (16.9)	
Visual Analogue Scale (VAS) – Alert			Included in ANOVA above
Pre-Testing	46.0 (21.0)	40.0 (24.0)	
Post-Testing	39.5 (24.3)	35.0 (22.1)	
Visual Analogue Scale (VAS) – Anxious			Included in ANOVA above
Pre-Testing	45.4 (20.6)	35.1 (29.7)	
Post-Testing	34.0 (29.8)	26.4 (27.8)	
Visual Analogue Scale (VAS) – Calm			Included in ANOVA above
Pre-Testing	49.6 (22.4)	48.5 (22.0)	
Post-Testing	49.2 (22.1)	56.9 (20.2)	

[11] 2 participants missing from 5-HT_4 group

In terms of side effects, two participants withdrew during the study: one due to abdominal pain (day 3 of study – placebo); one due to multiple symptoms including sleep disturbance, headache and brain fog (day 4 of study – 5-HT_4). Both resolved with 24 hours of discontinuing medication.

At the end of the study, participants guessed their study randomisation. Data suggested that participants in the 5-HT_4 were significantly less likely to correctly identify their randomisation compared to placebo (odds ratio (Pearson chi-square), p=0.043). Of those who guessed incorrectly in the 5-HT_4 group (9/26), two thirds suggested placebo and one third suggested citalopram (see Table 15).

Table 15: Results of randomisation guesses at the end of the study

	Placebo N=25*[12]	**5-HT_4 N=26**
Incorrect guess	7	9
Correct guess	11	3
Uncertain	7	14

5.3.3 Behavioural results of fMRI memory task: the effect of PF-04995274 on performance

All groups received training via the pre-scan task and performed well on the in-scan task (t(50) = -0.348, p=0.730; placebo (M=98.5%, SEM=0.51), 5-HT_4 (M=98.7%, SEM=0.26)). This indicates that all participants had adequately encoded the pre-scan images as required.

[12] 1 participant missing in placebo group

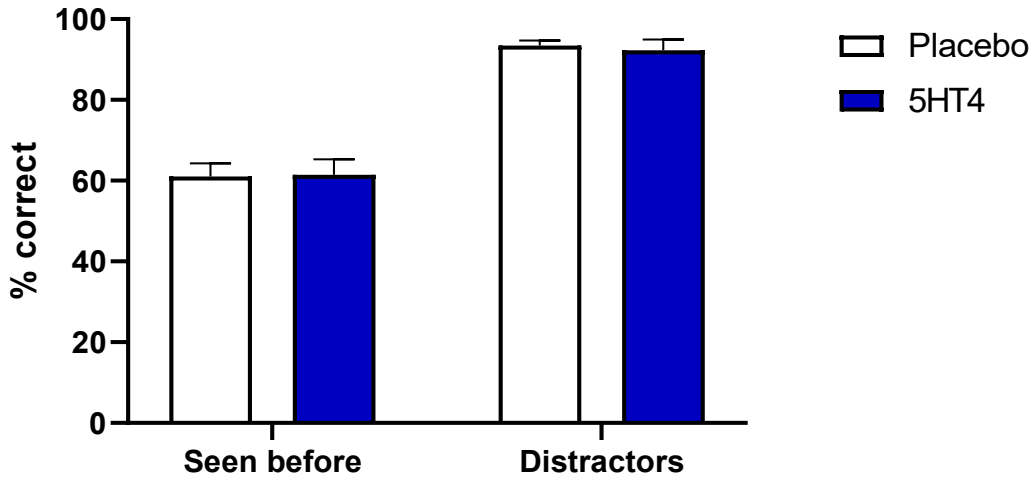
Administration of the 5-HT_4 agonist did not lead to improved memory performance in the post-scan recall test when compared to placebo (see Figure 16). There was no difference between groups when distinguishing images seen before (novel + familiar) versus distractors (F(1,46)=0.019, p=0.891, ηρ2=0.00; overall mean for placebo (M=77.2% SEM=1.82), 5-HT_4 (M=76.9%, SEM 1.89)), or when distinguishing each category of image (novel/familiar/distractor) (F(1,46)=0.016, p=0.901, ηρ2=0.00; overall mean for placebo (M=82.1%, SEM=1.45), 5-HT_4 (M=81.8%, SEM=1.51)). There was no interaction between image type (novel/familiar/distractor) and group (F(1,46)=0.095, p=0.76, ηρ2=0.00) (data missing for five participants (1 from placebo, 4 from 5-HT_4 = 48 (25:23) participants included)[13])).

Results were unchanged by adding in baseline HAM-D score or age as a covariate. For each category of image (novel/familiar/distractor), there was no effect of baseline HAM-D (F(1,45)=1.683, p=0.20, ηρ2=0.036), or age (F(1,45)=0.067, p=0.80, ηρ2=0.04) on the difference between groups when distinguishing images.

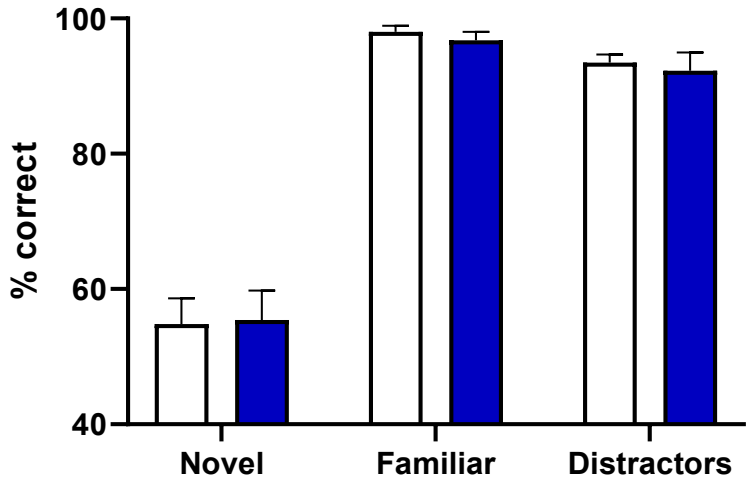
[13] This analysis includes the participant (in the 5-HT_4 group) excluded from image analysis due to significant movement. Results were similar when this participant was excluded from this analysis: F(1,45)=0.079, p=0.779. Results were also similar when I ran a sensitivity analysis excluding a statistical outlier from the analysis (also in the 5-HT_4 group): F(1,45)=0.038, p=0.531.

Figure 16: Post-scan recall task results (percentage total correct at identifying image type) divided by group: (A) seen before (novel+familiar) vs. distractors; (B) novel vs. familiar vs. distractors

A



B



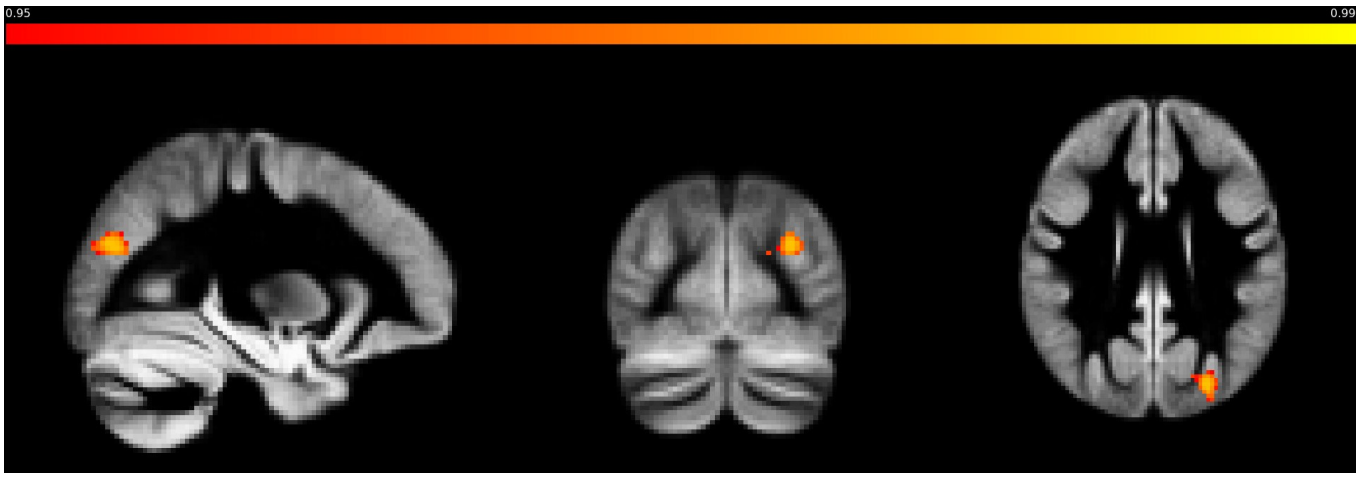
*White = placebo, blue = prucalopride. * = p<0.05, signifying a main effect of group on ANOVA both when comparing placebo vs prucalopride for **(A)** seen before (novel + familiar) vs. distractors; **(B)** novel vs. familiar vs. distractors.*

5.3.4 Analyses assessing for potential confounds in fMRI analyses

5.3.4.1 Grey matter

There were group-related differences in grey matter between the placebo and 5-HT_4 groups (FSLVBM: randomise: placebo > 5-HT_4, p=0.03; 5-HT_4 > placebo, p=0.99) involving the left lateral occipital cortex [placebo > 5-HT_4, peak voxel location: X=-28,-80,26, cluster size = 43 voxels] (see Figure 17). As pre-specified, whole-brain and hippocampal region of interest analyses include grey matter correction.

Figure 17: Group-related grey matter differences between placebo and 5-HT_4 groups (FSLVBM)



Sagittal, coronal, and axial images for FSLVBM at MNI location 45,54,45. Images thresholded at 1-p > 0.95. Red to yellow colours identify increases in grey matter differences (see colour bar included in image)

5.3.4.2 Perfusion

There was no difference between placebo and 5-HT_4 groups in regional blood flow (randomise: placebo > 5-HT_4, p=0.75; 5-HT_4 > placebo, p=0.21) or global blood flow (Oxford_ASL: p=0.40 (grey matter); p=0.24 (white matter)). Left and right hippocampal perfusion did not differ between groups (p=0.14 (LHC smoothed); p=0.16 (RHC smoothed) (see Table 16)). As pre-specified, whole-

brain and hippocampal region of interest analyses include cerebral perfusion correction, but were similar with and without.

Table 16: Hippocampal perfusion (smoothed and unsmoothed) across the right and left hemisphere (ml/100g/min)

		Left Hippocampus	**Right Hippocampus**
Smoothed	***Placebo***	50.4	50.8
	5-HT$_4$	53.7	53.8
Unsmoothed	***Placebo***	51.7	52.5
	5-HT$_4$	55.0	55.6

5.3.4.3 Region of interest task analyses

Activity between groups within the region of interest hippocampal mask did not vary as a function of baseline HAM-D scores ($F(1,49)=0.04$, $p=0.85$, $\eta p2=0.00$), or age ($F(1,49)=3.05$, $p=0.087$, $\eta p2=0.06$). However, activity within the mask did vary as a function of sex ($F(1,49)=11.93$, $p=0.00$, $\eta p2=0.20$), necessitating adding this covariate into region of interest analyses. Therefore region of interest analyses are also corrected for sex in addition to grey matter and perfusion as pre-specified.

5.3.4.4 Whole-brain task analyses

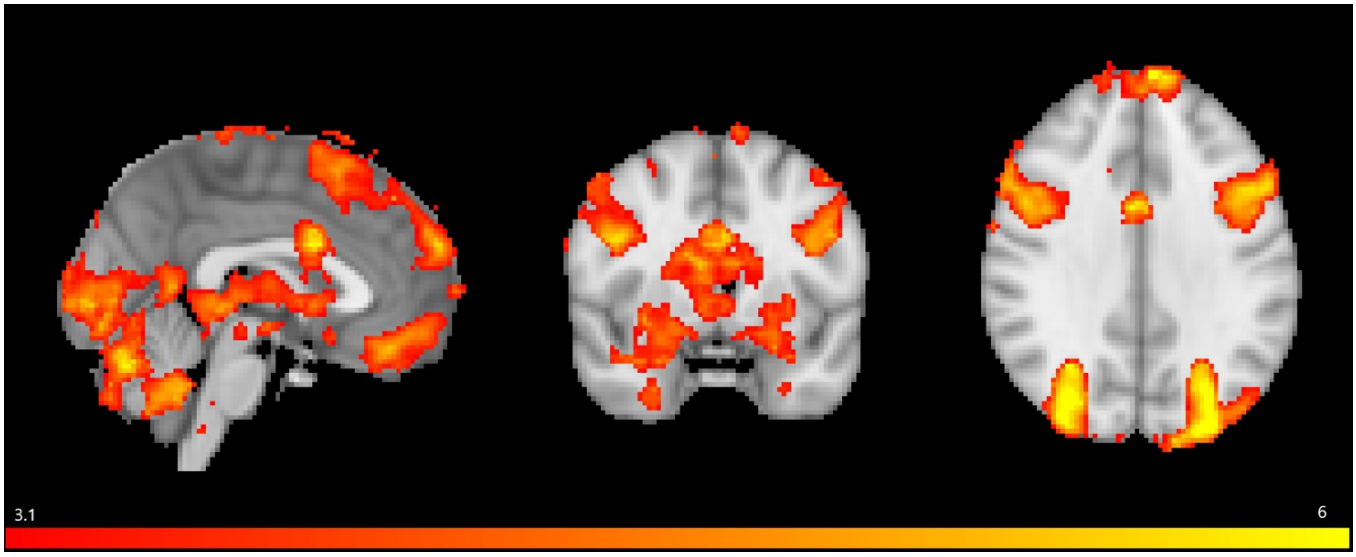
Whole-brain analyses were very similar when age, sex or baseline HAM-D were incorporated into analyses. Therefore, whole-brain analyses are reported corrected for grey matter and perfusion as pre-specified.

5.3.5 Memory Encoding Task fMRI: the effect of PF-04495274 on neural activity

5.3.5.1 Main effect of task

Consistent with previous reports (Filippini et al. 2009; Filippini et al. 2012), the novel versus familiar contrast revealed increased BOLD fMRI signal intensity bilaterally in the hippocampus, parahippocampal gyrus, and temporal fusiform cortex. Similar to Chapter Two, there was also increased activity to novel versus familiar pictures in association areas including the thalamus, regions in the frontal (inferior frontal gyrus, frontal pole, precentral gyrus, cingulate / paracingulate gyrus) and occipital lobes (lateral occipital cortex), cerebellum, basal ganglia (caudate, putamen) and amygdala (see Figure 18).

Figure 18: Regions with increased BOLD fMRI signal intensity for the main effect of task (novel > familiar)

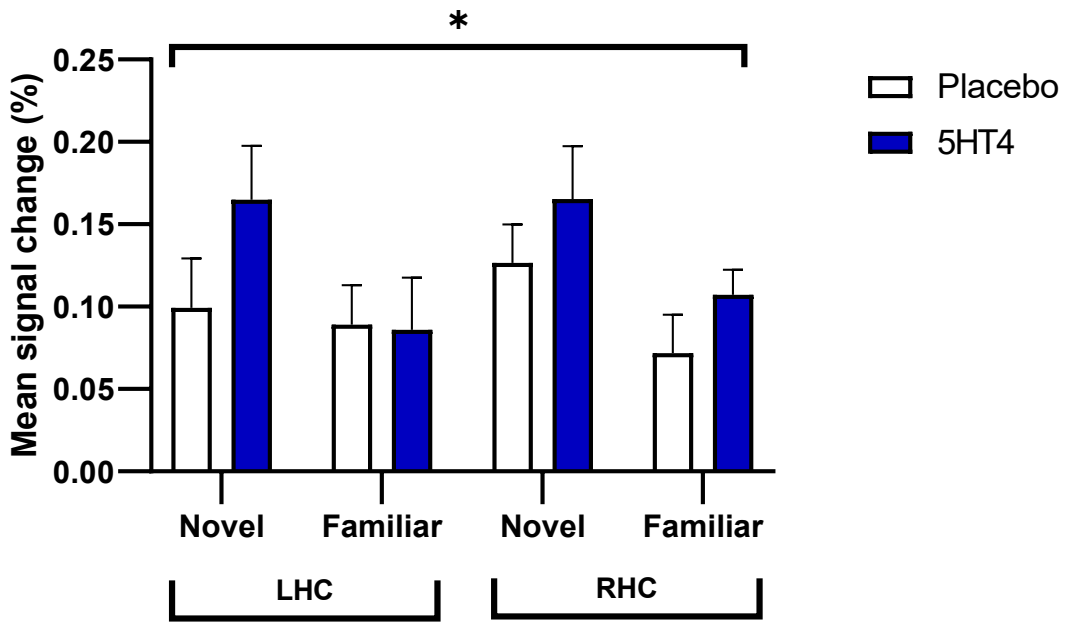


Sagittal, coronal, and axial images for the novel vs. familiar contrast at MNI location 45,65,52. Images thresholded at z > 3.1. Red to yellow colours identify increases in brain activation (see colour bar included in image)

5.3.5.2 Effect of treatment – region of interest analysis:

When investigating activity between groups within the hippocampal region of interest mask, there was a significant condition*hemisphere*group interaction ($F(1,49)=3.56$, $p=0.04$, $\eta p2=0.08$). Post-hoc pairwise comparisons identified that this significant interaction was predominately driven by the 5-HT_4 group having significantly increased activity to novel compared to familiar images, particularly in the left hemisphere (left: $p=0.035$; right $p=0.050$), which was not evident in the placebo group (left: $p=0.789$; right $p=0.053$).

Figure 19: BOLD percentage signal change extracted from the functional left (LHC) and right (RHC) hippocampal mask in response to novel and familiar images



*Error bars show standard error of the mean. * denotes significance within the ANOVA (p<0.05)*

However, there was no significant condition*group interaction for either hemisphere individually[14]: LHC novel vs. familiar: F(1,49)=1.80, *p*=0.19, np2=0.04; RHC novel vs. familiar: F(1,49)=0.00, *p*=0.99, np2=0.00. Furthermore, there was no significant difference in activity between the two groups in response to both novel and familiar images in the left and right hemisphere (F(1,49)=1.39, *p*=0.24, ηρ2=0.03; see Figure 19).

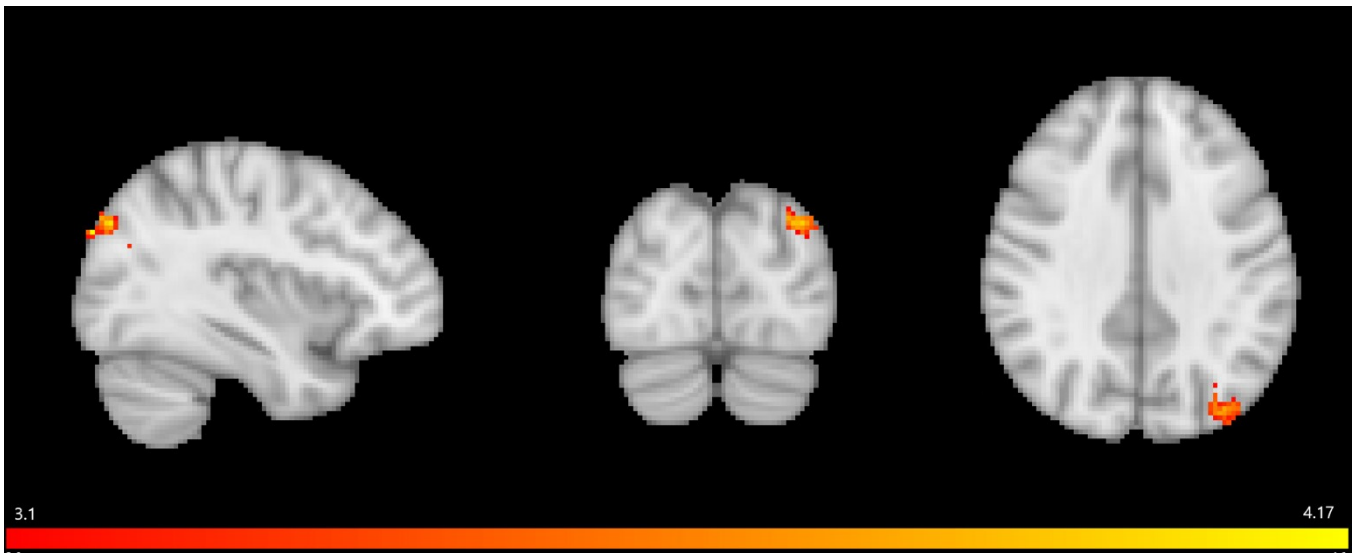
5.3.5.3 Effect of treatment – whole brain analysis

There was increased activity in the 5-HT_4 group compared with the placebo group in response to novel images (versus baseline) in the left lateral occipital cortex [5-HT_4 > placebo, Z=4.19, p<0.007, peak voxel location: X=-36, Y=-88, Z=32, cluster size = 151 voxels] (Figure 20(A)). Figure 20(B) represents the group-level extracted BOLD signal change for this activation cluster. There were no significant differences between the groups at a whole brain level for the novel > familiar contrast, or the familiar > baseline contrast. Whole-brain analyses were very similar when sex[15], HAM-D or age were incorporated into analyses.[16]

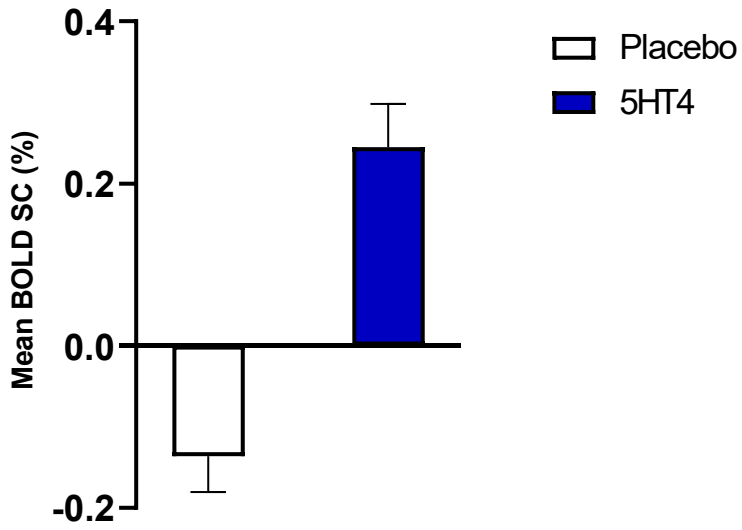
[14] Condition individually was also run in a similar ANOVA as a secondary analysis: LHC familiar vs RHC familiar: F(1,49)=1.81, p=0.18, np2=0.04; LHC novel vs RHC novel: F(1,49)=1.06, p=0.31, np2=0.02.
[15] Results for when ASL+GM+sex were included as covariates (for direct comparison with ROI analyses): [5-HT_4>placebo, Z=3.9, p<0.014, peak voxel location: X=-36, Y=-82, Z=36, cluster size = 131 voxels]
[16] Placebo and 5-HT_4 groups differed in grey matter in this region (placebo > 5-HT_4). Of note, cluster is still present here when whole-brain analyses were run without correction for grey matter.

Figure 20: Whole-brain activation in response to novel images (compared to baseline) in 5-HT_4 vs. placebo group

A



B



(A) *Sagittal, coronal and axial images depicting significantly increased activation in the 5-HT_4 group for the novel > baseline contrast in a left lateral occipital cortex cluster (peak voxel X=-36, Y=-88, Z=32, cluster size = 151 voxels). Images shown at MNI -36, -82, 34, thresholded at z > 3.1, p<0.05 corrected. Red to yellow colours identify increased in brain activation*

(B) *Group mean of the BOLD percentage signal change extracted from the left lateral occipital cortex cluster (novel > baseline). Error bars show standard error of the mean. Mean BOLD SC = Mean BOLD signal change.*

5.4 Discussion

In this study, a week's administration of the novel 5-HT_4 agonist, PF-04995274 increased neural activation for novel images in the left lateral occipital cortex in un-medicated depressed volunteers relative to placebo. It was also associated with a significant condition*hemisphere*group interaction within the hippocampus in a region of interest analysis (i.e. an increased activity to novel compared to familiar images within the left hippocampus). However, this did not result in improved behavioural cognition as assessed by the hippocampal memory task.

Therefore, in a pre-specified region of interest analyses, this study replicated the finding that 5-HT_4 receptor agonism in humans modulated hippocampal activity in the context of a memory task. Moreover, this study extended translation from healthy volunteers (see Chapter Two) into a clinical sample of depressed patients. The hippocampus is well-established as a memory encoding region. Furthermore, 5-HT_4 receptors are known to be present in this location (Pfizer 2011a), and animal data have consistently demonstrated that agonism of these receptors has an important role in facilitating hippocampal-dependent learning and memory (see Chapter One) with several mechanisms suggested to underlie this effect. For instance, when stimulated within the hippocampus, 5-HT_4 receptors can modulate glutamate activity via AMPA receptors (Chen et al. 2020), release acetylcholine (Hagena and Manahan-Vaughan 2017), and promote cell and spiny growth.

Interestingly, the pattern of hippocampal activation seen in this study is similar but subtly different from the prucalopride study (Chapter Two). With prucalopride, hippocampal activation

was increased for both forms of image (novel and familiar) compared to the placebo group, whereas in this study the change in activation was affected by image type (novel > familiar). This pattern of in-scan hippocampal activity seen with RESTAND is more similar to that seen in the original publications for this task (Filippini et al. 2009; Filippini et al. 2012), which used individuals with a genetic or age-related predisposition to Alzheimer's. This confirms the importance of assessing the role of 5-HT_4 receptor agonists specifically in a clinical population, rather than assuming healthy participant findings will generalise to those with depression.

The hippocampus is a particularly important for depression (Liu et al. 2017; Price and Duman 2020). There may be several reasons for this (Tartt et al. 2022), including its involvement in the stress response (Sheline et al. 2019), and its capacity for neurogenesis into adulthood (Eriksson et al. 1998). Reduced hippocampal volume in depression has been a fairly consistent and well-documented finding (Sheline et al. 2019), associated with markers of disease progression, such as the number of episodes, duration of illness, and treatment resistance (Belleau et al. 2019). Depression also affects hippocampal functioning i.e. its connections with other brain regions and involvement in brain processes such as mood and cognition (Tartt et al. 2022). In untreated depression, the hippocampus appears to have impaired connections to both the prefrontal cortex (Cao et al. 2012; Hao et al. 2020) (affecting cognition) and amygdala (Cullen et al. 2014; Hamilton and Gotlib 2008) (affecting mood) when compared with healthy controls. Moreover these connective abnormalities may correlate with depression severity (Cullen et al. 2014).

In whole-brain analyses, participants receiving PF-04995274 compared to the placebo group in RESTAND showed increased activity in a memory processing region, the left lateral occipital cortex (LOC). This is a region involved in the perceptual processing and encoding of objects

particularly relating to shape, size and position (Grill-Spector et al. 2001; Grill-Spector et al. 1999; Malach et al. 1995). Function also depends on laterality, with the left hemisphere involved in object naming as opposed to object matching in the right hemisphere (Large et al. 2007). In addition to encoding, the LOC is reactivated during memory processing of objects (Karanian and Slotnick 2015). Complexity for object memory appears to involve both the posterior subdivisions (specific for simpler aspects such as orientation) and anterior subdivisions (involved in more complex representations such as position in space) (Karanian and Slotnick 2015; Silson et al. 2013). This conceptual understanding of the LOC and its role is consistent with the results of this study, where in the scan participants were required to mentally name an object as an animal or landscape, press a button accordingly, and remember it for a later memory test (therefore requiring encoding of novel images seen). Furthermore, in unmedicated patients with depression, as in this study, magnetic resonance spectroscopy (MRS) studies show reduced levels of inhibitory GABA (Schür et al. 2016) especially in the occipital cortex (Bhagwagar et al. 2007; Sanacora et al. 2004). One laboratory have reported GABA occipital levels normalising with successful treatment of depression, including SSRI antidepressant treatment (Sanacora et al. 2002), stimulation therapies (Sanacora et al. 2003), and psychological therapy (Sanacora et al. 2006). Altogether, this suggests that the LOC is important in learning and memory relating to the specifics of objects, and that this cortex demonstrates abnormal neurochemical functioning in depression.

Intriguingly, these whole-brain findings overlap with the findings of a difference between the groups in terms of grey matter. That is, both involve a cluster within the left lateral occipital cortex. This cluster is still present when whole-brain results were not corrected for grey matter. By adding in grey matter maps as a nuisance regressor to the analyses, it was confirmed that the increase in activity seen here in the LOC cannot be explained by the difference in grey matter alone. There are two possibilities for this group difference relating to grey matter: either the

structure or topography (gyrification) is different between the groups. It is very interesting that the region where there was a grey matter difference between the groups in this study also became the focus of increased activation with the 5-HT_4 agonist. As this is the first fMRI study using PF-04995274 in humans, it is not possible to say if this finding is particular to this study population or whether it would generalise to other groups with un-medicated depression.

These whole-brain fMRI findings are different from the prucalopride study, where increased activity was found not in the left LOC but instead within the right angular gyrus. The angular gyrus (as detailed in Chapter Two) is thought to have a memory retrieval role in distinguishing objects that were (or were not) encoded as part of a schema (van der Linden et al. 2017). Activity here is reduced in those with cognitive impairment (Lau et al. 2016) and correlates with behavioural task performance (Jin et al. 2012). The different whole-brain findings between RESTAND and the prucalopride study may relate to the different populations (depressed versus healthy). Cognitive deficits are common in depression, do not respond well to SSRI treatment, and often persist after mood has improved (Etkin et al. 2015; Millan et al. 2012). In an unmedicated depressed population, where we would expect to see cognitive deficits (Rock et al. 2014), a potentially pro-cognitive medication may bring online different regions to improve cognition than would occur in a healthy population. That is, pro-cognitive medications should work in both clinical and healthy populations, but they may do this via different means depending on the baseline functioning; the caveat to this argument is that these two studies assessed two different 5-HT_4 receptor agonists as well as different participant populations.

This is the first study assessing the potential pro-cognitive effects of a 5-HT_4 receptor agonist in a clinical population. An important strength of RESTAND was that these participants with

depression were not on any antidepressant treatment, enabling assessment of PF-04995274 without interference from other serotonergic medication. However, perhaps partially related to the need for participants to be medication free, the average HAM-D score was relatively low at 16, and few included participants had severe depression. Nonetheless, severity of depression and severity of cognitive difficulties are poorly correlated (Dam et al. 2021). I also had the benefit of using a new 5-HT_4 receptor agonist that is well characterised in terms of brain receptor occupancy and dose via healthy human PET studies, (unlike prucalopride) and I was able to include a wide age range in the study (18 to 65) to maximise generalisability of any findings.

It is important to note that, in contrast with our previous prucalopride study, there was no effect of PF-04995274 on behavioural performance on the memory task. Depressed participants receiving PF-04995274 were not better able to distinguish images they had seen before (familiar images seen prior to as well as during the scan, and novel images seen first in the scan) from a set of previously unseen distractor images when compared to participants receiving placebo. This is in contrast to previous findings (Chapter Two, prucalopride study), where participants receiving the 5-HT_4 receptor agonist, prucalopride, did demonstrate improved recall for image type compared to placebo participants. It also is not consistent with the wealth of animal data where 5-HT_4 receptor agonists have shown improvements in learning and memory (see Chapter One and Two for detailed discussion).

This partial and variable translation of the earlier animal work and prucalopride studies from our laboratory (including work in this thesis) could be explained by several factors, including insufficient power. There is evidence that power may have been suboptimal in RESTAND as I demonstrated modulated activation to the hippocampus with PF-04995274 versus placebo on a

region of interest analysis, but this did not survive correction for multiple comparisons at the whole brain level. Interestingly, this was a very similar situation to Chapter Two.

In terms of behavioural data, to reliably detect an effect size of the magnitude seen with 5-HT_4 agonism in the prucalopride study post scan recall test (Chapter Two), the sample size required for an adequately powered study is 42 (G*Power, previous ηρ2=0.12 (effect size = 0.37), with an α=0.05, to give 90% power (1-β)). This suggests this study was just adequately powered to detect an effect of similar magnitude, as even after taking account of missing data there were 48 people in each group. Examining the performance of the RESTAND participants closely, the means and in-scan reaction times for both groups were similar to scores for the placebo group in the prucalopride study i.e. (i) seen before versus distractors results: prucalopride study prucalopride mean 81.0% (SEM 1.63), placebo mean 75.7% (SEM 1.70); RESTAND study 5-HT_4 mean 76.9% (SEM 1.89), placebo mean 77.2% (SEM=1.82); (ii) in-scan reaction times: prucalopride study prucalopride mean 0.69s (SEM 0.23); placebo mean 0.71s (SEM 0.03); RESTAND 5-HT_4 mean 0.71s (SEM 0.03), placebo mean 0.71s (SEM 0.03). Future studies could use behavioural tasks that are sensitive to impairments seen in depression, and therefore potentially more sensitive to change with a pro-cognitive agent in this population, and / or could assess cognition following a longer period of administration.

There may have also been other participant-level factors affecting these results. For example, although participants on antidepressants for this episode were excluded, previous use of antidepressants was not an exclusion criterion; number or type of previous antidepressant use was not collected systematically. However, those with previous use of ECT were excluded. Furthermore, a PET study has shown that 5-HT_4 receptor binding may be lower in patients with

unmedicated depression relative to healthy participants (Köhler-Forsberg et al. 2021). As pre-clinical studies of receptor occupancy with PF-04995274 have only been conducted in healthy volunteers, it is not possible to say if this is the optimal dose for receptor occupancy in un-medicated depression. It is also interesting that sex became a relevant factor in hippocampal activity in this study, as pre-clinical data involving occupancy and half-life were only conducted in men. Therefore, it is possible men and women may show differences in terms of receptor binding and processing of PF-04995274. For instance, the use of oral contraceptives in females appears to lead to lower 5-HT_4 receptor binding globally, with the largest difference present in the hippocampus (Larsen et al. 2020). Moreover, this altered binding may relate to differences in response to antidepressant treatment (Larsen et al. 2022). At this time, it is not possible to assess if this is relevant for these analyses. In view of this, and as evidence in this study indicates that hippocampal activation varied with sex, controlling for sex within the region of interest analyses seems optimal.

In conclusion, this study demonstrates that PF-04995274, a new 5-HT_4 receptor agonist, was able to partially replicate findings from the previous study of prucalopride (Chapter Two) and in a clinical population. That is, I replicated the finding that 5-HT_4 agonism increased activation in the hippocampus and related regions in healthy volunteers into a group of depressed patients. However, in RESTAND, unlike in healthy volunteers, this increase in neural activity did not lead to improved behavioural performance. It is uncertain whether this lack of behavioural effect may have been impacted by insufficient power, possibly relating to population and / or drug factors. Future work may be improved with larger sample sizes and / or increased information from PET studies of 5-HT_4 receptor agonist activity in clinical and more diverse populations.

CHAPTER 6

Prucalopride and the future risk of mental health outcomes: an emulated target trial

6.1: Introduction

As discussed in Chapter One, depression is a common and costly global condition, and many patients become treatment resistant or experience a therapeutic delay with first line antidepressants (Malhi and Mann 2018; WHO 2018). Thus, newer agents that act more quickly are sorely needed. Animal data suggests that 5-HT_4 agonists may have rapid antidepressant-like effects, behaviourally and neurochemically (Lucas et al. 2007). However, pilot data (Murphy et al. 2020) and work within this thesis (Chapter Three) has not as yet provided evidence for this effect in humans.

Prucalopride is a highly-selective 5-HT_4 receptor agonist, and a safe and efficacious treatment for chronic constipation in adults (Coremans 2008; Frampton 2009). It also has good brain penetrance. This makes it a potentially useful agent to examine real-world neuropsychiatric effects of 5-HT_4 receptor agonism. However, large-scale human clinical studies of this issue are lacking.

Analyses of existing clinical datasets offers the opportunity to investigate associations between medications and outcomes of interest using a large sample size in a real-world setting, where prucalopride has been extensively prescribed for its licensed indication (the treatment of chronic

constipation). Therefore, the primary aim of this study was to test whether extended prucalopride treatment was associated with a lower incidence of developing major depression within two years of initiating treatment. To control for any psychological effects mediated by the relief of constipation, I compared the effects of prucalopride with two other agents used to treat this condition, lubiprostone and linaclotide. While these agents are as clinically effective as prucalopride they have no known effect on 5-HT_4 receptors. Other mental disorders (cognitive disorders, anxiety, psychosis, substance misuse) were included as secondary outcomes.

To carry out this investigation I used a large electronic health records (EHR) network, the TriNetX Analytics network. This contains data from over 85 million patients mostly in the USA, from which cohorts of patients treated with each of the three drugs were identified and compared. This question lends itself well to an emulated target trial (Hernán et al. 2022) for two reasons: (i) all three drugs are third-line interventions for chronic constipation and the choice between the three is largely driven by clinician's preference thus limiting systematic indication and selection bias, and (ii) patients and clinicians are not aware *a priori* that these drugs might have differential effects on mental health thus limiting detection bias. In addition, propensity score matching was used to match cohorts at baseline for 108 covariates, and key sensitivity analyses were used to test the robustness of the results.

6.2 Methods

6.2.1 Study design and data collection

The TrinetX Analytics Networks were used for this retrospective cohort study: a cloud-based network that can access anonymised data from electronic health records in multiple healthcare organisations, mostly based in the United States (US) but also from Australia, the UK, India, Spain, Bulgaria, Malaysia and Taiwan. Full details about the network can be found in (Taquet et al. 2021a; Taquet et al. 2021c). In brief, this network allows access to over 85 million patients with data involving demographics, diagnoses and ICD-10 codes, medications, procedures, measurements (e.g. blood pressure, body mass index) and health care visits. Data is included from both primary and specialist healthcare organisations, and involves both patients insured and not insured under the standard US system. Each healthcare organisation remains anonymous within TriNetX, and is able to provide data with the necessary consents and approvals as long as research is the sole use. The process of data de-identification is attested by a qualified expert as defined in Section 164.514(b)(1) of the HIPPA Privacy Rule (Taquet et al. 2021a).

Researchers use the TriNetX online interface to build various cohorts by inputting specific inclusion and exclusion criteria. Different cohorts can then be matched for confounding variables using propensity score matching. These cohorts can then be compared for outcomes of interest over a selected time frame.

6.2.2 Cohorts

The primary cohort was defined as all patients with at least two prescriptions of prucalopride at least 12 months apart before the start date of analysis (29th November 2022). Two comparator cohorts were also created using medications recommended for the same clinical purposes: patients with at least two prescriptions at least 12 months apart of (i) linaclotide (a guanylate cyclase 2C agonist) and (ii) lubiprostone (a chloride channel activator). Having at least two

prescriptions 12 months apart was used as a proxy indicator that patients have been taking the medication for sufficient time. The index date (from which the follow-up started) was taken as the date of the first prescription of prucalopride (or linaclotide or lubiprostone).

The cohorts included patients of any age or sex remaining alive at the time of analysis. Patients with pre-existing diagnoses of common and / or serious mental illness within their health records before the index date were excluded; that is with either code F20-F29 (schizophrenia, schizotypal, delusional, and other non-mood psychotic disorders), F30-F39 (mood [affective] disorders), F40-F48 (anxiety, dissociative, stress-related, somatoform and other nonpsychotic mental disorders), or F01-F09 (mental disorders due to known physiological conditions including cognitive disorders).

All three medications (prucalopride, linaclotide, and lubiprostone) are approved by the FDA for use in the United States for chronic constipation and all three drugs are available under Medicaid. 5-HT_4 agonists, linaclotide and lubiprostone are third-line pharmacotherapy options in United States and other IBS-C guidelines (ASG 2022; BSG 2021).

6.2.3 Covariates

All cohorts were matched for disorders and medication that could be associated with differences in choices of anti-constipation treatment and / or with psychiatric disorders. In addition, matching occurred for any diagnoses or medication where the baseline incidence was greater than 5% in both cohorts and the difference between cohorts greater than 2% at the pre-matching stage.

Table 17: Covariates used for matching in primary analyses[17]

Age at Index	Parkinson's disease and other movement disorders	VACCINES
Female / Male	Demyelinating diseases of the central nervous system	TRICYCLIC ANTIDEPRESSANTS
White / Black	Systemic connective tissue disorders	BLOOD GLUCOSE REGULATORS
Unknown Ethnicity / Race	Other nervous system degeneration	omeprazole
Unknown Gender	Other anemias	OPIOID ANTAGONIST ANALGESICS
Other functional intestinal disorders	ANALGESICS	plecanatide
Gastro-esophageal reflux disease	ELECTROLYTES/MINERALS	ANTIMIGRAINE AGENTS
Gastroparesis	LAXATIVES including STIMULANTS	ANTIHYPERTENSIVES,OTHER
Essential (primary) hypertension	NON-OPIOID ANALGESICS	ESTROGENS & PROGESTINS
Irritable bowel syndrome	OPIOID ANALGESICS	ACE INHIBITORS
Noninflammatory female genital tract	ADRENAL CORTICOSTEROIDS	ANGIOTENSIN II INHIBITOR
Disorders of lipoprotein metabolism	ANTIASTHMA/BRONCHODILATORS	simethicone
Diseases of the eye and adnexa	ondansetron	CNS STIMULANTS
Other diseases of intestine	HYPEROSMOTIC LAXATIVES	CONTRACEPTIVES,SYSTEMIC
Benign neoplasms	AUTONOMIC MEDICATIONS	duloxetine
Other nutritional deficiencies	SEDATIVES/HYPNOTICS	esomeprazole
Chronic lower respiratory diseases	ANTACIDS	ANTIPSYCHOTICS
Diabetes mellitus	ANTIARRHYTHMICS	trazodone
Sleep disorders	ANTIDEPRESSANTS	ANTIDIARRHEAL AGENTS
Acute upper respiratory infections	ANTIULCER AGENTS	prochlorperazine
Osteoarthritis	ANTICONVULSANTS	dexlansoprazole
Other disorders of muscle	pantoprazole	scopolamine
Hernia	ANTILIPEMIC AGENTS	mirtazapine
Noninfective enteritis and colitis	ANTIANGINALS	bupropion
Chronic pain, not elsewhere classified	BETA BLOCKERS	venlafaxine
Disorders of thyroid gland	DIURETICS	citalopram
Diseases of arteries and arterioles	STOOL SOFTENER	paroxetine
Acute and chronic kidney disease	ANTIHISTAMINES,PHENOTHIAZINE	domperidone
Nicotine dependence	ANTIHISTAMINES,PIPERAZINE	escitalopram
Disorders of autonomic nervous system	THYROID MODIFIERS	sertraline
Malignant neoplasms of digestive tract	CALCIUM CHANNEL BLOCKERS	fluoxetine

[17] Capitals=covariates which contain subdivisions, non-capitals=terminal branch within covariate groupings. Listed in approximate order of incidence

Propensity score matching was achieved within TriNetX using a greedy 1:1 nearest neighbour and a caliper of 0.1. Matching for a covariate was considered appropriate if a standardised mean difference of 0.1 or less was observed between matched cohorts (Haukoos and Lewis 2015). Baseline characteristics of cohorts prior to matching (lifetime, 5 years and 1 year before inclusion) were checked.

This process resulted in matching for 108 variables including: age at index event, sex, race and ethnicity, body mass index / obesity, gastrointestinal (GI) diseases, metabolic disorders, diabetes mellitus, rheumatological disorders, neurological and neuropsychiatric disorders, cardiac and hypertensive disorders, endocrinological disorders, respiratory disorders, and GI and neuropsychiatric medication. The list (contracted considering space constraints) is detailed in Table 17. Matching remained consistent across all analyses, except where otherwise stated.

6.2.4 Outcomes

Primary outcome: The incidence of a first diagnosis of major depressive disorder (F32) was investigated within two years of the index date.

Secondary outcomes: The incidence of a first diagnosis of six other common and/or serious mental health outcomes was investigated (captured using the ICD-10 codes in brackets) within two years of the index date: (i) mood [affective] disorder (F30-F39), as an overarching outcome, as well as (ii) bipolar disorder (F31) specifically; (iii) anxiety disorder (F40-F48); (iv) dementia (any of F01-F03, G30, G31.0, G31.2, G31.83); (v) substance use disorder (F10-F19); (vi) psychotic disorder (F20-29).

6.2.5 Secondary analyses

6.2.5.1 Sensitivity analyses

As sensitivity analyses, it was assessed how primary and secondary outcomes might vary under the following conditions: (i) matching for number of health visits between cohorts to test whether differences in outcomes could be accounted for by differences in healthcare engagement as previously conducted in (Taquet et al. 2021b); (ii) investigating both first and any / recurrent diagnoses and thus not excluding from the cohort those with prior mental illness (iii) excluding those with prior neuropsychiatric illness as neurological and psychiatric outcomes can be closely linked (i.e. as in (Colbourne and Harrison 2022) in addition to psychiatric disorders excluding those with diagnoses of: impulse disorders (F63); extrapyramidal and movement disorders (G20-G26); other extrapyramidal and movement disorders (G25); primary insomnia (F51.05); and other insomnia not due to a substance or known physiological condition (F51.09)); (iv) excluding patients on either of the two most common SSRI antidepressants (within these cohorts, namely escitalopram and sertraline) as antidepressant usage in this high risk population was common. It should be noted, that, as in (Colbourne and Harrison 2022), for analysis relating to (ii), participants did not need to have the same diagnosis as an exposure and an outcome (e.g. they could receive a diagnosis of anxiety disorder prior to the index event, and a diagnosis of depression as an outcome).

6.2.5.2 Negative control outcomes

To assess for potential unmeasured confounding, matched cohorts were also compared in terms of a range of negative control outcomes, i.e. outcomes that are not expected to be influenced by differences in anti-constipation medications (Ali et al. 2019). 19 negative control outcomes were

selected, adapted from previous similar analyses using the TriNetX database (Colbourne and Harrison 2022). These were designed to include both emergency and primary care, and various body systems and mechanisms: cutaneous abscess, ganglion, ingrowing toenail, onycholysis, sebaceous cyst, seborrheic keratosis, trigger finger, viral warts, dog bite, acute myocardial infarction, adhesive capsulitis of shoulder, synovitis / tenosynovitis, blepharitis, fracture of finger, folliculitis, ankle sprain, paronychia, lower leg varicose veins, and lateral epicondylitis. The incidence of a first diagnosis of each negative control outcome was assessed individually within a cohort, and also as a composite of any of these outcomes (to increase statistical power and thereby increase the chance of detecting small but significant effects of residual confounding).

6.2.5.3 Additional comparisons

The two anti-constipation comparators (linaclotide and lubiprostone) were compared with each other in terms of their effect on mental health and negative control outcomes to assess potential equivalence in incidence.

Prucalopride was licensed by the FDA more recently than linaclotide and lubiprostone in the US (prucalopride 2018; lubiprostone 2006; linaclotide 2012), and the prescription of prucalopride may be affected by social factors relating to Medicaid availability. In order to control for this, linaclotide was compared with plecanatide; plecanatide is a drug with the same mechanism of action and indication as linaclotide, and the same Medicaid approval year as prucalopride (both approved for Medicaid in 2022). Cohort limitations prevented direct comparison of prucalopride and plecanatide. For analyses involving plecanatide, plecanatide use was not matched across cohorts.

The baseline characteristics of the linaclotide cohort before and after 2018 were also compared to check if this largest cohort varied substantially in composition before and after prucalopride was available in the US.

6.2.6 Statistical analysis

Cumulative incidences over the two-year follow-up were estimated using the Kaplan-Meier estimator. The Cox proportional hazard model and the log-rank test were used to compare matched cohorts in terms of hazard ratios (HR) with 95% confidence intervals (CI) (computed using the "survival" package 3.3-1 in R). The proportional hazard assumption was tested with the generalised Schoenfeld approach. E-values were calculated for all comparisons in the primary analysis: a large E-value implies that a large amount of unmeasured confounding would be required to explain away an effect estimate (VanderWeele and Ding 2017).

Statistical significance was set at two-sided $p<0.05$. Except for propensity score matching estimated within TriNetX, all statistical analyses were conducted using R version 4.2.1 between 29th November and 1st December 2022.

6.3 Results:

6.3.1 Cohort demographics and matching

Table 18: Demographics before matching for cohorts involved in primary analyses. Comorbidities and medications are lifetime prevalence.

Incidence in % unless otherwise stated

	Prucalopride	**Linaclotide**	**Lubiprostone**
Cohort size (n)	5235	135712	65282
Age at index (y, SD)	48.5, 18.6	54.4, 17.6	55.0, 19.1
Sex (M: F: O)	20.2: 79.8: 0.2	23.5: 76.5: 0.02	26.9: 73.1: 0.04
Ethnicity (W, B, unknown)	74.7, 10.6, 23.1	66.6, 19.9, 23.5	68.2, 17.1, 21.4
Gastro-oesophageal reflux disease	28.8	19.3	18.1
Primary hypertension	16.7	23.9	24.3
IBS	17.0	9.5	9.0
Lipidaemias	15.1	19.3	19.1
Disorders of thyroid gland	12.2	11.1	10.7
Chronic respiratory diseases	11.8	11.3	11.5
Diabetes mellitus	10.8	12.6	12.9
Chronic pain	8.7	8.0	8.1
Overweight, obesity	7.7	9.7	8.4
Ischaemic heart diseases	6.4	8.2	8.9
Iron deficiency anaemia	5.6	3.8	3.4
Acute and chronic kidney disease	4.6	6.1	6.6
Malignant neoplasms of digestive organs	1.7	1.4	1.4
Demyelinating diseases of the nervous system	1.5	1.1	1.3
Parkinson's disease	0.8	1.0	1.3
Laxatives	52.1	44.2	50.0
Opioid analgesics	49.2	50.0	56.1
Sedatives / hypnotics	46.8	39.9	44.1
Corticosteroids	47.5	43.1	42.1
Antidepressants	45.7	38.7	41.7
Antacids	37.0	35.3	39.1
Antilipaemic agents	28.2	34.3	36.3
Stimulant laxatives	27.2	21.0	25.3
Stool softeners	20.1	19.3	24.3
Thyroid modifiers	18.1	17.4	17.9
Antipsychotics	10.1	7.2	9.7
Duloxetine	9.9	8.4	9.3
Trazodone	9.5	8.8	10.6
Mirtazapine	6.4	2.7	3.4
Sertraline	6.3	5.3	5.3
Escitalopram	5.9	5.6	5.3
Fluoxetine	4.8	3.6	3.4
Citalopram	3.0	3.5	3.7

Table 19: Comorbidities and medications before matching for prucalopride versus linaclotide cohorts involved in primary analyses, restricted to 5 years and 1 year before analysis

Incidence in % unless otherwise stated

	5 years before		**1 year before**	
	Prucalopride	**Linaclotide**	**Prucalopride**	**Linaclotide**
Gastro-oesophageal reflux disease	27.1	17.1	21.2	14.0
Primary hypertension	15.7	22.9	11.4	18.3
IBS	16.4	8.7	13.2	8.0
Lipidaemias	13.8	18.0	9.9	14.4
Disorders of thyroid gland	11.1	10.1	7.7	7.9
Chronic respiratory diseases	10.7	10.8	7.3	7.5
Diabetes mellitus	10.2	12.4	8.2	10.0
Chronic pain	8.1	7.8	4.9	5.3
Overweight, obesity	7.2	7.9	4.5	6.3
Ischaemic heart diseases	5.8	8.4	4.1	5.9
Iron deficiency anaemia	5.1	3.1	3.2	2.4
Acute and chronic kidney disease	4.4	6.4	3.2	4.8
Malignant neoplasms of digestive organs	1.7	1.4	1.1	1.1
Demyelinating diseases of the nervous system	1.3	1.2	1.0	0.8
Parkinson's disease	0.8	1.3	0.7	0.9
Laxatives	50.5	49.8	39.7	35.3
Opioid analgesics	46.5	54.5	33.3	37.7
Sedatives / hypnotics	44.8	42.8	31.9	29.3
Corticosteroids	46.2	41.2	33.2	32.7
Antidepressants	44.1	41.1	34.6	31.8
Antacids	34.8	37.8	23.9	25.5
Antilipaemic agents	27.1	35.6	19.6	28.1
Stimulant laxatives	25.9	24.7	19.2	15.3
Stool softeners	18.5	23.4	12.1	12.9
Thyroid modifiers	17.6	17.6	13.1	14.5
Antipsychotics	9.8	9.5	7.0	5.7
Duloxetine	9.3	9.1	6.7	6.5
Trazodone	8.9	10.3	6.5	6.9
Mirtazapine	6.3	3.1	5.2	2.2
Sertraline	6.1	5.1	4.3	4.0
Escitalopram	5.7	5.2	3.9	4.2
Fluoxetine	4.4	3.3	3.1	2.7
Citalopram	2.8	3.5	2.0	2.5

Table 20: Comorbidities and medications before matching for prucalopride versus lubiprostone cohorts involved in primary analyses, restricted to 5 years and 1 year before analysis

Incidence in % unless otherwise stated

	5 years before		**1 year before**	
	Prucalopride	**Lubiprostone**	**Prucalopride**	**Lubiprostone**
Gastro-oesophageal reflux disease	27.1	18.5	21.2	13.3
Primary hypertension	15.7	25.0	11.4	19.1
IBS	16.4	9.3	13.2	8.0
Lipidaemias	13.8	19.4	9.9	14.3
Disorders of thyroid gland	11.1	10.7	7.7	7.9
Chronic respiratory diseases	10.7	11.6	7.3	8.2
Diabetes mellitus	10.2	13.4	8.2	10.5
Chronic pain	8.1	8.4	4.9	5.7
Overweight, obesity	7.2	8.8	4.5	5.7
Ischaemic heart diseases	5.8	9.0	4.1	6.6
Iron deficiency anaemia	5.1	3.4	3.2	2.4
Acute and chronic kidney disease	4.4	7.2	3.2	5.3
Malignant neoplasms of digestive organs	1.7	1.5	1.1	1.1
Demyelinating diseases of the nervous system	1.3	1.2	1.0	1.0
Parkinson's disease	0.8	1.3	0.7	1.1
Laxatives	50.5	49.1	39.7	42.7
Opioid analgesics	46.5	54.5	33.3	46.6
Sedatives / hypnotics	44.8	42.5	31.9	35.4
Corticosteroids	46.2	40.4	33.2	33.8
Antidepressants	44.1	40.1	34.6	35.9
Antacids	34.8	37.9	23.9	30.7
Antilipaemic agents	27.1	35.7	19.6	30.9
Stimulant laxatives	25.9	24.6	19.2	20.4
Stool softeners	18.5	23.7	12.1	18.9
Thyroid modifiers	17.6	17.3	13.1	15.4
Antipsychotics	9.8	9.2	7.0	8.3
Duloxetine	9.3	8.9	6.7	7.6
Trazodone	8.9	10.1	6.5	8.8
Mirtazapine	6.3	2.9	5.2	2.6
Sertraline	6.1	4.8	4.3	4.2
Escitalopram	5.7	4.8	3.9	4.2
Fluoxetine	4.4	3.1	3.1	2.7
Citalopram	2.8	3.4	2.0	2.9

Table 21: Main baseline demographics of matched cohorts comparing prucalopride with other anti-constipation agent for primary analyses.

Incidence in % unless otherwise stated. Comorbidites / medications are included as lifetime results.

	COHORT 1		**COHORT 2**	
	Prucalopride	**Linaclotide**	**Prucalopride**	**Lubiprostone**
Cohort size (n)	5207	5207	5119	5119
Age at index (y, SD)	48.5, 18.6	48.2, 18.0	48.7, 18.6	47.5, 19.4
Sex (M: F: O)	20.2: 79.7: 0.2	19.7: 80.1 0.4	20.3: 79.7: 0.2	18.9: 81.1: 0.2
Ethnicity (W, B, unknown)	74.7, 10.7, 23.2	76.4, 9.8, 22.7	74.6, 10.8, 23.2	75.9, 10.0, 22.7
Gastro-oesophageal reflux disease	28.7	28.8	28.2	29.1
Primary hypertension	16.8	16.9	16.8	15.5
IBS	16.9	17.0	16.5	16.4
Lipidaemias	15.1	15.4	15.4	15.0
Disorders of thyroid gland	12.2	11.6	12.0	12.0
Chronic respiratory diseases	11.8	11.8	11.7	11.4
Diabetes mellitus	10.7	11.0	10.7	10.4
Chronic pain	8.7	8.7	8.6	9.6
Overweight, obesity	7.7	7.6	7.5	7.5
Ischaemic heart diseases	6.4	6.4	6.5	6.3
Iron deficiency anaemia	5.5	5.6	5.6	5.6
Acute and chronic kidney disease	4.6	4.4	4.7	4.6
Digestive malignancy	1.7	1.7	1.7	1.4
Demyelinating diseases of the nervous system	1.5	1.5	1.5	1.5
Parkinsons disease	0.9	0.9	0.9	0.8
Laxatives	52.1	51.9	51.9	52.6
Opioid analgesics	49.1	49.3	49.0	48.9
Sedatives / hypnotics	46.8	48.2	46.6	46.2
Corticosteroids	47.4	48.5	47.1	47.8
Antidepressants	45.6	46.2	45.2	45.6
Antacids	36.9	37.0	36.8	37.6
Antilipaemic agents	28.2	28.7	28.4	27.2
Stimulant laxatives	27.2	26.8	27.0	27.9
Stool softeners	20.1	19.4	20.1	20.4
Thyroid modifiers	18.0	18.4	18.1	17.7
Antipsychotics	10.1	10.1	9.9	9.8
Duloxetine	9.9	10.3	9.8	9.9
Trazodone	9.5	9.9	9.5	9.9
Mirtazapine	6.3	6.2	6.1	5.2
Sertraline	6.2	5.8	6.2	6.3
Escitalopram	5.9	5.6	5.9	5.9
Fluoxetine	4.8	5.1	4.7	4.9
Citalopram	3.0	3.2	3.0	3.2

5235 patients had at least two prescriptions of prucalopride 12 months' apart. Before matching, the prucalopride cohort had a higher incidence of gastro-oesophageal reflux disease and irritable bowel syndrome (IBS) and a lower incidence of primary hypertension compared to the linaclotide and lubiprostone cohorts, although other disease and prescribing rates were similar. See Tables 18 to 20 for comorbidities and medications in unmatched cohorts for lifetime, 5 years and 1 year before analysis. Successful 1:1 matching was achieved between this prucalopride cohort and the linaclotide (135 712 patients) and lubiprostone (65 282 patients) cohorts resulting in cohorts with 5207 and 5119 patients respectively (see Table 21 for baseline characteristics in the matched cohorts)

6.3.2 Primary outcomes

6.3.2.1 Main analyses

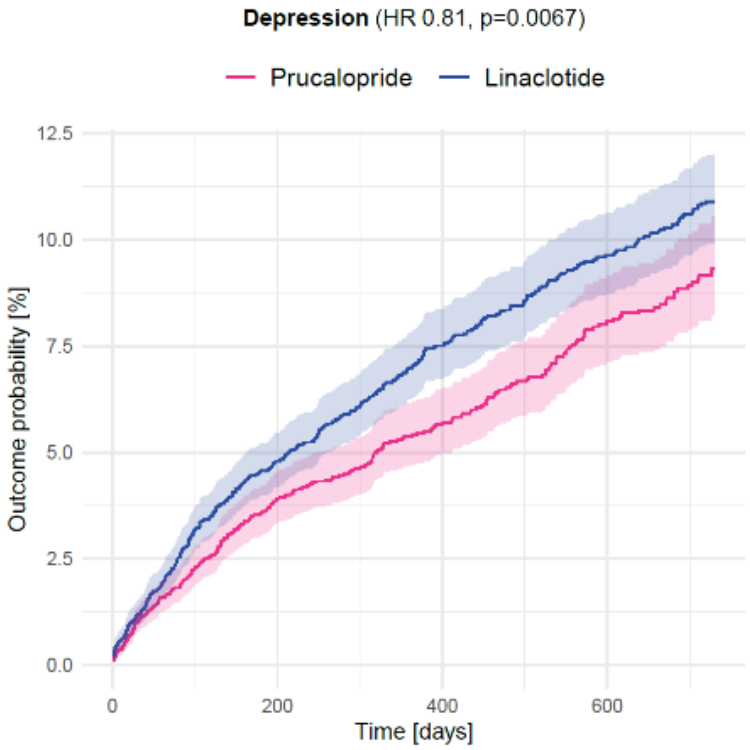
Table 22: 2-year follow up for primary outcomes

Outcomes for prucalopride versus (i) linaclotide and (ii) lubiprostone: percentage with each diagnosis during the 2-year exposure period and hazard ratio. Incidence shown with no prior mental illness. Bold indicates significant difference between cohorts in HRs. Proportional hazard (PH) assumptions tested with the generalised Schoenfeld approach where p<0.05 is significant for violations of proportionality in model.

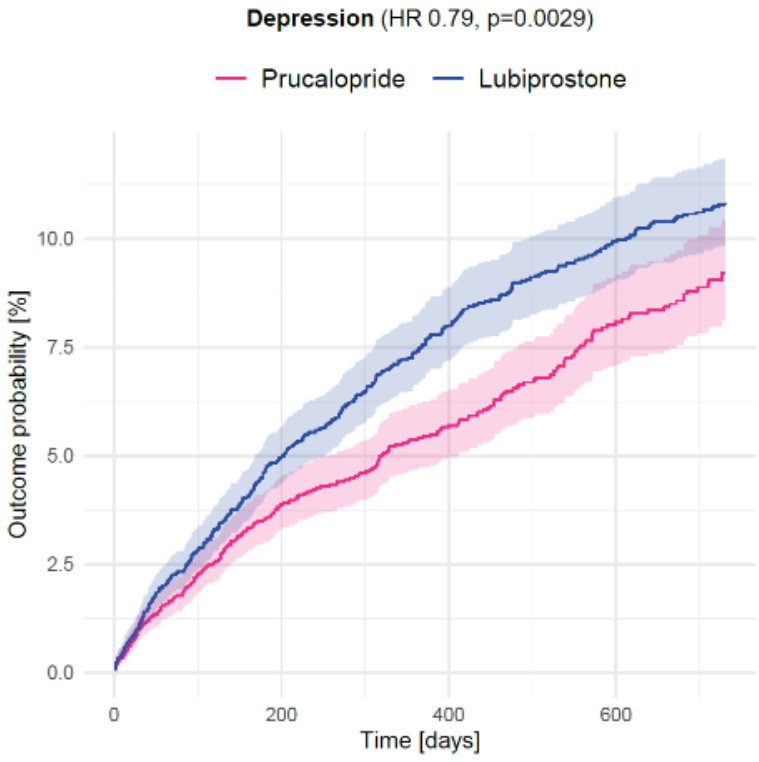
	Prucalopride incidence (%)	**Comparator incidence (%)**	**Hazard ratio (95% CI)**	**P values**	**E values**	**PH assumption**
(i) Prucalopride versus linaclotide	9.33 (8.23-10.56)	10.95 (9.95-12.04)	**0.81 (0.69-0.94)**	**0.0067**	1.78	0.080
(ii) Prucalopride versus lubiprostone	9.22 (8.12-10.45)	10.95 (9.98-12.00)	**0.79 (0.68-0.92)**	**0.0029**	1.84	0.066

Figure 21: Kaplan Meier curves showing outcome probability over 24 months versus time for prucalopride versus (A) linaclotide and (B) lubiprostone as per primary analyses for each primary outcome and cumulative negative control outcomes

A



B



In the following two years after the first prescription, those prescribed prucalopride as opposed to (i) linaclotide or (ii) lubiprostone for at least 12 months had a lower incidence of depression (prucalopride versus linaclotide: HRs 0.81 (95% CI 0.69-0.94), $p<0.07$; prucalopride versus lubiprostone: HR 0.79 (95% 0.68-0.92), $p<0.003$; see Table 22 and Figure 21).

Across both comparisons, E values around 1.8 were observed (Table 22), indicating that the significant hazard ratios are unlikely to be explained completely by residual confounding. The proportional hazard assumption was not violated for either comparison.

6.3.2.2 Sensitivity analyses for primary outcomes

As sensitivity analyses, it was assessed how the primary outcome might vary under various conditions. In brief these included matching for number of health visits, including patients with prior history of mental illness, excluding patients with neuropsychiatric illness, excluding patients receiving one of the two most common SSRIs (with no prior diagnosis of mental illness). I also repeated analyses for "first and any" diagnosis of the outcome (c.f. first only) and for a single prescription of anti-constipation agent. Across all conditions, cohorts remained of a similar size and results were very similar to primary analyses (see Table 23).

Table 23: 2-year follow up for primary outcome (depression) as per sensitivity analyses

Outcomes for prucalopride versus (i) linaclotide and (ii) lubiprostone: percentage with each diagnosis during the 2-year exposure period and hazard ratio. Incidence shown with no prior mental illness. Bold indicates significant difference between cohorts in HRs.

	Prucalopride versus linaclotide					**Prucalopride versus lubiprostone**				
	Prucalopride incidence (%)	**Linaclotide incidence (%)**	**Hazard ratio (95% CI)**	**P values**	**Cohort size**	**Prucalopride incidence (%)**	**Linaclotide incidence (%)**	**Hazard ratio (95% CI)**	**P values**	**Cohort size**
Matched for health visits	9.33 (8.23-10.57)	10.78 (9.78-11.88)	**0.83 (0.71-0.97)**	0.021	5207	9.21 (8.12-10.45)	10.44 (9.49-11.48)	**0.85 (0.73-1.00)**	0.048	5119
Assessed for recurrence of mental illness	42.12 (40.22-44.09)	44.66 (42.95-46.41)	**0.88 (0.82-0.95)**	0.0005	4292	41.98 (40.07-43.95)	48.00 (46.33-49.69)	**0.79 (0.74-0.85)**	<0.0001	4246
Exclusion of neuropsychiatric illness	9.12 (8.02-10.37)	10.79 (9.76-11.92)	**0.83 (0.71-0.97)**	0.022	4993	9.11 (8.00-10.37)	10.59 (9.61-11.67)	**0.85 (0.72-1.00)**	0.047	4904
Exclusion of two most common SSRIs	6.92 (5.90-8.09)	8.94 (7.94-10.06)	**0.77 (0.64-0.93)**	0.0071	4440	7.01 (5.98-8.20)	8.39 (7.46-9.45)	**0.83 (0.68-1.00)**	0.023	4365
Repeated for first and any outcomes	9.33 (8.23-10.56)	10.95 (9.95-12.04)	**0.81 (0.69-0.94)**	0.0067	5207	9.22 (8.12-10.45)	10.95 (9.98-12.00)	**0.79 (0.68-0.92)**	0.0029	5119
Single prescription of anti-constipation agent	9.27 (8.18-10.50)	11.25 (10.22-12.36)	**0.81 (0.69-0.94)**	0.0063	5218	9.22 (8.12-10.46)	11.67 (10.66-12.75)	**0.76 (0.65-0.89)**	0.0005	5213

6.3.3 Secondary outcomes and other analyses

6.3.3.1 Main analyses for secondary outcomes

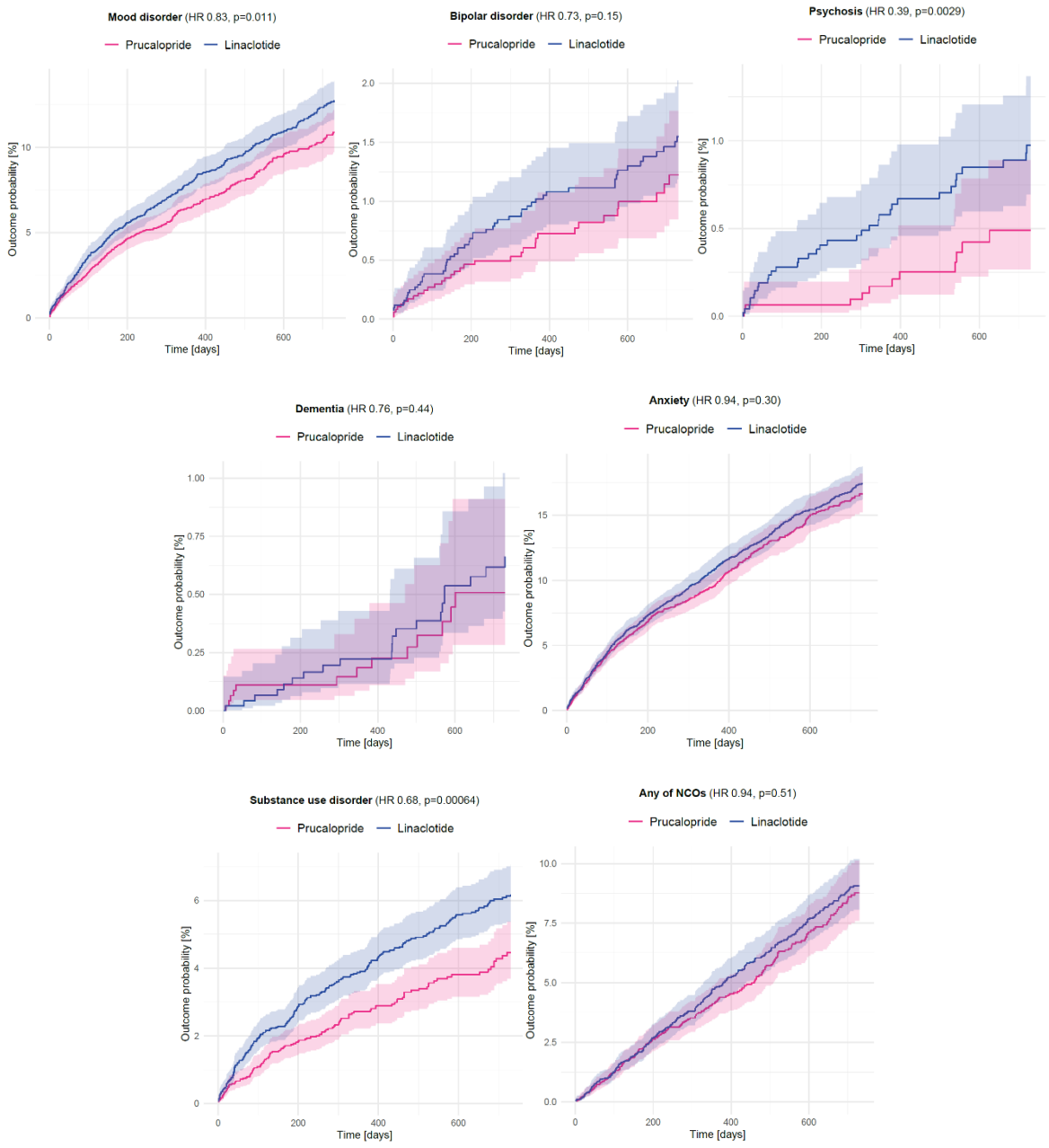
In the following two years after the first prescription, those prescribed prucalopride as opposed to linaclotide for at least 12 months had a lower incidence of mood disorder, psychosis, and substance misuse (HRs 0.39-0.83, *p*s<0.004; see Table 24 and Figure 22). No difference in bipolar disorder, anxiety disorder or dementia incidence was observed between the cohorts.

Table 24: 2-year follow up for secondary outcomes (prucalopride versus linaclotide)

Outcomes for prucalopride versus linaclotide: percentage with each diagnosis during the 2-year exposure period and hazard ratio. Incidence shown with no prior mental illness. Bold indicates significant difference between cohorts in HRs. Proportional hazard (PH) assumptions tested with the generalised Schoenfeld approach where p<0.05 is significant for violations of proportionality in model. NCO = composite negative control outcome

	Prucalopride incidence (%)	**Linaclotide incidence (%)**	**Hazard ratio (95% CI)**	**P values**	**E values**	**PH assumption**
Mood disorder	10.97 (9.79-12.28)	12.81 (11.73-13.98)	**0.83 (0.72-0.96)**	**0.011**	1.70	0.08
Bipolar disorder	1.22 (0.85-1.77)	1.55 (1.18-2.02)	0.73 (0.48-1.12)	0.15	N/A	0.59
Psychotic disorder	0.49 (0.27-0.89)	0.97 (0.69-1.37)	**0.39 (0.20-0.75)**	**0.0029**	4.56	0.29
Dementia	0.51 (0.28-0.91)	0.66 (0.43-1.02)	0.76 (0.38-1.53)	0.44	N/A	0.38
Anxiety disorder	16.66 (15.24-18.20)	17.45 (16.21-18.77)	0.94 (0.83-1.06)	0.30	N/A	0.09
Substance use disorder	4.54 (3.76-5.48)	6.23 (5.45-7.11)	**0.68 (0.55-0.85)**	**0.0006**	2.28	0.17
Any of NCOs	8.77 (7.59-10.13)	9.07 (8.06-10.19)	0.94 (0.78-1.13)	0.51	N/A	N/A

Figure 22: Kaplan Meier curves showing outcome probability over 24 months versus time for prucalopride versus linaclotide for secondary outcomes & cumulative negative control outcomes



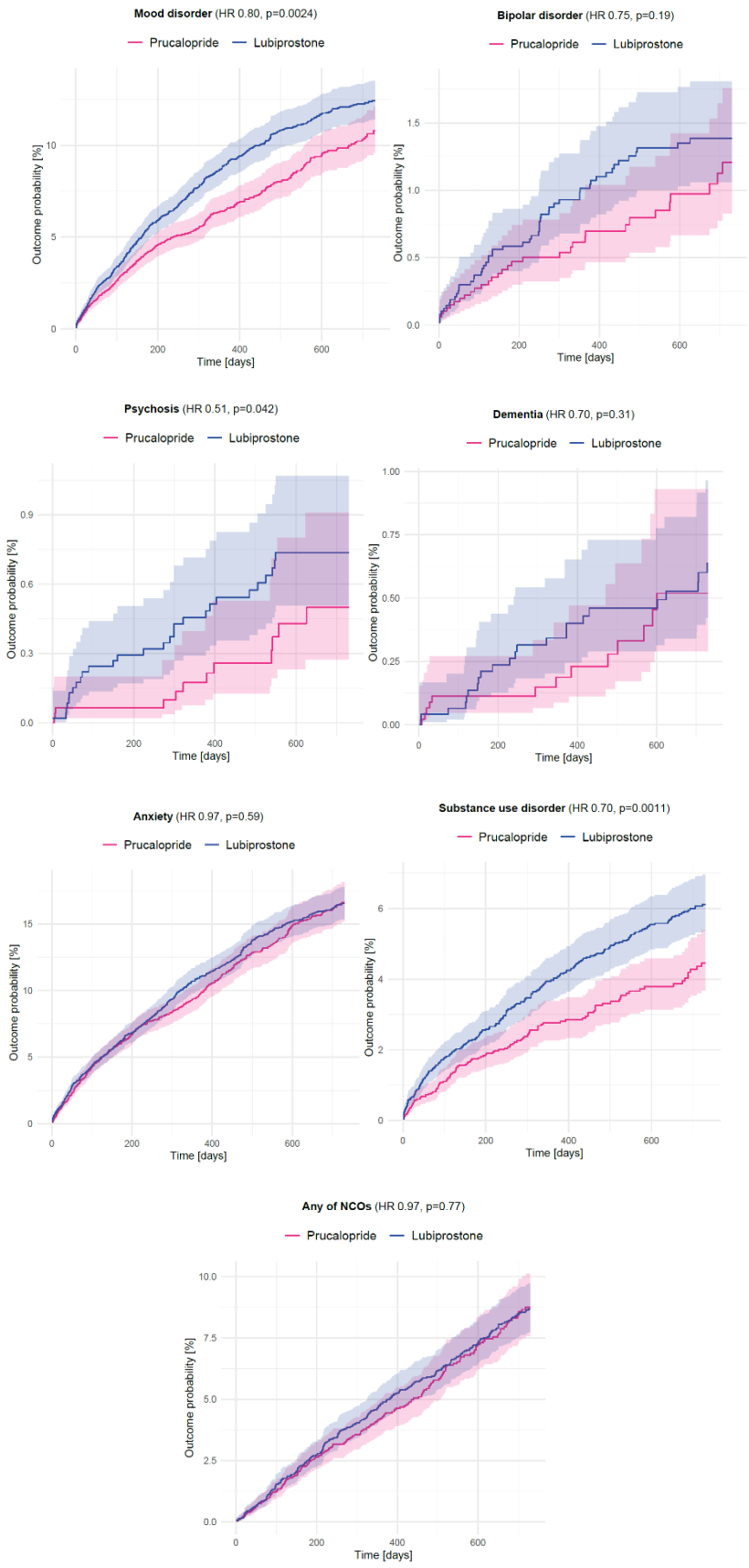
Similarly, in the following two years after the first prescription, those prescribed prucalopride as opposed to lubiprostone for at least 12 months had a lower incidence of mood disorder, psychosis, and substance misuse (HRs 0.51-0.80, *p*s<0.05; see Table 25 and Figure 23). No difference was observed in bipolar disorder, anxiety disorder or dementia incidence between the cohorts.

Table 25: 2-year follow up for secondary outcomes (prucalopride versus lubiprostone)

*Outcomes for prucalopride versus lubiprostone: percentage with each diagnosis during the 2-year exposure period and hazard ratio. Incidence shown with no prior mental illness. Bold indicates significant difference between cohorts in HRs. Proportional hazard (PH) assumptions tested with the generalised Schoenfeld approach where p<0.05 is significant for violations of proportionality in model. *= see Figure 24. NCO = composite negative control outcome*

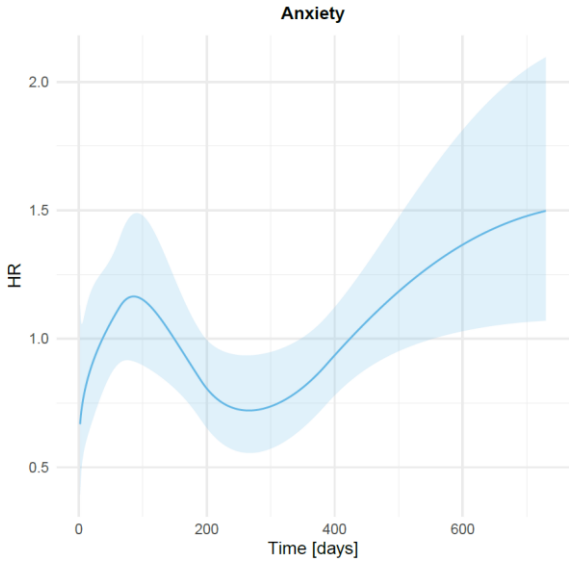
	Prucalopride incidence (%)	**Lubiprostone incidence (%)**	**Hazard ratio (95% CI)**	**P values**	**E values**	**PH assumption**
Mood disorder	10.79 (9.61-12.10)	12.49 (11.47-13.60)	**0.80 (0.70-0.93)**	**0.0024**	1.80	0.054
Bipolar disorder	1.21 (0.83-1.76)	1.38 (1.06-1.81)	0.75 (0.49-1.16)	0.19	N/A	0.75
Psychotic disorder	0.50 (0.27-0.91)	0.77 (0.54-1.12)	**0.51 (0.26-1.00)**	**0.042**	3.34	0.065
Dementia	0.52 (0.29-0.93)	0.64 (0.42-0.96)	0.70 (0.35-1.40)	0.31	N/A	0.26
Anxiety disorder	16.60 (15.16-18.16)	16.56 (15.39-17.81)	0.97 (0.86-1.09)	0.59	N/A	0.0038*
Substance use disorder	4.54 (3.75-5.50)	6.19 (5.44-7.05)	**0.70 (0.56-0.87)**	**0.0011**	2.22	0.41
Any of NCOs	8.85 (7.66-10.23)	8.72 (7.78-9.77)	0.97 (0.81-1.17)	0.77	N/A	N/A

Figure 23: Kaplan Meier curves showing outcome probability over 24 months versus time for prucalopride versus lubiprostone for secondary outcomes & cumulative negative control outcome



Across both comparisons for secondary mental health outcomes, E values between 1.70 and 4.56 were observed (Tables 24 and 25). This implies that the significant hazard ratios are unlikely to be explained completely by residual confounding. The proportional hazard assumption was not violated for any outcome where we found a significant difference between cohorts. It was violated for anxiety disorder with the prucalopride / lubiprostone comparison only (as shown in Table 25 and Figure 24), indicating that power may have been reduced for this result but this is unlikely to have affected overall findings.

Figure 24: Mental health outcome time-varying hazard ratio where there was evidence of non-proportionality of hazards in the main comparison between the cohort of patients receiving prucalopride and a matched cohort receiving an alternative anti-constipation agent.



Shaded area represents a 95% confidence interval

6.3.3.2 Sensitivity analyses for secondary outcomes

Sensitivity analyses for secondary mental health outcomes were also repeated as for primary outcomes. Cohort sizes for these analyses can be seen in Table 23. No significant differences in negative control outcomes were observed for any of these sensitivity analyses (i.e. prucalopride versus either comparator for any analysis). Individual results are detailed below:

Table 26: Matched for health visits

Outcomes for prucalopride versus linaclotide and versus lubiprostone when additionally matched for health visits and potential hazards influencing health status: percentage with each diagnosis during the 2 year exposure period and hazard ratio. Incidence shown without previous mental illness and 12 months prescription. Bold indicates significant difference between cohorts in HRs. NCO = composite negative control outcome

	Prucalopride versus linaclotide			**Prucalopride versus lubiprostone**		
	Prucalopride incidence (%)	**Linaclotide incidence (%)**	**Hazard ratio (95% CI)**	**Prucalopride incidence (%)**	**Linaclotide incidence (%)**	**Hazard ratio (95% CI)**
Mood disorder	10.89 (9.72-12.19)	12.43 (11.36-13.60)	**0.85 (0.74-0.98)**	10.87 (9.69-12.19)	11.81 (10.80-12.90)	0.88 (0.76-1.02)
Bipolar disorder	1.22 (0.85-1.77)	1.30 (0.98-1.74)	0.84 (0.54-1.30)	1.21 (0.83-1.76)	1.31 (0.99-1.73)	0.83 (0.53-1.29)
Psychotic disorder	0.49 (0.27-0.89)	0.59 (0.38-0.92)	0.68 (0.33-1.39)	0.50 (0.27-0.91)	0.78 (0.54-1.13)	**0.49 (0.25-0.97)**
Dementia	0.51 (0.28-0.91)	0.46 (0.32-0.81)	0.87 (0.42-1.81)	0.52 (0.29-0.93)	0.75 (0.52-1.11)	0.62 (0.32-1.21)
Anxiety disorder	16.70 (15.27-18.24)	17.92 (16.65-19.28)	0.92 (0.82-1.04)	16.68 (15.24-18.25)	17.11 (15.91-18.38)	0.97 (0.86-1.09)
Substance use disorder	4.46 (3.69-5.38)	6.09 (5.30-6.99)	**0.73 (0.59-0.92)**	4.54 (3.75-5.50)	6.49 (5.71-7.37)	**0.68 (0.55-0.85)**
Any of NCOs	8.87 (7.68-10.23)	9.22 (8.21-10.36)	0.91 (0.76-1.10)	8.82 (7.63-10.20)	7.44 (6.56-8.43)	1.16 (0.96-1.40)

With cohorts additionally matched for health visits, compared to the linaclotide group, the prucalopride group had a lower incidence of mood disorder and substance use disorder (HRs 0.73

– 0.85, *ps*<0.03). Compared to the lubiprostone group, the prucalopride group had a lower incidence of psychosis and substance use disorder (HRs 0.49 – 0.68, *ps*<0.05*)* (see Table 26).

Table 27: Recurrence of mental illness

Outcomes for prucalopride versus lubiprostone: percentage with each diagnosis during the 1-year exposure period and hazard ratio. Incidence shown with prior diagnosis of mental illness. Bold indicates significant difference between cohorts in HRs. NCO = composite negative control outcome

	Prucalopride versus linaclotide			**Prucalopride versus lubiprostone**		
	Prucalopride incidence (%)	**Linaclotide incidence (%)**	**Hazard ratio (95% CI)**	**Prucalopride incidence (%)**	**Lubiprostone incidence (%)**	**Hazard ratio (95% CI)**
Mood disorder	49.67 (47.74-51.62)	53.59 (51.86-55.33)	**0.88 (0.82-0.94)**	49.77 (47.83-51.73)	56.15 (54.48-57.83)	**0.81 (0.76-0.87)**
Bipolar disorder	9.19 (8.18-10.32)	9.95 (8.99-11.02)	0.91 (0.78-1.06)	9.30 (8.28-10.45)	11.08 (10.08-12.18)	**0.83 (0.72-0.97)**
Psychotic disorder	4.66 (3.89-5.57)	5.55 (4.81-6.39)	**0.80 (0.64-1.00)**	4.71 (3.94-5.63)	6.10 (5.36-6.94)	**0.69 (0.56-0.86)**
Dementia	1.72 (1.28-2.32)	2.54 (2.06-3.14)	**0.61 (0.43-0.87)**	1.74 (1.29-2.35)	2.28 (1.83-2.83)	**0.70 (0.49-1.00)**
Anxiety disorder	56.36 (54.42-58.31)	58.33 (56.61-60.06)	**0.93 (0.88-0.99)**	56.68 (54.73-58.65)	57.54 (55.88-59.20)	**0.93 (0.87-0.99)**
Substance use disorder	19.39 (17.92-20.97)	21.96 (20.57-23.42)	**0.84 (0.75-0.93)**	19.37 (17.89-20.95)	23.89 (22.49-25.36)	**0.78 (0.70-0.86)**
Any of NCOs	14.62 (12.84-16.62)	14.45 (12.94-16.12)	1.01 (0.85-1.20)	14.60 (12.81-16.62)	13.53 (12.14-15.08)	1.01 (0.85-1.20)

The effect of 12 months' prucalopride versus (i) linaclotide and (ii) lubiprostone in terms of recurrence of mental illness over 24 months follow up was also examined (i.e. where patients had prior mental illness as an inclusion criterion). In the following two years after the first prescription, those prescribed prucalopride as opposed to linaclotide had a lower incidence of mood disorder, psychosis, dementia, anxiety, and substance misuse (HRs 0.61 – 0.93, *ps*<0.05). In

the following two years after the first prescription, those prescribed prucalopride as opposed to lubiprostone had a lower incidence of all mental health diagnoses and subdivisions (HRs 0.69 – 0.93, *p*s<0.05) (see Table 27).

Analyses were repeated excluding patients in all cohorts with pre-existing neuropsychiatric disorder. In the following two years after the first prescription, for both sets of cohorts, those prescribed prucalopride as opposed to an alternative anti-constipation agent had a lower incidence of psychosis and substance misuse (prucalopride vs (i) linaclotide: HRs 0.44 – 0.67, *p*s<0.05; (i) lubiprostone: HRs 0.35 – 0.71, *p*s<0.05; see Table 28).

Table 28: Exclusion of neuropsychiatric illness

Outcomes for prucalopride versus (i) linaclotide and (ii) lubiprostone: percentage with each diagnosis during the 1-year exposure period and hazard ratio. Incidence shown with no prior neuropsychiatric illness and 12 months' prescription. Bold indicates significant difference between cohorts in HRs. NCO = composite negative control outcome

	Prucalopride versus linaclotide			**Prucalopride versus lubiprostone**		
	Prucalopride incidence (%)	**Linaclotide incidence (%)**	**Hazard ratio (95% CI)**	**Prucalopride incidence (%)**	**Lubiprostone incidence (%)**	**Hazard ratio (95% CI)**
Mood disorder	10.82 (9.63-12.15)	12.39 (11.29-13.58)	0.86 (0.74-1.00)	10.88 (9.67-12.23)	12.13 (11.08-13.26)	0.87 (0.75-1.01)
Bipolar disorder	1.28 (0.88-1.85)	1.35 (1.00-1.82)	0.92 (0.59-1.43)	1.31 (0.90-1.89)	1.22 (0.91-1.64)	0.95 (0.61-1.49)
Psychotic disorder	0.33 (0.16-0.71)	0.58 (0.37-0.91)	**0.44 (0.19-1.00)**	0.34 (0.16-0.73)	0.75 (0.51-1.11)	**0.35 (0.16-0.77)**
Dementia	0.46 (0.24-0.86)	0.52 (0.33-0.84)	0.72 (0.34-1.53)	0.47 (0.25-0.88)	0.38 (0.25-0.69)	0.98 (0.44-2.17)
Anxiety disorder	16.73 (15.27-18.31)	17.53 (16.25-18.90)	0.95 (0.84-1.07)	16.68 (15.20-18.28)	16.30 (15.10-17.58)	1.02 (0.91-1.16)
Substance use disorder	4.62 (3.81-5.59)	6.51 (5.70-7.44)	**0.67 (0.54-0.84)**	4.60 (3.78-5.59)	6.28 (5.49-7.17)	**0.71 (0.57-0.89)**
Any of NCOs	8.76 (7.55-10.15)	8.08 (7.13-9.16)	1.02 (0.84-1.23)	8.87 (7.64-10.29)	8.73 (7.77-9.81)	0.96 (0.80-1.16)

Analyses were run excluding any patient on one of two most common SSRI antidepressants (escitalopram / sertraline). In the following two years after the first prescription, those prescribed prucalopride as opposed to linaclotide had a lower incidence of psychosis and substance misuse (HRs 0.37 – 0.68, *p*s < 0.05). In the following two years after the first prescription, those prescribed prucalopride as opposed to lubiprostone also had a lower incidence of psychosis, dementia and substance misuse (HRs 0.72 – 0.80, *ps*<0.05) (see Table 29).

Table 29: Exclusion of two most common SSRIs

Outcomes for prucalopride versus (i) linaclotide and (ii) lubiprostone: percentage with each diagnosis during the 2-year exposure period and hazard ratio. Incidence shown with exclusion of three most common SSRIs and 12 months' prescription. Bold indicates significant difference between cohorts in HRs. NCO = composite negative control outcome

	Prucalopride versus linaclotide			**Prucalopride versus lubiprostone**		
	Prucalopride incidence (%)	**Linaclotide incidence (%)**	**Hazard ratio (95% CI)**	**Prucalopride incidence (%)**	**Lubiprostone incidence (%)**	**Hazard ratio (95% CI)**
Mood disorder	8.43 (7.32-9.70)	9.99 (8.93-11.16)	0.85 (0.71-1.01)	8.65 (7.51-9.95)	9.84 (8.83-10.95)	0.87 (0.73-1.03)
Bipolar disorder	1.14 (0.76-1.71)	1.31 (0.95-1.81)	0.84 (0.52-1.37)	1.16 (0.77-1.74)	1.57 (1.19-2.08)	0.71 (0.44-1.12)
Psychotic disorder	0.33 (0.16-0.69)	0.73 (0.48-1.12)	**0.37 (0.17-0.84)**	0.34 (0.16-0.71)	0.77 (0.52-1.16)	**0.38 (0.17-0.84)**
Dementia	0.33 (0.16-0.70)	0.67 (0.42-1.06)	0.48 (0.21-1.11)	0.34 (0.16-0.72)	0.71 (0.46-1.08)	**0.42 (0.19-0.95)**
Anxiety disorder	14.20 (12.74-15.80)	15.00 (13.72-16.40)	0.94 (0.82-1.09)	14.19 (12.73-15.82)	14.33 (13.12-15.64)	0.96 (0.83-1.11)
Substance use disorder	4.13 (3.33-5.11)	5.85 (5.02-6.81)	**0.68 (0.53-0.88)**	4.18 (3.37-5.18)	5.94 (5.13-6.86)	**0.67 (0.52-0.86)**
Any of NCOs	9.20 (7.87-10.74)	8.00 (6.98-9.15)	1.06 (0.87-1.30)	8.98 (7.66-10.53)	8.81 (7.76-9.99)	0.96 (0.79-1.18)

Analyses were repeated with outcomes assessed as *first or any* diagnoses. Compared with both linaclotide and lubiprostone, results were strikingly similar to when outcomes were assessed as *first* diagnoses for all mental health outcomes (see Table 30).

Table 30: Secondary outcomes repeated for first and any outcomes

Outcomes for prucalopride versus (i) linaclotide and (ii) lubiprostone: percentage with each diagnosis during the 2-year exposure period and hazard ratio. Incidence shown with exclusion of three most common SSRIs and 12 months' prescription. Bold indicates significant difference between cohorts in HRs. NCO = composite negative control outcome

	Prucalopride versus linaclotide			**Prucalopride versus lubiprostone**		
	Prucalopride incidence (%)	**Linaclotide incidence (%)**	**Hazard ratio (95% CI)**	**Prucalopride incidence (%)**	**Linaclotide incidence (%)**	**Hazard ratio (95% CI)**
Mood disorder	10.97 (9.79-12.28)	12.81 (11.73-13.98)	**0.83 (0.72-0.96)**	10.79 (9.61-12.10)	12.49 (11.47-13.60)	**0.80 (0.70-0.93)**
Bipolar disorder	1.22 (0.85-1.77)	1.55 (1.18-2.02)	0.73 (0.48-1.12)	1.21 (0.83-1.76)	1.38 (1.06-1.81)	0.75 (0.49-1.16)
Psychotic disorder	0.49 (0.27-0.89)	0.97 (0.69-1.37)	**0.39 (0.20-0.75)**	0.50 (0.27-0.91)	0.77 (0.54-1.12)	**0.51 (0.26-1.00)**
Dementia	0.51 (0.28-0.91)	0.72 (0.48-1.09)	0.66 (0.33-1.29)	0.52 (0.29-0.93)	0.66 (0.44-0.99)	0.67 (0.34-1.32)
Anxiety disorder	16.66 (15.24-18.20)	17.45 (16.21-18.77)	0.94 (0.83-1.06)	16.60 (15.16-18.16)	16.56 (15.39-17.81)	0.97 (0.86-1.09)
Substance use disorder	4.54 (3.76-5.48)	6.23 (5.45-7.11)	**0.68 (0.55-0.85)**	4.54 (3.75-5.50)	6.19 (5.44-7.05)	**0.70 (0.56-0.87)**
Any of NCOs	8.77 (7.59-10.13)	9.07 (8.06-10.19)	0.94 (0.78-1.13)	8.85 (7.66-10.23)	8.72 (7.78-9.77)	0.97 (0.81-1.17)

Analyses were conducted using cohorts of patients with a *single* prescription of prucalopride or linaclotide / lubiprostone. Results were very similar to main analyses (12 months' prescription of anti-constipation agents) (see Table 31).

Table 31: Primary analyses repeated for single prescription (intention to treat analysis)

Outcomes for prucalopride versus (i) linaclotide and (ii) lubiprostone: percentage with each diagnosis during the 2-year exposure period and hazard ratio. Incidence shown with single prescription. Bold indicates significant difference between cohorts in HRs. NCO = composite negative control outcome

	Prucalopride versus linaclotide			**Prucalopride versus lubiprostone**		
	Prucalopride incidence (%)	**Linaclotide incidence (%)**	**Hazard ratio (95% CI)**	**Prucalopride incidence (%)**	**Linaclotide incidence (%)**	**Hazard ratio (95% CI)**
Mood disorder	10.87 (9.70-12.17)	13.06 (11.96-14.25)	**0.83 (0.72-0.95)**	10.84 (9.66-12.16)	13.19 (12.13-14.33)	**0.80 (0.69-0.92)**
Bipolar disorder	1.18 (0.81-1.72)	1.76 (1.36-2.27)	**0.64 (0.42-0.98)**	1.25 (0.86-1.81)	1.31 (0.99-1.73)	0.86 (0.56-1.34)
Psychotic disorder	0.49 (0.27-0.89)	0.54 (0.34-0.86)	0.73 (0.35-1.52)	0.46 (0.25-0.87)	0.81 (0.56-1.16)	**0.44 (0.22-0.89)**
Dementia	0.51 (0.28-0.91)	0.81 (0.55-1.18)	0.56 (0.29-1.08)	0.52 (0.29-0.93)	0.96 (0.72-1.38)	**0.45 (0.24-0.85)**
Anxiety disorder	16.85 (15.43-18.40)	17.57 (16.32-18.91)	0.95 (0.85-1.07)	16.99 (15.53-18.57)	17.46 (16.26-18.74)	0.95 (0.85-1.07)
Substance use disorder	4.53 (3.75-5.47)	6.06 (5.29-6.94)	**0.71 (0.57-0.89)**	4.53 (3.74-5.49)	6.01 (5.27-6.86)	**0.72 (0.57-0.90)**
Any of NCOs	8.74 (7.56-10.09)	9.30 (8.28-10.44)	0.91 (0.76-1.10)	8.81 (7.61-10.19)	9.19 (8.26-10.30)	0.91 (0.76-1.09)

6.3.3.3 Negative control outcomes

No significant difference in risks of negative control outcomes were observed across both comparisons, either when assessed individually or as a composite (any of NCOs: HR 0.94-0.97, *p*s>0.05; Table 32).

Table 32: Negative control outcomes

Prucalopride versus (i) linaclotide and versus (ii) lubiprostone (12 months' prescription) for each negative control outcome over 24 months follow up

	Prucalopride versus linaclotide			**Prucalopride versus lubiprostone**		
	Prucalopride incidence (%)	**Linaclotide incidence (%)**	**Hazard ratio (95% CI)**	**Prucalopride incidence (%)**	**Lubiprostone incidence (%)**	**Hazard ratio (95% CI)**
Cutaneous abscess	0.84 (0.54-1.32)	0.63 (0.41-0.98)	1.35 (0.74-2.45)	0.86 (0.55-1.35)	0.55 (0.36-0.85)	1.36 (0.74-2.48)
Ganglion	0.43 (0.22-0.81)	0.34 (0.19-0.61)	1.11 (0.49-2.53)	0.43 (0.23-0.83)	0.60 (0.39-0.92)	0.68 (0.33-1.42)
Ingrowing toenail	0.20 (0.12-0.71)	0.35 (0.23-0.70)	0.49 (0.17-1.40)	0.21 (0.13-0.73)	0.39 (0.26-0.71)	0.44 (0.16-1.24)
Sebaceous cyst	0.39 (0.19-0.82)	0.46 (0.28-0.76)	0.58 (0.25-1.37)	0.40 (0.19-0.84)	0.58 (0.37-0.91)	0.53 (0.23-1.22)
Seborrheic keratosis	1.46 (1.03-2.06)	1.95 (1.60-2.60)	0.73 (0.49-1.10)	1.42 (1.00-2.01)	1.45 (1.10-1.91)	0.97 (0.63-1.48)
Trigger finger	0.78 (0.47-1.29)	0.74 (0.50-1.11)	0.86 (0.46-1.59)	0.76 (0.45-1.29)	0.68 (0.45-1.01)	0.91 (0.48-1.73)
Onycholysis	0.14 (0.053-0.39)	0.20 (0.095-0.44)	0.66 (0.19-2.26)	0.15 (0.054-0.40)	0.20 (0.087-0.44)	0.94 (0.26-3.32)
Viral warts	0.077 (0.025-0.24)	0.15 (0.06-0.35)	0.60 (0.14-2.52)	0.078 (0.025-0.24)	0.18 (0.082-0.41)	0.50 (0.12-1.99)
Myocardial infarction	1.12 (0.81-1.78)	1.11 (0.80-1.54)	0.99 (0.61-1.61)	1.19 (0.80-1.75)	0.91 (0.64-1.29)	1.29 (0.78-2.12)
Dog bite	0.11 (0.035-0.37)	0.11 (0.043-0.31)	0.83 (0.19-3.75)	0.12 (0.036-0.38)	0.072 (0.041-0.30)	1.20 (0.24-6.04)
Adhesive capsulitis	0.50 (0.29-0.84)	0.25 (0.16-0.57)	2.06 (0.90-4.71)	0.51 (0.30-0.86)	0.32 (0.20-0.64)	1.83 (0.84-4.01)
Synovitis / tenosynovitis	1.93 (1.41-2.65)	1.91 (1.48-2.46)	0.92 (0.62-1.35)	1.78 (1.28-2.48)	1.79 (1.42-2.34)	0.89 (0.60-1.33)
Blepharitis	0.63 (0.38-1.05)	0.71 (0.47-1.07)	0.94 (0.50-1.76)	0.65 (0.39-1.08)	0.75 (0.51-1.12)	0.94 (0.51-1.75)
Folliculitis	1.08 (0.71-1.64)	0.99 (0.70-1.40)	0.97 (0.57-1.63)	1.06 (0.69-1.63)	1.13 (0.82-1.55)	0.85 (0.51-1.43)
Paronychia	0.70 (0.44-1.14)	0.59 (0.47-1.10)	1.18 (0.63-2.21)	0.69 (0.42-1.13)	0.80 (0.56-1.16)	0.81 (0.45-1.45)
Varicose veins	0.80 (0.63-1.50)	1.12 (0.81-1.54)	0.69 (0.41-1.16)	0.81 (0.58-1.40)	0.67 (0.47-1.05)	1.29 (0.72-2.31)
Fracture of finger	0.19 (0.073-0.52)	0.11 (0.062-0.38)	1.74 (0.49-6.16)	0.20 (0.074-0.53)	0.12 (0.087-0.44)	1.76 (0.50-6.27)
Ankle sprain	0.29 (0.13-0.68)	0.31 (0.16-0.58)	0.76 (0.28-2.11)	0.30 (0.13-0.70)	0.39 (0.23-0.66)	0.51 (0.19-1.34)
Lateral epicondylitis	0.62 (0.42-1.24)	0.43 (0.26-0.73)	1.17 (0.56-2.44)	0.64 (0.43-1.27)	0.41 (0.25-0.68)	1.27 (0.61-2.64)

6.3.3.4 Additional comparisons

Similar analyses compared our two comparator anti-constipation agents with each other. There were 64492 patients in each cohort. Results were generally negative with some mixed findings across mental health and negative control outcomes: dementia had a significantly lower incidence in the linaclotide cohort and anxiety had a significantly lower incidence in the lubiprostone cohort. Composite negative control outcomes were not significantly different between the two cohorts. However, across the 19 individual NCOs, ankle sprain had a significantly lower incidence in the linaclotide group, and viral warts had a significantly lower incidence in the lubiprostone cohort (see Table 33). In summary, there was no consistent pattern of differences for either mental health or negative control outcomes comparing the linaclotide and lubiprostone cohorts.

Table 33: Linaclotide versus lubiprostone

Outcomes for linaclotide versus lubiprostone: percentage with each diagnosis during the 2-year exposure period and hazard ratio. Incidence shown without previous mental illness and 12 months prescription. Bold indicates significant difference between cohorts in HRs

	Linaclotide incidence (%)	**Lubiprostone incidence (%)**	**Hazard ratio (95% CI)**
Mood disorder	12.56 (12.25-12.88)	12.58 (12.28-12.88)	1.00 (0.96-1.03)
Depression	10.72 (10.43-11.02)	10.83 (10.55-11.11)	0.99 (0.95-1.03)
Bipolar disorder	1.43 (1.32-1.55)	1.35 (1.25-1.46)	1.07 (0.96-1.19)
Psychotic disorder	0.79 (0.71-0.88)	0.89 (0.81-0.98)	0.87 (0.76-1.01)
Dementia	1.41 (1.30-1.53)	1.57 (1.47-1.69)	**0.87 (0.78-0.97)**
Anxiety disorder	16.08 (15.74-16.44)	14.42 (14.11-14.74)	**1.12 (1.08-1.16)**
Substance use disorder	6.60 (6.36-6.85)	6.31 (6.09-6.54)	1.04 (0.99-1.10)
Myocardial infarction	1.50 (1.39-1.63)	1.57 (1.46-1.69)	0.94 (0.85-1.05)
Dog bite	0.12 (0.094-0.16)	0.093 (0.069-0.13)	1.32 (0.88-1.98)
Ganglion	0.37 (0.32-0.44)	0.37 (0.32-0.43)	1.02 (0.82-1.27)
Ingrowing toenail	0.42 (0.36-0.48)	0.37 (0.32-0.43)	1.13 (0.92-1.40)
Sebaceous cyst	0.41 (0.35-0.48)	0.47 (0.41-0.53)	0.86 (0.71-1.06)
Seborrhoeic keratosis	1.47 (1.35-1.59)	1.41 (1.30-1.52)	1.05 (0.94-1.18)
Trigger finger	0.63 (0.56-0.71)	0.61 (0.54-0.69)	1.03 (0.87-1.22)
Onycholysis	0.15 (0.12-0.19)	0.18 (0.14-0.22)	0.87 (0.63-1.21)
Viral warts	0.20 (0.16-0.25)	0.14 (0.11-0.18)	**1.41 (1.02-1.96)**
Cutaneous abscess	0.54 (0.47-0.61)	0.44 (0.38-0.51)	1.22 (1.00-1.47)
Adhesive capsulitis of shoulder	0.33 (0.28-0.39)	0.31 (0.26-0.36)	1.05 (0.83-1.33)
Synovitis and tenosynovitis	1.46 (1.35-1.58)	1.37 (1.27-1.49)	1.07 (0.96-1.20)
Blepharitis	0.56 (0.50-0.64)	0.63 (0.56-0.70)	0.91 (0.77-1.08)
Folliculitis	0.89 (0.80-0.99)	0.84 (0.76-0.93)	1.05 (0.91-1.21)
Paronychia	0.59 (0.52-0.67)	0.52 (0.45-0.59)	1.14 (0.95-1.36)
Varicose veins	0.95 (0.86-1.05)	0.90 (0.82-0.99)	1.05 (0.92-1.21)
Finger fracture	0.20 (0.16-0.25)	0.21 (0.17-0.25)	0.95 (0.71-1.27)
Ankle sprain	0.31 (0.27-0.38)	0.45 (0.39-0.51)	**0.69 (0.55-0.86)**
Lateral epicondylitis	0.42 (0.36-0.49)	0.34 (0.29-0.40)	1.24 (1.00-1.54)
Any of NCOs	8.67 (8.39-8.97)	8.51 (8.24-8.78)	1.02 (0.97-1.07)

Linaclotide was also compared with plecanatide in terms of mental health and negative control outcomes. Plecanatide is an anti-constipation agent with the same mechanism of action as linaclotide and was newly-approved for Medicaid in 2022 as tier 4 (i.e. similar to prucalopride).

The matched cohorts contained 11 894 patients. There were no significant differences on any mental health outcome or the composite negative control outcome (see Table 34).

Table 34: Plecanatide versus linaclotide

Outcomes for plecanatide versus linaclotide: percentage with each diagnosis during the 2 year exposure period and hazard ratio. Incidence shown without previous mental illness and 12 months prescription. Bold indicates significant difference between cohorts in HRs

	Plecanatide incidence (%)	**Linaclotide incidence (%)**	**Hazard ratio (95% CI)**
Mood disorder	11.39 (10.67-12.15)	11.56 (10.87-12.29)	0.99 (0.91-1.09)
Depression	9.61 (8.94-10.32)	10.12 (9.47-10.81)	0.95 (0.86-1.05)
Bipolar disorder	1.12 (0.90-1.38)	1.10 (0.89-1.35)	1.04 (0.78-1.39)
Psychotic disorder	0.53 (0.38-0.73)	0.56 (0.42-0.75)	0.87 (0.58-1.33)
Dementia	0.82 (0.63-1.07)	0.90 (0.71-1.13)	0.85 (0.61-1.20)
Anxiety disorder	16.39 (15.55-17.27)	16.62 (15.81-17.46)	1.00 (0.93-1.08)
Substance use disorder	4.86 (4.38-5.40)	5.61 (5.11-6.16)	0.88 (0.76-1.01)
Any of NCOs	8.02 (7.35-8.74)	8.43 (7.79-9.12)	0.93 (0.82-1.04)

As substance misuse was significantly lower in the prucalopride group versus linaclotide and lubiprostone across all analyses, and as this outcome reflects a large spectrum of disorders (F10-19), the risks of each substance were analysed individually to gain further insight. This analysis was performed as per primary analyses, although we also matched for number of previous health visits to capture differences in healthcare utilisation that might result from socioeconomic differences. When compared to linaclotide and lubiprostone, prucalopride resulted in a significant reduction in incidence for nicotine misuse (prucalopride versus (i) linaclotide (HR 0.55 (0.41-0.75) and (ii) lubiprostone (HR 0.51 (0.38-0.69)).

Table 35: Main baseline demographics of unmatched cohorts comparing linaclotide pre 2018 (before FDA approval of prucalopride) versus 2/1/2018 onwards

Incidence in % unless otherwise stated. Comorbidites and medications are included as lifetime results.

	COHORT 1	
	Pre-2018	**2018 onwards**
Cohort size (n)	46791	98314
Age at index (y, SD)	53.2, 17.3	55.3, 17.6
Sex (M: F: O)	21.9: 78.1: 0.02	24.5: 75.4, 0.02
Ethnicity (W, B, unknown)	69.9, 17.5, 19.0	64.3, 22.0, 24.5
Gastro-oesophageal reflux disease	18.5	21.8
Primary hypertension	22.1	27.4
IBS	10.3	10.8
Lipidaemias	17.3	22.2
Disorders of thyroid gland	10.8	12.1
Chronic respiratory diseases	10.2	12.4
Diabetes mellitus	11.5	14.5
Chronic pain	6.1	10.2
Overweight, obesity	8.3	12.2
Ischaemic heart diseases	7.2	9.2
Iron deficiency anaemia	3.1	4.5
Acute and chronic kidney disease	5.1	7.8
Malignant neoplasms of digestive organs	1.1	1.7
Demyelinating diseases of the nervous system	1.2	1.0
Parkinsons disease	1.0	1.1
Laxatives	42.8	44.7
Opioid analgesics	50.1	48.7
Sedatives / hypnotics	39.7	38.2
Corticosteroids	38.3	44.6
Antidepressants	37.8	37.2
Antacids	32.5	35.2
Antilipaemic agents	30.0	35.9
Stimulant laxatives	18.0	22.1
Stool softeners	17.6	19.6
Thyroid modifiers	16.9	17.1
Antipsychotics	6.3	7.2
Duloxetine	8.3	8.1
Trazodone	8.4	8.7
Mirtazapine	2.5	2.7
Sertraline	5.2	4.8
Escitalopram	5.0	4.8
Fluoxetine	3.4	3.2
Citalopram	3.9	2.7

Finally, the linaclotide cohort was examined (as the comparator with the largest population) before and after FDA approval of prucalopride (before 2018 and 2018 onwards). The linaclotide cohort was similar in composition either side of this divide (see Table 35).

6.4 Discussion

Using electronic health records (EHRs), the prescription of at least 12 months' prucalopride compared with either linaclotide or lubiprostone (alternative anti-constipation agents but without 5-HT_4 receptor agonist action) was associated with a reduced incidence of depression at two years after the first prescription. Similar results were observed for some secondary outcomes, most consistently substance misuse and psychosis. However, prucalopride use, when compared to alternative anti-constipation agents, rarely affected incidence of anxiety disorders.

This is the first study of its kind examining a 5-HT_4 receptor agonist and its potential to affect depression and other mental health outcomes for two years following initiation of treatment. The robustness of these findings are supported by the consistency of the results across various sensitivity analyses. When I *only* included patients with previous mental illness in order to examine the effect of prucalopride on risk of recurrence, results for primary and secondary outcomes were even more striking. However, these participants are not necessarily showing reduced recurrence of depression with prucalopride; instead these findings relate to mental illness more broadly (i.e.) patients may have had a prior diagnosis of anxiety or psychosis. Importantly, prucalopride does not appear to reduce the incidence of non-mental health outcomes compared to linaclotide or lubiprostone.

Results for primary and secondary mental health outcomes remained fairly consistent when I matched for number of health visits (which is a method of controlling for general healthcare usage), and also when I excluded (i) prior neurological outcomes in addition to mental health outcomes (as these may overlap with psychiatric disorders), and (ii) prescription of the most commonly-used SSRI (selective serotoninergic reuptake inhibitor) antidepressants. Prior to matching, cohorts within this study had similarly large proportions (circa 40%) of patients with prior lifetime antidepressant usage. This may be because SSRIs can be prescribed for indications other than depression (i.e. IBS, menopause, chronic pain), or it may indicate that, similar to other chronic disease populations, these cohorts may be at higher risk of future mental illness than those without a chronic disease. Therefore, to optimise generalisability and reduce bias of findings, those with lifetime antidepressant use were not excluded from main analyses and instead usage was matched across cohorts. However, even when those on the most common SSRIs were excluded as a sensitivity analysis, findings were similar.

One possible confound within this analysis was that prucalopride was licensed and available in Medicaid more recently that the two other comparators (i.e. linaclotide and lubiprostone). This may have influenced the sociodemographic profile of those able to access it, and so in order to control for this additional comparison analyses were undertaken. There was no difference in incidence of mental health outcomes at two years when comparing linaclotide with another anti-constipation agent, plecanatide, which was approved by Medicaid at the same time as prucalopride, and there was no discernible effect on mental health outcomes when we compared linaclotide and lubiprostone directly. This suggests that the findings cannot be explained by a difference in drug availability between prucalopride and linaclotide / lubiprostone, or by other differences specific to each comparator agent. Neither linaclotide nor lubiprostone have commonly-occurring recognised psychiatric side effects, and both are peripherally acting (have

minimal brain involvement) with little bioavailability (little systemic action). Therefore, these comparators are ideal due to their similar indications and population to prucalopride, and likely lack of neuropsychiatric effects.

5-HT_4 receptor agonists have demonstrated rapid antidepressant potential in multiple rodent studies (Lucas et al. 2007; Mendez-David et al. 2014; Pascual-Brazo et al. 2012). These rapid effects are supported mechanistically, with increased BDNF production after only a few days (c.f. weeks for SSRIs) and direct raphe action (Lucas 2009; Lucas et al. 2007). Initial experimental work in humans did not find an antidepressant-like profile on measures of emotional processing (Murphy et al. 2020) (Chapter Three); however, these studies were conducted in healthy volunteers using a low dose of prucalopride (1mg, half the licensed dose). This epidemiological study of over 5000 patients who have recently taken prucalopride for at least one year appears to support the animal data that there may be a beneficial effect versus similar anti-constipation agents in terms of depression, and that this appears to be consistent for at least two years after the first prescription.

There is also evidence that 5-HT_4 receptors are implicated in other mental disorders, such as dementia, anxiety, psychosis and substance use disorders. 5-HT_4 receptor agonists have been suggested as a potential target for the treatment of dementia due to their molecular mechanisms of action (acetylcholine release, hippocampal cell proliferation, APP processing) (Consolo et al. 1994; Mohler et al. 2007; Rebholz et al. 2018; Siniscalchi et al. 1999) and pro-cognitive effects in animal models (Fontana et al. 1997; Hagena and Manahan-Vaughan 2017; King et al. 2008; Lamirault and Simon 2001; Mohler et al. 2007; Quiedeville et al. 2015). This has been supported by recent human translational work (Murphy et al. 2020) (Chapters Two and Three). Although in the current analysis, prucalopride did not reduce incidence of dementia across most analyses,

dementia is a rare outcome compared to depression or substance misuse (i.e. 0.5% incidence versus 5-10% incidence over two years) and it was not possible to meaningfully filter our results to the over 65s (where dementia is proportionally focussed) due to the relatively low number of older patients taking prucalopride.

Prucalopride seems to have relatively little impact on incidence of anxiety disorder compared to comparator agents in our study. 5-HT_4 receptors in the hippocampus have been suggested to have a role in anxiolysis (Chen et al. 2020; Faye et al. 2019; Mendez-David et al. 2014), although these effects do not seem to require hippocampal neurogenesis (unlike SSRIs)(Mendez-David et al. 2014) and instead a separate mechanism involving AMPA receptor modulation may be involved (Chen et al. 2020). However, the literature in animal models of anxiety is difficult to separate from antidepressant potential due to limitations in these models in translating rodent behaviour into clinical syndromes. Further work will elucidate whether 5-HT_4 receptor agonists can have a role in anxiolysis in humans.

5-HT_4 receptors may also have role in psychosis and schizophrenia. Impaired 5-HT_4 receptor activity has been suggested to decrease neuronal survival via reduced trophic serotonergic effects (Cho and Hu 2007), which may be particularly relevant in schizophrenia (Eggers 2013). More specifically, the severity of reduced hippocampal volume in first episode psychosis correlates with the density of 5-HT_4 hippocampal receptors (Park et al. 2021). As cognitive impairments are common in psychosis, and appear to link to both development and progression of the disorder (Jonas et al. 2022; Karcher et al. 2022), the pro-cognitive potential of prucalopride seen in animal studies and earlier within this thesis may be of interest in the treatment of psychosis.

The consistent and compelling findings in terms of reduced substance misuse (specifically nicotine addiction) were outside the original hypothesis. This findings may relate to: (i) an element of residual confounding for socioeconomic status or (ii) prucalopride may be reducing predisposition to addiction, specifically nicotine. I attempted to mitigate against the first of these possibilities by matching for healthcare use, and finding anti-constipation agents with similar costs. There may be biological plausibility for prucalopride ameliorating an individual's addiction potential by improving particular aspects of cognition (i.e. improving executive functioning may improve an individual's control over otherwise automatic impulses and habit-seeking behaviours (Lechner et al. 2019)). However, detailed studies on 5-HT_4 receptor activity within the wider context of substance misuse remain pending.

Occam's razor would suggest that the beneficial effects of prucalopride across various mental illnesses as seen here may most parsimoniously be explained by adaptation of a unifying common pathway. For 5-HT_4 receptors two such explanations may exist: (i) improving cognition, or (ii) the gut-brain axis.

First, cognitive deficits are common in various mental illnesses, are poorly managed with existing treatments, and often persist after remission of primary symptoms (Etkin et al. 2015; Millan et al. 2012) (and see Chapter One). Therefore, cognitive impairments are likely to be separable mechanistically from other symptoms, such as mood in depression (Rock et al. 2014), and persistence may increase risk of relapse (Halahakoon et al. 2019). Moreover, at the neurobiological level, cognitive control as a brain network has been proposed as a transdiagnostic neuroimaging correlate across major psychiatric disorders (McTeague et al. 2017). In this way, attempting to improve cognition with a specific agent may be a clinical target in its own right to improve quality of life, and potentially improve longer-term prognosis and outcomes in

depression and other mental illnesses. However, a single code to examine cognition directly is not available in electronic health records, hampering the ability to test this within these analyses.

Second, in terms of the gut-brain axis as detailed in Chapter One, focus has centred on two potential and possibly overlapping mechanisms for this connection: the microbiome (Margolis et al. 2021) and serotonin (Shine et al. 2022). The microbiome (the billions of commensal micro-organisms that line our gut) has consistently shown an important role in communication between the gut and the brain (Margolis et al. 2021). Of particular relevance for our findings, this impact is known to extend to involvement in cognition (Gareau 2014) and mood (Cryan and Dinan 2012). Separately, serotonin levels in the brain may be intimately connected with, and possibly an extension of, those found in the gut. Variations in serotonergic tone may facilitate different levels of cognitive functioning with higher serotonin levels enabling recruitment of networks to enhance cognitive flexibility (Shine et al. 2022). In this way, these varied systems connecting the gut and the brain, all of which may be affected by 5-HT_4 receptors, are likely to have relevance to our findings.

There are also some unavoidable limitations relating to EHRs that should be considered. First, this type of analysis may be subject to errors in coding, and patients may seek healthcare outside the TrinetX network of organisations. It is possible patients identified as without mental illness may have had a previous episode not recorded appropriately, or received a diagnosis outside the network. This caveat is unlikely to affect the main conclusions of this study since both the analysis of "*first or any diagnoses"* and the analysis excluding patients who ever used one of the two most common SSRIs (which might captured additional cases of depression) showed very similar results. Nonetheless, as some psychiatric medications are prescribed for non-psychiatric purposes particularly relevant for our cohorts, such as the menopause and IBS, these medications were

included in matching to ensure consistency across our cohorts. Second, we cannot be sure that patients were actually taking anti-constipation medications as prescribed. However, this might cause false negatives rather than false positives, which therefore would not alter the conclusions of this study in terms of positive findings. In addition, the numbers at first prescription of prucalopride and after 12 months are very similar and suggest stability of usage (5245 patients with one prescription versus 5235 taking for at least 12 months, accessed 29/11/2022-1/12/2022).

Third, another concern of pharmaco-epidemiological studies is confounding by indication: that is those who are less likely to develop the outcome (i.e. mental illness) are more likely to receive the intervention (prucalopride). To guard against obvious indication bias, I have selected two comparator treatments (linaclotide and lubiprostone) with the same indication and recommended in guidelines at the same stage of treatment as prucalopride (i.e. constipation refractory to laxatives). In addition, results based on negative control outcomes show that any indication bias would need to specifically affect mental health outcomes. As well as matching for a range of demographic, diagnostic and medication factors, additional mitigations were instituted in supplementary analyses to ensure cohort equivalence. Prucalopride was not available in Medicaid until 2022 unlike the two comparators, and thus the prucalopride cohort may be substantially different in terms of social circumstances. To mitigate this, the number of healthcare visits were matched as a proxy of healthcare usage, and linaclotide was compared with plecanatide (an anticonstipation medication also receiving first Medicaid approval in 2022). Patient numbers within TriNetX are too small to compare plecanatide directly with prucalopride. However, plecanatide and linaclotide have the same mechanism of action, and thus it is reassuring that no such difference in incidence of mental health or negative control outcomes was found (indicating limited socioeconomic residual confounding). Furthermore, costs are fairly

similar for prucalopride, linaclotide, and plecanatide (around $500-600 per 30 tablets). Lubiprostone is slightly cheaper as generic versions are available, but the consistency of results when matching for health visits implies this had little effect on results. As prucalopride and lubiprostone are both used for opioid-induced constipation, we have also matched for analgesics generally and for opioid analgesics specifically.

Another method to address confounding by indication is to compare cohorts on negative control outcomes (NCOs), a set of outcomes that we would not expect to show differences between cohorts if populations are similar. A large number of these were purposefully selected to cover various body systems and levels of clinical emergency, and to reach a similar incidence to mental health outcomes cumulatively. In every analysis involving prucalopride, no individual NCO showed a significant difference in risk between prucalopride and the comparator agent. This was the same when NCOs where measured cumulatively across all analyses. This provides extra weight to the robustness of these findings.

Unfortunately, the TriNetX database is restricted to healthcare organisations predominately in the US. Therefore, comparable cohorts were created for the US population specifically, and these results may be less generalisable to other countries where these anti-constipation agents are not readily available (including the UK). In addition, all cohorts are predominately female, white and middle-aged. Due to this focus, and the overall cohort size, data are insufficient to undertake analyses divided by sex and age, and thus it is not possible to comment on whether effects are generalisable across the population. Furthermore, patients were followed up for two years, and so it is not possible to comment on the extended duration of any effects beyond this time period.

An important point to consider is whether prucalopride is substantially more effective than comparators as an anti-constipation agent, and thus results could be at least partially an artefact of a relatively greater improvement in constipation symptoms. To mitigate this possibility of a difference in magnitude of constipation improvement between cohorts, patients were only included in cohorts if they had two prescriptions at least 12 months' apart. This would imply that patients in all cohorts were finding symptomatic relief from their agent, as they had surpassed the recommended trial period of three to four months.

Bearing in mind these limitations, there is evidence that prucalopride prescription is associated with reduced incidence of future diagnosis of depression and other mental illnesses in this high risk population (both with and without recorded histories of mental illness). However, the data are observational and show association rather than causation. Nonetheless, they raise the possibility that when prescribing prucalopride for constipation, there may be a complementary and beneficial effect on mental health, compared to alternative anti-constipation agents linaclotide or lubiprostone. Potential mechanisms may relate to general effects on cognition or modifications of the gut-brain axis (either via serotonin or the microbiome), or to more specific effects occurring with individual mental health outcomes (e.g. depression). This study identifies the need for further exploration into 5-HT_4 receptor agonists and their role in preventing and ameliorating future symptoms of mental illness, ideally with larger observational cohorts or with the use of well-designed randomised trials.

CHAPTER 7

General Discussion

7.1 Summary of findings

This thesis aimed to investigate whether it was possible to translate the neurocognitive effects of 5-HT_4 receptor agonists from animals into humans. To achieve this, a set of studies were conducted in different study populations.

First, in healthy participants, the effects of 5-HT_4 receptor agonism versus placebo in an fMRI experimental medicine study were assessed. This involved two task paradigms (hippocampal memory encoding and implicit face emotion) and a resting-state scan. In Chapter Two, 5-HT_4 receptor agonism was found to be associated with increased activation of the hippocampus and the right angular gyrus (a related memory processing region) whilst participants were undertaking the memory task compared to placebo. Furthermore, prucalopride participants also demonstrated improved cognitive scores on the behavioural memory test, in that after the scan, compared to placebo participants, they were more accurate in stating whether they had (or had not) seen a picture in the scan.

In Chapter Three, the prucalopride group again performed better on the cognitive aspect of the behavioural faces task (stating accurately whether the faces were male or female). At a neural level, prucalopride participants had reduced activation in a set of regions analogous to the default mode network (DMN) during the face emotion task. In Chapter Four, during a resting-state fMRI scan, the prucalopride group showed enhanced resting-state functional connectivity (rsFC)

between the central executive network (CEN, a cognitive network) and a cluster involving the posterior and anterior cingulate cortices (PCC / ACC) on network analysis. Seed analysis focussing on regions found to be important in previous 5-HT_4 receptor work in humans identified greater rsFC between the ACC and its connections (namely the left lateral occipital cortex) and reduced rsFC between the hippocampus and other regions of the DMN.

Second, in participants with depression not receiving antidepressant medication, I assessed if it was possible to replicate the findings of Chapter Two in this different population (i.e. the effect of 5-HT_4 receptor agonism on the memory encoding fMRI task). A different 5-HT_4 receptor agonist was used, which had the advantage of PET data to guide dose selection to ensure adequate receptor occupancy in humans. In Chapter Five, 5-HT_4 receptor agonism led to increased activation in the left lateral occipital cortex (a region activated when encoding and retrieving memories for objects that matter) when compared to placebo, as well as modulating activity in the hippocampus. Therefore, this latter finding did replicate from healthy volunteers into a depressed population. However, in contrast with the effect seen with prucalopride in Chapter Two, there was no effect of PF-04995274 on behavioural performance of the memory encoding task.

Third, 12 months' prescription of prucalopride was compared with other anti-constipation agents in terms of depression and other mental health outcomes over two years using a live electronic health records database. Here, chronic use of prucalopride resulted in reduced incidence of depression (and other various mental illnesses) over the next two years after the first prescription.

Taken together, these findings suggest that across different experimental medicine paradigms, 5-HT_4 receptor agonism (even at a low dose) appears to improve cognition in humans. There are a number of possible functional mechanisms that these studies suggest might underpin this pro-cognitive effect, including increasing / modulating activation of the hippocampus and other memory regions, and appropriately reducing activation within and connectivity between regions of the DMN. Interestingly, although the experimental medicine studies in this thesis did not provide evidence of an antidepressant-like profile of prucalopride on an emotional processing tasks, large dataset findings indicate that 5-HT_4 agonism (using a higher dose of prucalopride) may reduce future incidence of depression.

7.2 5-HT_4 agonism and neurocognitive effects

7.2.1 5-HT_4 receptor agonism and cognitive effects in humans

As detailed throughout this thesis, there is a wealth of animal data that 5-HT_4 receptor agonism is important for hippocampal-dependent learning and memory, in both healthy (Hagena and Manahan-Vaughan 2017; King et al. 2008) and disease models (Guo et al. 2019b; Wang et al. 2022). As well as behavioural effects, this animal data is supported by a series of studies on potential mechanisms that may underlie these benefits. These include the release of acetylcholine in brain regions associated with cognition (Peñas-Cazorla and Vilaró 2015) and having specific effects on the hippocampus (such as promoting spiny growth (Restivo et al. 2008), potentiating theta oscillations (Johnson et al. 2012; Wang et al. 2022), and inhibiting apoptosis (Hashemi-Firouzi et al. 2021). Early human work identified that a single administration of low dose prucalopride was associated with signs of cognitive enhancement in healthy volunteers, compared to placebo (Murphy et al. 2020).

In this thesis, it was demonstrated in healthy volunteers that 5-HT_4 receptor agonism in humans increases hippocampal activity alongside other memory processing regions in the context of a memory task (see Chapter Two). Importantly, this finding of modulated hippocampal activity was replicated in an un-medicated depressed population (see Chapter Five). As this translated across both populations, it indicates that the 5-HT_4 receptor holds promise as a novel target for the treatment of cognitive impairment, and in particular memory-related dysfunction.

Consistent with the effects of prucalopride and PF-04995274 on hippocampal function, the work contained in this thesis highlights that 5-HT_4 receptor agonism can improve performance on a range of behavioural measures of cognition after six days administration in healthy humans (see Chapter Two). However, this pro-cognitive behavioural effect did not translate into those with unmedicated depression (see Chapter Five). As discussed in Chapter Five, there are several explanations for this discrepancy. First, there is evidence for increased sensitivity of pharmacofMRI to detect changes at the neural level when these are not identifiable at the behavioural level (Wandschneider and Koepp 2016). Therefore, it is possible that Chapter Five shows a partial translation (at the neural level only) because of other factors that impacted on the drug's ability to produce a pro-cognitive behavioural effect. One factor that may have affected statistical power is the diversity of the RESTAND population related to characteristics that may affect cognition and / or drug physiology, such as age, baseline HAM-D score and brain 5-HT_4 receptor binding (see Methodological Considerations for further discussion and possible suggestions for future work). In addition, behavioural data was missing for four individuals from the 5-HT_4 group, reducing the 5-HT_4 group size for this analysis to 23 participants (versus 25 in the placebo group). Therefore, although the power calculation in Chapter Five suggested that 21 participants per group should be sufficient based on data from Chapter Two, this effect size was

estimated on the basis of work in a young healthy population with minimal variation in terms of age, and no complicating factors such as disease severity or comorbid medication. Chapter Five also discussed that the pre-clinical data on receptor occupancy involved only healthy males, and thus the dose required to achieve >80% occupancy with PF-04495274 may be different in a group of mostly females with un-medicated depression, consistent with known variations in 5-HT_4 receptor binding with sex and depression.

There are two other alternative explanations for the lack of effect of PF-04995274 on behavioural memory performance. First, it is uncertain whether 5-HT_4 receptor agonism in depression leads to improvements in memory as ascertainable by this behavioural task. That is, the neural effects of 5-HT_4 receptor agonism seen in the depressed sample may correlate with improved behavioural cognition assessed with a different and / or more sensitive measure. Surprisingly, the overall performance of the depressed sample across both participant groups in the post-scan memory recall test in the RESTAND study was better than the placebo group in the healthy volunteer study, indicating that this task may not be sensitive to cognitive deficits in depression. Second, there may be differences between the agonists used in these studies. Both agonists used (prucalopride and PF-04995274) are highly-specific, but subtle differences are likely to exist relating to receptor activity and agonistic functioning, and this may underlie (at least partially) any differences in behavioural response. See Methodological Considerations for further discussion of each of these points. Nonetheless, the work in this thesis, together with previous preclinical and human work establishes the 5-HT_4 receptor as a very interesting target for improving cognition, and likely to be of relevance in various contexts of health and disease.

7.2.2 5-HT_4 receptor agonism and effects on mood in humans

Animal models also suggest that 5-HT_4 receptor agonism appears to improve deficits in terms of anhedonia and anxiety behaviours (see Chapter One) (Gomez-Lazaro et al. 2012; Lucas et al. 2007; Mendez-David et al. 2014; Pascual-Brazo et al. 2012). Of particular interest, these effects seem to occur much more quickly than similar studies using SSRIs (Lucas et al. 2007), and to be produced with the same and also different mechanisms. That is, similar to SSRIs, 5-HT_4 receptor agonists cause increased release of neurotrophic proteins, such as BDNF and CREB (although this occurs more quickly), and after an appropriate delay, hippocampal neurogenesis (Lucas et al. 2007). However, these animal models also demonstrate rapid modulation of glutamatergic signalling, which is not apparent with SSRIs (Chen et al. 2020).

Experimental medicine data in humans, both prior to this thesis (Murphy et al. 2020), and included in this thesis (see Chapter Three), are not suggestive of 5-HT_4 receptor agonism having an an antidepressant-like profile on emotional processing. However, consistent with the preclinical work, the analysis of data from electronic health records contained in this thesis (Chapter Six) suggests that 5-HT_4 receptor agonism reduces the incidence of future depression in patients with constipation (a group known to be at high risk of mood disorder). This evidence was sufficiently reliable that it was replicated over the two year follow up period in every analysis completed as part of this study (in all main and sensitivity analyses). This indicates that 5-HT_4 receptor agonism and beneficial effects on mood may be translating on a broader population scale in humans with potentially long-term benefits.

It may seem surprising that there is a difference in findings between these two study methodologies. However, there may be some explanations for this. The experimental medicine studies with prucalopride only used a low dose (1mg, half the licensed dose). This was to reduce the risk of side effects in healthy volunteers, especially those relating to gastrointestinal

symptoms. However, because the patients in the TriNetX study were receiving prucalopride for constipation, they are likely to have been taking the licensed 2mg dose (although details regarding dosage are not available in the database). It is possible that neural and behavioural effects in experimental studies would require, or be more easily visualised, at the 2mg dose. Furthermore, the TriNetX study involved a much larger population (around 5000 patients on prucalopride), which will have improved statistical power. Another possibility is that prucalopride may be having a beneficial effect on future mood via a mechanism that does not involve emotional processing. Whilst this is an established mechanism of conventional antidepressant action (Harmer et al. 2009), the effects of prucalopride on future incidence of depression could have been mediated primarily via effects on other cognitive processing (e.g. non-affective cognition) or via a mechanism that is independent of cognition (e.g. via gut-brain interactions).

7.3.3 5-HT$_4$ receptor agonism and effects on functional connectivity in humans

As detailed in Chapter One, depression is associated with functional changes within and between resting-state neural networks compared with healthy volunteers. In particular, those affected are part of the so-called triple network model (Menon 2011) due to the "flip-flop" interactions that occur between them during higher-order processing (i.e. the default mode (DMN), central executive (CEN) and salience networks (SN)) (Bhaumik et al. 2017; Dong et al. 2019; Neufeld et al. 2018). Abnormal connections between these specific networks may be linked to suboptimal cognitive performance (Shen et al. 2018). In healthy volunteers, pro-cognitive medication appears to lead to both increased and decreased connectivity including generally *reduced* resting-state functional connectivity (rsFC) between regions of the DMN (Becker et al. 2022) and *increased* rsFC in regions involved in cognition outside of the DMN, particularly parts of the prefrontal cortex (Miskowiak and Petersen 2019; Petersen and Miskowiak 2021).

Consistent with this, this thesis found that in healthy volunteers, six days' prucalopride enhanced inhibition of areas within the DMN whilst performing directed cognitive tasks with improved accuracy (Chapter Three). When assessing rsFC within networks directly as part of the same study, this thesis also confirms that sub-acute administration increased rsFC between a cognitive network, the CEN, and important regions for cognition and emotion, the posterior and anterior cingulate cortices (PCC / ACC). Focussing on regions of interest based on the previous results with prucalopride, prucalopride also increased rsFC between the ACC and processing regions, including the lateral occipital cortex. This was of particular interest as in Chapter Five (the RESTAND study), the left lateral occipital cortex was found to have increased activation with agonism of the 5-HT_4 receptor using a different agent (PF-04995274), suggesting a level of reproducibility between the different agonists and methodologies. In seed analyses, prucalopride also was associated with reduced rsFC between the hippocampus and other regions of the DMN. Again, this finding supported previous results in this thesis, as in Chapter Three during an implicit faces task, prucalopride reduced activation to various regions analogous with the DMN.

Thus, this thesis describes the first data on the effects of prucalopride on rsFC in humans. Furthermore, the findings are consistent with previous evidence of the effects of pro-cognitive medication on rsFC in healthy humans, and are consistent with other data in this thesis showing the neural effects of 5-HT_4 agonism. These findings also provide data supporting the hypothesis that prucalopride may have pro-cognitive activity in humans, and furthermore, add insight into other potential mechanisms by which this may be occurring.

7.3 Methodological considerations

7.3.1 Modelling pro-cognitive and antidepressant mechanisms using different populations

Experimental medicine studies are a vital part of the drug translational pathway. Undertaking clinical trials is expensive and resource-intensive, requiring large numbers, clinical populations, and sufficient follow up period for clinical outcomes to occur. Experimental medicine determines an appropriate proxy that is necessary and sufficient to assess the outcome in a rapid manner in a healthy population. One such example is rapid shifts in biases from negative to positive information processing with antidepressant medication for depression. This speed occurs despite the delay in clinical response that is typical with SSRIs, and these behavioural and neural effects occur in healthy participants as well as in those with low mood. In this way, experimental medicine studies allow rapid detection of drugs with clinical antidepressant activity in both healthy and depressed populations (Arnone et al. 2009; Pringle et al. 2011; Rawlings et al. 2010). This model also applies when assessing potential pro-cognitive agents using cold "cognitive" measures (Horder et al. 2009; Smith et al. 2018).

In this thesis, the translational pathway from positive findings in animal models to potential medication with proven therapeutic benefit in humans has been followed step-wise: first with experimental medicine studies in a healthy population, and then a planned replication in a clinical population. This latter step is an important part of the pathway, but there are additional considerations when performing experimental medicine studies in clinical samples compared to healthy volunteers. As might be expected, the RESTAND population was more diverse than the prucalopride study population in terms of demographics such as age and severity of depression (as assessed by HAM-D), both of which may affect cognitive functioning. These factors may have added noise into the RESTAND data, and although this improved real-world generalisability of findings, it may have affected power within the small numbers of an experimental medicine

study. In future studies, the experimental medicine model in this population of people with depression could be improved by selecting participants who are more similar to each other, or increasing the sample size to allow for greater baseline variation.

As discussed in Chapter Five, there is also limited information about 5-HT_4 agonist receptor occupancy in those with depression. For instance, we know the dose of PF-04995274 required to occupy 80%+ brain 5-HT_4 receptors in healthy individuals, but we do not know whether this is affected by depression (for this drug or prucalopride). Drug activity at this receptor may be different in a clinical population due to complex alterations in intrinsic serotonergic tone, availability of 5-HT_4 receptors, and 5-HT_4 receptor binding. An assessment of 5-HT_4 receptor agonist activity in those with depression with PET studies would help guide future work.

A question that should be considered is, if 5-HT_4 receptor agonists *are* pro-cognitive, whether they have the same behavioural outcome in healthy and clinical populations. Petersen and Miskowiak (Petersen and Miskowiak 2021) propose five criteria for a valid biomarker, including that it should produce "similar effects in patients with neuropsychiatric disorders and controls". There is evidence from the work contained in this thesis that 5-HT_4 receptor agonism is able to modulate hippocampal activation (and activation to other memory processing regions) in both healthy and depressed populations (Chapters Two and Five). However, it did not improve behavioural cognition in both. Petersen and Miskowiak (2021) also suggest that changes in functioning in the dorsal prefrontal cortex and the default mode network could be a transdiagnostic biomarker for pro-cognitive effects. Consistent with this, the work contained in this thesis provides initial evidence that prucalopride altered functioning within the default mode network in a healthy volunteer sample (Chapters Three and Four). We will be able to explore if this finding can be replicated using the RESTAND resting-state data in the future.

It is also important to note that the RESTAND study was conducted before and during the Covid-19 pandemic. This meant that participants who took part after the study restarted in October 2020 had a slightly altered experience, namely that psychiatric assessments were conducted remotely. This should have not otherwise affected data collection, which remained in-person.

7.3.2 Using pharmacological fMRI in experimental medicine studies

Using BOLD fMRI to assess the capacity and breadth of a pharmacological manipulation to modulate brain activity is a well-established mode of research (Wandschneider and Koepp 2016). It allows us to assess the impact of medications on the human brain *in vivo* and translate findings from preclinical research. In this thesis, two different studies using this method are described: the prucalopride study (Chapters Two to Four) and RESTAND (Chapter Five). Another advantage of BOLD fMRI is that researchers can monitor brain activity both at rest (while participants are asked to relax) and during tasks designed to stimulate particular regions of the brain. In this way, it was possible to assess for the first time whether administration of a 5-HT_4 receptor agonist may be having neural effects in humans, particularly related to cognition and emotional processing, and the details of any effects, as per the hypothesis of this thesis.

Furthermore, BOLD fMRI is a very sensitive tool (Wandschneider and Koepp 2016). For example, in the RESTAND study (Chapter Five), it was found that 5-HT_4 receptor agonism modulated hippocampal activity (a replication of Chapter Two), but (unlike in the prucalopride study) there was no associated improvement in behavioural cognition. The high sensitivity of this method allows us to detect even small but statistically significant changes in brain activation – and thus identify whether a drug may have neurocognitive effects – even when those changes in brain

activity may be too small to result in behavioural change detectable by cognitive tasks. There are several possible explanations for this lack of behavioural effect in RESTAND. Predominately, this involves factors potentially affecting power (see Chapter Five) including differential receptor binding in depressed versus healthy volunteers (see 7.3.1). From a pharmacological point of view, power could be increased by ensuring the dose administered does result in optimal receptor occupancy for this clinical population and increasing the duration of administration.

There are some limitations of this method that need to be considered. First, BOLD assesses physiological events occurring in brain regions as a proxy of brain activity. Therefore, to confirm that any changes of the BOLD signal detected by fMRI did relate to brain activity and not neurovascular changes, fMRI analyses in this thesis were controlled for perfusion (measured using arterial spin labelling) where possible. I also checked whether perfusion was different between groups globally and regionally, including the hippocampus. Other methods to improve the validity of analyses included accounting for other structural differences between our participant groups (such as grey matter), and only undertaking region of interest analyses on pre-specified regions specific to the task.

7.3.3 Difficulties with study design

A common issue with between group study designs (such as that used in the prucalopride study (Chapters Two to Four) and RESTAND (Chapter Five)) is that study groups may not be the same in terms of characteristics (demographics, clinical factors). Randomisation attempts to mitigate this and blinding aims to eliminate bias on the part of the researcher and participant in terms of drug / group choice.

In the prucalopride study and RESTAND, the design included stratification for gender, to ensure an equal spread of males and females across the groups. Minor differences between groups are not normally a significant problem, especially in experimental medicine studies of healthy volunteers such as the prucalopride study. To allow us to maximise power, an age restriction was also applied (18 to 40). As described in Chapter Two, due to chance, there were fewer participants in the 5-HT_4 group who had English as a second language compared to the prucalopride group. Fortunately tasks of reading English were not involved, and posthoc analyses did not suggest this affected the results.

However, in RESTAND, participants were drawn from a different population: they had a current diagnosis of major depressive disorder and were not taking antidepressant medication. As discussed in Chapter Five and 7.3.1, the broad HAM-D and age range within groups may have affected statistical power. While the RESTAND study over-recruited to ensure at least 25 participants in each group after exclusions, analyses suggested that it was underpowered. For example, the modulation of hippocampal activity did not meet significance on whole brain analyses (did not survive after multiple corrections at the voxel level) but was significant on region of interest analyses. It is possible that power could be increased in future studies by either recruiting a larger sample, or restricting the population in terms of characteristics so that the depressed individuals are more similar to each other within and across groups.

Pharmaco-epidemiological studies (such as the TrinetX study (Chapter Six)) have some advantages over experimental medicine studies (such as enabling assessment of large datasets over a longer period of time) but there are also some limitations. Rather than being controlled in their design, by their nature pharmaco-epidemiological studies are observational. That is, participants are not selected, randomised or stratified, and thus large numbers are necessary to

avoid analytic concerns related to noise and confounds in the sample. However, the corollary of this is that whole populations are available for inclusion in the study, thus minimising the selection bias that may otherwise exist in smaller experimental studies. Probably one of the most notable concerns with pharmaco-epidemiological studies is the risk of confounding by indication. For instance, in Chapter Six, this would be seen if those receiving prucalopride were somehow less likely to get adverse mental health outcomes in a systematic manner. Therefore, as discussed in Chapter Six, comparator cohorts were selected based on alternative medications for the same indication, and all cohorts were matched across domains assessed to be important for this population. It was also checked that results were not affected by drug availability or cost, and that cohorts did not differ on a range of physical health outcomes related to the intervention.

7.3.4 Generalisability to other 5-HT$_4$ agonists

Across this thesis, two different 5-HT$_4$ agonists have been examined: prucalopride in healthy volunteers, and using data from electronic health records, and PF-04995274 in unmedicated depressed volunteers. As previously discussed in 7.3.1, data exists in terms of dosage and optimal receptor occupancy in healthy participants for PF-04995274 but this information is lacking for prucalopride. Furthermore, in the prucalopride study, a low dose (1mg) was used to minimise gastrointestinal side effects, whereas in RESTAND 15mg of PF-04995274 was used as this was shown to be safe, acceptable and to achieve greater than 80% occupancy of brain 5-HT$_4$ receptors in healthy volunteers.

As the prucalopride study and RESTAND were otherwise mirror studies (the same design apart from the population and 5-HT$_4$ agonist studied), this might suggest that of the two, the prucalopride study might be less likely to show evidence of translation of neurocognitive effects.

With the caveat that in this thesis I am only examining data from the hippocampal memory encoding task, comparing across the two studies suggests that the stronger translation with a behavioural and neural demonstration of a pro-cognitive effect was seen with prucalopride. Some possible explanations for this relating to study populations and design are discussed earlier in this chapter (7.3.1, 7.3.3). However, another important possibility for the differential translation seen in this thesis is the different drugs used in the two studies, due to subtle variations in pharmacokinetics and pharmacodynamics. For this reason, it is difficult to be certain to what extent these findings are generalisable to other 5-HT_4 receptor agonists. Further research into different 5-HT_4 agonists and their pharmacological actions in the human brain will help us to unpick this in more detail.

7.4 Conclusions

This thesis presents the results of three different studies investigating 5-HT_4 receptor agonism with the aim of translating the neurocognitive effects seen in animals into humans.

Using an experimental medicine model, 5-HT_4 receptor agonism with low-dose prucalopride in healthy volunteers suggested pro-cognitive potential (the prucalopride study). That is, prucalopride improved behavioural memory recall for previously-seen images and gender discrimination on an implicit emotional task compared to placebo. Neurally, this was associated with increased activation to hippocampal-dependent memory regions, and increased resting-state functional connectivity (rsFC) between the posterior cingulate cortex / anterior cingulate cortex and the central executive network. It also reduced activation within a set of regions indicative of the default mode network, and reduced rsFC between the hippocampus and other

default mode network regions. The prucalopride study did not find evidence for potential antidepressant activity with this medication in humans.

Focussing on potential cognitive effects, the next step of translation investigated 5-HT_4 receptor agonism with PF-04995274 in patients with un-medicated depression (RESTAND). Initial findings from the hippocampal memory encoding task appear to show a partial replication from the healthy volunteer data: PF-04995274 modulated activity within the hippocampus compared to placebo but there was no effect on behavioural memory scores. However, PF-04995274 did also result in increased activity to the left lateral occipital cortex. This is another memory processing region, supporting evidence of other potential pro-cognitive activity with PF-04995274. Furthermore, this particular finding was interesting for two other reasons: (i) this region showed increased rsFC with the left and right rostral ACC with low-dose prucalopride (in the prucalopride study), indicating another element of replication across the two studies; and (ii) this region showed reduced grey matter / different gyrification in the PF-04995274 group compared with the placebo group in RESTAND. Further studies are needed to explore the potential importance of this finding.

The final step of translation was to explore whether chronic use of prucalopride (at 2mg daily) could improve long-term mental health outcomes, namely depression, using electronic health records (TriNetX). Intriguingly, considering results from the experimental medicine studies, this pharmaco-epidemiological study suggested that 12 months' prescription of prucalopride *was* associated with reduced incidence of depression at two-year follow up.

There are some limitations that need to be considered when appraising the evidence from this thesis. In particular, the prucalopride study used a low dose, which may have affected power. RESTAND may also have been underpowered due to variation within study groups or nuances in 5-HT_4 receptor agonism due to the clinical nature of the population, and the fMRI memory encoding task may not be ideal for use in depressed patients. The TriNetX study may also have been underpowered to detect effects in certain disorders, such as dementia due to few older patients on this medication.

Nonetheless, the results from this thesis indicate that 5-HT_4 receptor agonism shows potential in humans in terms of both pro-cognitive activity and reducing the risk of future depressive episodes. Further studies in clinical populations with 5-HT_4 receptor agonists (accounting for methodological considerations discussed in this thesis) are needed to elucidate if there is a therapeutic niche for these medications. Potential research avenues needed to progress the translation of 5-HT_4 receptor agonists into therapeutic medications include (i) delineating the optimal dose for individual agents to achieve >80% receptor agonism in psychiatric disorders; (ii) confirming the duration of action of clinically-effective 5-HT_4 receptor stimulation in humans; and (iii) undertaking studies regarding breadth of potential clinical actions. This latter path is needed to clarify first which psychiatric disorders may be helped by 5-HT_4 receptor agonists' likely pro-cognitive actions and which subdivisions of cognition are targeted. Second, it is important to assess the extent of potential effects on mood in clinical populations to clarify the specific subgroups that may receive the most therapeutic benefit.

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Appendix

Full details of fMRI methods for Chapter Four

fMRIprep: Results included in Chapter Four come from preprocessing performed using fMRIPrep 20.2.0 (Esteban et al. 2019) , which is based on Nipype 1.5.1 (Gorgolewski et al. 2011; Gorgolewski et al. 2018).

Anatomical data preprocessing: The T1-weighted (T1w) image was corrected for intensity non-uniformity (INU) with N4BiasFieldCorrection (Tustison et al. 2010), and used as T1w-reference throughout the workflow. The T1w-reference was then skull-stripped with a Nipype implementation of the antsBrainExtraction.sh workflow (from ANTs), using OASIS30ANTs as target template. Brain tissue segmentation of cerebrospinal fluid (CSF), white-matter (WM) and gray-matter (GM) was performed on the brain-extracted T1w using fast (FSL 5.0.9, RRID:SCR_002823, (Zhang et al. 2001). Volume-based spatial normalization to two standard spaces (MNI152NLin6Asym, MNI152NLin2009cAsym) was performed through nonlinear registration with antsRegistration (ANTs 2.3.3), using brain-extracted versions of both T1w reference and the T1w template. Head-motion parameters with respect to the BOLD reference (transformation matrices, and six corresponding rotation and translation parameters) were estimated using MCFLIRT (Jenkinson et al. 2002).

Functional data preprocessing: For each of the 1 BOLD runs found per subject, a reference volume and its skull-stripped version were generated by aligning and averaging the first echo of 3 single-band references (SBRefs). Head-motion parameters with respect to the BOLD reference

(transformation matrices, and six corresponding rotation and translation parameters) are estimated before any spatiotemporal filtering using mcflirt (FSL 5.0.9, (Jenkinson et al. 2002). A B0-nonuniformity map (or fieldmap) was estimated based on a phase-difference map calculated with a dual-echo GRE (gradient-recall echo) sequence, processed with a custom workflow of SDCFlows (Glasser et al. 2013). The fieldmap was then co-registered to the target EPI (echo-planar imaging) reference run and converted to a displacements field map with FSL\u2019s fugue and other SDCflows tools. Based on the estimated susceptibility distortion, a corrected EPI (echo-planar imaging) reference was calculated for a more accurate co-registration with the anatomical reference. The BOLD reference was then co-registered to the T1w reference using flirt (FSL 5.0.9, (Jenkinson and Smith 2001)) with the boundary-based registration (Greve and Fischl 2009) cost-function. Co-registration was configured with nine degrees of freedom to account for distortions remaining in the BOLD reference. A T2* map was estimated from the preprocessed BOLD by fitting to a monoexponential signal decay model with nonlinear regression, using T2*/S0 estimates from a log-linear regression fit as initial values. For each voxel, the maximal number of echoes with reliable signal in that voxel were used to fit the model. The calculated T2* map was then used to optimally combine preprocessed BOLD across echoes following the method described in (Posse et al. 1999). The optimally combined time series was carried forward as the preprocessed BOLD. The BOLD time-series were resampled into standard space, generating a preprocessed BOLD run in MNI152NLin6Asym space. Several confounding time-series were calculated based on the preprocessed BOLD: framewise displacement (FD), DVARS and three region-wise global signals. The three global signals are extracted within the CSF, the WM, and the whole-brain masks. Additionally, a set of physiological regressors were extracted to allow for component-based noise correction (CompCor, (Behzadi et al. 2007). Principal components are estimated after high-pass filtering the preprocessed BOLD time-series (using a discrete cosine filter with 128s cut-off) for the two CompCor variants: temporal (tCompCor) and anatomical (aCompCor). The head-motion estimates calculated in the correction step were also placed within the corresponding confounds file. The

confound time series derived from head motion estimates and global signals were expanded with the inclusion of temporal derivatives and quadratic terms for each (Satterthwaite et al. 2013). Frames that exceeded a threshold of 0.5 mm FD or 1.5 standardised DVARS were annotated as motion outliers. All resamplings can be performed with a single interpolation step by composing all the pertinent transformations (i.e. head-motion transform matrices, susceptibility distortion correction when available, and co-registrations to anatomical and output spaces). Gridded (volumetric) resamplings were performed using antsApplyTransforms (ANTs), configured with Lanczos interpolation to minimize the smoothing effects of other kernels (Lanczos 1964). Non-gridded (surface) resamplings were performed using mri_vol2surf (FreeSurfer).

Tedana: Tedana version 0.0.9 was used to produce a denoised optimally combined timeseries file for each participant. TE-dependence analysis was performed on input data from fMRIprep. A user-defined mask was applied to the data. An adaptive mask was then generated, in which each voxel's value reflects the number of echoes with 'good' data. A monoexponential model was fit to the data at each voxel using log-linear regression in order to estimate T2* and S0 maps. For each voxel, the value from the adaptive mask was used to determine which echoes would be used to estimate T2* and S0. Multi-echo data were then optimally combined using the T2* combination method (Posse et al. 1999). Principal component analysis based on the PCA component estimation with a Moving Average (stationary Gaussian) process (Li et al. 2007) was applied to the optimally combined data for dimensionality reduction. A series of TE-dependence metrics were calculated for each component, including Kappa, Rho, and variance explained. Independent component analysis was then used to decompose the dimensionally reduced dataset. Next, component selection was performed to identify BOLD (TE-dependent), non-BOLD (TE-independent), and uncertain (low-variance) components using the Kundu decision tree (v2.5; (Kundu et al. 2013)). This workflow used numpy (Van Der Walt et al. 2011), scipy (Jones et al. 2001), pandas (McKinney 2010), scikit-learn

(Pedregosa et al. 2011), nilearn, and nibabel (Brett et al. 2010). This workflow also used the Dice similarity index (Dice 1945).

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