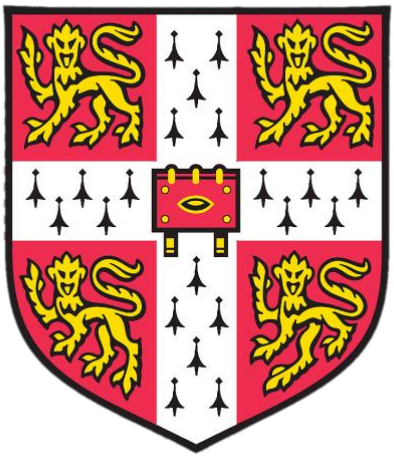
From Acute Injury to Chronic Disease: Exploring the Neurological Consequences of Mild Traumatic Brain Injury through Functional Neuroimaging



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This dissertation is submitted in October 2023 for the degree of *Doctor of Philosophy*.

For my Dad,
Richard Jeremy Woodrow (1967 – 2019)

Preface

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the preface and specified in the text. My work was funded by the Medical Research Council Doctoral Training Programme (MR N013433-1) and the Pinsent Darwin Trust.

A large group of researchers, clinicians, and technicians, across many institutions and hospitals, have contributed to collection of data analysed within this thesis, many of whom are listed in Appendix 2.1.

The study outlined in **Chapter 4**, with information regarding cohort selection given in **Chapter 2** and respective image preprocessing in **Chapter 3**, has been peer-reviewed and published as:

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And finally, the study outlined in **Chapter 3,** with cohort selection from **Chapter 2**, is currently in submission as:

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I further state that no substantial part of my thesis has already been submitted before for any degree or other qualification except as declared in the preface and specified in the text. It does not exceed the prescribed 60,000-word limit for the Clinical Medicine and Clinical Veterinary Medicine Degree Committee.

Abstract

Traumatic brain injury (TBI) is a global health crisis, with incidence rates growing rapidly. Further, TBI is the leading cause of injury-related death and disability, with lifelong impacts on the individual and their families. Of increasing concern is mild TBI (mTBI), which is overexpressed in the population yet lacks adequate attention in current clinical practice. Despite many experiencing long-term consequences of so-called 'mild' TBI, the relative paucity of clinical understanding and care of these patients has created a disconnect between injury and outcome. Thus, we are currently unable to explain the neurological underpinnings of poor outcome after mTBI, predict who might experience long-term effects, or sufficiently treat these patients. In this thesis, I aim to re-frame 'mild' TBI as a non-trivial and long-term disease, using multiple neuroimaging methods to better understand and prognosticate the outcomes of these individuals.

Using data from collaborative multi-centre project CENTER-TBI, Chapters 2-5 explore the acute and enduring neurological effects after even the 'mildest' TBI. Expressly, those individuals within hospital settings who do not present existing markers of poor outcome such as damage on computerised tomography (CT) or pre-injury neuropsychiatric conditions. Even with such 'mild' injury, Chapter 2 finds that this does not necessitate mild outcome, as 47% of our mTBI group show incomplete functional and/or symptomatic recovery at 6 months post-injury. This chapter further identifies that common structural neuroimaging methods or blood-based biomarkers are not associated with poor outcome in this cohort, necessitating the need for novel markers of chronic outcome. Chapter 3 establishes mTBI as a global functional disorder, using resting-state functional magnetic resonance imaging. Explicitly, all resting-state networks intrinsic to healthy brain function show vast alterations in functional connectivity. Additionally, these networks show injury-induced changes in how they are spatially distributed across the brain using a novel measure of component distribution complexity. Both measures show preliminary associations between disrupted network functional connectivity and poor outcome, but require further acute biomarkers to successfully differentiate chronic outcome.

Chapter 4 begins to focus investigations on a globally connected subcortical structure, previously ignored in TBI; the thalamus. In the same cohort of mTBI, I find acute thalamic hyperconnectivity, with specific vulnerabilities of individual thalamic nuclei. These acute fMRI markers differentiate those with versus without chronic post-concussive symptoms, additionally with time- and outcome-dependent relationships in a sub-cohort followed longitudinally. Moreover, chronic emotional and cognitive symptoms are associated with acute changes in thalamic functional connectivity to known serotonergic and noradrenergic targets, respectively. This begins to bridge the gap between macrostructural and microstructural investigation; translating findings from acute imaging into treatment-relevant targets and aiming for each field to mutually influence the other for therapeutic development. Chapter 5 additionally finds that thalamocortical connectivity is exacerbated in the special interest group of repeat mTBI. These results further establish thalamic pathophysiology as a marker of acute injury and outcome, and has important implications for both public and professional sports players.

In the final experimental Chapter 6, I further explore the evolving and potentially lifelong thalamic neuronal consequences of TBI, across all severities. Using rarely-collected ^{11}C-flumazenil positron emission tomography (PET), the thalamus shows unique markers of selective neuronal loss extending many years post-injury, which additionally mirror regions of cortical damage. These thalamic markers are related to multiple adverse outcomes, thereby substantiating that the thalamus can link the injury event with the long-term disease of TBI.

Overall, this thesis establishes functional neuroimaging as an invaluable tool for better understanding and prognosticating mTBI. Moreover, I propose the thalamus is a common source of injury, outcome, and long-term disease following TBI. It thus demands greater recognition and investigation in the TBI community, which is beginning to take flight. So-called 'mild' TBI is neither trivial nor temporary, and has traditionally been dismissed in public and clinical settings. For the many individuals experiencing long-term symptoms, multidisciplinary teams must work together to form new therapeutic pathways, towards a precision medicine approach. Only then will future research and healthcare professionals be able to sufficiently care for this growing population.

Acknowledgements

Thank you to my Dad, for giving me everything you could, for your unwavering support, and for building me up into the woman I am today. From daily spelling tests in the car rides to school, to teaching yourself A-level maths in case I needed help; I owe my entire academic career to you. You didn't get to see me start this PhD, but I know you will be watching as I finish it.

To my Grandad, who scanned and printed every essay, painting, and certificate (on multiple types of paper for a linen or glossy finish). I dread to think how much ink you would use re-printing this one. Thank you for encouraging me and inspiring me to take pride in whatever I do. I promised I would work hard, and I would make you proud.

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Chapter 1

Introduction

This thesis aims to elucidate functional consequences of even the mildest of traumatic brain injuries presenting to hospital settings. In doing so, three key messages will be conveyed. Firstly, many patients can experience long-term consequences of even a 'mild' traumatic brain injury. Secondly, a lack of structural damage does not preclude a lack of functional damage. Finally, the thalamus can link the injury event to the long-term disease of traumatic brain injury, across injury severities and timepoints. Following this, we must aim to better translate research in functional imaging from academia to the patient, and to the public.

1.1 Traumatic brain injury

1.1.1 What and where

Traumatic brain injury (TBI) is defined as an alteration in brain function, or other evidence of brain pathology, caused by an external force- Menon et al., 2010[1].

This definition of TBI was developed in 2010 by The Demographics and Clinical Assessment Working Group of the International and Interagency Initiative toward Common Data Elements for Research on Traumatic Brain Injury and Psychological Health[1]. Following years of conflicting definitions of TBI across countries and research settings, this definition offered essential clarification for reporting of TBI, and the comparison and interpretation of research; such as the present thesis.

With a clear definition of TBI, we can begin to understand its global challenges. In my introduction, I will summarise the key messages surrounding TBI epidemiology, individual experience, and its socioeconomic impacts. I will also aim to portray the challenges of understanding, treating, and prognosticating TBI, as highlighted in a recent special issue on traumatic brain injury commissioned by Lancet Neurology in 2017[2]. The aim of this commission was to call to action global efforts in research and clinical care, to better address this growing healthcare challenge. A specific focus is given to *mild* TBI in my introduction, as is the topic of this thesis, to provide context of what is missing in the field, and how the research in this thesis can begin to fill these gaps.

Epidemiology

Globally, over 60 million TBIs occur each year[3], and are a leading cause of injury-related death and disability[4]. It is estimated that TBI caused 8.1 million years lived with lifelong disability in 2016 alone[5], and TBI is projected to remain the greatest cause of disability amongst neurological diseases until 2030; 2-3 times the impact of cerebrovascular disorders and Alzheimer's disease[6]**.** Moreover, TBI incidence rates increased by 3.6% between 1990-2016 [5], suggesting that these impacts on death and lifelong disability are continuing to grow. In addition to the enduring physical, emotional, and cognitive disabilities that may impact the lives of TBI survivors and their families, TBI presents a great socioeconomic burden, representing 0.5% of the *total* global output (US$400 billion)[2]. This substantiates TBI as a global health crisis.

Such increases are not internationally uniform, however, as shown in **figure 1.1** from a study investigating global incidence of TBI[3]. The greatest increases in TBI are seen in low-middle-income countries (LMICs) attributable to rising road traffic incidents, and high-income countries (HICs) presenting greater incidence of falls in elderly populations[4,5,7]. Each of these patterns faces unique challenges. Reporting in LMICs may not fully characterise the incidence of TBI, stemming from unclear definitions of TBI, variable reporting and standards of care, limited access to timely healthcare, and those with mild injury or concussion failing to present to hospitals at all[2]. Even so, LMICs have three times the incidence rate of TBI compared to HICs[3]. For example, India presents the highest TBI rate in the world, accounting for almost 1 in 5 global TBIs[5]. These statistics are compounded by patients not reaching hospital care soon enough after injury, therefore missing an optimal window of care[8], and high in-hospital mortality rates estimated at 24.6%[9]. Nevertheless, a recent review found quality of TBI reporting and research to be very low in most cases, implying these figures could be a vast underestimation of the true effects of TBI in India[9]. This largest population of TBI is under-researched and under-reported.

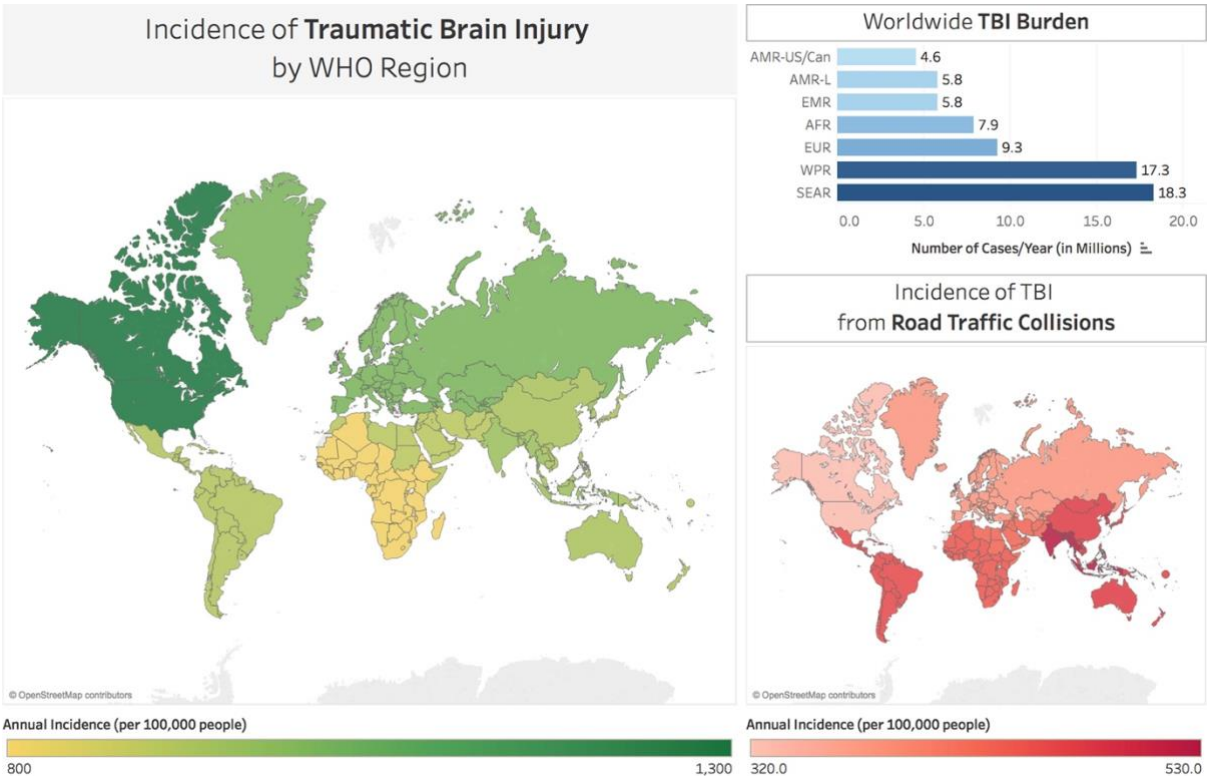


Figure 1.1. Global incidence rates of TBI. Values are per 100,000, colour coded by World Health Organisation region. Additionally highlights TBI incidence from road traffic collisions prevalent in low-to-middle income countries. Figure from Dewan et al (2018).

The challenges faced by HICs are quite different to LMICs. The number of TBIs in elderly populations are increasing at a greater rate than expected by population ageing[4]. This is a particular problem, as age is one of the clearest predictors of poor outcome after adult TBI[10,11]. For example, in multicentre European project CENTER-TBI, persons over 65 years of age accounted for 28% of all TBIs across Europe yet accounted for 50% of the mortality rate[12]. These statistics may be inflated by standards of clinical care and current prejudices towards older persons. Perceptions that age is universally associated with poor outcome have been linked to less aggressive treatment from more junior clinicians, reduced likelihood of prompt imaging investigation and transfer to specialist facilities, and more treatment-limiting decisions[13]. In contrast, those receiving prompt and aggressive treatment have shown good outcomes in 39% of elderly populations[14]- a very substantial proportion which should not be dismissed. Increasing life-expectancy and activity in older populations may only exacerbate these inflated mortality rates, and may soon also affect LMICs who transition towards older populations[15]. There is also a global increase in the number of paediatric TBIs[5], which can have substantial impacts on long-term development[16] and is the leading cause of death of children and adolescents in HICs[2]. For

the purposes of this thesis, adult TBI (>18 years old) is distinctly focussed upon, as is commonplace in the literature to separate adult and paediatric groups.

These demographic characteristics of worldwide adult TBI have been changing in recent years from what was previously viewed as a 'young-man's disease'. A previous review of TBI epidemiology conducted in 2003 concluded that greatest frequencies of TBI particularly affected young males in adolescence and young adulthood[17]. However, more recent studies of 10-year global burden of disease found an age-sex interaction of incidence[5]. Whilst young males indeed showed greater rates of TBI than female counterparts, greatest incidence rates were in elderly populations, where sex did not differ. This is particularly important to consider, as females are regularly underrepresented in TBI studies[18], and male rodents are primarily used as the experimental injury model[19]. Moreover, there are an increasing number of females in sport and military service, and emerging awareness that many sustain but do not report TBI due to intimate partner violence[18]. These factors alter this traditional view of TBI as a young man's disease, and suggest older populations and both sexes are at risk.

Biological sex is furthermore known to have impacts on outcome after TBI. A review of existing literature found that there was a general trend for worse outcome in females than in males[19], albeit findings were mixed across the small pool of previous studies. A key caveat to such reviews however is the limited sample sizes for female participants in historical studies, and the lack of hormonal status measurement unique to females which has shown preliminary links to outcome[18]. Studies sufficiently powered to find effects have indeed shown that young female participants have higher risk of mortality than males of the same age, but this pattern was reversed in later life coinciding with postmenopausal status[18]. There is nevertheless limited research on the interaction of hormones, sex, and outcome after TBI to draw clear conclusions, and is an emerging area of demand for future research within the TBI community. Importantly, research is acknowledging the need to study female TBI given its relationship to poor prognosis[18,19], particularly in high rates of postconcussive symptom reporting after mild TBI[20].

Severity

TBI is often categorised into 'mild', 'moderate', and 'severe' injury. The characterisation is commonly performed using the Glasgow Coma Scale[21] (GCS), which combines

assessments of eye-opening (scale 1-4; 1= no response, 4= normal response), verbal (scale 1-5), and motor (scale 1-6) responses, to rapidly evaluate levels of consciousness at the scene of injury. Total score on these components thereby distinguishes a severe (GCS 3-7), moderate (GCS 8-12), and mild (GCS 13-15) injury. Using these boundaries, an estimated 80-90% of TBIs are classified as mild[3,22,23], although non-reporting of mild injuries may bring this figure even higher. Such assessments can be useful in clinical settings to guide treatment pathways and outcome prognosis (**figure 1.2**). Indeed, the GCS has been validated in its relationship to other markers of brain injury, whereby more severe injury related to decreased glucose metabolism[24], increased abnormalities on neuroimaging[25], and increased serum concentrations of blood biomarkers for inflammation and neuronal injury[26]. Moreover, poorer outcomes of mortality and disability are associated with decreased GCS[27]. This makes the GCS invaluable for initial assessment, particularly in emergency situations.

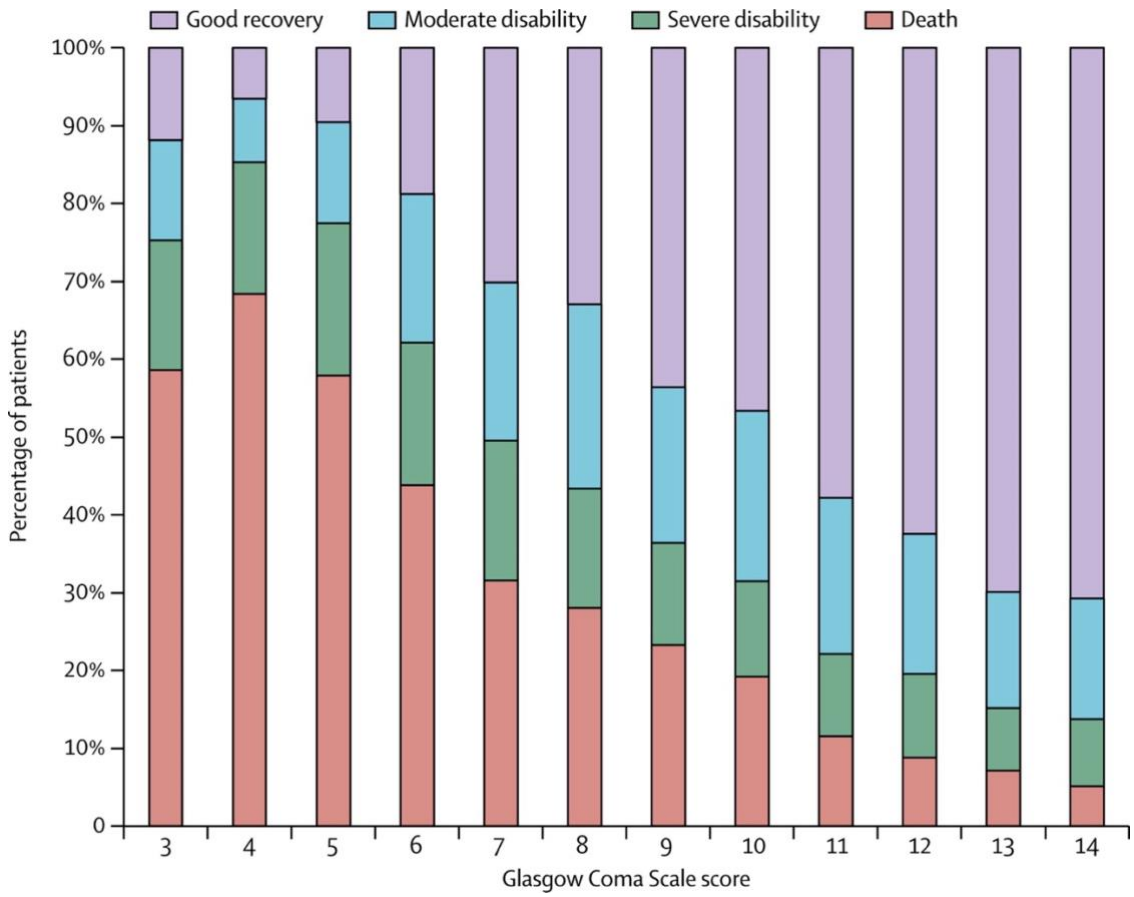


Figure 1.2. Mortality and outcome 6 months after injury in relation to GCS. Figure derived from Teasdale et al (2014)[28]. Outcome is in accordance with the Glasgow Outcome Scale: Good recovery: able to return to work or school; Moderate disability: able to live independently; unable to return to work or school; Severe disability: able to follow commands/unable to live independently.

It should be noted, however, that initial physical manifestations of TBI range from minor disorientation to full loss of consciousness and coma. This range means that designing and implementing a universal definition and scaling system for all TBI severities is difficult. The GCS is particularly basic for mild injury which may not present loss of consciousness as its primary symptom. For example, there has been great debate on the inclusion of GCS-13 being labelled as 'mild', including its exclusion in some Australian mild TBI criteria[29], given its substantially higher rates of intracranial injury compared to GCS 14 or 15[30]. Given mild TBI accounts for such vast proportions of TBI, further assessments may be useful in our future understanding of these diagnostic criteria. Indeed, the GCS was initially developed as a complimentary measure to other clinical assessments such as computerised tomography (CT) imaging[28]. Moreover, there are currently no refined criteria of severity which are universally adopted[2], which creates challenges for accurate severity diagnosis. For instance, some criteria require measurement of loss of consciousness and focal neurological defecits[31], or post-traumatic amnesia, whilst others are made retrospectively using CT imaging[32]. Further complexity arises from the use of pre-hospital intubation and sedation, when an accurate GCS or estimate of consciousness should be ascertained prior to these interventions[28].

The idea of injury 'severity' could also be explained by other factors aside from this single acute measurement. These may include need for neurosurgical intervention, level of treatment required, length of hospital stay, or functional/symptomatic/quality of life outcome, to name a few. These acute and long-term markers of severity do not necessarily align- for example, acutely life-threatening epidural hematomas (localised bleeding) often result in good outcome if treated rapidly, whereas diffuse injury commonly missed on routine imaging protocols are associated with long-term disability[33]. Moreover, a 'mild' injury may induce more severe consequences if the individual has experienced multiple prior injuries or has existing risk-factors for poor outcome (discussed further below in 1.1.2 Heterogeneity of TBI, and 1.2.2 Prognostication). Whilst informative of acute risk of mortality and intracranial management, the GCS may fail to answer these aspects of injury 'severity' which evolve over time within the individual. Some authors suggest a risk assessment approach to classification[33] (low, medium, high-risk) which can change over time irrespective of initial GCS, to better capture the ongoing prognoses and care of individuals in clinical settings.

For the purposes of this thesis, the GCS alone will be used to categorise severity of TBI, as is most common in existing literature and clinical practice. There is nevertheless a need for a universal and well-defined criterion for TBI severity. It is becoming clear in the literature this requires a multidimensional assessment to best guide diagnosis and clinical intervention.

In sum, TBI is a growing global health problem, most commonly occurring as mild TBI. There exists a complex interplay between sociodemographic factors such as age, sex, and geographical location. These are made more challenging by undetermined universal diagnostic criteria, which may fail to fully capture the lived experience of long-term injury.

1.1.2 Heterogeneity of TBI

A further obstacle is the intrinsic heterogeneity of TBI. There are infinite possible manifestations of primary injury dependent on the type, intensity, duration, and direction of external forces causing a TBI[2]. For instance, contact with an external object during a fall or assault may cause penetrative injury, contusions (bruising) and/or hematomas (localised bleeding) at the region of impact. These may additionally exhibit a *coup-contrecoup* pattern of damage. Such focal injury types can further vary at the location or depth of brain tissue, whereby a haematoma can be termed epidural, subarachnoid, subdural, intraventricular, or intraparenchymal[34], which may require different interventions and care. These focal injuries may not necessarily present in all TBI individuals, however, particularly after mild TBI. In contrast, rapid acceleration-deceleration common in road traffic collisions can cause rotational and sheering forces widespread across the brain, which may manifest as diffuse axonal injury (DAI)[35]. Given the mechanical loading of the brain during injury, DAI may particularly present in central subcortical structures. Whilst DAI is not routinely diagnosed in mTBI, there is indeed emerging evidence that on a microscopic level there may be some degree of DAI after all severities of TBI[35]. Evidence for DAI in these mild cases has been particularly localised at inhibitory parvalbumin interneurons in mTBI rodent models[36], undetected by routine imaging strategies. This literature is developing however, and often remains undiagnosed in milder injury types. Thus, the primary injury itself can greatly differ between

individuals, being focal or diffuse, or combinations of both. This is what the injury brings to the individual.

There are further aspects that the *individual* brings to the *injury*. In the short-term, it is important to distinguish primary injury mechanisms from secondary injury. Secondary inflammation following injury is substantially driven by host response, and increases in intracranial pressure can impair blood flow and brain structure, leading to oxygen deprivation and ischemia which can cause knock-on effects above the initial insult itself[2]. Additional individual differences can impact long-term implications of TBI. For example, genetic components such as apolipoprotein E (APOE) alleles, are thought to influence outcome, whereby presence of the ε4 allele has been linked to worse 2-year outcome[37] and increased risk of dementia post-TBI[38]. However, the study of genetic influences of TBI is in its infancy and results are not universally replicated[39]. There are also concepts of neurocognitive reserve and neuronal plasticity, sometimes termed 'resilience' to injury. Some studies have proposed that greater brain volume and pre-injury cognitive ability may be associated with more successful recovery[40]. These can be understood as brain reserve capacity and cognitive reserve respectively. Further studies have found that pre-injury intelligence quotient (IQ) is *neuroprotective* rather than supporting faster recovery[41], however others found that higher pre-injury IQ and younger age related to greater cognitive recovery at follow-up[42]. Intrinsically, lower IQ and total brain volume are associated with older age, which may underly to an extent the relationship between age and poor outcomes, but this has not been sufficiently studied[40]. Consequently, many individual differences can impact the short-term recovery to injury and lifelong vulnerability to ongoing neurodegeneration.

Thus, each TBI is a product of the injury *and* the individual (**figure 1.3**). Initial injury severity indeed has clear implications to outcome prognosis and mortality, however further secondary effects and individual differences in injury response may manifest over months or even the lifetime following TBI. This has led TBI experts to suggest that rather than a single acute event, TBI can be best understood as a collection of disease processes, which may persist or evolve over the lifetime[2]. Thus, each facet of injury and outcome may require its own individualised management. This inherent heterogeneity of TBI triggered some authors, including one of my own supervisors, to term TBI as,

"*...the most complex disease in our most complex organ.*"- Maas, Menon et al (2015)[43]

It is therefore unsurprising the great challenge of prognosticating and treating TBI patients, given no two are the same. Recent literature has surged in a 'precision medicine' approach, aiming to evaluate an individual's variance in genes, environment, and lifestyle, to maximise treatment effectiveness on a person-by-person basis. This multidimensional approach may be necessary to help us better understand and treat each individual, rather than their unifying diagnosis.

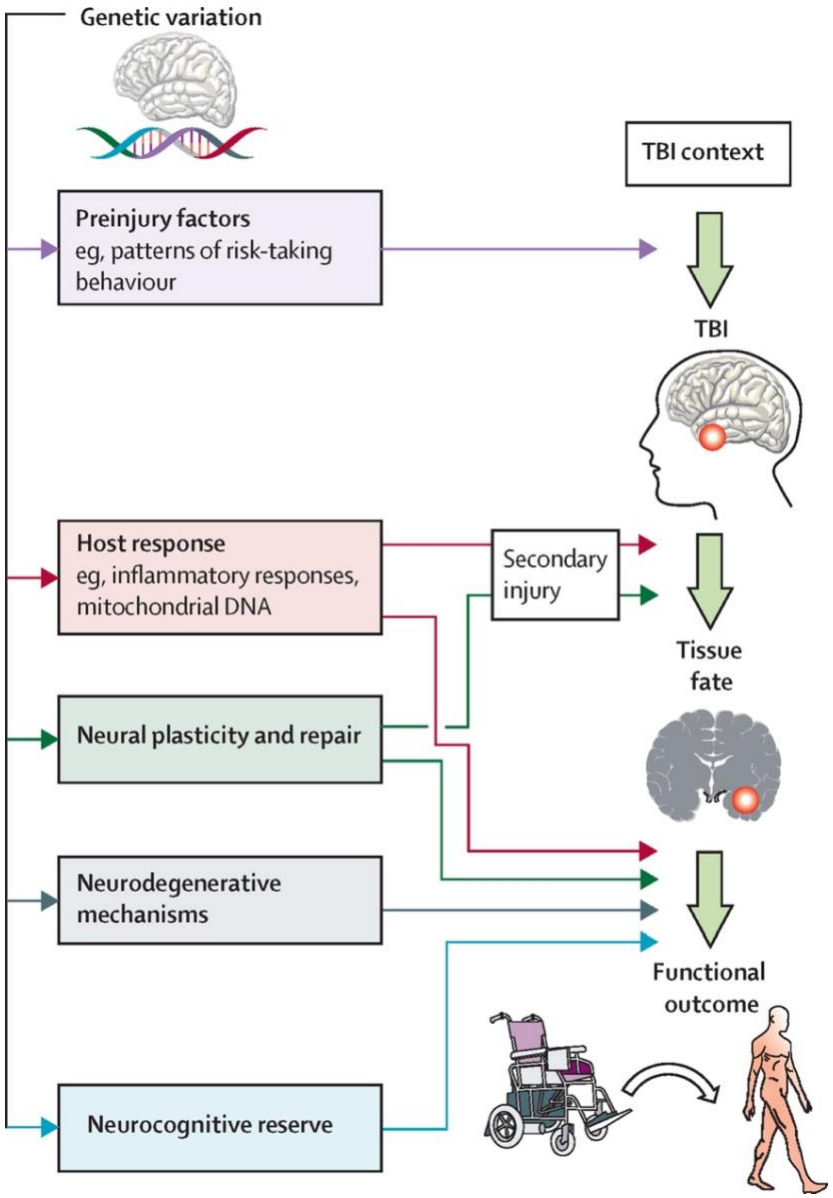


Figure 1.3. Heterogeneity of TBI and impact on outcome. Figure from Maas Menon 2017[2], demonstrating that functional outcome is dependent on complex interplay of injury-specific and subject-specific factors. Each individual therefore experiences a different injury and outcome, which requires a multidimensional approach.

1.1.3 Mild TBI: perception versus reality

Having defined the problem of traumatic brain injury, I would like to return to the specific challenges of *mild* TBI (mTBI). For the purposes of this thesis, mTBI is defined as a GCS of 13-15. It should be recognised that as many as 38 different definitions were found internationally by The World Health Organization (WHO) Collaborating Centre Task Force on Mild Traumatic Brain Injury[44], creating ongoing challenges for diagnosis and research. As discussed, mTBI accounts for 80-90% of all TBIs[3,22,23], albeit this number may be much higher due to underreporting to hospital settings. Nevertheless, mTBI has historically garnered little academic attention compared to moderate and severe TBI, which may be attributed to its perception as a 'mild' disorder.

From a public perspective this is an understandable conclusion; having witnessed countless sports professionals return to play after a knock to the head and celebrating their commitment to the game. This is compounded by the interchangeable use of '*concussion*' and '*mTBI*', leading to a dismissal of its long-term effects. For those looking for a trusted source of medical information in the UK, www.nhs.uk, the first paragraph under 'Head Injury and Concussion'[45] as of August 2023 reads,

"*Most head injuries are not serious, but you should get medical help if you or your child have any symptoms after a head injury. You might have concussion (temporary brain injury) that can last a few weeks.*"

This conveys two key messages- mTBI is *not serious*, and its effects are *temporary*.

Such views have similarly perpetuated in modern clinical practice, for example Griffinstein (2012) writes; "*mTBI is a self-contained condition that resolves quickly without special treatment, a generally accepted conclusion by fair-minded neuropsychologists*"[46]. These perceptions directly influence clinical care if internalised and acted upon. A recent study compared predictions of 6-month recovery by clinicians to later rates of real-world recovery, in a sample of over 200 mTBI cases presenting to the emergency department[47]. Whilst clinicians predicted 90% would fully recover by 6 months, only 50% achieved this full functional and symptomatic recovery. Such low accuracy remained irrespective of the clinicians' level of experience, clearly demonstrating the disconnect between clinician

perception and patient experience. Thus, many mTBI patients may not be adequately assessed and cared for post-injury, particularly beyond the acute phase of their illness.

Several studies have since validated these high rates of 'incomplete' recovery following mTBI. Functional 'recovery' is often assessed using the Glasgow Outcome Scale Extended (GOSE[48]). The GOSE rates patient function into 8 categories from death (1) to upper-good recovery (8) and is commonly dichotomised to classify a 'good' versus a 'poor' outcome[49]. For mTBI, such dichotomisation occurs at GOSE-8 (upper good recovery; return to daily life) versus ≤7 (between GOSE-1; death, to GOSE-7; return to work with some injury-related problems). Whilst dichotomisation removes precision of patients within eight categories down to two and has particularly been criticised for its lack of sensitivity and ceiling effects in mild populations[49], it has clinical value in distinguishing who has fully recovered versus who has not. In the largest study to-date from TRACK-TBI, 54% of over 1000 mTBI patients presented 'incomplete' functional recovery (GOSE≤7) at 12-months post-injury[50]. Even in the 'mildest' injury phenotype within their data collection, i.e., no evidence of structural damage on routine neuroimaging, a staggering 73% of these patients presented incomplete recovery at 2-weeks post-injury, and 56% perpetuated at 6-months post-injury[51]. One would expect *all* patients to fully recovery from a mTBI if its effects were 'not serious' and 'temporary', however even this broad diagnostic tool shows this is not reality.

Further diagnostic tools have looked to *postconcussive symptoms* following mTBI, to better probe nuances of patient experience. Postconcussive symptoms can include depression, cognitive impairment, headaches, and fatigue, and are often recorded using the Rivermead Postconcussion Questionnaire (RPQ[52]). The RPQ is a self-report measure of experienced severity of 16 most-commonly cited post-concussion symptoms compared to pre-injury levels, on a five-point scale from 0 indicating 'not experienced' to 4 as 'a severe problem'. Using self-reported symptom ratings, the RPQ is simultaneously a more nuanced look into individual patient experience and suffers from a lack of standardisation across individuals. As the RPQ is scaled in relation to experience of each symptom *pre*-injury, this introduces a potential effect of recall bias, particularly if it has been some time since injury or if the individual is suffering from memory or cognitive difficulties post-injury. Moreover, simply presenting the above symptoms as common post-injury may prompt one participant to overestimate their subjective

experience or memory of that symptom differentially to another participant experiencing the same objective symptom severity. Self-report measures are an invaluable window into real lived experience but must be used with an understanding of their limitations. *Postconcussive syndrome* is a further diagnostic classification defined in the International Classification of Diseases 10th Revision (ICD-10), as reporting three or more postconcussive symptoms at greater than pre-injury levels (e.g., sleep disturbances of greater impact following TBI, even if experienced pre-TBI). Using this criteria, independent large-scale studies have indeed found high prevalence rates of postconcussive symptoms after mTBI; 34-43% in European study CENTER-TBI at 6-months post-injury[53], and 47% in American study TRACK-TBI at 12-months post-injury[54]. The latter also highlighted specific persistence of cognitive symptoms, such as poor concentration and taking longer to think, which were closely followed by fatigue, headache, and irritability. Thus, symptom persistence in the mTBI population is a clear burden to everyday life in a substantial subset of patients.

Mild TBI can have additional impacts beyond the clinical setting. For instance, over half (53%) of 415 mTBI participants had not returned to work 2-weeks post-injury, whereby 17% remained out of work at 12-months post injury[55]. These employment consequences are significant and may contribute to additional long-term psychological and socioeconomic strain. Additional studies have found mTBI survivors experience lower levels of life satisfaction and community integration 3-years post-injury[56] and even report decreased romantic relationship satisfaction[57]. Thus, socioeconomic impacts of mTBI are vast and lingering well-beyond the injury event.

Finally, there are known lifelong impacts of TBI which increase the risk of developing neurodegenerative diseases[58–60]. This has recently been shown even following mild TBI in a meta-analysis of long-term follow-up studies, whereby history of mTBI increased risk of Alzheimer's disease development by 18%[61]. Accumulating studies suggest that pathological changes occurring after a mTBI can interact with the healthy aging process, to increase vulnerability to a wide range of neurodegenerative conditions[62,63], such as Alzheimer's disease and Parkinson's disease. This is particularly amplified in cases of repetitive head injury, such as sports professionals and veterans, with one study finding a 56% increased risk of Parkinson's disease[64].

Thus, these 'mild' injuries should not be dismissed as a simple resolving event, but a lifelong disease process[2].

Public perceptions of mTBI have nevertheless been developing in recent years, particularly in the sports community. Since the seminal case study on retired National Football League (NFL) player Mike Webster[65], and its subsequent Hollywood movie adaptation '*Concussion*', the long-term effects of repetitive sports concussion have exploded in public awareness. For example, a recent post-mortem study of over 200 American football players found that *chronic traumatic encephalopathy* (CTE), a progressive neurodegenerative disease causing behavioural and mood problems and dementia, was present in 87% of cases which increased in likelihood to 99% of NFL players[66]. CTE was initially characterised by Martland in 1928 as 'punch drunk' syndrome in professional boxers[67], however in the 21st century our understanding is beginning to change the way sports concussions are approached. Charities such as Headway UK now promote '*Concussion Aware*' in sport with the tagline, '*if in doubt, sit them out*', to encourage players to avoid returning to play immediately after a head collision[68]. These have now been implemented in new guidelines for grassroots sports published by the UK Government in April 2023, requiring a minimum of 24hrs rest after a concussion. This is being supported by major UK sports associations such as the Football Association, Rugby Football Union, and Association for Physical Education in schools. Nevertheless, this increased awareness is only growing in the sports community, whilst other single event mTBIs remain underestimated as a serious medical event.

These statistics are undeniable; that mTBI is *neither trivial nor temporary*. This suggests we need greater awareness of the long-term effects of even a single mTBI, and prognostication tools to help clinicians provide the best care and support to those patients who need it.

1.2 Current understanding of mTBI

1.2.1 Treatment

As discussed, each individual mTBI can vary vastly in its injury and patient characteristics. This creates a challenge for treatment- no two injuries are the same, and

thus identical treatments may not have the same effectiveness. Current 'treatment' is commonly education and reassurance about possible symptoms one might experience post-TBI, such as from a clinician or information leaflet[69]. This easily implementable approach however has not proven successful in improving patient recovery or symptom severity. As shown in a recent systematic review[69,70], only two studies have found long-term benefits of educational intervention, which were at high risk of bias. Thus, there is a need to find effective treatments for mTBI patients to support a more successful long-term prognosis and reduced symptomatology.

The first line of support could be pharmacological intervention to target specific symptoms post-TBI. However, twenty *different* pharmacological interventions have been investigated, such as methylphenidate and sertraline, with little replication or overlap between studies. Moreover, these have aimed to ameliorate outcomes ranging from cognition and memory to depression, fatigue, and pain[71]. This demonstrates the range of investigated agents and target outcomes, meaning few pharmacological agents have been sufficiently investigated or validated. This is further compounded by the range of interventional timepoints; only 4 studies have exclusively targeted the first 24hours post-injury, whilst others ranged up to 77 weeks[71]. As mTBI transitions from being acute to chronic, multiple drug targets may be beneficial at different therapeutic windows. However, it is the early window of opportunity (<24hrs) which is thought to hold greatest preventative therapeutic benefit, as the pathophysiology of TBI increases in complexity over time causing drugs to lose their efficacy[72]. A systematic review of pharmacological interventions ultimately concluded that there is not enough evidence to support clinical decision making for pharmacological interventions post-mTBI[71], and indeed there are no FDA-approved medications for cognitive or neuropsychiatric problems[73].

Despite this, some drugs are prescribed 'off-label' based on small, randomised control trials. These include the neurostimulant methylphenidate for cognitive dysfunction, and selective serotonin reuptake inhibitor sertraline for post-injury depression[73]. For example, the first randomised control trial of methylphenidate treatment found benefits to fatigue and cognition after 30-weeks of daily administration, compared to the placebo group[74]. A further study followed patients who had been taking methylphenidate for an average 5.5 years and found no adverse safety effects from its prolonged use and found marked deterioration if treatment was suspended[75]. These studies suggest long-term use

of methylphenidate may be beneficial for commonly reported symptoms of cognitive slowing and fatigue but are yet to reach sufficient sample sizes in formal clinical trials for their widespread use[71,73]. Without further clinical trials and robust evidence to support pharmacological interventions, populations experiencing long-term symptoms remain unsupported by drug intervention[71].

Psychological interventions have the additional benefit that individual injuries can be helped with individualised therapies. However, they have the disadvantage that only the presenting symptoms are being treated rather than an underlying pathology[69]. Nevertheless, mental health comorbidities and coping styles such as fear avoidance are regularly associated with poor outcome after mTBI[76], suggesting a psychological intervention may be beneficial. Cognitive behavioural therapy (CBT) has been most researched as a potential postconcussive treatment[76], however literature is limited with several recent reviews finding inconsistent benefits of CBT[69,77,78]. For example, a randomised control trial found no difference between effectiveness of 5 CBT sessions compared to 5 telephone counselling sessions on return-to-work post-injury, and surprisingly found *benefit* of telephone counselling in symptom reporting and severity[79]. This suggested that a lower-effort early-intervention is equally, if not more, beneficial than individualised CBT. As this study did not include a control group however, we are unable to ascertain how effective either of these interventions are compared with routine information leaflets. One early study nevertheless found relative benefit of telephone counselling compared to routine treatment[80]. Preliminary studies have suggested CBT might be more beneficial for specific symptoms such as headache[81] and sleep[82,83], rather than tackling general postconcussive symptoms. There is however sparse research on psychological interventions for mTBI as a whole, and their impact on long-term outcomes further than 6 months[69,78].

To summarise, currently no single intervention can be recommended post-mTBI due to a dearth of high-quality evidence in the field and a lack of replication in formal randomised control trials[69]. Thus, there are a marked lack of treatment avenues for mTBI patients, complicated by the heterogeneity of injury. To aid in future clinical trial development, we need to know which patients might benefit from therapeutic intervention, and which might reach a full recovery naturally. This will enable earlier intervention in high-efficacy therapeutic windows to enable a preventative approach to

long-term symptoms, rather than curative for the later-presenting symptoms. Knowing which patients will have a good versus poor prognosis, however, is not a small problem.

1.2.2 Prognostication

Prognostic models aim to predict long-term outcome based on acute measurements. They can also be valuable at predicting risk and thus facilitating risk management, informing communication with patients and relatives on potential outcomes and beneficial interventions, and indeed facilitating clinical trials. There are two major prognostic models which have been extensively tested and externally validated; Corticoid Randomization after Significant Head Injury (CRASH)[84] and International Mission on Prognosis and Analysis of Clinical Trials (IMPACT)[85]. Both models predict mortality (at 14 days or 6 months post-injury), and include key variables of age, pupillary reactivity, some element of GCS (total GCS in CRASH, and motor score only in IMPACT), and CRASH additionally includes a variable of major extracranial injury. Each model has been extended using CT-identified elements (described in subsequent section 1.2.3), such as traumatic subarachnoid haemorrhage and epidural hematoma. Prognostic models are commonly assessed by *sensitivity* (true positive rate), *specificity* (true negative rate), *false positive/negative rate*, and most informatively *area under the curve* (AUC, range 0-1). This describes model performance comparing true and false positives at different classification thresholds, where higher values indicate greater classification accuracy.

In a recent large-scale validation study with CENTER-TBI, both IMPACT and CRASH showed high performance rates at predicting mortality and unfavourable outcome in moderate and severe TBI[86]. The IMPACT model achieved AUCs between 0.77 and 0.88, and CRASH achieving AUCs between 0.66-0.88, supporting use of either model in real-world clinical settings. Further studies have found high false-positive rates (20.8-33.3%) with these models[87], however this is a preferable trade-off to having high false-negatives in clinical settings to ensure at-risk patients are not missed or under supported.

A major caveat to these models however is their inability to support *mild* TBI. The IMPACT model was purely created for GCS<12 patients and thus is inapplicable for mTBI (GCS 13-15). In contrast, the CRASH model does include mTBI patients, but has shown poor calibration for 'unfavourable outcome' in validation study[88], as this was defined by CRASH

as GOSE<5 which only applied to 11% of the mTBI cohort. Thus, the vast majority of TBI (80-90% mTBI) is not sufficiently served with these predictive models which were either not created for this population at all, or use uninformative boundaries of good and poor outcome for this population.

Several further efforts have aimed to create more informative models specifically for mTBI populations[89–92]. For example, the UPFRONT model[89] aims to predict GOSE=8; a useful threshold for mTBI patients who have the potential to reach full functional recovery. An additional version predicts presentation of 3 or more postconcussive symptoms. These models consider further predictor variables of mental health or previous TBI history, neck pain, and early symptoms of headache, nausea, and dizziness. The two models predicting functional or symptomatic recovery achieved an AUC of 0.77 and 0.75 respectively in internal validation[89] and have reached similar levels of success in external validation study[88]. Nevertheless, UPFRONT has an important drawback- its success has been attributed to the inclusion of *two-week post-injury symptoms* (e.g., depression, anxiety, postconcussive symptoms, coping styles). In real-world environments, recording these predictors two-weeks post-injury is unfeasible as it could requires patients to return to hospital settings leading to drop-out, and greater clinician workload for follow-up appointments. The best prognostic markers and models should be acquired as soon as possible post-injury, to align with current clinical care.

A final model to consider in the context of this thesis is from Falk and colleagues (2021)[92]. They looked explicitly at GCS-15 mTBI and prediction of 1-month incomplete recovery at GOSE≤7, achieving AUC of 0.79. This model intentionally used easily obtainable predictors of age, CT abnormality, history of depression, self-report moderate/severe headache, difficulty concentrating, and photophobia, all collected on the day of injury. This model has not yet been externally validated but demonstrates future developments supporting the growing mTBI population.

There are several further models not discussed in the context of this thesis[90,91,93], which will be important to future clinical care of mTBI patients. Whilst those most rigorously tested and validated have been developed for the moderate and severe populations, the need for mTBI-specific models with relevant outcome characteristics is being recognised. These models most commonly use predictors of age, initial injury severity,

CT abnormality, history of mental health conditions, and history of previous TBI, each associated with poor outcome. Improving the prognostic accuracy of these models requires additional biomarkers of poor outcome, with sufficient sensitivity for mTBI. One readily developing method to this end is using imaging-derived phenotypes from routine and advanced neuroimaging. Indeed, the addition of CT imaging has improved performance of several prognostic models in moderate and severe TBI[86,93,94]. The subsequent section will discuss some possible neuroimaging modalities in depth, and which might be most applicable to a mTBI context to develop new biomarkers of outcome.

1.2.3 Structural neuroimaging

CT

The first line of radiographic investigation following TBI is a computed tomography (CT) scan. This collects multiple X-ray images taken from different angles on a rotating X-ray tube, and computationally combines them using tomographic reconstruction to produce cross-sectional images of internal anatomy. As in traditional X-ray images, CT shows the greatest visibility for bone, but shows poor resolution for soft tissue. In instances where internal damage is a concern, CT has great benefit in identifying skull fracture or subdural or epidural hematomas to prompt surgical intervention[2,95], but is unable to evaluate parenchymal contusions, diffuse axonal injury (DAI), and limited ability to detect intracranial hypertension[96,97]. Owed to its cost-effectiveness and rapid acquisition, CT scans have enabled neurologists to better understand and characterise acute head injury in routine clinical care, and rapidly triage patients for medical intervention.

Findings on CT are commonly classified in severity according to two main systems: Marshall CT Score[32], and the later-developed Rotterdam CT Score[94]. Marshall Scoring grades patients in one of six categories based on CT findings, retrospectively if surgical evacuation is performed, whereas Rotterdam Scoring adds up scaling factors for the presence or absence of CT findings to a total score out of 6 (**table 1.1**). Both were developed for the purpose of aiding prognosis of TBI with higher scores indicating greater CT abnormality and worse prognosis. For instance, Rotterdam scores predict post trauma 6-month mortality rate as follows: score 1, 5%; score 2, 7%; score 3, 16%; score 4,

26%; score 5, 53%; and score 6, 61%[94]. Subsequently, these higher values on these scoring systems or simply presence of CT abnormalities have aided existing prognostic models and consistently contribute to poorer outcome across TBI severities[27,88,98].

Table 1.1. Common CT classification systems.

Marshall Score	Marshall Description	CT Finding	Rotterdam Scoring	Definition
I	No visible intracranial pathology	Basal Cistern	0	Normal
II	Midline shift of 0 to 5 mm, basal cisterns remain visible, no high or mixed density lesions >25 cm3		I	Compressed
			2	Absent
III	Midline shift of 0 to 5 mm, basal cisterns compressed or completely effaced, no high or mixed density lesions >25 cm3	Midline Shift	0	Absent or <5mm
			I	>5mm
IV	Midline shift >5 mm, no high or mixed density lesions >25 cm3	Epidural Mass Lesion	I	Present
			0	Absent
V	Any lesion evacuated surgically	Intraventricular or subarachnoid haemorrhage	I	Present
VI	High or mixed density lesions >25 cm3, not surgically evacuated		0	Absent
			+I	Sum Score (/6)

Thus, there have been great clinical benefits of CT implementation, particularly in moderate and severe TBI populations for prompting surgical intervention. This is not necessarily the case in mild TBI, who display CT abnormalities in only 5-23% of individuals, compared to 67% of moderate and 97% of severe TBI patients[47,99,100]. Historically, this absence of damage on CT led to a dismissal of *any* brain pathology in mTBI and thus the dismissal of persistent symptoms. Exemplifying this is an excerpt from Larrabee (p.196, 1997)[101], which highlights the stereotype that no damage on CT equates to no brain damage to underly 'real' symptomatology;

"*Symptom base-rate data show poor specificity for several so-called "post-concussion" symptoms, which occur frequently in non-brain damaged populations. Persistence of "post-concussive" complaints is considered as a function of somatization*".

This perspective still perpetuates today, exemplified by the disparity between low accuracy rates of clinician prediction of outcome and patient experience. Regularly, mTBI patients with CT findings are termed '*complicated*' versus '*uncomplicated*' injury in those without CT damage, although this has been criticised for negating the complexity of possible outcomes in this latter population[102]. A more favourable convention in recent literature has been to describe CT-positive (CT+) and CT-negative (CT-) individuals, and indeed acute CT abnormality in mild TBI populations has been established as a risk factor for adverse outcome[102]. A recent meta-analysis of CT+ mTBI patients showed small but clinically relevant rates of clinical deterioration (11.7%), neurosurgical intervention (3.5%) and death (1.4%). Moreover, damage on CT remains an important prognostic factor for poor outcome in mTBI[89,90,92,100]. Its benefits are thus established and routinely used in clinical care.

However, existing models are insufficient for mTBI populations as discussed[88], and only a minority of patients display such findings despite far greater rates of adverse outcome[54]. Thus, CT alone is unable to characterise the vast majority of mild TBI, or their adverse outcomes.

MRI

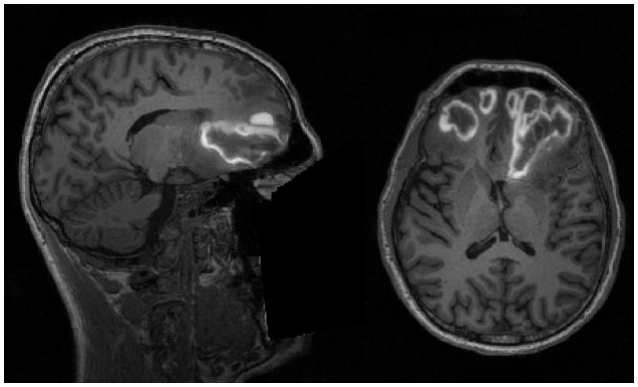
A second line of investigation is Magnetic Resonance Imaging (MRI), developed by Paul Lauterbur (1973)[103] and Sir Peter Mansfield (1977)[104] for which they received Nobel honours in 2003. At the centre of this technique is a strong magnetic field, and differential resonant properties of tissues which enable their distinction in different images.

In all organic matter there are free protons, i.e., hydrogen (H+) ions. These are particularly abundant in water, and as such, in brain tissue. As shown by Gerlach & Stern in 1922[105], these subatomic particles have a fundamental angular momentum (*spin*) in a random orientation. When exposed to a strong magnetic field, termed B0, protons will align to B0 in either a high-energy state (parallel and in opposite direction to the field), or a marginal majority in the low-energy state (parallel and in same direction as the field), creating net magnetisation to B0 termed *longitudinal* magnetisation[106]. Additionally, protons will *precess* at a specific frequency (*Larmor frequency*) out of phase with one another. When a radiofrequency pulse is applied at this Larmor frequency, often at an angle of 90°,

protons in the low-energy state will move towards the high-energy state and become perpendicular to B0 and precess in-phase with one another. This produces *transverse* magnetisation. Once the radiofrequency pulse is removed, the protons will begin *relaxation* back towards their low-energy state and fall out-of-phase. This relaxation induces a release of energy, or magnetisation decay, which is detected as a change in voltage by the MRI scanner receiver coils[106].

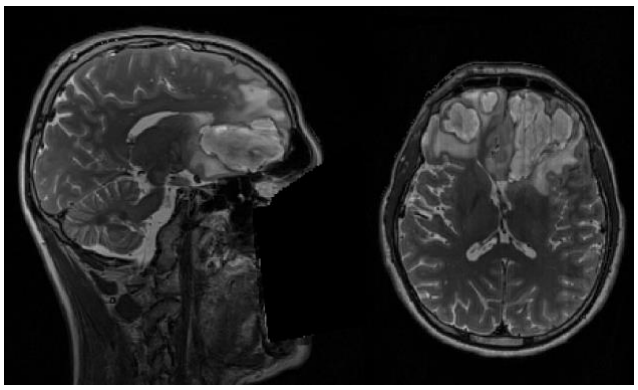
Importantly for MRI, different tissues have different relaxation properties, enabling them to be distinguished[107]. A T1-weighted image measures the time taken for longitudinal magnetisation to return to $1\text{-}e^{-1}$ (*T1 relaxation*), which differs between tissue-types. For instance, protons in water-rich regions such as CSF appear as lower intensity due to slower T1 relaxation, versus lipid-rich regions such as white matter appear with higher intensity due to fast T1 relaxation. These T1-weighted images are thus chosen for high-resolution anatomical images of the brain[107].

A complementary measure, T2 relaxation, instead describes the time for transverse magnetisation to decay to e^{-1}[106]. The loss of phase coherence of protons during transverse relaxation occurs for two main reasons. Firstly, this is due to field inhomogeneity in B0, measured by a third value T2* relaxation. As T2* is the driving factor underlying functional MRI, this will be explained in greater depth in **Section 1.3.1**. The second reason for loss of phase coherence is that a proton's spin is affected by local variations in the magnetic field caused by the small magnetic fields of neighbouring nuclei, termed *spin-spin* interaction[106]. Thus, CSF which is comprised of small molecules which are far apart experience less spin interaction and have longer T2 values, versus grey matter which has large macromolecules and thus greater spin-spin interaction and shorter T2 values. T2-weighted images are therefore useful for identifying haemorrhages[107]. An extended version of T2-weighted imaging is Fluid Attenuated Inversion Recovery (FLAIR), which enables additional contrast between abnormalities and healthy CSF and thus is used in identifying pathology[107]. An example of T1 and T2-weighted MRI and FLAIR are shown below in **figure 1.4**.



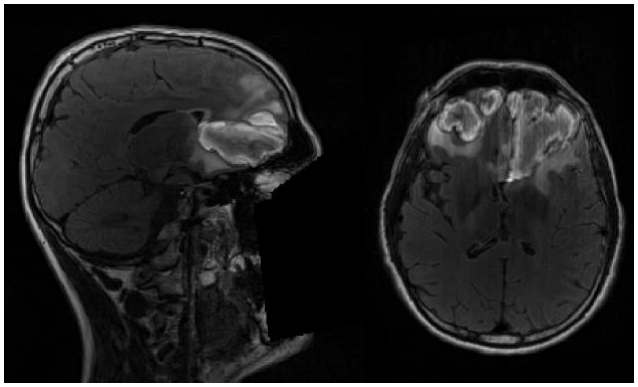
T1-weighted MRI

- Longitudinal relaxation
- CSF = dark
- Grey matter = grey
- White matter = bright



T2-weighted MRI

- Transverse relaxation
- CSF = bright
- Grey matter = grey
- White matter = dark



FLAIR

- Transverse relaxation, longer TR and TE
- CSF = dark
- Pathology = brightest

Figure 1.4. **Example TBI patient with acute MRI** (male, aged 37 years). Patient presented to the emergency room with GCS= 9 following violence/assault. Imaging was performed 72 hours post-injury and displays mass lesion in the frontal cortex with traumatic subarachnoid haemorrhage and traumatic axonal injury. These images demonstrate the relative value of each acquisition sequence for identifying healthy anatomy and pathology following injury.

Compared to CT, MRI has far greater discriminatory power of soft tissue types and can create three-dimensional images which is beneficial for examination of lesions and other pathology not visible on CT. It is also considered safer as there is no exposure to ionising radiation. Some disadvantages however are the increased cost and acquisition time for MRI versus a CT scan, meaning this is not performed routinely unless specified by a clinician for advanced investigation or used in research settings. MRI is also unsuitable

for those with contraindications such as metal implants which cannot be inside the strong magnetic field.

In mTBI, MRI has been 4-5 times more sensitive at detecting abnormalities than CT[108]. For example, a study by the TRACK-TBI group found that 27% of mTBI patients with negative CT showed abnormality on MRI[109], thereby demonstrating its additional diagnostic power. Such methodological developments of MRI began to change the perspective that CT-negative patients experienced no neurological damage. Rather, CT may not be sufficiently sensitive for this population. Additionally, having at least one contusion or four foci of haemorrhagic axonal injury identified on MRI was associated with poor functional outcome on the GOSE at 3 months post-injury, *after* controlling for CT findings[109]. Thus, MRI may have additional diagnostic and prognostic value compared to CT in mild TBI populations.

Nevertheless, there remain low incidence rates of MRI abnormality in mTBI, as with CT. A recent review of neuroimaging in mTBI stressed that even in chronic stages, pathology on MRI is either subtle or absent entirely[110]. This low prevalence rate is compounded by numerous studies which have failed to associate presence of abnormality with adverse neuropsychological outcome in mTBI[110–113]. Thus, CT and MRI are unable to fully characterise mTBI, and the debate surrounding so-called "uncomplicated" mTBI continued.

Quantification of MRI

Quantitative MRI assessments may have additional value for detecting more subtle differences in mTBI[114]. From structural MRI, common measures are volume, thickness, and surface area, and more recent developments of shape and contour analysis. This region of interest (ROI) perspective posits that deviation of values from demographically matched healthy controls indicates some level of injury-related damage which could be affecting ROI structural integrity and thus behaviour[113].

Whilst moderate and severe TBI have long been characterised by whole-brain atrophy at rates of around 5% per year post-injury[115], a recent review was inconclusive in the case of mild cohorts[115]. As discussed, injuries can vary in type, intensity, and direction, which may render ROI-level approaches underpowered for assessing more subtle differences in

mTBI. There are nevertheless recent studies which have found morphological differences in some more vulnerable regions, such as the corpus callosum, thalamus, pituitary-hypothalamic area, basal ganglia, amygdala, and hippocampus[113]. These central structures may be highlighted in cross-sectional studies because they represent a commonality between patients; irrespective of type or direction of injury forces, there is great stress and strain within this 'cone of vulnerability'[113]. The unique mechanical forces experienced by these regions has been empirically demonstrated in both simulation studies[116,117] and *in vivo* measurements of brain deformation during very mild posterior-anterior head deceleration in healthy volunteers[118,119].

Importantly, volumetric changes represent just one facet of mTBI[113]. These particularly vulnerable regions are often termed 'hubs', due to their far-reaching influence on other cortical, subcortical, and cerebellar regions. For example, the thalamus is known to be structurally connected to the entire cortex[120] and influence whole-brain functional dynamics[121] . Thus, primary thalamic injury could have widespread secondary effects on cortical function and thus long-term behavioural changes, which cannot be characterised by a single acute MRI alone.

More globally-directed volume losses have since been reported in the post-acute phases[122–124] , and in numerous longitudinal studies extending to a year post-injury and beyond[125–127]. Such techniques may also benefit our understanding of mTBI progression by tracking changes in healthy or lesioned tissue. For example, Richter et al[128] found global decreases in white matter volume at two-weeks post injury after mTBI despite no changes in imaging performed within 72hrs, suggesting there are secondary neuroanatomical substrates of injury within white matter.

Nevertheless, imaging at late time points has limited prognostic application, and could represent the consequences of pathophysiology rather than mapping injury processes. Thus, volumetric measures aid our post-acute understanding of mTBI, but appear to lack specific prognostic value in this mild population.

DTI

A further development in neuroimaging which has attracted great research interest in the TBI community is Diffusion MRI (dMRI). Its sensitivity to the anisotropic diffusion of water molecules has enabled the development of several biophysical modelling approaches to estimate microstructural features of white matter structures[129]. Diffusion Tensor Imaging (DTI)[130] is the most widely used model to analyse dMRI data. This technique summarises the diffusion of water molecules as a three-dimensional equiprobability ellipsoid where the amount of diffusion (eigenvalues of the DT) in each orthogonal direction (eigenvectors of the DT) define the ellipsoid itself, which also captures the principal diffusion direction. This is under the model that intracellular molecules will show restricted, anisotropic diffusion perpendicular to white matter fibres, whereas extracellular molecules show isotropic, or unrestricted, diffusion. Further combinations of the DT eigenvalues can reflect voxel-wise tissue properties, such as its mean diffusivity (MD; average magnitude of diffusion in three axes) and fractional anisotropy (FA; scalar value between 0 and 1 from isotropic to anisotropic diffusion). There are however several limitations of DTI; crossing fibres within a voxel cannot be resolved, and MD and FA are inherently non-specific to microstructural features[131]. For instance, reduced FA could indicate both reduced neurite density or increased neurite orientation dispersion, and DTI alone is unable to reconcile this.

Nevertheless, this technique has proven useful in identifying microstructural white matter damage *in vivo* not visible on traditional MRI. In the mTBI literature, many studies have found acute microstructural disruption associated with cognitive and behavioural deficits[132]. Until recently, DTI measures had not reached predictive value in mTBI due to inconsistent findings across studies; reporting decreases, increases, and no alterations in FA[96,132]. Further studies with far greater sample sizes have aimed to address these shortcomings in the literature. The TRACK-TBI group studied n=391 mTBI patients at two weeks and six months post-injury, and found globally increased AD and MD, and reduced FA, at both timepoints[133]. Furthermore, increased AD and MD at two weeks were associated with incomplete recovery at 6 months (GOSE<8) demonstrating the prognostic utility of DTI. These findings were echoed in a smaller study by CENTER-TBI, finding globally increased MD and reduced FA at three days and thirty-one days post-injury[128]. These authors further distinguished differential phenotypes of recovery based

on DTI findings and showed that those with increasing MD and decreasing FA over time (i.e., progressive injury) also showed deterioration in postconcussive symptoms.

One caveat to these data is the inclusion of both CT-positive and negative patients. As discussed, many mTBI patients do not present visible abnormalities on CT or MRI. A previous study from a smaller TRACK-TBI cohort found that whilst mTBI as a whole demonstrated the characteristic changes of increased FA and decreased MD compared to healthy controls, the subset of CT/MRI negative patients displayed no significant differences in DTI at all compared to controls[134]. Further studies have found only those CT-negative patients who experienced loss of consciousness show differences in acute MD compared to controls, and neither loss nor maintenance of consciousness groups showed changes in FA[135]. Thus, DTI is a useful tool for prognostication in TBI, but could relate to the existing biomarker of CT-status. Developments in the DTI literature are ongoing to fully discern its prognostic utility, such as using more sensitive analysis methods like NODDI (neurite orientation dispersion and density imaging). These are in their infancy given the differential acquisition parameters required for NODDI, and thus small sample sizes currently collected, but show promising results in increasing our understanding of WM damage[136].

Thus far we have only considered changes in brain structure following injury. These studies from quantitative MRI and DTI solidified that mild TBI is not void of long-term neurophysiological consequences, but was rather limited by methodological constraints of earlier neuroimaging methods. With greater advancement of image analysis, we are beginning to better understand how the brain is altered following even the mildest injury, and consequently, how we can proceed to treat these patients. We next consider a further level of complexity; brain function. As MRI and DTI have built upon the foundations of CT imaging for head injury, finding greater change at each methodological advancement of investigation, functional MRI is our next tool for exploration.

1.3 A functional disorder

1.3.1 What is fMRI?

Functional MRI (fMRI) enables us to measure brain function over time noninvasively and indirectly, whereby neural activity is coupled to cerebral blood flow. This can be used to investigate how different regions of the brain respond and interact to a stimulus or task, how regions interact with one another at rest, and how these might be altered in disease when compared to brain function of healthy controls.

As discussed in section 1.2.3, T2* relaxation is a measure of transverse relaxation of protons including that which occurs due to field inhomogeneity in B0. Importantly for fMRI, this can be affected by the magnetic properties of oxygenated and deoxygenated haemoglobin in the blood, which intrinsically vary with changes in neural activity in normal physiology [137,138]. When oxygenated haemoglobin becomes deoxygenated, thought to occur with energy demands of neural activity, it changes from being diamagnetic to paramagnetic thereby changing its magnetic susceptibility. This creates local distortions in the magnetic field which decreases the net MR signal. However, neural activity increases blood flow to a greater extent than oxygen metabolic rate, thereby increasing the availability of oxygenated haemoglobin and thus increasing the MR signal. This is termed the *blood oxygen-level dependent* (BOLD) signal.

In this sense, fMRI is an *indirect* measure of brain function as it measures the magnetic changes associated with neural activity rather than the activity itself[139]. There are further criticisms of BOLD-fMRI, as several interacting physiological variables (e.g., cerebral blood flow, cerebral blood volume, cerebral metabolic rate of oxygen) all increase with neural activity, but have conflicting effects on the BOLD signal, thereby clouding the true source of BOLD increases and decreases[139]. Nonetheless, fMRI has been invaluable in advancing our knowledge of the brain in health and disease.

One of the most influential methods in neuroscience was classical neuropsychology; mapping lesions to their corresponding cognitive and behavioural deficits[140]. This promoted the idea of localised function- that specific regions are specialised for different functions. Such ideas date back much further in history to the ancient Greeks' debate on

the location of consciousness being in the heart or the brain, and Galen's formal propositions of phrenology[141]. Lesion case studies, however, gave biological credibility to such localisation claims, in modalities such as language[142], decision making[143], and memory[144]. Task-based fMRI advanced this understanding by enabling investigation of *presence* of behaviour and functional change, rather than the *absence* of behaviour to structural loss. Lesion studies were limited in this sense, as they needed to occur by chance in a novel region without overlap or secondary damage infiltrating results. Whereas fMRI allowed researchers to probe increasingly specific stimuli and behaviours with greater control of study design in wider populations, as opposed to chance lesion case-studies. One of the most famous examples of this in fMRI research is the 'fusiform face area' showing greater BOLD activation to images of faces than houses[145].

Perhaps an even greater contribution of functional MRI has been the transition from localised towards distributed function. Where before lesions and their corresponding deficits were confined to isolated ROIs, fMRI increasingly found groups of regions all coordinating a change in activity in response to a stimulus or even at rest[146–149], suggesting a distributed approach across the brain to everyday function. The most salient example of this is the hallmark of *resting-state* fMRI (rsfMRI), functional connectivity.

Functional connectivity works under Hebbian theory that colloquially is known as *cells that fire together, wire together*[150]. More formally, different brain regions which show temporal correspondence in activity are thought to work in harmony for a particular stimulus/task, and thus can be thought of as functionally connected. Even at rest, when the participant is asked to lay in the fMRI scanner without a task or stimulus, common sets of distributed regions show temporal activity correspondence of baseline BOLD signal thus comprising *resting state networks*. This spontaneous activity once thought to be noise in fMRI data, is now known to be a fundamental signature of functional organisation[151].

First characterised in the 'default mode network' (DMN)[152,153] there are now multiple functional networks consistently shown across populations at rest and task[154,155]. Some of these canonical networks are shown in **figure 1.5**, according to the seven-network parcellation proposed by Yeo and colleagues (2011)[156]. A salient example of the utility of functional connectivity was shown by Boes and colleagues[157] who performed a lesion

mapping study of participants with a specific set of neurological symptoms. They found that merely 26% of participants had overlapping lesion regions, but importantly, those with similar symptom profiles had lesions within the same functional networks (>90% overlap). This demonstrates the utility of modern imaging techniques compared with previously limited anatomical study to better understand behaviour and disease. Indeed, network organisation is now accepted as an intrinsic organisational property in the brain which show derangements in disease and are related to behavioural deficits[158], and additionally show relationship with gradients of biological development[159].

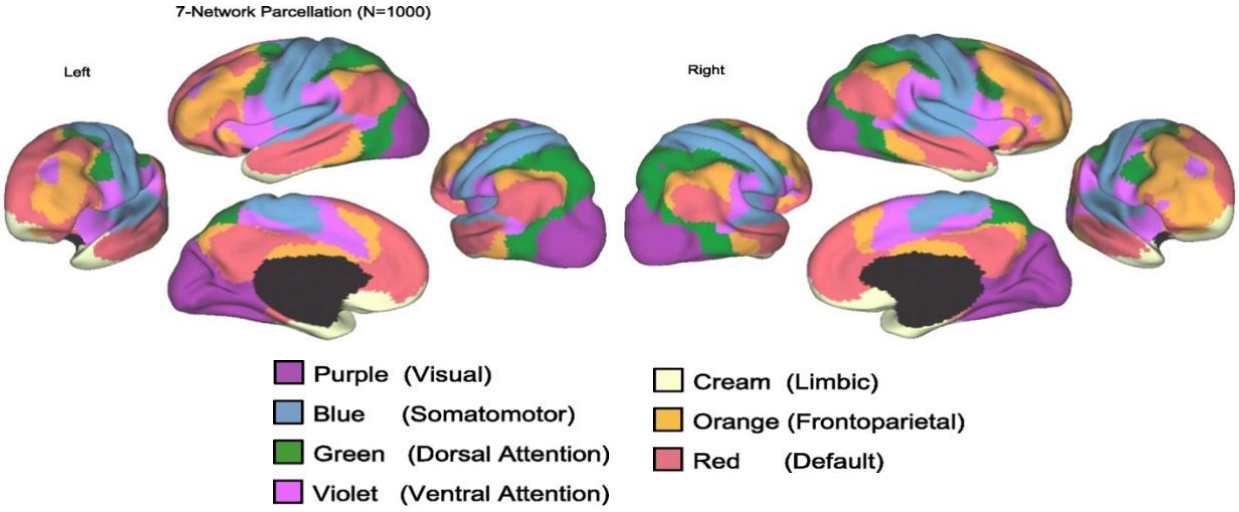


Figure 1.5. Resting state networks. Figure adapted from Yeo et al., 2011[156], showing their seven-network definition of canonical resting-state networks.

Studies using rsfMRI have been steadily increasing since 1995, of which around half consider clinical populations[158]. This technique is particularly useful in clinical cases as it requires no underlying abilities to perform a task, for example can still be performed in cases of coma. Functional connectivity analyses have since expanded much further than their original networks. We now investigate mathematical properties of these networks using graph theory, look at how every voxel in the brain is connected to every other voxel, and even track dynamic changes of functional connectivity over time into functional 'states', to name a few. With advancements of larger open-access datasets and increased computational power, functional investigations have exploded into an entirely new domain of computational neuroscience which we are only beginning to fully appreciate.

1.3.2 Function versus structure

A question which has probed researchers over the last few decades is how such a rich and flexible functional experience arises from a more fixed set of anatomical restraints[160]. Specifically, how does brain function arise from brain structure.

The proposition of harmonious recruitment of regions had been growing over time, leading researchers to develop the idea of a *structural connectome*[161]- how distal regions are anatomically connected. This is based on connecting white matter fibres between distal regions, often obtained using DTI. We now understand the structural brain to have highly connected 'hubs', often termed the 'rich club', which are densely connected with other brain regions and one-another[162]. With the advent of rsfMRI, researchers began to wonder how these functional signatures might arise from more well-established anatomical constraints. There was an early belief that structural and functional connectivity would have high correspondence[163,164]. That is, the dynamic environmental demands faced by the functional brain are supported by structural connectivity, and this underlying structure places restraints on brain connectivity, dynamics, and human cognition[164].

However, numerous studies investigated the relationship between structure and function and did not find a linear relationship[164–168]. One of these was from Honey, Sporns, and colleagues (2009)[165], who compared rsfMRI and DTI in the same individuals. They found that whilst increasing structural connectivity related to increasing functional connectivity, there were aspects of functional connectivity unexplained by structural connections alone. Plainly, functional connectivity was observed between regions with little-to-no underlying direct structural connectivity. This was partially explained by indirect structural connections and interregional distances; however, the fact remained that functional connectivity could not be completely inferred from structural connectivity. These findings have since been corroborated that functional connectivity may be supported by the anatomical structure of the human brain, however linear relationships do not explain the full functional connectome[163,166,167]. Further biophysical models and machine learning approaches have since advanced much further than these linear relationships, finding greater structure-function coupling than previously thought[168–170], including using the strength of structure-function coupling to predict

cognitive performance[168]. These results suggest that we are unable to discern whether the modest relationship between structure and function is a natural property of the brain, or if modelling is not yet advanced enough to successfully capture this relationship. Regardless, structural connectivity most commonly explains ranges of only 50-60% of the variance in large-scale functional brain networks[168].

These discussions beg the question: why does this matter for mild TBI? We have discussed that mTBI patients rarely display anatomical deficits underlying the greater proportion of chronically symptomatic patients. This concluded that routine structural imaging is insufficient. Presently, we discuss that *function* is not linearly related to *structure*, i.e., a lack of structural deficit does not necessitate a lack of functional deficit. Consequently, as functional signatures can have greater correspondence to complex behaviour, it is not unfounded to hypothesize mTBI as a *functional* disorder. This notion will be explored in existing literature and is the focus of the second experimental chapter of this thesis.

1.3.3 Functional changes after mTBI

A vast literature has begun to characterise injury-induced *functional* changes after mTBI with behavioural relevance, initially focussing on resting-state networks. For instance, Palacios and colleagues (2017)[171] found reduced within-network and between-network connectivity of multiple resting-state networks associated with six-month outcomes, and these patterns differed between patients with versus without damage on CT. Such studies demonstrate the relative utility of early rsfMRI in these CT-negative patients, compared with previously discussed methodologies.

The network literature is not without its flaws however, as a recent review highlighted great inconsistencies across the field with little converging results over the last 10 years of investigation[132]. Taking the DMN as example, studies have found increased DMN-Salience network functional connectivity related to better executive function[172], decreased within and between connectivity of several networks including DMN predictive of 6-month outcomes[171], increased and decreased connectivity within and between DMN and other networks[173], and no DMN alterations at all [174], all in the acute

phase of mTBI. These contradictory findings remain, despite the DMN being the most highly studied network in mTBI literature[132].

These discrepancies may be partially explained by the great heterogeneity of mTBI populations[2], discussed previously, which require a large and well-defined sample to account for this increased variance. However, samples are most commonly between 20-50 participants per group [132]- except for one recent study [175]- which reduces power within a study thereby limiting our ability to find consistent effects across studies. There is furthermore great variation between studies in image analysis methodology, inclusion criteria of CT damage or neuropsychiatric history, and demographic factors such as age and sex; both of which have known but incompletely understood impacts on recovery[19,176]. Inevitably, different approaches and patient characteristics across the field will produce a range of potentially conflicting results.

There is nevertheless a growing consensus that acute functional changes after mTBI occur on a *global* scale, as opposed to affecting one single network. This connectomic scale of alterations was proposed by Iraji and colleagues[177], who demonstrated connectivity increases and decreases affecting whole-brain functional networks which differentiated mTBI from controls with 100% specificity and 93.75% sensitivity. Convincingly, only 2/30 of their mTBI patients showed any structural abnormality on CT or MRI despite these vast global functional changes. Indeed, several studies of acute mTBI, particularly those with larger sample sizes, have reported functional changes *globally* affecting resting-state networks[171,178–180]. This global approach may be deliberately useful for cases of mTBI, as individual injuries can vary substantially, rendering regional approaches applicable to some patients and not to others. Whereas a global approach may encompass widespread changes regardless of injury location, duration, or type, particularly in cases without regional structural damage. As discussed, some regions may show selective vulnerability to injury forces[113]. One of these, the thalamus, is additionally a globally connected region which interacts with the entire cortex and integrates information for functional networks[121]. Consequently, the thalamus may be an important region of interest which shows *both* universal vulnerability to primary injury and global reach. Despite this potential, the thalamus has not yet been sufficiently studied in a mTBI population[181].

Further research into global properties of functional networks has used graph theory approaches and suggest the intrinsic makeup of networks is distorted. An ideal network is defined as having small worldness- lying between complete order and disorder with highly integrated hubs and fewer long-range connections[182]. Studies investigating such network properties find less efficient networks in acute mTBI, exemplified by increased path length and disrupted small worldness[172,174,183]. Moreover, mTBI patients were found to spend less time in highly efficient networks and more time in states with disrupted small worldness when analysing functional connectivity over time[174] (termed, dynamic connectivity). A review of graph theory metrics in all TBI severities concluded that hyperconnectivity was a common signature of injury, alongside disrupted global integration characterised by reduced network efficiency[184]. Thus, an increasing body of evidence suggests that functional alterations occur after mTBI on a *global* scale, particularly affecting the efficiency of network communication.

However, there remains great discussion about the nature of connectivity changes being hyper- or hypo-connected. Initial hyperconnectivity post-injury is an increasingly common finding in the literature[185,186]. This may be caused by specific neuronal damage[187] leading to less signal variability and thus increased 'connectivity' by timecourses of activation becoming more similar, or perhaps an adaptive response aiming to overcome such injury. A main proponent of this adaptive hypothesis suggests that hyperconnectivity is acutely beneficial by increasing the connections through 'hub' regions which are highly interconnected throughout the brain and display high metabolic efficiency[185,188]. This aims to combat network inefficiency caused by damage to any node in the functional connectome, whilst reducing metabolic costs in a metabolically disrupted neuronal environment post-injury. Multiple studies of moderate and severe TBI have directly tested and support this adaptive hyperconnectivity hypothesis, proposing it as a compensatory response[188–190]. However, the mild TBI literature faces greater speculation on what is adaptive or maladaptive[191]. As discussed, studies regularly report either hyper- or hypo- connectivity, or indeed both simultaneously, in mTBI. There is a need to further investigate connectivity changes after mTBI and their relationship to outcomes, to fully substantiate claims of adaptivity in this population.

Importantly in this discussion of hyper- versus hypo-connectivity, timing of imaging post-injury has great potential to impact findings given the evolution of pathoanatomical

changes in the acute and post-acute timepoints. Initially adaptive hyperconnectivity mechanisms in moderate and severe TBI may become maladaptive if chronically persistent, by increasing vulnerability to secondary pathology due to elevated metabolic stress[188]. A developing literature in mild TBI similarly proposes there are longitudinal changes in brain function which may vary depending on good or poor outcome. This model by Boshra and colleagues (2020)[186], shown in **figure 1.6**, suggests that changes in mTBI transition from acute hyper- to chronic hypo- connectivity as initially adaptive mechanisms fatigue from persistent overstimulation. In contrast, they suggest successful recovery is characterised by long-term recovery of connectivity to healthy levels. Such longitudinal changes in connectivity may partially explain the mixed findings presented in the current mTBI literature. Particularly some older publications from the early 2010s have used conflicting timepoints post-injury within the same study, ranging as far as 13-136 days post-injury in a small sample of only 30 patients[173]. Thus, longitudinal functional investigation of well-defined timepoints post-mTBI is needed to clarify the literature.

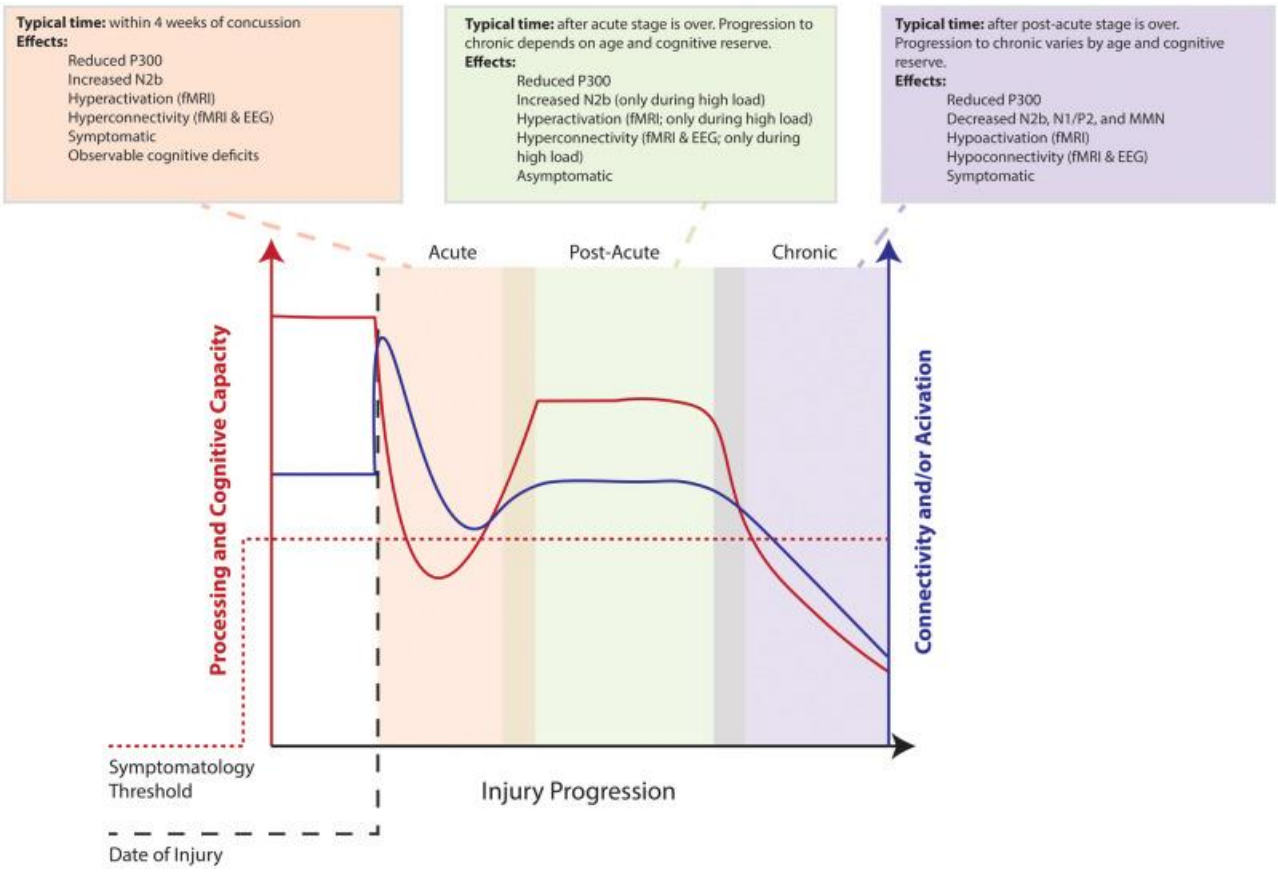


Figure 1.6. Model of longitudinal functional connectivity changes in mTBI. Theorised model from Boshra et al (2020)[186]. Overlap of acute, post-acute, and chronic timepoints reflects unclear transitions in the literature, and relating to age and cognitive reserve.

Regardless, the model proposed by Boshra et al[186] was based on their findings using electroencephalography (EEG) rather than rsfMRI and is yet to be empirically validated. Particularly in the field of rsfMRI, few studies thus far have presented longitudinal follow-up of clinical outcomes[171,174] or serial imaging[175,192,193] in mTBI cohorts, with only one study extending beyond 6 months post-injury[193]. These authors found some potential of reducing connectivity of the DMN from 1-3 months to relate to persistent postconcussive complaints but did not find clear longitudinal changes in mTBI. Concurrent imaging and outcome assessment with a longitudinal design will be the most powerful tool in understanding ongoing functional changes and hopefully symptom recovery after mTBI, and how these might relate on an individual level.

In sum, the current literature on functional changes after mTBI is mixed but appears to suggest a global scale of alterations. Importantly, even mTBI patients without damage on routine imaging display these widespread outcome-relevant functional changes acutely after injury, highlighting the diagnostic and prognostic potential of rsfMRI. There is a need for clarification of this literature as current studies across the mild population are vast but variable, with small samples, and insufficient clinically relevant follow-up.

1.3.4 What is missing?

As discussed in this section on neuroimaging methods, increasing sensitivity has been beneficial for more specific and mild phenotypes of TBI. For example, how can one explain the young person with a negative CT and no neuropsychiatric history, who continues to experience fatigue and depression years after their injury? Such cases account for a large proportion of mTBI, who are currently in a gap of clinical understanding. Emerging literature suggests that rsfMRI is a useful tool for studying this population.

A clear obstacle between functional imaging and patient outcomes, however, is how this translates into clinical care. Currently, CT and structural MRI are cost-effective and accessible in most modern care systems, with clear clinical benefit for surgical intervention[96]. Conversely, functional imaging is largely confined to research settings due to its cost, acquisition time, lack of normative standards for individual patient comparison, and required expertise for analysis[96]. Moreover, there have not yet been

sufficient patient benefits in research settings to outweigh these obstacles, despite its "*undeniable potential for refining characterisation of TBI*"[2]. This is not to say that fMRI has yielded no benefit, as our understanding of these conditions has greatly increased in the academic community. Nevertheless, this benefit is yet to reach the patient, causing some to question the purpose of further funding and effort into these endeavours[194]. If patient benefit is the goal, fMRI has failed to perform.

There is also some degree of mistrust of fMRI research. Following media sensation, Eklund and colleagues' 2016 paper called into question the methods of 40,000 fMRI papers with up to 3,500 potentially incorrect conclusions based on false positives[195]. This hangover into neuroscience has created a further gap between "basic" neuroscience and computational scientists.

An emerging idea is that we have been asking clinical questions which fMRI is struggling to answer[196]. Particularly, fMRI measures were initially developed to understand cognitive neuroscience questions using group averaging, and not necessarily for identifying single-subject clinical biomarkers[196]. Moreover, group-level differences may not represent the great variance seen in fMRI at the individual-level[197]. A growing notion therefore is to use fMRI as a tool to increase our understanding of disease to form new hypotheses and targets for therapeutic neuromodulation or electrical stimulation, rather than as a target for routine clinical investigation. In this scenario, understanding the neuronal correlates of outcome in these patients may promote translational research into therapeutic targets to best *treat* these patients. We can understand these correlates at earlier timepoints than CT and MRI, which instead show *post*-acute changes, and thus clinically intervene in an earlier 'window of opportunity' to gain greatest recovery from injury. This is currently mere ideology and has not been put into practice.

Thus, there are two major gaps in using rsfMRI for mTBI patients; firstly, clarification of a mixed functional literature to better understand how the mTBI brain has changed, and secondly, how this information can be translated from academia into patient care.

1.4 Thesis aims

This introduction has identified the "problem" of mTBI:

Mild traumatic brain injury is overexpressed in the population yet lacks adequate attention in clinical care and neuroimaging.

The relative paucity of clinical understanding and care of these patients has created a disconnect between injury and outcome, whereby we are currently unable to predict who might experience long-term effects or sufficiently treat these patients. This lack of biological understanding has previously been hindered by methodological constraints, misclassifying so-called mild injury as lacking damage. Based on literature discussed, rsfMRI may hold greater potential for characterising and prognosticating CT-negative mTBI than structural imaging alone (CT, MRI, DTI). This growing functional literature has suggested a brain-wide scale of acute functional alterations, particularly affecting resting-state networks and central hub regions such as the thalamus, which requires further exploration in a large and well-defined sample. Thus, my overarching hypothesis for this thesis is:

Mild TBI demonstrates brain-wide acute functional changes in resting-state networks and thalamocortical interaction, with direct association to long-term functional and postconcussive outcomes.

To further explore this hypothesis, the aims of this thesis are as follows,

i) Define mild TBI as a predominantly *functional* disorder, by demonstrating marked acute functional change in the absence of gross volume, diffusion, and alternative biomarker change.
ii) Identify specific acute correlates of chronic outcome using rsfMRI, focussing on resting-state networks and thalamic functional connectivity.
iii) Track how these correlates vary with time and with good/poor outcome; over 12 months, several years, and in the special case of repeat injury.
iv) Translate findings from acute imaging into treatment-relevant targets using complimentary positron emission tomography (PET), thereby bridging a gap between macrostructural and microstructural investigation.

Chapter 2

The ‘Mildest’ mTBI Cohort

2.1 Introduction

As discussed, 'mild' TBI is often conflated with a non-serious and short-term injury, which quickly resolves within a few weeks. We are beginning to understand that this is very much not the case, as previous large-scale studies have consistently found high rates of incomplete recovery (GOSE) and chronic postconcussive symptoms in mTBI[50,54,198]. Despite this, there is an absence of well-performing prognostic models with sufficiently specific boundaries for good/poor outcome for the mTBI population[88,199]. Thus, novel biomarkers are needed to aid prognostication of outcome after mTBI.

In the introduction to this thesis, neuroimaging techniques were presented as a potential avenue for novel biomarker development, including MRI, DTI, and rsfMRI. Whilst these have shown mixed success at prognosticating outcome, many studies have included populations of mTBI who present existing markers of poor outcome, such as evidence of damage on CT, neuropsychiatric history, or history of previous concussion. Thus, it remains impossible to disentangle whether poor outcomes and associated neuroimaging findings are attributable to these risk factors, or to the TBI. It is important to design a cohort to investigate outcome in the 'mildest' TBI within hospital settings, who simultaneously present acute mild injury severity (GCS 13-15) and do not present existing markers of poor outcome and thus considered 'low-risk'. This therefore encompasses a mild injury phenotype spanning both diagnostic and prognostic criteria. Previous studies have not necessarily been able to apply such strict inclusion criteria due to the combination of limited data collection, particularly of advanced neuroimaging, and the intrinsic heterogeneity of mTBI.

This is now made possible by multicentre European project Collaborative European NeuroTrauma Effectiveness Research in TBI (CENTER-TBI)[43]. This observational prospective cohort project was conducted as part of the larger International Initiative for Traumatic Brain Injury Research (InTBIR) and had two main aims. Firstly, to better characterise TBI in a European context, and secondly, to identify effective intervention and management strategies for TBI care. To do this, the core CENTER-TBI study collected data from 4509 individuals at all severities and stages of TBI progression from 2014 to 2017, across 18 different countries. These were recruited at hospital settings: after presentation to the emergency room (ER; 19%), after admission but not to intensive care

(34%), or after admission to intensive care units (ICU; 47%)[12]. Observational data regarding initial injury and outcome were collected, including routine clinical measurements such as GCS, GOSE, and CT if required by local criteria on initial presentation. However, CENTER-TBI also collected non-routine observational data, such as blood-based biomarkers, DNA, outcome measures of clinical, cognitive, and psychological importance, and advanced neuroimaging beyond CT and MRI. Moreover, this was collected longitudinally in patients, up to 2 years post-injury. For example, the project included 13 sites across Europe acquiring longitudinal rsfMRI which was collected at 6-, 12-, and 24-months post-injury. CENTER-TBI thus provides a unique opportunity to investigate the behavioural and neuronal consequences of 'mild' TBI in a large and well-defined cohort, with longitudinal neuroimaging and outcome measurements at regular follow-up.

One of the specific aims of CENTER-TBI was to, "*develop multidimensional approaches to characterisation and prediction of TBI*"[43]. I therefore begin the experimental section of this thesis by introducing the primary study cohort investigated in Chapters 3 and 4, which work towards this aim. This cohort was designed to investigate mTBI in adults. Given the vast potential of rsfMRI to identify novel biomarkers for mTBI already discussed[2], this modality is the primary focus of subsequent chapters in this thesis. The present chapter substantiates my focus on rsfMRI and the need for functional biomarkers, by presenting the prevalence of poor outcomes in this mild cohort, which are not sufficiently differentiated by alternative potential biomarkers.

2.2 Method

2.2.1 Participant inclusion

Data were obtained from subjects recruited to the MRI sub-study of CENTER-TBI between December 2014 and December 2017 (https://clinicaltrials.gov/ct2/show/NCT02210221), version CENTER CORE 3.0. Ethical approval was obtained in accordance with relevant laws and regulations for each recruiting site, and informed consent was given by each participant either directly or by legal representative/next of kin. Further details of sites and ethical approvals can be found at https://www.center-tbi.eu/project/ethical-approval. Investigators for the CENTER-TBI MRI sub-study are referenced in **Appendix 2.1.**

Inclusion criteria for this cohort were aged 18-70 years with no history of previous concussion or neuropsychiatric disease (depression, anxiety, schizophrenia, attention deficit/hyperactivity disorder, developmental learning disability, epilepsy, sleep disorders, or substance abuse). Patients additionally sustained a mTBI (Glasgow Coma Scale (GCS) 13-15), required a head CT according to local criteria on initial presentation, showed no CT abnormalities, and were recruited at the ER or admission strata, excluding those in ICU. CT abnormalities were identified using CENTER-TBI coding for this variable within their database, to exclude those with CT abnormality marked as 'present'. These criteria were chosen to include the 'mildest' mTBI presenting to hospital settings, thereby excluding those with pre-existing markers of poor outcome, and excluding those who presented to intensive care units who are known to experience worse injury and outcome[12], and may have experienced substantially different inpatient care. Additionally, patients had undergone T1-weighted MRI and rsfMRI in the acute phase post-injury, to enable later inclusion in functional neuroimaging investigations. Imaging was defined as 'acute' if it was collected at 'Early' or '2 week' timepoints according to CENTER-TBI timepoint coding. Acquisition protocols for CENTER-TBI imaging data are described in the central CENTER-TBI resources at https://www.center-tbi.eu/project/mri-study-protocols. Matched healthy controls were recruited from the same centres as patients, and contemporaneously imaged on the same MRI systems. This resulted in n=155 mTBI patients and n=108 healthy control subjects. A consort diagram detailing patient inclusion at each stage can be seen in **figure 2.1.**

Centralised quality control was undertaken within CENTER-TBI to include imaging data of sufficient quality for academic research[43]. However, remaining quality concerns required attention. Visual inspection of each orthographic view of the T1 and first rsfMRI volume presented three quality concerns: i) missing/incorrectly reconstructed data, ii) structural abnormalities violating inclusion criteria, and iii) susceptibility artefacts on functional imaging. In cases i and ii, these subjects were excluded from further analyses. In case iii, orbitofrontal and temporal regions showed site-specific susceptibility artefacts (mTBI site-03 = 18/26 affected, site-10 = 8/10). These artefacts are caused by differences in magnetic susceptibilities of tissue types producing different precessional frequencies and thus B0 inhomogeneities. Distortion correction applies an opposing fieldmap to the distortion, however this was not possible with these data as one single-

band functional image was recorded. Fieldmaps must be calculated using the phase difference between two echoes in a double-echo sequence, or the difference in distortion between two acquisitions with opposite phase-encoding directions. An experimental 'fieldmap-less estimation' tool[200] was trialled on a further subset of 20 participants, however alterations were inconsequential. Therefore, all subjects in site 10 and affected subjects in site 3 were excluded, as detailed in **figure 2.1.** Of the original n=4509 TBI patients and n=171 control participants collected within CENTER-TBI, my final cohort thus included n=108 patients and n=76 healthy controls following quality control and site exclusion, as shown in **figure 2.1** below.

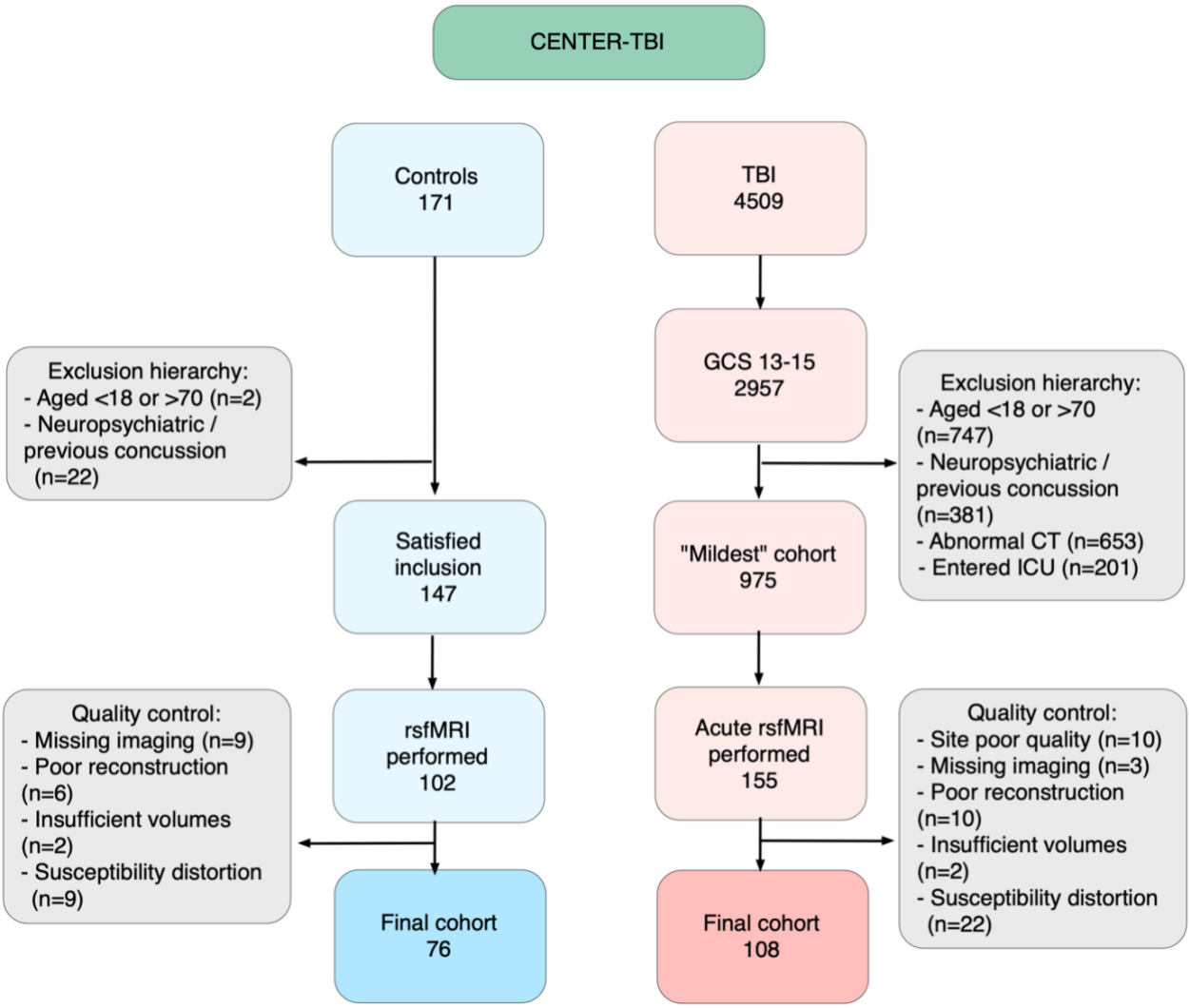


Figure 2.1. Consort diagram for participant inclusion. All data were obtained from CENTER-TBI CORE v3.0. Two streams describe the number of participants excluded at each stage of the inclusion hierarchy, with controls shown in blue and TBI in coral. Grey boxes present further information regarding the number of participants excluded for each criterion. The final cohort included comprised n=76 healthy controls and n=108 patients with mTBI.

2.2.2 Outcome measurements

CENTER-TBI collected a vast array of clinical, cognitive, and psychological outcome measures[43]. These have previously been investigated across the spectrum of TBI severities, finding that psychological as opposed to cognitive outcomes best distinguish this milder end of the injury spectrum[201]. I therefore focussed investigations on two common outcome measures of functional recovery (GOSE) and symptomatic recovery (postconcussive symptoms), at the commonly used recovery time of six months post-injury. This helps us to understand the long-term burden of mTBI.

As presented in the introduction to this thesis, GOSE is a measure of functional outcome which is commonly dichotomised into good versus poor outcome. In mTBI, this occurs at GOSE-8 (upper good recovery; return to daily life) versus ≤7 (between GOSE-1; death, to GOSE-7; return to work with some injury-related problems), respectively. This has clinical value in distinguishing recovery but has been criticised for its lack of specificity, low sensitivity, and ceiling effects in mild populations[49]**.** I therefore also investigated postconcussive symptoms using the RPQ. This is a self-report measure of experienced severity of 16 most-commonly cited post-concussion symptoms compared to pre-injury levels, on a five-point scale from 0 indicating 'not experienced' to 4 as 'a severe problem'. The RPQ can additionally be used to binarize groups into good and poor outcome- as with GOSE. Clinical postconcussion syndrome is commonly defined by the International Classification of Diseases, Tenth Revision, as having a history of TBI and three or more postconcussive symptoms. However, this is not universally agreed upon resulting in several further evaluation methods[202,203] and discrepancies in what constitutes an 'experienced' symptom[204]. Prevalence rates of postconcussive syndrome in mTBI populations vary substantially depending on the classification method used, however the most common method in the literature aligns with ICD-10 criteria with an 'experienced' symptom rated at 2[205]. Using this definition, the present cohort was split into postconcussive symptom positive (PCS+) or negative (PCS-) at 6-months post-injury, to enable results to be best compared with existing literature.

2.2.3 Alternative biomarkers

To demonstrate the relative value of functional neuroimaging, it is first important to demonstrate that existing potential biomarkers are not sufficient at prognosticating outcome in this cohort. In a highly overlapping mTBI cohort derived from CENTER-TBI, it was previously shown that there is a lack of gross structural change following mTBI using structural MRI[206]. This previous project identified no changes in total brain volume, white matter volume, or grey matter volume, between mTBI and healthy controls. Thus, the absence of CT abnormality defined in my inclusion criteria, or gross structural abnormality on T1-weighted MRI in an overlapping CENTER-TBI cohort, suggests further exploration is required.

The first alternative biomarkers I explored were global microstructural metrics obtained from DTI, as these have shown prognostic value to other CENTER-TBI cohorts[128], albeit not necessarily the 'mildest' cohort as CT abnormalities were not an exclusion criterion. DTI data for the present cohort were again obtained in the acute phase post-injury at the same timepoint as rsfMRI data. Values were taken as a subset of data included in a previous study on mTBI[128]. The metrics investigated were global FA (scalar value between 0 and 1 from isotropic to anisotropic diffusion) and global MD (average magnitude of diffusion in three axes). Mean values were obtained within each subject's cortical grey matter, non-cortical grey matter, and white matter, as determined by subject-specific masks obtained during image segmentation. These were extracted from acquisitions with additional B0 if available for each patient, as this provided superior quality of preprocessing in their pipelines[128].

The second alternative biomarkers I explored were early blood-based serum levels, which have proven successful in previous studies in predicting damage on CT in mTBI[207,208], and adding additional prognostic value to existing models in more severe injury[209]. Six key blood-based biomarkers were available within CENTER-TBI, specifically, neuron-specific enolase (NSE), S-100 calcium-binding protein B (S100B), glial fibrillary acidic protein (GFAP), Tau, ubiquitin C-terminal hydrolase -L1 (UCH-L1), and neurofilament light chain (NFL). Each of these has been previously linked to different aspects of injury. NSE was historically the most frequently investigated marker associated with poor outcome and is involved in glycolysis (the metabolic pathway that converts glucose into pyruvate)[210].

However, recent studies have suggested that S100B is more beneficial than NSE in understanding injury and outcome[210]. S100B is a biomarker of astroglial breakdown, and thus far is the only marker to be implemented in TBI guidelines in Scandinavia to triage CT[211]. Other markers investigate neuroaxonal damage (NFL), neuronal cell body injury (UCH-L1), neurodegeneration (Tau), and astrogliosis/astroglia injury (GFAP)[212]. Whilst these markers have shown utility in triaging the need for CT and injury monitoring in moderate and severe TBI, there may be limited prognostic utility in milder types of injury[213]. However, GFAP has shown the greatest predictive value of CT abnormality and some prognostic utility in mTBI, above all other biomarkers combined[208].

Blood-biomarker values were extracted from CENTER-TBI (CORE v3.0) and collected within 30 days post-injury for inclusion. A clinical-use automated system was used to measure S100B and NSE, using an electrochemiluminescence immunoassay kit (ECLIA). These were run on the e 602 module of Cobas 8000 modular analyser (Roche Diagnostics, Mannheim, Germany) at the University of Pécs, Hungary. Whereas, serum Tau, GFAP, UCH-L1, and NFL were analysed using an ultrasensitive immunoassay using digital array technology (Single Molecule Arrays, SiMoA)-based Human Neurology 4-Plex B assay (N4PB). These were run on the SR-X benchtop assay platform (Quanterix Corp., Lexington, MA) at the University of Florida, USA.

2.2.4 Multicentre harmonisation

The increasing prevalence of multicentre studies such as CENTER-TBI has placed a greater need for harmonisation. That is, the simple fact of having different scanners at different locations can induce site-differences which can be comparable to the differences between patients and controls[214]. Harmonisation aims to ameliorate these effects, whilst retaining biologically relevant information such as clinical group, sex, or age. Three main types of harmonisation can be employed. The first is to harmonise imaging acquisition protocols across sites, to start with the most similar images as possible. This was completed within CENTER-TBI[43], however previous studies have shown that significant site-effects can remain despite protocol harmonization between scanners[215,216], which can importantly mask true effects of disease[217]. It is important to consider the issue of multisite acquisition in the context of the present cohort, as participants were acquired from eleven different site locations across Europe

encompassing fourteen different scanners (**Appendix 2.2)**. The ratio of TBI to controls per site/scanner also varied substantially, meaning great care is needed to remove site effects whilst retaining biologically relevant information.

Two further types of harmonisation can be applied; to harmonise the whole images themselves or harmonise the imaging-derived phenotypes[214]. The former is often impractical in large-scale studies, due to the vast computational resources required to harmonise every image in a database prior to sharing. For this reason, harmonisation is most commonly applied on extracted imaging features, such as FA or MD. Using such methods should be done with caution as they could alter imaging-derived phenotypes inappropriately if not used correctly, as highlighted by Richter et al., (2022)[218].

I therefore performed additional harmonisation of imaging-derived data using an empirical Bayesian method ('ComBat'). This procedure has been successfully applied in previous diffusion-imaging[219], cortical thickness[220], and rsfMRI studies[221,222], to reduce scanner-specific variation whilst retaining important biological information. Importantly in the present context, is has been successfully applied for small sample sizes per site and biological variation across sites as presented in the current cohort who vary in the ratio of TBI to controls per site. Covariates of group, age, and sex were included in the models to preserve biologically relevant information and avoid overcorrection. This harmonisation process is used throughout this thesis and is described in relevant sections of each chapter.

Harmonisation was applied to DTI metrics (FA, MD), for differences in site/scanner. In doing so, n=1 control subject was excluded due to being the only participant from that site/scanner, and thus unable to include in harmonisation pipelines which require at least n=2 subjects per site.

2.2.4 Statistical analysis

All statistical analyses were conducted using R (v.4.1.2) at a false discovery rate (FDR)-corrected significance level of $P \leq 0.05$ unless otherwise stated. Missing demographic data typical of large datasets were handled by multiple imputation using the Multivariate Imputation by Chained Equations algorithm[223] with $n = 5$ imputations. This modelled

missing data using existing age and sex with a logistic regression model for binary data (missing sex; n = 2 controls) applied within groups to avoid potential group effects.

Control and mTBI groups were initially compared for two-tailed differences in age (Fisher's exact) and sex (chi-squared). Tests were chosen to account for the categorical nature of recruitment in CENTER-TBI protocols[43] which aim to combat possible differences in admission rates during study recruitment. However, age is hereafter treated as a continuous covariate in all statistical analyses. These groups were further compared in diffusion-weighted metrics following statistical harmonisation for site/scanner. Controls and patients were compared using a linear model with covariates of sex and age.

Outcome groups (GOSE, PCS) were then compared for covariates of age and sex using identical tests as described above, and additionally for time since scan of injury (independent samples t-test) and baseline GCS (Fisher's exact). Groups were then compared in DTI metrics and blood-based biomarkers using additional covariates of time since injury and baseline GCS within the linear model.

2.3 Results

2.3.1 Clinical characteristics

Patient and control groups did not differ in age ($X^2$1= 2.2, p=.34) or sex ($X^2$1=0.2, p=.64). Clinical characteristics for these groups can be seen in **table 2.1**. Imaging was performed at a mean of 13.74 (SD 9.86) days post-injury in the mTBI group, and 6-month outcomes collected at a mean of 197 (SD 33.0) days post-injury. These demographics were largely similar to the wider cohort of mTBI collected within CENTER-TBI, with identical inclusion criteria except presence of imaging data (**Appendix 2.3**). Thus, my cohort can be considered a representative sample of 'mildest' mTBI across Europe who present to hospital settings.

Table 2.1. Baseline demographic, injury, and outcome measures by group.

	Control (n=76) n (%)	**mTBI (n=108)** n (%)
Age		
18-35	26 (34.2)	29 (26.9)
36-55	36 (47.4)	50 (46.2)
55-70	14 (18.4)	29 (26.9)
Sex		
Male	46 (60.5)	69 (63.9)
Female	30 (39.5)	39 (36.1)
Glasgow Coma Score		
15	-	88 (81.5)
14	-	19 (17.6)
13	-	1 (0.9)
Injury Cause		
Road Traffic Incident	-	51 (47.2)
Incidental Fall	-	38 (35.2)
Other Non-intentional injury	-	7 (6.5)
Violence/Assault	-	7 (6.5)
Act of Mass Violence	-	1 (0.9)
Unknown	-	4 (3.7)
Strata		
Emergency Room	-	48 (44.4)
Admission	-	60 (55.6)
6 Month GOSE		**n=106**
Score (n)	-	1 (1) 4 (1) 5 (2) 6 (18) 7 (26) 8 (58)
Complete	-	58 (54.7)
Incomplete	-	48 (45.2)
6 Month PCS	-	**n=98**
PCS+	-	31 (31.6)
PCS-	-	67 (68.4)

2.3.2 Mild injury does not necessitate mild outcome

Importantly, 45.3% (n=48/106) of TBI patients were not fully functionally recovered 6-months post-injury, according to the GOSE. Whilst these statistics did appear to improve over time, with a greater proportion reaching a full functional recovery by 12 months, a substantial proportion of participants remained functionally burdened well after their injury. **Figure 2.2** shows the dichotomisation of GOSE scores at 3, 6, and 12 months, in complete (GOSE-8) and incomplete (GOSE≤7) recovery in the present cohort. Group membership on outcome categories at 6-months was not related to age ($X^2 1=0.6$, p=.75), sex ($X^2 1= 0.1$, p=.76), or baseline injury severity as measured by GCS (Fisher's exact, p=.79).

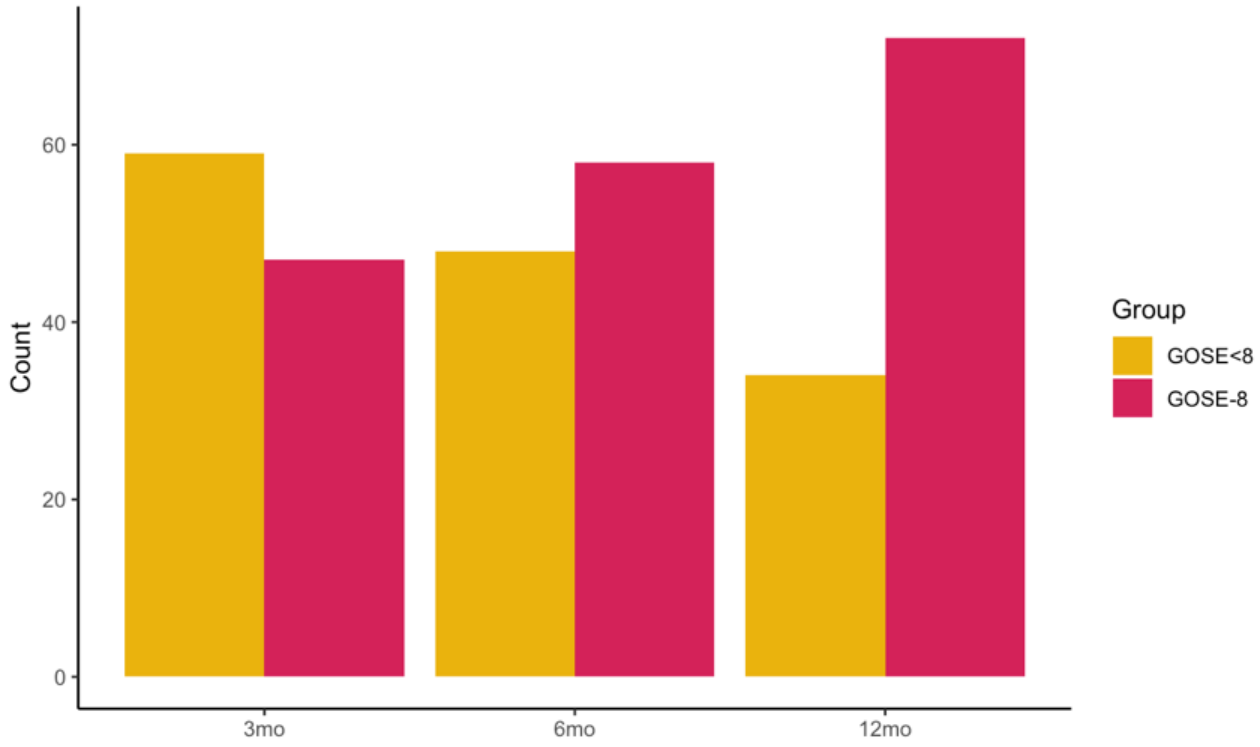


Figure 2.2. GOSE status at 3, 6, and 12 months. Data are coloured by functional outcome group. Each timepoint had GOSE reported for the same cohort of n=106 patients.

The second outcome measure considered was postconcussive outcome, as measured using the RPQ where n=98 patients had completed this at 6-months post-injury. Strikingly, 31.6% of the mTBI cohort were classified as PCS+. Group membership was not related to age ($X^2$1=0.2, p=.90), sex ($X^2$1=0.001, p=.97), or baseline GCS (Fisher's exact, p=.69). The PCS+ group was largely a subset of the incomplete functional recovery group, as n=28/31 PCS+ were also classified as GOSE≤7. Thus, n=51 participants were not functionally and/or symptomatically recovered at 6 months, representing 47.2% of the cohort.

Reports for each postconcussive symptom within the RPQ are presented below in **figure 2.3**. Most prevalent symptoms reported at greater than pre-injury levels were fatigue (n=34/98), poor concentration (n=25/98), and headaches (n=25/98).

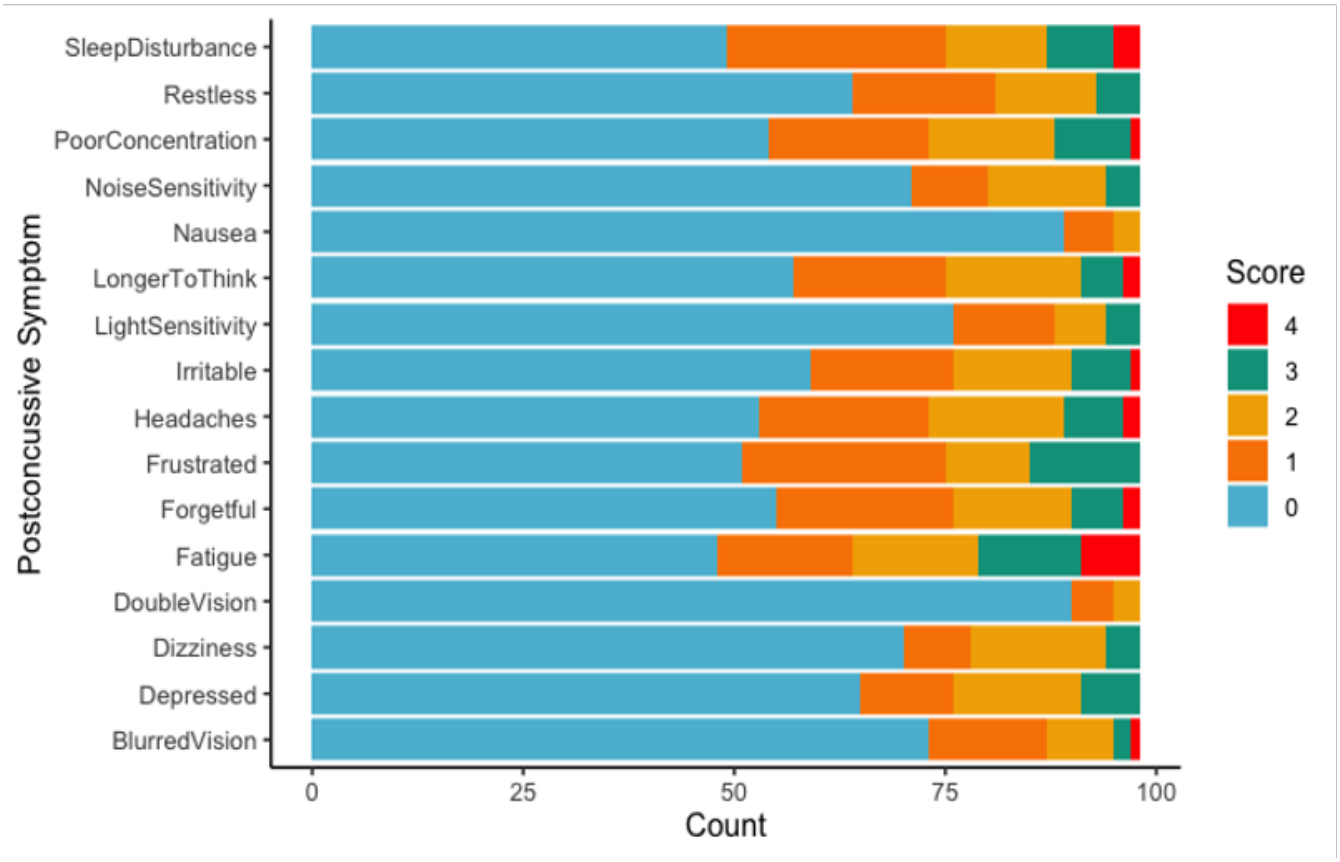


Figure 2.3. Rivermead postconcussive symptom reporting. Measured as count per response. Responses to postconcussive items on the RPQ recorded at 6 months post-injury. Score indicates symptom experience compared to pre-injury levels on a five-point scale: 0 = 'not experienced at all', 1 = 'no more of a problem', 2 = 'a mild problem', 3 = 'a moderate problem', 4 = 'a severe problem'.

Based on chronic PCS groups, total RPQ score was compared from baseline admission to 6 months to ascertain whether baseline scores were related to later chronic outcome. Baseline scores were collected for n=85/98 of the patients with 6-month scores, at 1.02 +/- 1.85 days post-injury. As expected, total RPQ score at 6 months in this PCS+ group was significantly higher than in the PCS- group using a Mann Whitney U-test (W = 25 p<.001). Importantly however, total RPQ scores showed no statistical difference between the two groups at baseline (W = 658, p = .19). This is shown in **figure 2.4**, demonstrating the differential trajectories of recovery versus persistence in the two groups. Thus, baseline symptomology is unable to predict later symptom emergence/persistence in this cohort, and other markers of outcome require investigation.

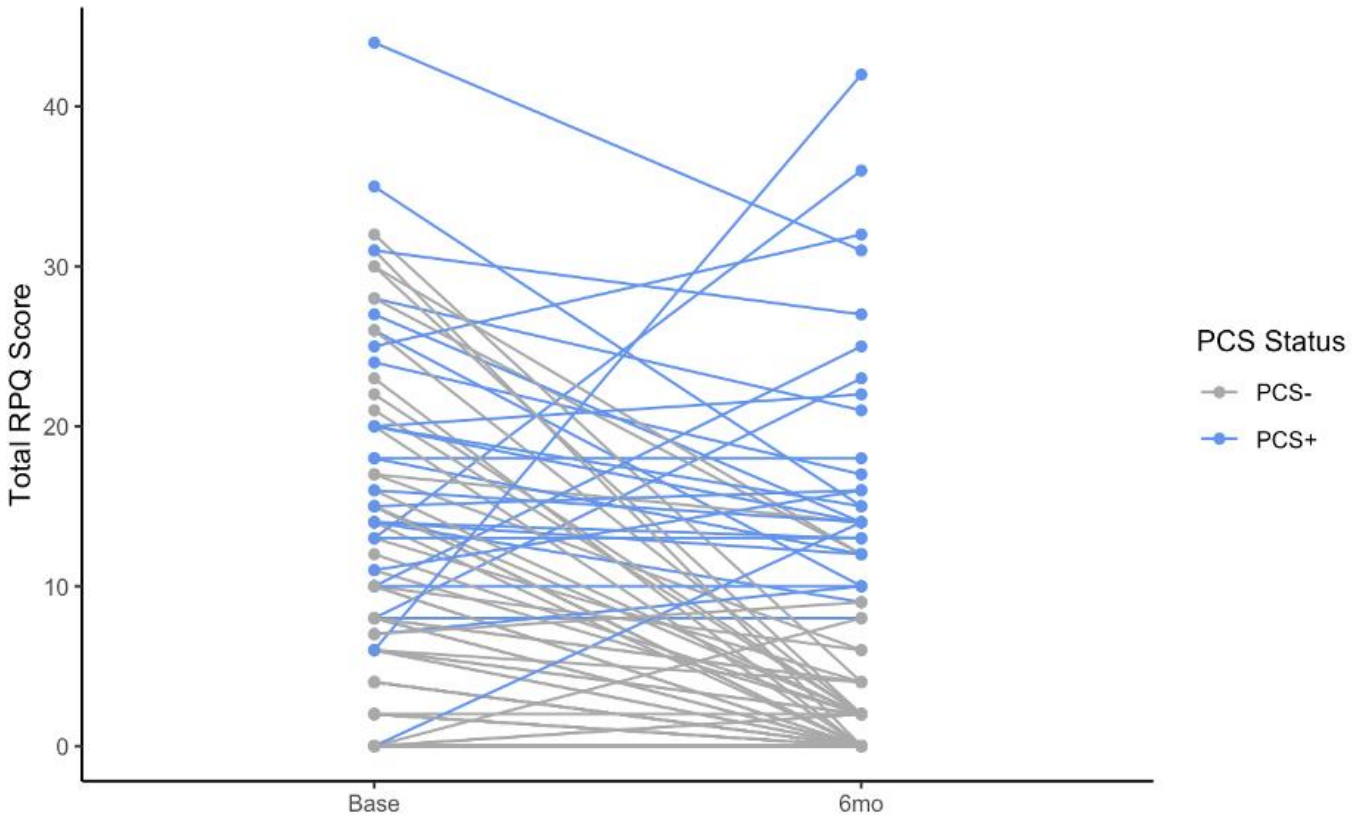


Figure 2.4. Change in postconcussive symptoms from baseline to 6-months. Total RPQ score per patient, color-coded by 6-month postconcussive (PCS) group. Lines join individual patient scores at each timepoint to demonstrate symptom change from no significant difference between groups at baseline, to significantly higher RPQ scores in the PCS+ (presenting 3 or more symptoms) group at 6 months.

Finally, alcohol intoxication has been shown to influence both injury and outcome in previous studies of mTBI[224,225], thus, I further assessed this in my mTBI cohort. Importantly, no participants presented pre-injury substance use disorders as specified in my exclusion criteria. Alcohol involvement at time of injury was reported in 16/108 individuals with mTBI, however baseline injury severity was not significantly different in this group to the non-alcohol involvement injury group ($X^2 2=0.7$, p=.41), nor were 6-month outcomes (GOSE $X^2 1=0.5$, p=.50; PCS $X^2 1=0.1$, p=.79). A behavioural questionnaire conducted at 6-months post-injury indicated that no subjects were consuming more than six alcoholic drinks once per month, further suggesting there is limited concern for influence of alcohol on this mTBI cohort's injury or recovery.

A further influential variable on injury and outcome may be prescription medication received In my mTBI cohort, 34/108 reported medication use prior to injury including that for hypertension (n=12), anti-allergy medication (n=8), asthma (n=6), diabetes (n=4),

high cholesterol (n=3), eczema (n=2), and hormone replacement therapy (n=1). The small sample sizes and range of medications reported meant none were explicitly tested for their influence on outcome. However, some links have been made between high blood pressure and its management, with mTBI history and cognitive decline particularly in males[226]. Moreover, I have not considered the influence of post-injury medication and treatment as these proved difficult to consistently track over time in all individuals. This is a limitation of my cohort, however questions regarding the impact of specific medications on injury and outcome would be better assessed using the vast CENTER-TBI database, rather than my small sub-cohort requiring functional neuroimaging.

2.3.3 The need for functional biomarkers

DTI metrics were available for a subset of my cohort (n=107/108 mTBI, n=56/76 controls). There were extracted from acquisitions with additional B0 if available (n=140 with additional B0, n=23 without). These yielded no significant differences in FA or MD across any global measures of cortical grey matter, non-cortical grey matter, or white matter, in TBI versus controls, or related to 6-month outcomes, with p-values shown in **figure 2.5**. Thus, one can deduce a lack of global microstructural white matter damage across this cohort of mTBI.

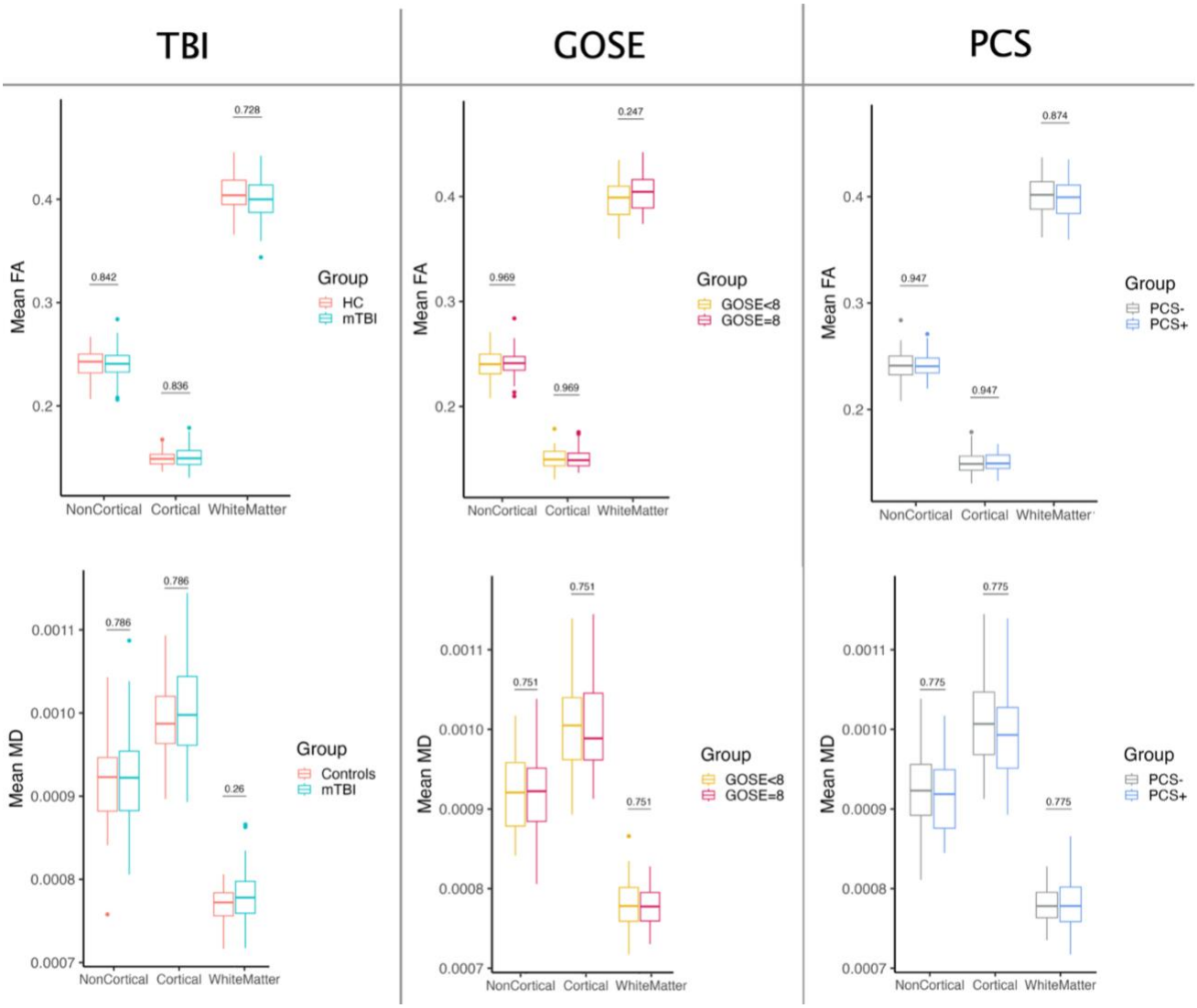


Figure 2.5. Global diffusion tensor microstructure values acutely after injury. Boxes show white matter metric of fractional anisotropy (FA- top) and mean diffusivity (MD- bottom), and group comparison between patients and controls (left), complete vs incomplete functional recovery (centre), and complete vs incomplete symptomatic recovery (right). P-values are given for each test after FDR correction.

The final biomarkers of interest were blood-based serum levels. Values were obtained from CENTER-TBI (CORE v3.0) for six markers, for an average of 84 mTBI participants per marker (range 79-92) and collected at a mean of 2.98 (SD 6.80) days post-injury. Values were not available for the healthy control cohort. These showed no significant differences between GOSE or PCS groups in NSE ($F_{1,86}$=1.74, p =.38; $F_{1,82}$=0.07, p =.79), S100B ($F_{1,87}$=4.08, p=.28; $F_{1,83}$=1.35, p =.58), GFAP ($F_{1,76}$ =1.33, p =.38; $F_{1,72}$=0.76, p =.58), Tau ($F_{1,76}$=0.02, p =.89; $F_{1,72}$=0.19, p =.79), UCH-L1 ($F_{1,72}$=0.76, p=.46; $F_{1,68}$=1.00, p =.58), or NFL ($F_{1,75}$=1.56, p =.38; $F_{1,71}$=1.50, p =.58), respectively after FDR-correction for multiple comparisons.

2.4 Discussion

This chapter presented the 'mildest' mTBI cohort which present to hospital settings; having no damage on CT, no neuropsychiatric history or history of concussion, and did not enter the ICU. Using high-quality data from CENTER-TBI[43], I have designed such a cohort with strict inclusion criteria with sufficient sample sizes for large-scale investigation of neuroimaging data. Despite being labelled as mild in injury severity, almost half of this mTBI cohort showed incomplete 6-month recovery, which was not related to common variables of age, sex, or baseline injury/symptom severity. These high rates of incomplete recovery in this group corroborate that of previous large-scale studies in mTBI[50,51,54,198]. For example, TRACK-TBI found a that 56% of their CT-negative mTBI patients presented incomplete functional recovery at 6-months post-injury according to GOSE[51], and 34-43% of all mTBI patients in CENTER-TBI displayed persistent postconcussive symptoms at 6-months post-injury. Thus, 'mild' injury does not always lead to mild outcome.

This specific group is arguably the most likely to achieve a full recovery as they do not present existing variables of poor outcome. They are thus at greatest risk of the disconnect between clinical predictions of outcome and real-world experiences of poor outcome[198], and may be insufficiently supported in clinical settings. Novel biomarkers are required to better support this growing population. In this chapter I investigated two types of emerging biomarkers- global diffusion metrics derived from DTI and blood serum levels. These measures have previously shown prognostic value for more severe TBI, and particularly for CT positive mTBI[128,207,208].

In the present cohort without such damage on CT, however, these did not display any differences between outcome groups in global microstructural metrics (FA, MD), nor any single blood-based marker. There has been great interest in DTI for better characterising and prognosticating mTBI, with recent success in large-scale studies finding globally increased AD and MD and reduced FA at both two weeks and six months post-injury, associated with incomplete recovery[133]. However, these findings were not consistent when considering subgroups of CT/MRI negative patients[134], or CT-negative patients who did not experience loss of consciousness[135]. As such, the present results align with previous suggestion that global diffusion metrics may not hold the same prognostic

potential for CT-negative mTBI than that shown for CT-positive. Similarly, blood-based biomarkers have proven successful in predicting damage on CT in mTBI[207,208], with prognostic value in more severe injury[209]. Particularly GFAP has shown prognostic utility in mTBI[208], however I found no single marker had significant difference between outcome groups in this cohort.

These results suggest that existing biomarkers and baseline injury characteristics are insufficient in prognosticating the high rates of poor outcome presented in this cohort. Thus, *functional* neuroimaging warrants further investigation for novel biomarkers of poor outcome.

2.4.1 Limitations

The limitations of my results are threefold. Firstly, blood-based biomarker levels can vary substantially over time post-injury. Data available for this cohort had large variation in mean time since injury, and I aimed to keep sample sizes as large as possible by their inclusion. I therefore cannot exclude the possibility that acute blood-biomarker levels had early changes from control levels, which were not detectable in these data.

Secondly, only global FA and MD values were investigated in this cohort, and not values within individual tracts or regions which could be more informative. For instance, specific central tracts subject to high sheering and strain forces during injury, such as thalamocortical radiations, would be interesting to consider in future study. However, I chose to focus on global metrics alone as a previous study identified such local white matter changes in tandem with global alterations in an acute mTBI cohort including those with damage on CT[128]. Given that my CT-negative cohort did not show global microstructural change, combined with the heterogeneity of mTBI and its proposition as a global disorder, I did not explore local changes further. Moreover, growing evidence suggests mTBI is a global disorder[177], due to its vast variety in injury location, duration, and context, making little overlap between individual injuries. Global metrics hence appeared more informative than local measures in this instance. Thus, these results are merely to demonstrate null findings using a global approach in DTI for this cohort, to support my focus on functional neuroimaging techniques.

Finally, in my definition of 'mildest' TBI I am limited to those presenting to hospital settings and who have undergone CT. These patients are thus the mildest individuals from the CENTER-TBI dataset following my strict inclusion criteria, but may not represent the full burden of mTBI who do not present to hospitals. Moreover, I have only considered the simple definition of GCS 13-15 for mild TBI, and not considered further variables such as loss of consciousness. Whilst this variable has shown to influence prognostic ability of one previous DTI study[135], using the standard GCS definition best aligned with existing literature and common clinical practice. This decision was to ensure my future results were interpretable for real-world populations and easily replicable in other datasets who may not have such vast data collection as CENTER-TBI.

2.4.2 Conclusion

To reiterate, this 'mild' group is currently expected to recover post-injury, yet we are unable to discern the almost 50% who experienced incomplete recovery. The absence of neuropsychiatric disease, previous concussion, visible structural damage, microstructural abnormality, or difference in these blood biomarkers in our poor outcome groups underlined the need for novel biomarkers to help prognosticate chronic outcome. The following two chapters explore novel *functional* biomarkers using rsfMRI, and their relationship to long-term outcome.

Chapter 3

Globally disrupted functional network connectivity in mild traumatic brain injury

3.1 Introduction

Having substantiated the need for novel biomarkers in mTBI, I next looked to resting-state functional MRI (rsfMRI). This chapter reconciles a previously mixed literature using this technique and proposes mTBI as a *functional* disorder with *global* neurological consequences. We begin to better understand the acute sequalae following injury and its prognostic potential, by relating acute imaging to 6-month functional outcome.

3.1.1 Previous literature

As discussed in the introduction to this thesis (**Chapter 1**), several groups have looked to rsfMRI to characterise the acute neurological consequences of mTBI but have thus far reported mixed findings[132]. Studies have reported both increases[172] and decreases[171], or indeed no change [174], in functional connectivity of resting-state networks (RSNs, e.g., default mode network) post-injury, leading to questions regarding the utility of functional imaging in a clinical setting for this population. As previously discussed, these discrepancies may be partially explained by the great heterogeneity of mTBI populations[2]. A large and well-defined sample is required to account for this increased variance, whereas studies have been limited by small sample sizes commonly between 20-50 participants per group[132] with the exception of one recent study with n=91 mTBI but merely n=23 controls[175]. There is furthermore great variation between studies in image analysis methodology (seed-based versus data-driven), time since injury within cohorts, and demographic factors such as age and sex; both of which require further investigation of their effects on recovery[19,176]. Such a range of approaches and cohorts will inevitably incur a range of results with potentially conflicting conclusions.

Nevertheless, some key variables appear to align between studies. Those studies that report *hyper*connectivity take a distributed approach, using whole-brain measures, studying younger cohorts, and more homogenous populations such as sports-related concussion[192,227,228]. Studies reporting *hypo*connectivity focus on seed-based differences between and within networks, in older and more variable cohorts, such as those recruited at hospital settings[171,175,178].

A further movement within the mTBI literature is recognising the *global* functional effects post-injury[177]. This global approach may be deliberately useful for cases of mTBI, as individual injuries can vary substantially, rendering regional approaches applicable to some patients and not to others. Indeed, several studies of acute mTBI, particularly those with larger sample sizes, have reported functional changes affecting all resting-state networks[171,178–180], substantiating a connectome-scale of alterations post-mTBI. There is thus a need for clarification of this literature as current studies across the mild populations are numerous but with variable findings, small samples, and insufficient clinically relevant follow-up.

A data-driven approach is arguably the most beneficial for clarifying this mixed literature. This avoids researcher biases of network inclusion, which have historically been largely cortico-centric[132]. This means regions central to mechanical forces experienced during injury, such as the thalamus and general subcortex[113], have been ignored in network approaches despite their known integral role in network function[121]. One data-driven whole-brain approach to researching networks is Independent Component Analysis (ICA), which separates fMRI data into spatially independent components with similar timecourses of activation. These components can then be identified as resting-state networks, derived directly from the data. This approach contrasts the traditional seed-based approach of network definition, which uses binary masks of a network based on existing parcellations, which is decided upon by the researcher. ICA may furthermore be superior for mTBI due to its whole-brain definition of networks. Each independent component's (IC) spatial map is constructed from z-scores of temporal coactivation for every voxel in the brain to that component. The inclusion of each voxel therefore implicates the whole brain in network definition. This provides a more globally integrated approach to network neuroscience than extracting average timeseries' from binary region of interest (ROI) masks, which ignore regions outside of network masks and often exclude the subcortex and cerebellum entirely.

Several authors have used ICA to study acute mTBI, most recently finding widespread connectivity changes within and between all studied RSNs partially related to cognitive impairment[178], thus echoing the connectomic scale of functional changes in mTBI increasingly being found in larger studies. A recent review of ICA approaches in acute mTBI concluded that, whilst a promising technique for mTBI investigation, evidence is

somewhat conflicting as in the wider mTBI literature[191]. They found that of the sixteen studies using ICA for rsfMRI, six presented *hyper*connectivity, four presented *hypo*connectivity, five presented both simultaneously, and one reported no functional changes post-injury. Nevertheless, they highlight that functional *hyper*connectivity may be adaptive in the face of injury, as multiple studies found that increased acute connectivity was associated with fewer postconcussive symptoms. This relationship was found with concurrent symptoms shortly after injury in frontal DMN regions[229], and related to 6-month symptoms of CT negative patients in posterior regions of large-scale networks including visual and DMN[171]. Nevertheless, authors concluded that more research is required to fully understand the mal/adaptive nature of such changes in mTBI, and their relationship to outcome[191]. They further concluded that ICA approaches to functional connectivity may aid our identification of diagnostic and prognostic markers in mTBI.

ICA has also complemented and extended classic seed-based connectivity findings in two acute mTBI studies using these approaches concurrently. Iraji and colleagues (2015)[230] found decreased within- and increased between-network connectivity of the DMN, with widespread alterations with hippocampus, amygdala, and thalamus, in both approaches. Findings were bolstered using ICA which showed that within-DMN voxel coactivation and brain-wide spatial extent of DMN was reduced in acute mTBI, providing additional network-specific measures unobtainable from their ROI-based study. Similarly, Madhavan and colleagues (2019)[175] found decreased seed-based connectivity of DMN, visual, and motor regions, related to symptom severity at 3 months, but this outcome was not predicted within ICA networks. As ICA-derived networks explicitly separate between and within-network connectivity whereas seed-based functional connectivity measures these components combined[231], their seed-based results more strongly suggest that altered *between*-network connectivity underpins symptom prognosis due to lack of significant results within ICA networks[175].

These studies have demonstrated that ICA can yield more specific results than seed-based approaches alone, but so far have offered mixed results regarding functional change in acute mTBI, and how these might be related to outcome. Such findings warrant further exploration using ICA to find early prognostic markers on a brain-wide scale. Moreover, no study has attempted to link such markers to *treatment* of mTBI

patients, thereby limiting findings to a purely research setting. Translational investigations are needed to push the rsfMRI field forward from research into clinical practice. This aims to bridge gaps between neuroimaging and preclinical work for future treatment development, but also translates findings into a more accessible framework for clinicians. This has the power to influence clinical practice now, by increasing awareness that mild TBI can have vast functional consequences which may require ongoing care.

3.1.2 Aims and hypotheses

I therefore aim to study whether functional connectivity of resting-state networks has diagnostic and prognostic utility for CT-negative mTBI and attempt to clarify a mixed existing literature on acute functional change. This utilises the largest acute mTBI sample with rsfMRI to-date, employing data-driven methods to explore its relationship to 6-month recovery. I additionally design a novel marker of component distribution complexity to better understand whether resting state network architecture is affected by injury. Finally, preliminary associations are made with the activity of neuromodulatory brainstem nuclei to probe therapeutic possibilities of underlying functional change.

My hypotheses are as follows:

1) Mild TBI can be described as a *functional* disorder.
2) Acute functional changes occur on a global scale, affecting all studied networks in their definition and between-network connectivity.
3) Acute functional changes differentiate 6-month functional outcomes, with neurotransmitter-relevant associations for future study.

3.2 Method

3.2.1 Cohort

The cohort for this chapter was defined in **Chapter 2** and includes n=108 mild TBI patients with 3T structural (T1w) and functional (rsfMRI) data acquired in the acute timepoint post-injury, and n=76 healthy controls. Imaging was performed at a mean of 13.74 (SD 9.86) days post-injury, and 6-month outcomes collected at a mean of 197 (SD 33.0) days

post-injury. All data were obtained from CENTER-TBI (CORE 3.0). Acquisition protocols for these imaging data are described in the central CENTER-TBI resources at https://www.center-tbi.eu/project/mri-study-protocols, and summarised for this cohort in **Appendix 3.1,** which were centrally harmonised as far as possible.

This chapter primarily focuses on outcome as measured by 6-month GOSE, which was recorded for 106/108 mTBI participants. Importantly, 45.3% (n=48/106) of 'mild' TBI patients were not fully functionally recovered 6-months post-injury. I additionally considered 6-month postconcussive symptom groups, completed by n=98 participants, whereby 31.6% were classified as PCS+.

3.2.2 Imaging preprocessing

For preprocessing T1w MRI and rsfMRI data, I first considered an in-house pipeline using SPM 12[232]. However, such an approach required manual origin setting of every image. This is unfeasible in large datasets and can heavily impact the success of coregistration of these images and their respective normalisations if not correctly performed. Additionally, the SPM pipeline was originally created for handling lesioned brains, which was deemed unnecessary given the mild and non-lesioned nature of my cohort. Rather, I aimed to introduce a standardised and data-driven approach to my analysis, which could be undermined by in-house preprocessing conventions.

Thus, preprocessing was largely performed using fMRIprep[200] (v1.5.4). This combines best-judged aspects of different software for a standardised and freely accessible preprocessing pipeline. Differences in preprocessing have the potential to influence group-level results[233,234], and thus using this standardised approach is beneficial in enabling greater replication between groups who use identical preprocessing parameters. fMRIprep provides 'minimal' preprocessing of neuroimaging data, including structural MRI and fMRI, as a baseline for neuroimaging researchers to then make study-specific preprocessing decisions. For example, which denoising parameters to include and how much spatial smoothing should be applied, which do not have defined criteria within the wider rsfMRI literature[234] and can vary depending on the research question. Each of these preprocessing steps is now explained in turn. A standardised boilerplate of

the preprocessing pipeline automatically generated by fMRIprep is given in **Appendix 3.2** for additional reference.

Firstly, T1-weighted structural MRI data were corrected for non-uniformity in image intensity using N4BiasFieldCorrection[235], distributed with ANTs 2.2.0[236]. These images were then skull-stripped, also using ANTs, and segmented into three tissue classes using FSL's FAST (FSL 5.0.9). These were white-matter, grey-matter, and cerebrospinal fluid. Structural data were then spatially normalised to the ICBM 152 Nonlinear Asymmetrical template version 2009c (MNI152NLin2009cAsym) standard-space template using nonlinear registration with antsRegistration (ANTs 2.2.0), using brain-extracted versions of the T1-weighted reference image for each individual and the T1 template. This was applied to bring all images into an identical space for group-level analysis.

Next, a reference volume of each rsfMRI image was generated and skull-stripped using a custom methodology of *fMRIprep*. This reference image was subsequently coregistered to the T1-weighted reference image (in subject-space) using FLIRT (FSL 5.0.9)[237] with boundary-based registration cost-function. This used 9 degrees of freedom to account for distortions remaining in the rsfMRI reference image. Head motion was then estimated by applying calculated transforms to correct for 6 directions of rotation and translation calculated using MCFLIRT (FSL 5.0.9)[237]. This was applied in subject space by resampling. Corrected images were then normalised into standard space, using the previously calculated transformations applied to T1-weighted images.

Additionally, functional data were denoised by signal regression, and spatially smoothed with a 6mm gaussian kernel. Denoising included covariates of average white matter and cerebrospinal fluid timeseries', rigid-body head motion (12 DOF), and temporal high-pass filter. These are estimated within fMRIprep, whereby high-pass filtering uses a General Linear Model with the Discrete Cosine Transformation approach to produce a basis of discrete cosines for frequencies slower than 0.008 Hz. Any volumes identified as motion outliers (exceeded 0.5 mm framewise displacement or 1.5 standardised DVARS) were removed from the data, and any data exceeding n=164 volumes were also removed from the end of acquisition for group-level analysis. No subjects were removed entirely following censoring, as all presented over 4 minutes of uncontaminated data[234].

Higher motion parameters are a common signature of patient populations, and as such should be treated with caution given the known effects of motion on functional connectivity estimates[238]. However, this poses a conflict; we need to avoid spurious motion-induced changes in connectivity estimates whilst still including a representative patient sample which may intrinsically display greater motion. Simply excluding all patients with high motion may be systematically excluding a group of interest and introduce sampling bias. There are several alternative methods for signal denoising and motion regression, such as CompCorr[239] and ICA-AROMA[240], which are under constant debate within the rsfMRI literature[233,234]. I chose to use signal regression and volume censoring as this combination has been shown as the most effective and stringent method of motion correction in resting-state data whilst retaining control over what is being discarded as noise[234]. There is less control in data reduction techniques and whether the quality of signal-to-noise is being equally separated across subjects and across groups. Moreover, additional data reduction and noise removal was performed during subsequent steps of network definition described below, further supporting my use of a minimal denoising strategy during preprocessing.

3.2.3 Network definition

A data-driven approach was used to derive and define resting-state networks. This approach is preferable to seed-based techniques in this context, as it provides a reproducible pipeline for future research, is not restricted to pre-defined network masks, and further avoids assumptions that neurotypical (i.e., similar to healthy controls) network definition is appropriate in this clinical population on an individual level. I therefore used Independent Component Analysis (ICA), which separates fMRI data into spatially independent components (ICs) with similar timecourses of activation. Group-level spatial ICA was performed using the Group ICA fMRI toolbox (GIFT v3.0c[241]) in three stages: a) data reduction, b) group-level ICA, and c) subject-level back reconstruction. An overview of this process is shown below in **figure 3.1**. A grey matter mask was created to constrain the analysis region (MNI152NLin2009cAsym 2mm grey matter probability map, threshold=0.2), and the first 5 volumes for each participant excluded to ensure scanner stabilisation.

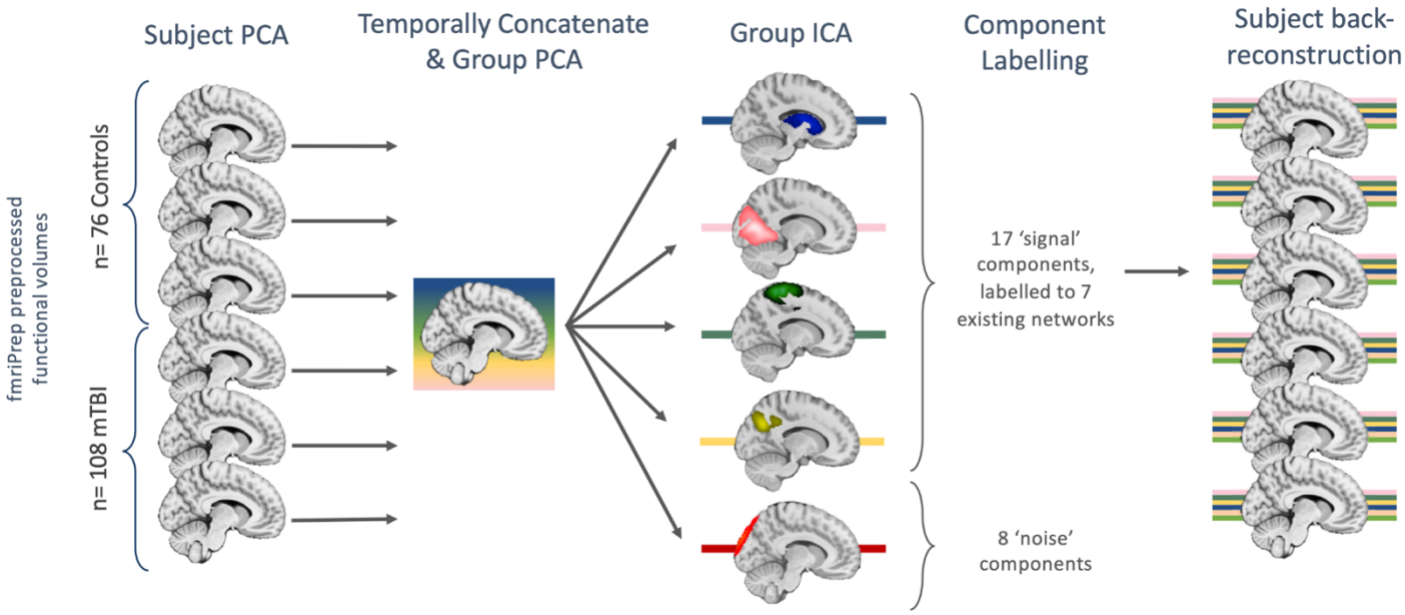


Figure 3.1. Overview of group-ICA approach to network derivation and definition. PCA = principal component analysis, ICA = independent component analysis.

The ICA approach was as follows; GIFT's standard two-stage data-reduction was applied using principal component analysis to reduce subject data to 40 principal components and group-level reduction to 25 principal components, whereby subject data were stacked for covariance matrix computation at the group-level. The Infomax algorithm[242] was then implemented for spatial ICA to obtain 25 group-level ICs. This number was chosen as 20-30 ICs can reliably estimate IC networks[243], and the common data-driven estimation method of 'minimum description length' vastly overestimated the number of components (~170 ICs). Group ICA was applied on all subjects simultaneously, as individual ICAs per group has been shown as less sensitive at detecting group differences[244] and could introduce incomparable networks between groups.

To ensure the reliability of the Infomax algorithm for these data, the ICASSO Toolbox[245] was used to repeat group ICA 20 times, using bootstrapping. The quality of each IC was quantified, and ICs all exceeded a quality index of 0.9 (range 0-1, i.e., very high quality). Finally, the best estimate of these ICs derived from ICASSO were back reconstructed to subject-level components. For each subject this produced a spatial map of voxelwise coactivation (functional connectivity of voxel-to-IC average timecourse) and a weighted average component timecourse (i.e., more implicated voxels have greater weight in average timecourse calculation) for each component, which were converted to z-scores.

Group-level ICs were classified by 7 independent reviewers, and scored as 2 (signal), 1 (unknown), or 0 (noise). Classification to one of the three categories was influenced by the recently proposed hierarchical decision pathway[246] which is designed for subject-level denoising but adaptable to group-level ICs. This pathway states ICNs should largely present grey matter activation, low spatial overlap with vascular/ventricular motion and susceptibility artefacts, and dominant low-frequency fluctuation. Each IC was visually inspected using combined information from spatial activation maps (z>1.0; axial montage and peak voxel z-score ortho), average timeseries, and frequency spectra to determine IC classification. ICs were excluded as noise if the mean rating was <1 (n=8 components).

Mean IC ratings are shown in **figure 3.2**. This further displays each ICs with dynamic range (absolute maximum-minimum normalised power on IC frequency power spectra) and fraction of low frequency fluctuations. RSNs are commonly assumed to be comprised of low-frequency fluctuations alone, however some studies have demonstrated the value of looking at higher-frequency fluctuations of networks[247]. These results exemplify the necessity of visual inspection rather than thresholding based on fraction of low frequency fluctuations, as this provided no clear signal/noise distinction. ICs were excluded as noise if the average rating was <1, i.e., components 3, 4, 6, 12, 15, 19, and 23.

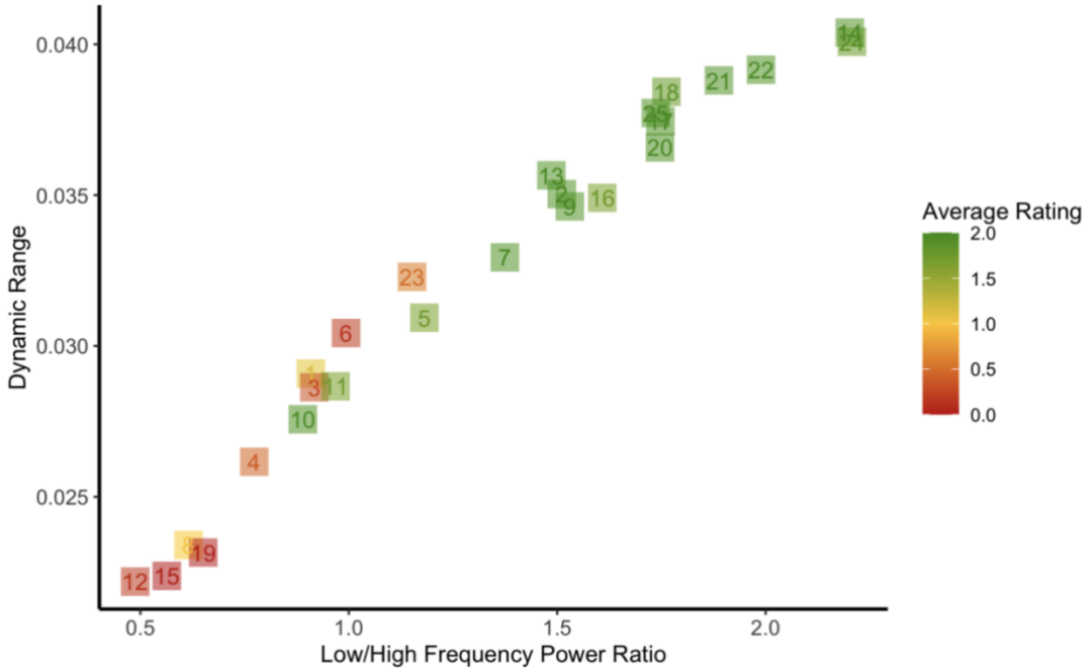


Figure 3.2. Relationship of dynamic range and frequency power ratio. Each independent component is labelled with its component number and coloured by mean rating as signal (2), unknown (1), or noise (0).

Remaining 'signal' ICs were labelled as belonging to a network (e.g., 'visual') using GIFT's Component Identifier tool. This applies spatial correlation of the IC to known resting-state network maps and presents maximum Pearson's correlation with any of these templates. The 'Neuromark 53' template was used[248] as it additionally incorporates subcortical and cerebellar masks, which have traditionally been ignored in cortico-centric research. All ICs were assigned to the network with highest correlation value and checked by visual inspection. The final networks can be seen below in **figure 3.3**. For subsequent analyses, each component was investigated separately, with results considered in the context of its respective network.

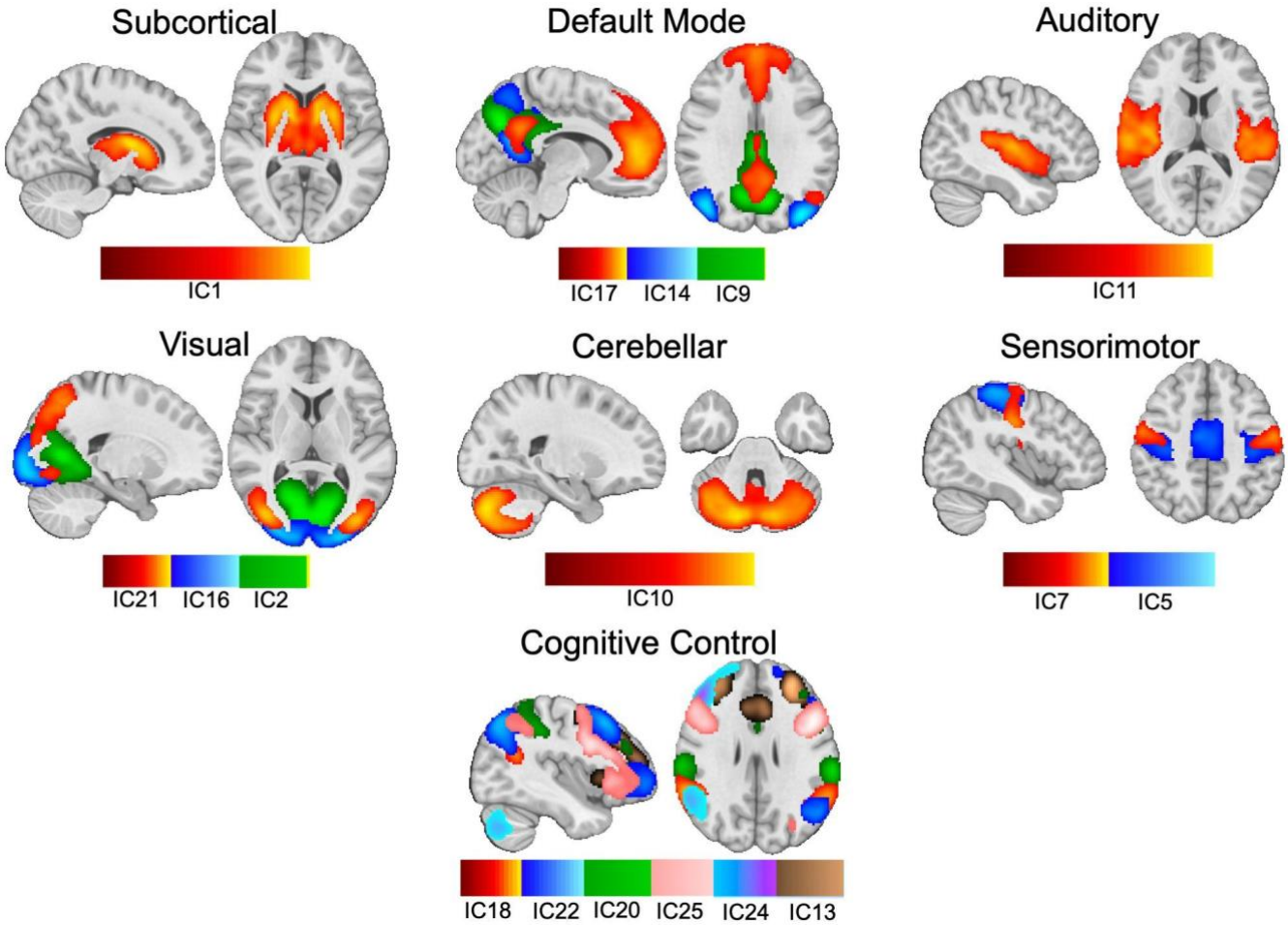


Figure 3.3. Group mean resting-state networks derived from ICA approach. Individual components are shown as z-score maps of highest voxelwise network involvement, with lower threshold z=1.0. These are labelled and grouped into their respective canonical networks.

3.2.4 Statistical analysis

All analyses were conducted at FDR-corrected p<0.05 using Benjamini-Hochberg in R (v.4.1.2), within test, unless otherwise stated. Three key measures of resting-state networks were investigated between groups with additional covariates of sex and age; between-component connectivity, within-component connectivity, and a novel measure of component distribution complexity, which are described in turn. Additional efforts were made to harmonise data for multicentre acquisition across n=14 site/scanners included in this cohort using an empirical Bayesian method ('ComBat'). ComBat application is required prior to statistical testing, and thus was performed on subject-level IC maps (voxelwise, as in[219]), between-component connectivity measures (FC coefficients, as in[221]), and entropy values per subject. This process is described in full in **Chapter 2.**

Firstly, between-component connectivity was obtained using Pearson's correlation between every pair of *i* components' timecourses for each of *j* subjects to produce an *i* x *i* x *j* connectivity matrix of correlation coefficients, followed by Fisher's Z transformations. These values were tested for significant differences between patients and controls with a two-sample t-test for each component pair (n=136 comparisons). Secondly, within-component connectivity differences were assessed by comparing voxelwise z-score of IC spatial maps between groups using SPM12[232]. All analyses between groups were constrained to the area of highest implicated voxels using a one-sample t-test (FWE p<0.01). Clusters were considered significant if surviving thresholds of p<0.001 at the voxel-level with further FWE-correction of p<0.05 at the cluster-level.

Finally, I investigated a novel measure of *component distribution complexity*. As discussed, network definition may vary between individuals, particularly in clinical populations, making data-driven approaches an attractive method. This does not however substantiate IC definition as the 'gold standard', as these are often thresholded to include only the most highly coactivating voxels to that network using a one-sample t-test, as performed above. This arguably removes an attractive aspect of ICA; in that it proposes a global perspective to network definition; each component's spatial map is constructed by voxelwise z-scores of temporal coactivation to that component's average timeseries. Thus, every voxel in the brain is implicated in each IC prior to thresholding, supporting

the brain-wide nature of coordinated neural activity, and can be harnessed to assess how networks may be differentially defined in clinical populations.

In healthy control populations, the distribution of these z-scores is commonly positively skewed and heavy-tailed, whereby the most implicated voxels for a component represent the upper heavy tail. An example of this is shown later in **figure 3.6** with further explanation of the corresponding results. An identical distribution may not be present in clinical populations if their underlying brain network composition has been altered by injury or disease. I thus wanted to investigate whether this z-score distribution is altered in mTBI, using Shannon Entropy. This describes the (un)predictability of possible outcomes of a random variable: the more predictable the variable's outcome, the less entropy it has. Conversely, a distribution has high entropy when its outcome is less predictable. In other words, entropy quantifies the extent to which the probability mass of the distribution is concentrated (low entropy) or distributed among many possible outcomes with relatively similar probability (high entropy)[249].

This measure can be used to compare the distribution of voxelwise coactivation z-scores between groups, and investigate whether this distribution is altered or neurotypical in mTBI patients. A higher entropy would indicate greater spread of voxelwise recruitment across the brain, rather than being concentrated to defined regions within a network. Such a result would indicate the very definition of network recruitment has altered on a brain-wide scale, becoming less well-defined and differentiated. I emphasize that this entropy measure is spatial in nature (the complexity of network recruitment), as opposed to other common measures of temporal entropy (the complexity of a timeseries).

Specifically, I reasoned that a distribution of voxelwise z-scores with higher entropy would indicate less differentiation between the highest-scoring voxels (i.e., those traditionally designated to form an intrinsic network) and the rest of the brain. To probe such potential differences, entropy calculations were repeated more specifically for voxels within the thresholded masks of network inclusion created during voxelwise analyses with SPM (one-sample t-test, as described earlier), and voxels outside of this mask, thereby representing *within*-IC and *outside*-IC entropy respectively. These therefore present the distribution of voxelwise recruitment both within and excluding

the commonly defined regions within a network component, to explicitly test whether outside-IC regions are being differentially affected in IC definition.

Entropy of the underlying distribution of voxelwise z-scores was calculated for each participant for each component, by constructing a histogram of i binned z-scores, whereby,

$$Entropy = -\sum_i (p(i) \times log_2(p(i)))$$

Number of bins was constant and bin width was determined by taking the absolute minimum and maximum z-score across all subjects and dividing this range by the number of bins. This was repeated at n=20,50,100,500 bins to assess stability of comparisons. Shannon Entropy was used rather than alternative distribution measures (e.g., variance, kurtosis), due to the non-normality of these data being a positively skewed and heavy-tailed distribution in healthy controls.

3.2.5 Relationship to 6-month outcome

I finally compared acute measures showing significant alterations in mTBI patients to 6-month functional outcome. All significant analyses above were repeated to compare outcome groups (GOSE, PCS) including additional covariates of initial GCS and time since injury to scan, and further compared to controls in between-component and entropy analyses.

Post-hoc analyses further explored whether significant connectivity increases uniquely found in GOSE-8 versus controls' between-component functional connectivity showed relationship to neuromodulatory systems. This was performed as neuromodulatory systems are integral to large-scale cortical connectivity profiles[250,251], and have shown systems-level changes in severe TBI[252]. As several between-network connectivity pairs were uniquely found to be associated with *good* outcome (GOSE-8), their significant relationship with specific neuromodulatory systems could link adaptive connectivity change to potential treatment targets for future investigation. This translational effort aims to extend rsfMRI to inform other areas of neuroscience and therapeutic investigation to better understand and promote positive outcomes after mTBI.

To probe neuromodulatory systems, average timecourses were extracted for n=5 neuromodulatory brainstem nuclei (Dorsal Raphe, Median Raphe, Locus Coeruleus, Pedunculopontine Nucleus, Ventral Tegmental Area) using the Harvard Ascending Arousal Network Atlas[253]. These nuclei have known differential neuromodulatory roles in brain function and arousal. Functional connectivity between these nuclei and outcome-relevant ICs were calculated and harmonised as described previously and compared between outcome groups and controls.

3.3 Results

3.3.1 'Mild' injury causes global alterations in between-network functional connectivity

Group ICA was successful in deriving seven complete resting-state networks across n=25 independent components, as shown in **figure 3.3** in Methods. Results from between-component comparisons between mTBI and controls are shown in **figure 3.4**, with significant group-differences indicated with an asterisk. This shows a pattern of both increases and decreases in connectivity, not confined to any one network label, and encompassing *all* 7 networks. Importantly, these are almost exclusively between components of *different* networks, except for IC13-IC20 showing reduced between-component connectivity in mTBI which are both labelled as 'cognitive control'. This suggests widespread between-network connectivity alterations in acute mTBI.

Matrices of between-network connectivity pre- and post-harmonisation are presented in **Appendix 3.3**, to exemplify the success of this procedure in reducing site-specific variance whilst retaining biologically relevant variance and increasing sensitivity to difference detection between groups.

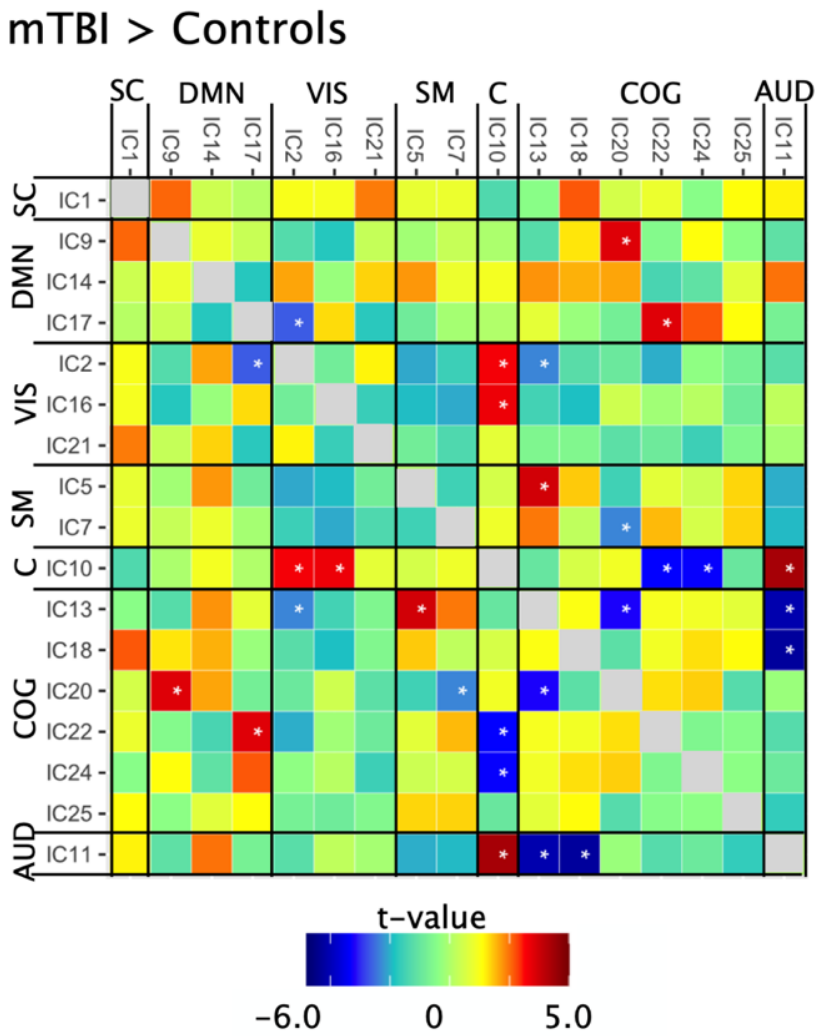


Figure 3.4. Between-component functional connectivity mTBI > controls. All comparisons are FDR corrected, with significant pairwise effects at p<0.05 indicated with a white asterisk. The t-value for each test is colour-coded, whereby increased connectivity in mTBI is shown in red, while blue squares indicate decreased connectivity in TBI when compared to control subjects. Labelled networks are shortened to SC (subcortical), DMN (default mode network), VIS (visual), SM (sensorimotor), C (cerebellar), COG (cognitive control), and AUD (auditory).

Results from within-component functional connectivity changes after mTBI demonstrate less-pronounced change than between-component results, echoing above results of acute changes between rather than within networks. These isolated clusters are presented in **figure 3.5**, showing results for all ICs within that network in each figure, and no data is shown for the cerebellar component as this found no regions of connectivity change. Of note, clusters of significant change are largely found at the borders of component definition which are shown in purple.

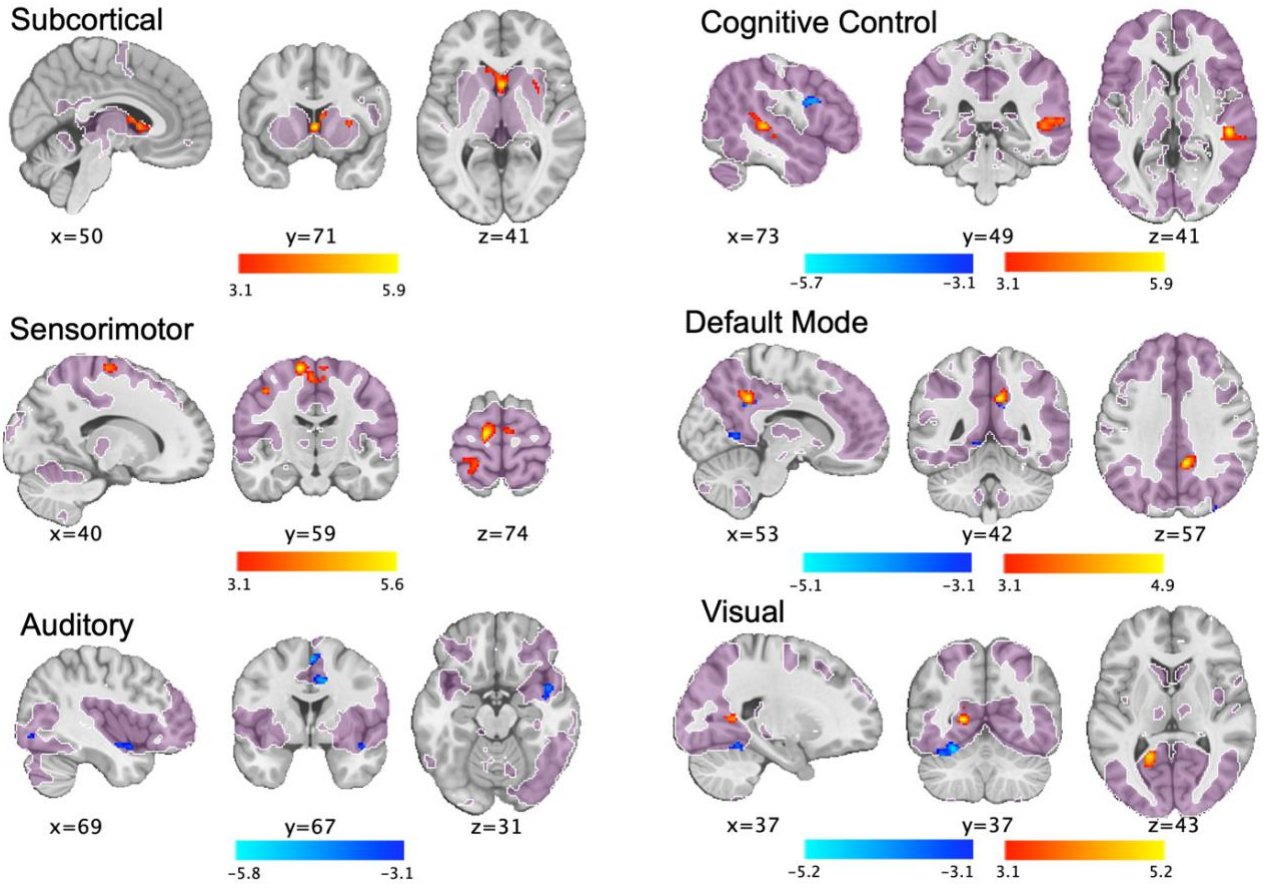


Figure 3.5. Within-component functional connectivity. Comparisons between control and mTBI groups. For each network, purple regions show combined masks from one-sample t-test (FWE $p<0.01$) for each of the respective components, i.e., regions constraining group-comparisons. Areas of significantly higher within-component connectivity in mTBI compared to controls are presented in red, and areas of lower within-component connectivity are presented in blue, with each describing z-scores of the statistical tests. Significant results are determined at $p<0.001$ at the voxel level, and $p<0.05$ FDR-corrected at the cluster-level. Cerebellar network is not shown due to lack of significant voxelwise change between groups.

3.3.1 Increased brain-wide recruitment of network resources

Component distribution complexity, as measured using Shannon entropy, was compared between patient and control groups. These results, shown in **figure 3.6A**, demonstrate that in *every* component the distribution of brain-wide z-scores had significantly higher entropy in mTBI patients than in controls. This entropy measure describes the complexity of a spatial distribution, rather than temporal entropy of a timeseries. These significant differences were stable across the number of bins, further examples of which

are presented in **Appendix 3.4**. Additional analyses assessed within- and outside-IC entropy for each component. These comparisons found that distribution complexity did not significantly differ between groups within-ICs, aside from IC11 (AUD) which showed higher entropy in mTBI patients (**figure 3.6B**). However, outside-IC entropy was significantly higher in patients in 13/17 components (**figure 3.6C**), encompassing all networks except sensorimotor. Differences were again stable across the number of bins as presented in **Appendix 3.4**.

3.3.2 Hyperconnectivity and monoaminergic neuromodulation as an adaptive response to injury

Each acute imaging-derived phenotype identified to be significantly different between patient and control groups was subsequently related to 6-month outcome. Namely, by comparing patient outcome groups GOSE-8 to GOSE≤7. No significant differences were found in within-network connectivity, or in between-network connectivity surviving strict multiple comparisons corrections (**Appendix 3.5**).

However, three measures showed significantly higher entropy in GOSE≤7 than GOSE-8 after FDR correction; whole-brain entropy of DMN component IC9 [$F(1,100)=7.4$, $p=.008$], and both whole-brain entropy of Cerebellar IC-10 [$F(1,100)=10.5$, $p=.002$] and outside-IC entropy of Cerebellar IC-10 [$F(1,100)=5.2$, $p=.025$], proposing these as possible acute markers of long-term recovery. All entropies were significantly higher in GOSE≤7 than in controls except outside-entropy of IC2 (Visual), and most entropies were significantly higher in GOSE-8 than in controls. These results are shown in **Appendix 3.6.**

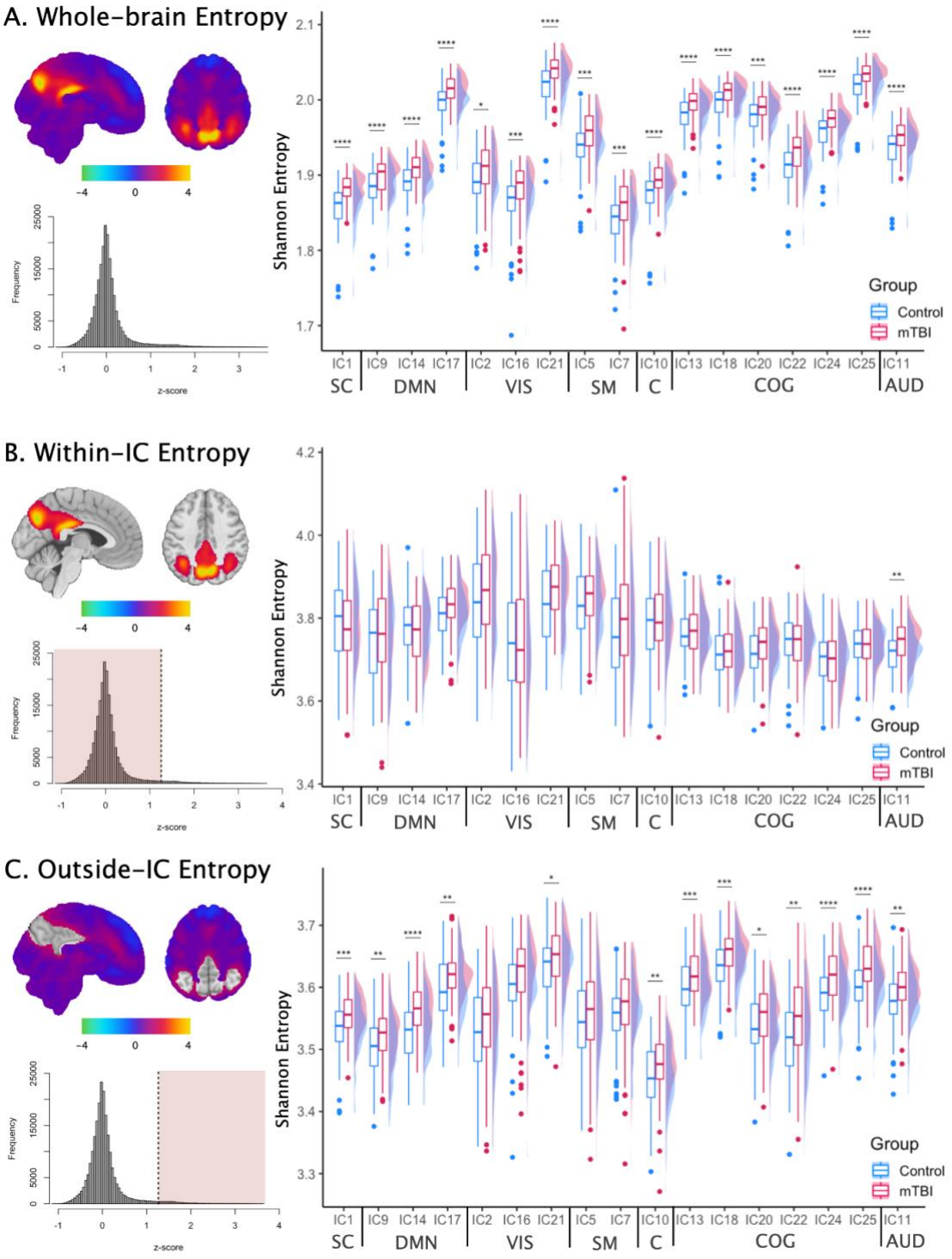


Figure 3.6. Exploratory measure of Shannon entropy to compare the distribution complexity of component definition. Left column displays regions being investigated in each test, using IC9 as an arbitrary example; whole-brain z-scores in A, within-component z-scores in B, and outside-component z-scores in C. Example histogram shows heavy-tailed distribution of z-scores (n=100 bins), with red regions excluded from analysis. Thresholds of within/outside independent components (dotted line) are determined by one-sample t-test. Right-hand column displays between-groups comparisons of these measures respectively. Shannon entropy values (y-axis) are measures of distribution complexity, and are here displayed for n=100 bins. Significantly different comparisons after FDR-correction are shown with; $p<0.05$ = *, $p<0.01$ = **, $p<0.001$ = ***, $p<0.0001$=****.

Due to strict corrections for multiple comparisons, some effects may have been insufficiently powered to overcome this. Entropy values showed a high degree of multicollinearity to one-another; thus, a principal component analysis (PCA) was run for dimensionality reduction of the whole-brain entropy and low-voxel entropy values to more specifically study the two GOSE groups. Scree plots suggested these could be reduced to one principal component, encompassing 59.4% and 41.7% of the total variance in the whole-brain and low-voxel entropies respectively. This first principal component was compared between the GOSE groups using an unpaired samples t-test, and found that whilst low-voxel entropy did not differ [$t(106)=0.71$, $p=.482$], GOSE≤7 showed a borderline significantly higher entropy across the whole brain compared to GOSE-8 [$t(106)=1.91$, $p=.052$]. Whole-brain network entropy may therefore also aid classification of 6-month outcome in future predictive modelling approaches.

To further assess any relationship between acute imaging and long-term outcome, patient groups were additionally compared to healthy controls. Between-network connectivity measures showed differences in mTBI participants compared to controls when considering favourable/unfavourable outcome, as shown in **figure 3.7**. Both outcome groups presented widespread significantly altered functional connectivity compared to controls in the acute phase, suggesting that even those with 'mild' injuries and positive outcomes undergo acute functional consequences. Crucially however, the GOSE-8 group showed both increases and decreases compared to controls, whereas those with incomplete recovery (GOSE≤7) showed only decreases in acute connectivity, suggesting a potentially adaptive nature of *increased* between-network functional connectivity in this acute phase around 2-weeks post-injury.

These n=7 hyperconnected pairs in the favourable recovery group encompassed n=11 ICs and were further explored with respect to brainstem neuromodulation by calculating brainstem-IC functional connectivity (**figure 3.8**). This was to assess whether potentially adaptive acute functional connectivity could be linked to brain-wide neuromodulatory systems, which could subsequently be harnessed for therapeutic benefit. When compared to controls, both outcome groups showed significant increases and decreases in this functional connectivity, implicating the dorsal raphe (DR) and pedunculopontine nucleus (PPN). Crucially, only the favourable outcome group (GOSE-8) showed increases in median raphe to auditory, and ventral-tegmental area (VTA) connectivity to sensory

and cognitive control ICs (**figure 3.8A**). As these nuclei are implicated in serotonergic and dopaminergic neuromodulation respectively, these results suggest that upregulated monoaminergic neuromodulation, particularly VTA involvement, may support an adaptive response to injury. No significant differences were found between outcome groups (**Appendix 3.5**).

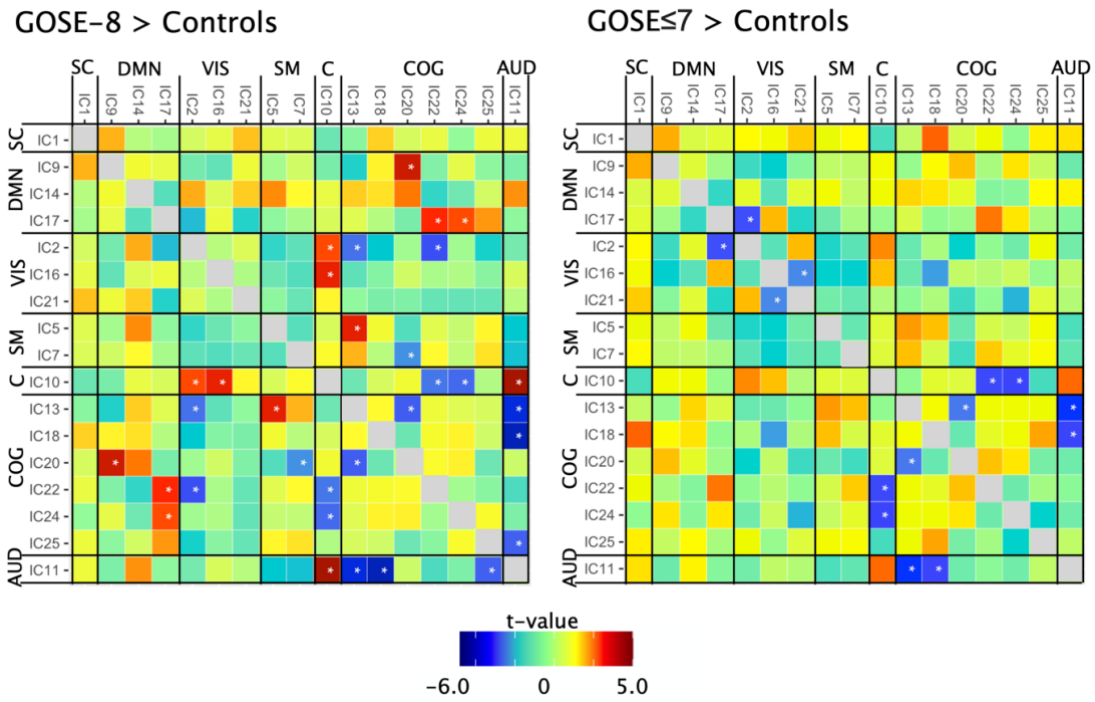


Figure 3.7. Acute between-network connectivity and 6-month outcome. All comparisons are FDR corrected, with significant pairwise effects at p<0.05 indicated with a white asterisk. The t-value for each test is colour-coded, whereby increased connectivity in mTBI is shown in red, while blue squares indicate decreased connectivity in each respective TBI outcome group when compared to controls. Labelled networks are shortened to SC (subcortical), DMN (default mode network), VIS (visual), SM (sensorimotor), C (cerebellar), COG (cognitive control), and AUD (auditory).

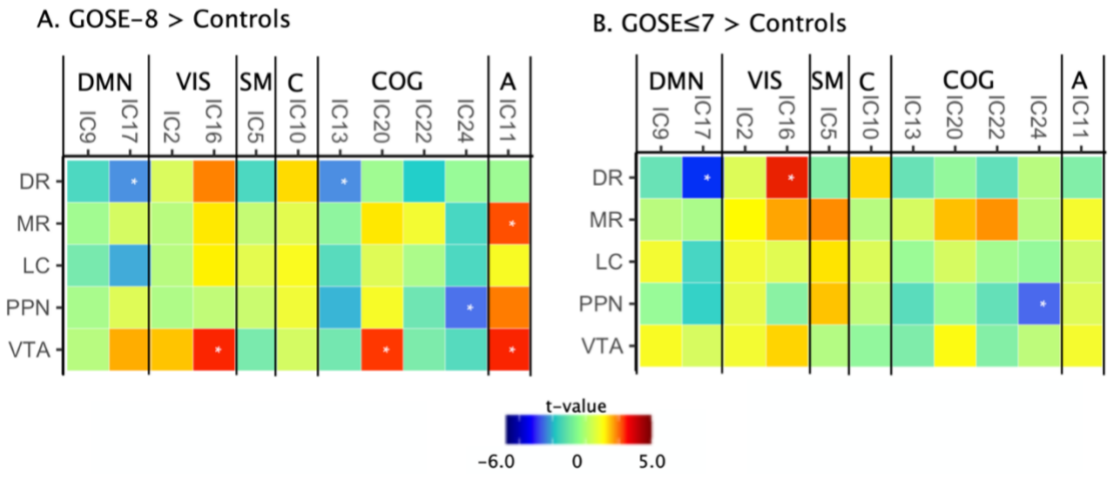


Figure 3.8. Brainstem-to-component functional connectivity. All comparisons are FDR corrected, with significant pairwise effects at p<0.05 indicated with a white asterisk. The t-value for each test is colour-coded, whereby increased connectivity in the respective mTBI outcome group is

shown in red, while blue squares indicate decreased connectivity in mTBI when compared to control subjects. Labelled networks are shortened to DMN (default mode network), VIS (visual), SM (sensorimotor), C (cerebellar), COG (cognitive control), and AUD (auditory). Neuromodulatory brainstem nuclei are labelled as DR (dorsal raphe), MR (median raphe), LC (locus coeruleus), PPN (pedunculopontine nucleus), and VTA (ventral tegmental area).

All measures of between-network functional connectivity and entropy were additionally compared in postconcussive symptom outcome groups (PCS+ vs PCS-). No significant differences were found in between-network connectivity or entropy measures between PCS+ and PCS-, suggesting that acute network-level measures are not as useful in assessing later postconcussive symptoms in this cohort. In comparison to healthy controls, both groups showed comparable patterns of increases and decreases in between-network connectivity across a variety of network pairs, similar to the mTBI vs control comparison presented in **figure 3.4.** These only reached significance after strict multiple comparisons correction in the PCS- vs control comparison, and not when considering PCS+. Whilst this paradoxically suggests that the adverse outcome group (PCS+) was more like healthy controls, the smaller sample size of this group (n=31) compared to PCS- (n=67) may have limited the power to find significant effects, explaining the lack of significant change in the PCS+ group. These matrices as presented below in **figure 3.9,** with further entropy-related results presented in **Appendix 3.7**. Overall, acute network-level measures were not sufficient to associate later postconcussive symptoms.

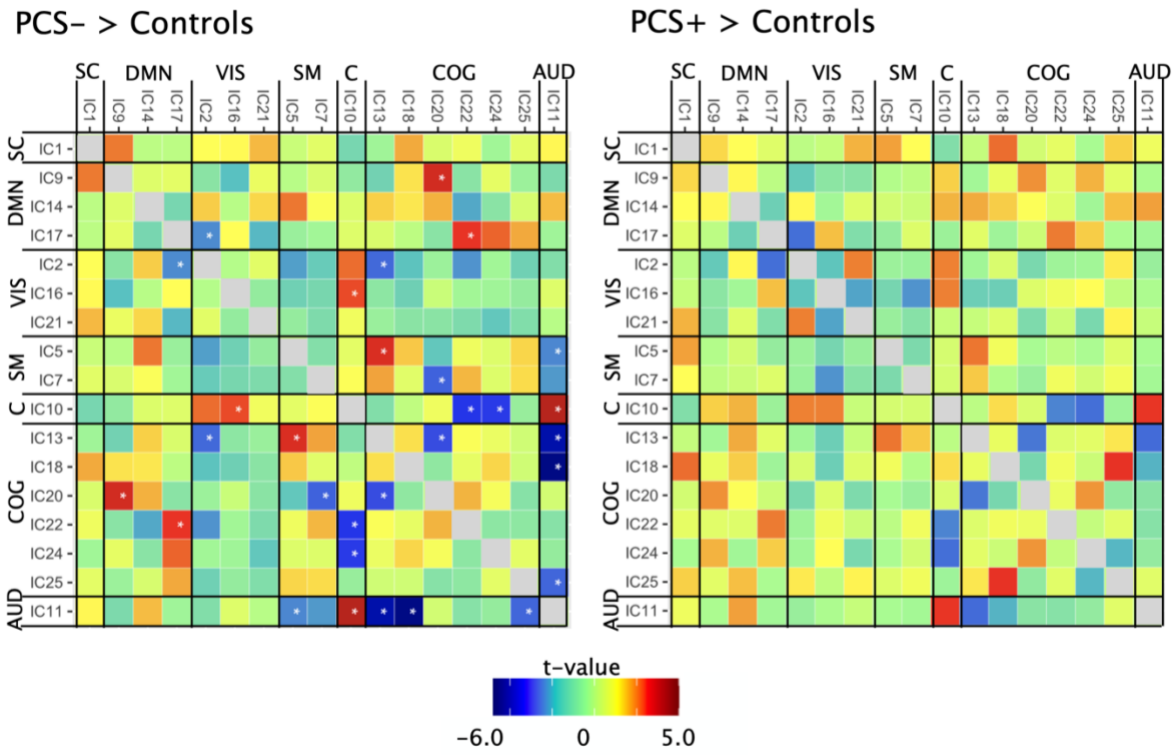


Figure 3.9. Acute between-network connectivity and 6-month postconcussive outcome. All comparisons are FDR corrected, with significant pairwise effects at $p<0.05$ indicated with a white asterisk. The t-value for each test is colour-coded, whereby increased connectivity in mTBI is shown in red, while blue squares indicate decreased connectivity in each respective TBI outcome group when compared to controls. Labelled networks are shortened to SC (subcortical), DMN (default mode network), VIS (visual), SM (sensorimotor), C (cerebellar), COG (cognitive control), and AUD (auditory).

3.4 Discussion

In the largest sample to date studying acute functional network alterations in mTBI, I here provide clarification to a previously mixed literature. Using data-driven methods, I propose acute mTBI is a *global functional* disorder, predominantly characterised by substantial between-network changes even in 'mild' CT-negative injuries. Critically, this cohort was designed to establish changes in functional connectivity that are directly attributable to global brain dysfunction due to the TBI (i.e., what the injury brings to the patient) by excluding patients with increased vulnerabilities for persistent symptoms (such as pre-existing mental health issues: what the patient brings to the injury). Further, the exclusion of patients with structural abnormalities on CT allowed the exploration of purely functional network dysfunction unrelated to structural injury. This functional

perspective thus shows great potential for a growing mTBI population, who are insufficiently characterised by routine structural imaging alone, and are often dismissed as malingering or suffering from a more general umbrella diagnosis of Functional Neurological disorder[254]. These results characterise more specific imaging substrates for such incomplete recovery, which may have implications for diagnosis, prognosis, and selection of therapeutic targets.

My primary finding is that between-network functional connectivity was significantly altered around 2-weeks after mTBI. This showed both increases and decreases in connectivity acutely after injury compared to controls, whereby all investigated networks were implicated, particularly affecting connectivity between components of *different* networks. Combined with minimal clusters of within-network change in voxelwise analyses, these results directly support the largest previous study of acute mTBI which proposed between-network changes were most salient for understanding functional consequences of injury[175]. Their findings explicitly noted DMN, visual, and motor networks to be most implicated. I indeed found high involvement of these networks, as 8/14 significant changes implicated one or more of these networks in between-component pairs. However, I further propose mTBI elicits *global* changes, as all networks investigated here were significantly altered in mTBI in the acute phase. This provides support to previous studies demonstrating alterations across a variety of networks[171,230,255], proposing a connectomic scale of alterations[177]. Moreover, going beyond previously cortico-centric approaches, I found significant changes in both cerebellar and subcortical domains, which have not been sufficiently explored in mTBI functional connectivity research, but have shown significant changes after injury when investigated[230,255,256]. Given the known vulnerability of some subcortical structures, such as the thalamus[113], these warrant further investigation and whether more specific injury-induced changes can be used to discern outcome.

Global functional changes were seen irrespective of functional outcome, suggesting that injury, no matter how mild, can induce vast neurological disruption. A recent paper assessing outcomes between different severities of injury used a similarly defined mTBI cohort as a proxy for healthy controls (GCS-15, GOSE-8, and CT-)[257] given that they showed minimal long-term cognitive deficits, emphasizing the mild nature of this cohort. However, I here show that even these mild groups with good outcome show global

functional changes acutely after injury. These findings suggest clinical practice of mTBI patient care could benefit from functional markers to better understand and care for this growing population. Thus, mTBI can be functionally considered a global disorder, for which rsfMRI is an invaluable tool.

A global disorder therefore requires a global approach, which I aimed to investigate using ICA. Several authors have used ICA to study acute mTBI, with mixed findings largely encompassing widespread functional change with preliminary behavioural associations[171,230,255]. Here, I capitalised on the global nature of IC-derivation to explore a novel measure of component distribution complexity. This demonstrated that the very definition of networks was changed; with increased brain-wide recruitment of resources after injury, and less differentiation of voxels in terms of intrinsic network membership. To the best of my knowledge, previous studies have not looked at this aspect of data-driven components, instead focussing on the most implicated regions and negating the brain-wide nature of coordinated neural activity. I found that peripheral regions of a network, i.e., areas of the brain *outside* of those commonly thresholded network regions, had higher entropy acutely after mTBI, whilst within-IC entropy remained unchanged. This pattern indicates that non-canonical contributions to ICs are distributed more broadly in patients than controls, suggestive of a more continuous distribution that is less clearly peaked around zero – reflecting diminished differentiation. This interpretation is further supported by previous work within my lab group on mTBI, which found a global *increase* in mean intrinsic connectivity demonstrating that regions behave more similarly to each other, rather than providing independent contributions[206]. It is essential to note that the measure of component complexity encompasses the spatial distribution of network recruitment, rather than temporal complexity of a timeseries. Intriguingly, the same previous project on mTBI observed that the increase in spatial complexity is accompanied by a decrease in the brain-wide temporal complexity of time-series, correlating with increased global connectivity[206]. Indeed, these combined increases in global connectivity and decreased temporal complexity were replicated in the present dataset (**Appendix 3.8**). Together, these results suggest that mTBI patients' brain organisation becomes less diverse both over time (with more stereotypical activity) and in space, with higher correlation between regions and broader contributions to ICs.

Indeed, the intrinsic properties of networks have consistently shown global alterations following mTBI. Previous studies have found long-term decoupling of structure-functional relationships[258], less efficient networks exemplified by increased path length and disrupted small worldness[172,174,259], and more time spent in states with disrupted small worldness studied using dynamic functional connectivity (measured over time)[174]. Such graph-theoretical measures quantify network properties *post*-network-definition, which are complemented by the present *peri*-definition metrics, corroborating a global breakdown in intrinsic network properties, and proposing that the very definition of functional networks in mTBI populations has changed.

Importantly, the acute functional measures showed preliminary associations to long-term recovery, measured by 6-month GOSE, suggesting a potentially adaptive nature of between-network *hyperconnectivity*. Such acute increases in connectivity are a common response to injury and have been associated with positive outcomes in moderate and severe cohorts[185,188], but are far less understood in mild cohorts[191]. The neural explanation for why increased connectivity could constitute an adaptive response is not yet understood- suggested to exploit latent anatomy or offload neural demand to non-damaged 'hub' regions to maintain healthy brain function, or perhaps an attempt to stabilise injury-induced irregularities by increasing coordination between non-damaged regions. Nevertheless, when viewed from a behavioural standpoint, *adaptive* acute hyperconnectivity has been shown in some mTBI studies, particularly in default mode, frontoparietal, and cerebellar networks[191]. In support, I found that acute functional hyperconnectivity between networks was uniquely present in those with *favourable* recovery at 6 months (GOSE-8) compared to controls, and not in those with *unfavourable* recovery (GOSE≤7). Furthermore, these hyperconnected pairs almost exclusively involved DMN and Cerebellar components, suggesting these may have behavioural relevance.

These networks were further implicated as clinically relevant in my novel functional imaging measure: component distribution complexity. Patients with incomplete recovery at 6 months showed increased acute entropy in DMN and cerebellar components compared to both controls and complete-recovery groups. Crucially, only this complexity measure successfully differentiated outcome groups within the mTBI cohort, with the incomplete recovery group moving further away from the network architecture

seen in healthy controls. This proposes unique prognostic value of network distribution complexity for long-term outcome using acute functional imaging. The relative importance of DMN and cerebellar regions to functional recovery has similarly been found by previous papers investigating functional network alterations in mTBI[191]. Of note, various measures of DMN-related functional connectivity have been shown to be associated with executive function[172], have predictive value for symptom severity[175], and prognostic value for persistent postconcussive symptoms[193], particularly overlapping precuneal regions in the latter, which highly corresponds to my IC-9 found to differentiate outcome groups in whole-brain entropy. The cerebellum has been far less investigated in the context of mTBI functional connectivity[260], partially due to the cortico-centric approach of some resting-state network definitions. However, when it is studied, the cerebellar network is the only network which has consistently found injury-induced hyperconnectivity after mTBI in a recent review of ICA approaches[191]. Acute functional cerebellar changes have additionally shown relationship to 6-month learning performance[171], postconcussive complaints[173], and appear integral to classification of mTBI groups using machine learning[260]. Given the global scale of functional alterations in mTBI, it is perhaps unsurprising that these two networks show particular relevance to injury and outcome, as both the cerebellum and precuneus have shown key roles in a variety of networks and their coordination of information[261,262], thereby having globally directed roles in brain function.

Finally, my exploration of neuromodulatory links to 'adaptive' hyperconnectivity converged on the importance of monoaminergic-driven brainstem nuclei. These neuromodulatory systems are integral to large-scale cortical connectivity profiles[250,251], and have shown systems-level changes in severe TBI[252], echoed here in this mild cohort. Namely, the VTA showed increased functional connectivity to several ICs, uniquely in mTBI participants with favourable outcome. Whilst tentative, such acute dopaminergic upregulation may constitute an adaptive response to injury, not present in those with unfavourable outcome. Indeed, dopamine levels and dopaminergic systems are vastly altered following injury in animal models of TBI[263,264], and several therapeutic developments in dopaminergic agonists show benefits for neuropsychiatric outcomes in clinical trials[265,266]. Whilst this does indeed suggest that acute increases in dopaminergic involvement may elicit neuroprotective effects, this remains a complex issue in terms of

mal/adaptivity[267]. The underlying mal/adaptive nature of acute functional changes and their relationships to neuromodulation remains speculative within TBI literature and requires further research to fully understand functional sequalae and their relationship to recovery in longitudinal study.

3.4.1 Limitations

Whilst this study is successful in providing a baseline for acute mTBI research, it has several limitations. Firstly, the GOSE was considered as an outcome measure with crude boundaries of what constitutes 'favourable' versus 'unfavourable' outcome. Although this tool is clinically relevant for assessing overall functional outcome, it has been criticised for insufficient specificity for mTBI populations and ignores the nuances of what constitutes a 'favourable' outcome for the patient[49,88]. I additionally investigated postconcussive symptom groups, however no significant associations were found between acute network connectivity and long-term symptoms. This suggests that whilst canonical resting-state networks have utility for prognosticating general functional outcome, they may not for general postconcussive outcome. Postconcussive symptoms are diverse, and each individual symptom may recruit different networks or regions within that network. Thus, failure to find significant effects may be attributed to a lack of specificity in these analyses, looking only to those experiencing any three or more symptoms. This may be compounded by strict multiple comparisons correction across all possible networks rather than targeting analyses to known influential networks/regions for that symptom, and a lower sample size of the postconcussive groups reducing power to find effects. I did not investigate further specific outcome measures, as a baseline was required for the field before more specific questions and behavioural associations can be systematically investigated. Furthermore, I only present acute imaging around the 2-week timepoint, and future research should seek to understand the evolving consequences of mTBI, which may have time-dependent and outcome-dependent relationships[186].

Finally, a limitation of my novel measure using Shannon Entropy is that it is defined by predictability. An entirely random distribution is unpredictable and would present high entropy but is not in itself a complex distribution. An ideally complex network is instead defined as having small worldness- lying between complete order and disorder with

highly integrated hubs and less well-connected long-range connections[182]. Therefore, my current findings are unable to distinguish whether IC distribution entropy indicates increased randomness or increased complexity. This measure may additionally compliment graph-theory approaches, although literature combining graph-theory with ICA is currently developing[268]. I nevertheless hope this global and data-driven approach will be adopted by future research in this field.

3.4.2 Conclusion

Mild TBI can thus be characterised as a global and functional disorder. This chapter provides clarification to a mixed field of mTBI literature, demonstrating that even in this 'mild' injury cohort there are vast acute alterations of increased and decreased functional connectivity. This substantiates the relative benefit of functional imaging above structural imaging alone in understanding mTBI, as the latter was unable to prognosticate injury or outcome in this cohort (**Chapter 2)**. Whereas, rsfMRI is shown here to aid our understanding of injury and begin to prognosticate which individuals would benefit from long-term care, particularly in these patients who otherwise show no visible structural damage in routine imaging. Crucially, no single network or region was found to be implicated in injury above all others, with some behavioural relevance of DMN and cerebellar components. Given the vast array of injury types, mechanisms, and directions of injury patients experience, it may be unsurprising there is a lack of specificity in these findings, which instead point to brain-wide change. Better prognostic markers may be elucidated by capitalising on such a global approach; by taking whole-brain measures and investigating globally connected hubs, such as the thalamus. Future research should also aim to further combine neuroimaging with treatment-informing investigation to pave the way for preclinical study in this population, particularly with respect to monoaminergic neuromodulatory systems.

Chapter 4

Acute thalamic connectivity precedes chronic post-concussive symptoms in mild traumatic brain injury.

4.1 Introduction

I have now discerned a *functional* landscape of global changes following mTBI, in the absence of structural changes. This is informative in the context of my 'mild' cohort, as it substantiates the claim that all head injuries can have global functional consequences. Whilst this has clinical implications in that no patient should be dismissed as 'return to normal', it lacks further purpose for the patient and their trajectory post-injury. This chapter begins to look beyond functional networks approaches, to seek a *diagnostic* marker of injury, a *prognostic* marker of later outcome, and how this can *translate* beyond academic settings.

In **Chapter 3**, I proposed that *increases* in connectivity may be adaptive in the face of injury, with preliminary associations to better outcome using the GOSE. I further proposed relevance of the DMN and Cerebellar components' entropy in discerning good and poor outcome. However as discussed, whilst the GOSE is clinically useful, it has been criticised for its lack of granularity for mild TBI populations. When considering chronic postconcussive symptoms, the global network measures identified in **Chapter 3** failed to identify clear acute markers distinguishing those with, versus without long-term symptoms. This suggests alternative measures are needed to better understand and identify postconcussive symptom targets. As identified in **Chapter 3**, not any one network was subject to functional alterations following mTBI, substantiating this as a *global* disorder. Thus, it follows that a globally connected 'hub' region may further our understanding.

One such hub is the thalamus; a subcortical grey matter structure located in the centre of the brain, in the dorsal part of the diencephalon. The thalamus is often described as the brain's 'relay station' due to its reciprocal connections throughout the entire cortex, enabling its pivotal role in information transfer between motor, sensory, and associative cortical regions[121,269]. Importantly, the thalamus is not solely involved in sensory relay, but additionally in coordinating complex cognition across the cortex and its networks [121,269]. This was recently highlighted by Shine and colleagues, who argue that the thalamus can impact brain-wide processing in four main ways (**figure 4.1**); by promoting regional activity, modulating regional coupling, facilitating changes in the topology of networks, and regulating neural variability[269]. The thalamus therefore exhibits hub-like behaviour

by allowing and modulating information exchanges throughout the brain. Thus, the thalamus must be re-established as a region of interest in traditionally cortico-centric neuroimaging research to fully understand brain-wide control of information processing[269].

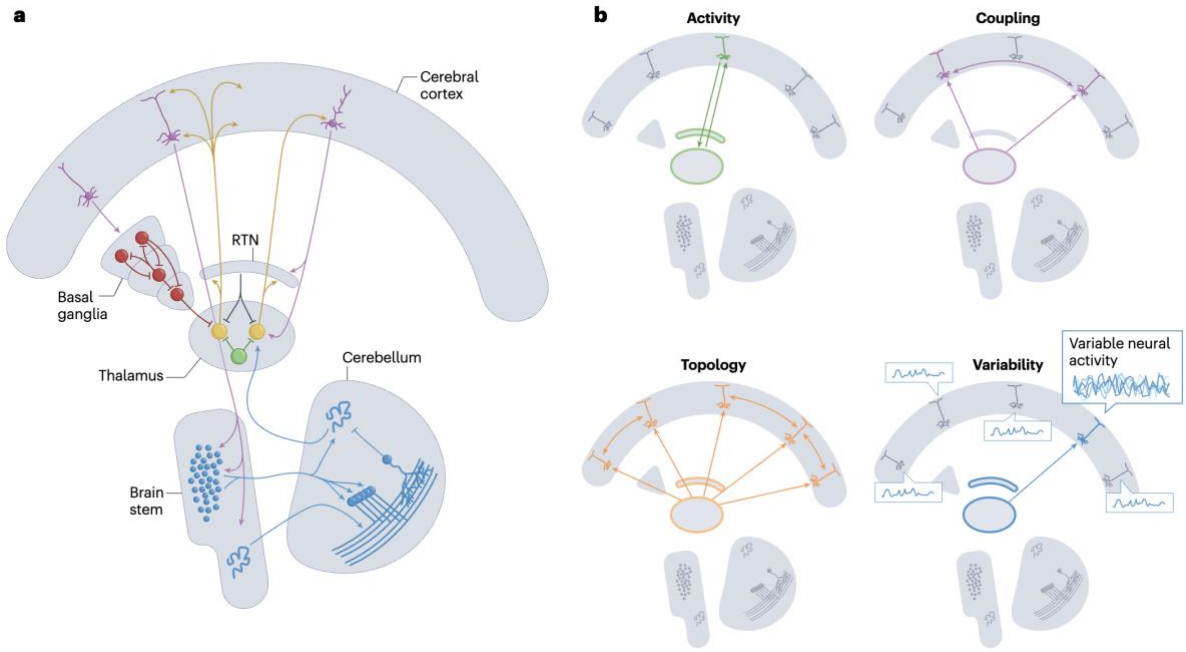


Figure 4.1. Functional neuroanatomy of the thalamus. A) schematic of the thalamus and its relationship to cortical, subcortical, brainstem, and cerebellar structures. Different populations of neurons within the thalamus (yellow) interact with different cortical layers (purple), inhibitory input from the basal ganglia (red), excitatory input from the cerebellum (blue), and neuromodulatory input from the brainstem. Local inhibition occurs at the level of the reticular nucleus (RTN) and GABAergic interneurons (green). B) diagrams describing four ways the thalamus can impact brain-wide information processing and systems-level change. Figure and underlying model from Shine et al., (2023)[269].

Importantly, the thalamus is not a single grey-matter structure, but a diverse population of nuclei. Human post-mortem studies have long parcellated the bilateral thalami into several nuclei since the work of Mann (1905)[270]. Most cited today is Morel and colleagues (1997)[271], who additionally used multiarchitectonic parcellation based on three calcium-binding proteins. These have delineated the thalami into distinct nuclei based on their anatomic structure and neuronal function, thereby indicating their distinct biological significance. Due to inter-individual differences in thalamic composition and competing 'schools' of thought in anatomical nomenclature first noted over 75 years ago[272], there are many non-overlapping thalamic parcellations with varying number of distinct nuclei. These discrepancies are now trying to be reconciled in relevant literature[273].

Thalamic nuclei are also known to have differential structural connectivity to the cortex[274], and can thus be further grouped into relay (first order) versus association (higher-order) nuclei[88]. These first-order nuclei predominantly receive driver inputs from ascending sensory pathways[274,275]; for example, the lateral geniculate nucleus provides a direct pathway from the optic nerve to primary visual cortex. In contrast, higher-order nuclei receive both driver and modulatory inputs from the cortex[274,275], for example the mediodorsal and central-lateral nuclei which have implications in wakefulness and consciousness[276,277]. This grouping into two 'orders' of nuclei, however, does not encompass the intralaminar nuclei which are inherently non-specific to cortical input, nor the reticular nucleus which forms an inhibitory GABAergic sheath around the largely excitatory thalami to locally inhibit thalamic activity alongside inhibitory interneurons[274]. Aside from thalamocortical interactions, distinct neural populations in the thalamus additionally receive input from the cerebellum, basal ganglia, and neuromodulatory brainstem nuclei (**figure 4.1**), which can further impact neural processing and cortical interaction[278]. Thus, the thalamus is highly diverse in its neural architecture, and is not one singular structure but multiple nuclei with distinct and overlapping functions which have brain-wide implications.

Importantly, a recent study found that *all* thalamic nuclei, both first-order and higher-order, are functionally connected to *multiple* functional networks[121]. They therefore described the thalamus as a "global kinless" hub, capable of multimodal integration across a variety of networks, thereby subserving a variety of cognitive functions.

Given the complexity of thalamic connections, focal thalamic injury can disrupt network organisation of functional networks, thus substantiating its integral role in healthy global function[121,279]. Most recently, electrical stimulation of the anterior thalamus in humans mirrored normative resting-state networks running through that stimulation site, suggesting that direct stimulation can target brain networks on a thalamocortical level[280]. Hence, the thalamus and its nuclei may be a useful lens for better understanding globally directed disorders, such as mTBI.

I chose to focus on the thalamus for three further clinically relevant reasons expanded upon below: i) the thalamus is highly vulnerable to injury, ii) many postconcussive symptoms are phenomenologically linked to thalamic function, and iii) a small previous

literature suggests specific thalamic damage in the face of injury, but has not been further investigated in a large sample.

4.1.1 Previous literature

The thalamus is particularly susceptible to injury-induced damage, as the highest sheer stress levels experienced during TBI are localised to midline and subcortical regions including the thalamus in both simulation studies[116,117] and *in vivo* measurements of brain deformation during mild posterior-anterior head deceleration in healthy volunteers[118,119]. In such human studies, the brain was observed to move and compress against the skull along the initial direction of motion and subsequently the opposite direction, known as *coup-contrecoup* injury. However, motion was constrained by the basal tethering region in both unidirectional and angular acceleration, thereby causing high sheer strain feels on subcortical structures. This is summarised in **figure 4.2.** These studies therefore suggest primary injury mechanisms uniquely affect this region, independent of the mechanism or direction of injury. This has led one recent paper to describe the midbrain region including the thalamus as the 'cone of vulnerability'[113].

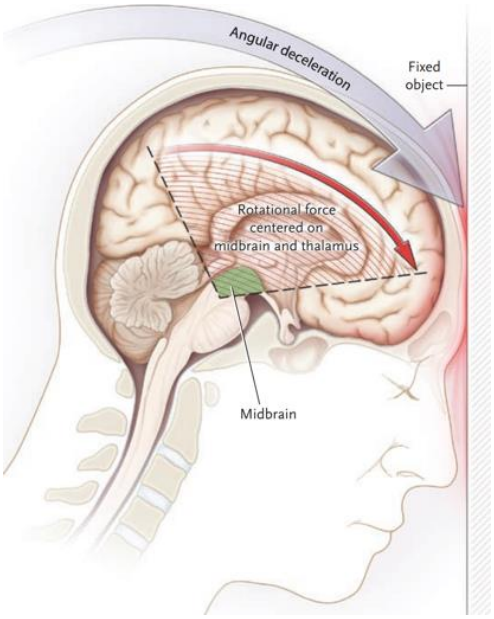


Figure 4.2. Mechanical forces during injury. Example of traumatic brain injury with rapid deceleration during collision with a fixed object, producing maximal forces on the midbrain and thalami. Image from Roper et al., 2007.

Indeed, thalamic dysfunction has additionally long been implicated in common postconcussive symptoms such as headache[281] sleep disturbances[282] fatigue[283] and

cognition[284]. In mTBI cohorts, the thalamus has shown chronic volumetric loss associated with greater symptom reporting[123], and fatigue[285]. Evidence from advanced imaging modalities at subacute and chronic timepoints has found a relationship between post-TBI depression and loss of structural integrity of the dorsolateral prefrontal-thalamic tract[286], worse neuropsychological scores associated with decreased mean kurtosis in the thalamus[122], and poorer cognitive performance associated with lower cerebral blood flow[284]. Thus, the thalamus could be an important region of interest in pathogenesis and prognosis following mTBI[181].

However, much of the evidence given above has been found in the chronic phase post-injury, with greater emphasis in the literature on moderate and severe TBI. For markers to have both diagnostic and prognostic utility in mild TBI cohorts, thalamic changes must be demonstrated in the acute phase.

A handful of studies have previously investigated thalamic functional connectivity after mTBI in the acute/subacute phase, and suggested injury-induced thalamic *hyper*connectivity. This increased connectivity with the thalamus was widespread, found sub-acutely in anterior prefrontal cortex and supramarginal gyrus[230], and acutely in posterior cingulate, dorsal anterior cingulate cortex, bilateral medial temporal regions, the default mode network, and primary sensory regions[187]. Further evidence suggested such widespread changes may be due to a breakdown in thalamocortical communication evidenced by subacute reductions in thalamic topographical efficiency[287]. Other small studies have correlated thalamic functional change with symptomatology in mTBI. Increased spread and asymmetry of thalamic resting-state networks were both linked to increased concurrent subacute depression, postconcussive symptoms, and impaired cognitive performance[256]; and increasing functional connectivity between the thalamus and dorsal attention network over 6weeks-4months correlated with decreases in self-reported pain and postconcussive symptoms[288]. These reports support a relationship between widespread thalamic hyperconnectivity and persistent postconcussive symptoms, potentially driven by selective thalamic vulnerability.

However, these studies of thalamic changes following mTBI are few in comparison with the wealth of studies reporting network-level dysfunction. The thalamus has been traditionally ignored[269], both in mTBI research and the wider neuroimaging community.

This may be attributed to previous ideas that its sole function was sensory relay- thus being irrelevant for higher cognitive function-, and proximity to sources of noise in fMRI (i.e., the ventricles) making thalamic signal less reputable in acquisitions with lower spatial resolution and less advanced preprocessing/denoising pipelines. With a new understanding of its brain-wide importance for healthy information processing[269], there is a renewed interest in thalamic function after injury which warrants further investigation.

Current literature lacks studies with sufficient sample sizes and longitudinal follow up and has not yet investigated the role of biologically-relevant subdivisions of the thalamus. Individual nuclei have different biological properties, primary functions, and cortical connectivity, and may therefore have differential prognostic specificity and therapeutic relevance. Moreover, previous studies included patients with pre-existing risk factors for post-TBI symptoms, clouding the neuroimaging consequences of these factors from those due to TBI. Finally, none of these studies relate imaging-derived measures associated with symptoms to their neurochemical basis or potential therapeutic targets. This is an unmet need, as current treatments for postconcussive symptoms lack both evidence-based support and a clear biological framework[289].

4.1.2 Aims & hypotheses

This chapter therefore aims to elucidate acute thalamic changes after mTBI. I investigate both structural volume changes and functional connectivity changes from individual thalamic nuclei in mTBI versus healthy controls, and their relationship to postconcussive outcome. Additionally, I follow a sub-cohort of mTBI with longitudinal imaging and postconcussive symptom reports, to understand possible trajectories of connectivity and symptom change. Finally, I explore possible therapeutic targets of functional changes using a novel method correlating changes with healthy average PET maps.

My hypotheses are as follows:

1) There are acute functional, but not structural, thalamic changes after mTBI. This is characterised by thalamocortical *hyper*connectivity.
2) Acute thalamic hyperconnectivity is associated with 6-month postconcussive symptom presentation.

3) Thalamic connectivity changes over time towards hypoconnectivity in those with persistent symptoms.

With the additional exploratory hypothesis:

4) Thalamic functional changes are associated with specific neurochemical systems.

4.2 Method

4.2.1 Cohort

These analyses used an identical cohort as in **Chapter 3**; n=108 mTBI patients and n=76 healthy controls. Additionally, a subset of this mTBI cohort had serial structural and functional imaging at 6- and 12-months post injury, thus were followed longitudinally. All data were preprocessed according to parameters given in **Chapter 3 section 3.2.2**, and thus are not repeated here. Briefly, data were preprocessed using fMRIprep's standardised pipeline including steps of bias correction, segmentation, coregistration, motion correction, and spatial normalisation to standard MNI space. Data were then denoised via signal regression of nuisance covariates, and spatially smoothed with a 6mm gaussian kernel.

Six-month outcomes assessed functional and symptomatic recovery using the GOSE[48] and Rivermead Postconcussion Symptom Questionnaire (RPQ[52]). As before, these measures were binarized to 'complete' (GOSE-8) versus 'incomplete' (GOSE ≤7) recovery, and postconcussive symptom (PCS) positive or negative. Postconcussive symptoms were further explored using the three-factor structure of RPQ encompassing cognitive, emotional, and somatic domains[202]. Groups were defined on those who presented (>=1) or did not present (<1) that factor by taking a mean of the relevant RPQ items. These arguably lenient groupings were used due to the mild nature of the cohort to ensure any presenting symptoms would be captured, and sample sizes were suitable for group comparisons. A summary of methods used in this chapter are presented below in **figure 4.3.**

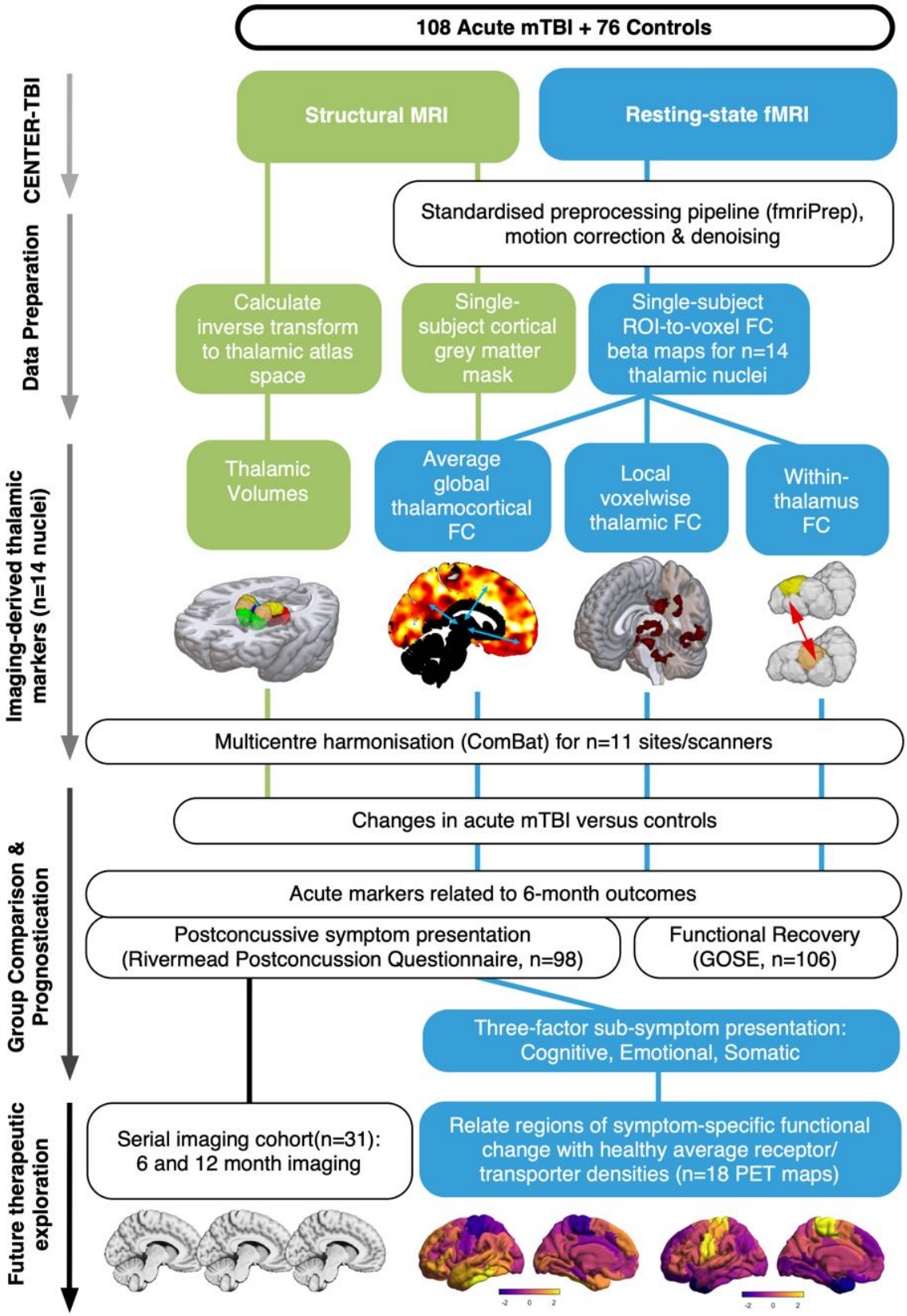


Figure 4.3. Methodological summary for Chapter 4. Flow diagram includes steps regarding structural MRI (green) and resting-state fMRI (blue), with additional analysis steps presented in white.

4.2.2 Thalamus subdivisions

The left and right thalamus and seven thalamic nuclei per hemisphere (n=16 regions of interest; ROIs) were investigated using the probabilistic atlas defined by Najdenovska and colleagues[290]. This was obtained from a large and healthy sample (n=70) with T1-weighted and T2-weighted MRI and DWI, to group regions with similar microstructural properties according to their clustering algorithm, which was applied for individual-level parcellation and combined into an average atlas, used here. I chose to use this thalamic parcellation as their data-driven results were further validated with histological comparison, the atlas was proven to be a successful substitution for individually segmented thalamic nuclei when DWI data is unavailable[290], and more than seven subdivisions seemed unfeasible given the spatial resolution of fMRI data to give sufficient specificity in clinical populations. Atlas standard space was transformed into preprocessed standard space with FSL FLIRT, using affine transformation with 12 degrees of freedom, 180-degree search angle and trilinear interpolation, and applied to thalamic maximum probability masks. These were re-binarized at values >0.5 to avoid transformation-induced overlaps which could impact results. Nuclei are shown below in **figure 4.4**.

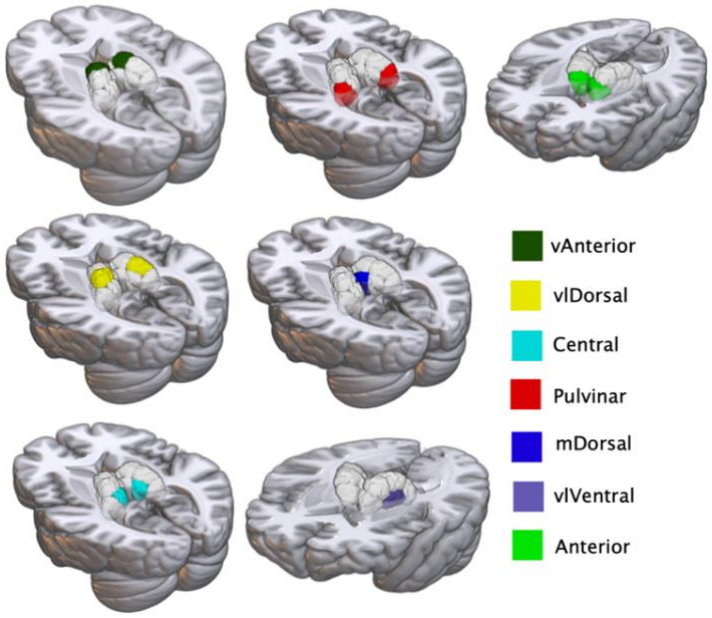


Figure 4.4. Thalamic nuclei masks. Colour shows bilateral thalamic 'group' whereby labelling indicates a known anatomical nucleus or nuclei group, except for the 'Central' group which represents the central lateral, lateral posterior, and some anterior medial pulvinar. Thalamic groups were analysed in each hemisphere individually, to form 7 subdivisions per hemisphere. Abbreviations: vAnterior (ventral anterior group), vlDorsal (ventral lateral posterior, dorsal division group), mDorsal (medial dorsal group), vlVentral (ventral-lateral ventral group). Derived from Najdenovska and colleagues.

4.2.3 Thalamic volume

For volume extraction, each raw T1-weighted (T1w) scan was first corrected for scanner bias field inhomogeneities[235] and spatially normalised to the MNI ICBM152 T1w template corresponding to thalamic atlas space[291] via affine and non-linear registration[236]. To estimate spatial normalisation quality, the zero-normalised cross correlation (ZNCC) was computed between aligned T1w scans and the T1w template image:

$$ZNCC = \frac{1}{N} \sum_{x,y} \frac{(x_i - \mu_x)(y_i - \mu_y)}{\sigma_x \sigma_y}$$

with N being the number of voxels within the brain mask of image x (the projected T1w scans) and image y (the T1w template), and μ and σ representing the mean and standard deviation respectively. A high ZNCC value corresponds to high similarity between image intensities and indicates a successful spatial alignment between scans. Importantly, ZNCC did not differ between patient and control groups following harmonisation ($F_{1,180}$=1.07, p=.30). The inverse of the found transformations were used to project the thalamus atlas with nearest neighbour interpolation from MNI template space to each subject's individual T1w space. Volumes of thalamus (left and right) and its nuclei were computed by summing up the voxels of the back-projected atlas regions and multiplied by single-voxel volume. Eventually thalamic volumes were normalised by the total brain volume, estimated via automated brain extraction[292].

4.2.4 Thalamic functional connectivity

Three lines of thalamic functional connectivity were investigated. Firstly, average thalamocortical connectivity was investigated using the CONN toolbox v.20.b[293], as previous work in mTBI has found widespread functional alterations across the cortex. For each participant, this obtained beta maps of ROI-to-voxel connectivity for all n=16 thalamic ROIs, and a mean calculated within a mask for each individual's cortical grey matter. Secondly, local brain-wide functional connectivity changes were assessed using the previously calculated beta maps and studied for voxelwise connectivity differences between groups using SPM12[232]. Finally, functional connectivity between thalamic ROIs was calculated by correlating each pair of average timecourses (first 5 volumes removed)

to obtain a correlation coefficient, and Fisher's r-to-z transform applied. Each of n=184 subjects therefore had a 16x16 matrix of within-thalamus connectivity values.

4.2.5 Association to neurotransmitter systems

Neurotransmitter systems become strongly dysregulated following injury[252]. Consequently, to better understand potential underpinnings of altered connectivity and to characterise possible therapeutic avenues for chronic symptomatology, we explored whether clusters of significant change from group comparisons might be related to specific neurotransmitter receptors and/or transporters.

Receptor densities were estimated using group-averaged PET receptor/transporter maps obtained from healthy volunteers for a total of 18 receptors and transporters, across 9 neurotransmitter systems, as detailed in recent work by Hansen and colleagues[294]. These included dopamine (D1, D2, DAT), norepinephrine (NET), serotonin (5-HT1A, 5-HT1B, 5-HT2A, 5-HT4, 5-HT6), acetylcholine ($\alpha 4\beta 2$, M1, VAChT), glutamate (mGluR5), GABA (GABA-A), histamine (H3), cannabinoid (CB1), and opioid (MOR). Volumetric PET images were registered to the MNI-ICBM 152 nonlinear 2009 (version c, asymmetric) template, averaged across participants within each study, then parcellated and receptors/transporters with more than one mean image of the same tracer (5-HT1b, D2, VAChT) were combined using a weighted average. See Hansen *et al*[294] for detailed information about each map.

Both the PET maps and the statistical maps of seed-to-voxel correlation t-scores were then parcellated into discrete cortical regions according to the recent local-global cortical functional atlas of Schaefer[295] scales 100 and 200, and the multimodal cortical parcellation of Glasser[296] with 360 cortical regions.

4.2.6 Statistical analyses

All statistical analyses were conducted using R (v.4.1.2) at an FDR-corrected significance level of $p \leq 0.05$ unless otherwise stated. Harmonization for site/scanners was applied prior to statistical analysis on each imaging-derived value type individually, using the same parameters described in **Chapter 2**. Further support for harmonization validation and denoising quality control are given in **Appendix 4.1.**

For thalamic volume, average thalamocortical functional connectivity, and within-thalamus functional connectivity, each variable was compared between cohort groups (controls vs patients) using a linear model with type III SS to assess the significance of group membership while controlling for covariates of sex and age. Thalamic volumes were additionally controlled for spatial normalisation quality (ZNCC) within the model. Variables with significant differences were then similarly compared between outcome groups (GOSE, PCS), further accounting for age, sex, time since injury, and baseline GCS in the linear model.

Final mTBI vs control comparisons of voxelwise functional connectivity used SPM12[232] and ran a one-sample t-test (FWE p=0.01, implicit mask) to establish the most implicated voxels across participants' beta-maps. Second-level analysis was constrained by the one-sample results' mask and ran two-sample t-tests between control and mTBI groups. These tests were conducted with thresholds set at $p<0.001$ (uncorrected) at the voxel level and FWE-corrected $p<0.05$ at the cluster level and repeated for all n=16 thalamic ROIs. Results informed further investigation of functional connectivity differences between outcome groups (GOSE, PCS) according to the same statistical criteria.

Seed-to-voxel t-maps with significant clusters were further parcellated and correlated with z-scored PET maps, to assess their spatial correspondence. This focussed on three nuclei of interest identified previously, and with significant clusters of group differences. The statistical significance of correlations was tested against a rigorous null model that considers the spatial dependency of the data by using spatial autocorrelation-preserving permutation tests, termed spin tests[297,298]. Parcel coordinates were projected onto the spherical surface and then randomly rotated and original parcels were reassigned the value of the closest rotated parcel. This procedure was performed with 10,000 repetitions, thereby obtaining a null distribution with preserved spatial autocorrelation. This spin test embodies the null hypothesis that neurotransmitter density and thalamic seed-based functional connectivity are spatially correlated with each other only because of inherent spatial autocorrelation. Significantly associated PET maps at the mTBI-Control level were taken forward to comparisons between outcome groups, and only in those maps where significant voxelwise differences were found. All p-values were corrected for multiple comparisons using FDR-correction within-test and required replication across all three parcellation schemes for additional robustness of my results.

In all comparisons found to be significant between PCS outcome groups, postconcussive symptom types were further explored. This used the three-factor structure of RPQ encompassing cognitive, emotional, and somatic domains[202]. Patients were thus split into those with or without cognitive (Cog+ n=38; Cog- n=60), emotional (Emo+ n=38; Emo- n=60), or somatic symptoms (Som+ n=23; Som- n=75). Group membership was not related to age ($X^2$1=0.4 p=.82; $X^2$1=0.2, p=.91; $X^2$1=1.1, p=.59), sex ($X^2$1=0.4, p=.54; $X^2$1=2.2, p=.14; $X^2$1=1.9, p=.17), time since injury for scan (*t*96=0.7, p=.48; *t*96=0.3, p=.79; *t*96=1.2, p=.25), or baseline GCS (Fisher's Exact p=1; p=1; p=.81) in cognitive, emotional, or somatic groups respectively. The cognitive and emotional sub-groups showed significant overlap of patient inclusion ($X^2$1=42, p<.001; n=30 with both cognitive and emotional symptoms) but are investigated here as separate phenotypes.

The final analyses investigated longitudinal changes in the serial imaging cohort. These were compared in demographic characteristics and imaging-derived variables to the non-follow-up cohort to ensure continuity between acute and longitudinal findings. All data were preprocessed and analysed with covariates as before, calculating thalamic volume and average thalamocortical connectivity from each nucleus, but without statistical harmonisation for site differences due to smaller sample size reducing its success across the n=4 sites present. Significant variables were further compared to PCS status using a two-way mixed-ANOVA, where PCS status was defined based on previously described criteria being met at 6 and/or 12 months (between-subjects), and time of imaging was acute or 12 months post-injury (within-subjects). Significant interaction effects were further explored for effects of PCS group, with a post-hoc within-subjects linear model with covariates of age, sex, initial GCS, and time between acute and 12-month imaging.

4.3 Results

4.3.1 Functional, but not structural, thalamic changes are seen in the acute phase of mTBI

Several lines of evidence suggested widespread functional alterations in acute mTBI, despite no differences in thalamic volume (**table 1**). First, average global functional connectivity between the thalamic ROIs and cortical GM were significantly different

between the two groups, with significant nuclei-specific global hyperconnectivity from the bilateral ventral anterior (vAnterior; Left $F_{1,180}$=10.5, p=.02; Right $F_{1,180}$=8.34, p=.02) and right ventral lateral dorsal (vlDorsal; $F_{1,180}$=9,89, p=.02) in patients compared to controls after FDR correction (**figure 4.5A; table 1**). I found further evidence for vulnerability of these specific nuclei when considering within-thalamus connectivity. Across 23 pairs of nuclei, patients showed increased connectivity compared to controls (**figure 4.5B**), and additionally the averaged connectivity to the rest of the thalamus for the left and right vAnterior and the right vlDorsal thalamic nuclei was significantly higher in patients versus controls (**table 4.1, figure 4.5C**). No decreases in within-thalamus functional connectivity were found in mTBI.

Table 4.1. Mild TBI versus controls comparisons in structural and functional imaging.

Thalamic ROI	**T1w Volume F-test (*1,179*)**	**Thalamocortical FC F-test (*1,180*)**	**Average within-thalamus FC F-test (*1,180*)**
Left Thalamus	*F*=0.9, *p*=.96	*F*=4.1, *p*=.10	-
Right Thalamus	*F*=0.02, *p*=.97	*F*=2.7, *p*=.20	-
Left-hemisphere nuclei			
Pulvinar	*F*=0.1, *p*=.97	*F<0.01*, *p*=.99	*F*=2.0, *p*=.24
Anterior	*F*=3.7, *p*=.32	*F*=4.7, *p*=.08	*F*=1.5, *p*=.25
mDorsal	*F*=0.05, *p*=.97	*F*=0.7, *p*=.61	*F*=1.5, *p*=.25
vlDorsal	*F*=0.4, *p*=.97	*F*=6.0, *p*=.06	*F*=4.8, *p*=.08
Central	*F<0.01*, *p*=.99	*F*=0.4, *p*=.63	*F*=2.6, *p*=.24
vAnterior	*F*=1.7, *p*=.80	***F*=10.5, *p*=.02**	***F*=7.8, *p*=.03**
vlVentral	*F*=0.01, *p*=.97	*F*=1.4, *p*=.41	*F*=1.4, *p*=.26
Right-hemisphere nuclei			
Pulvinar	*F*=4.5, *p*=.31	*F*=1.3, *p*=.41	*F*=5.2, *p*=.08
Anterior	*F*=6.6, *p*=.18	*F*=5.7, *p*=.06	*F*=1.1, *p*=.30
mDorsal	*F*=0.1, *p*=.97	*F*=0.4, *p*=.64	*F*=1.8, *p*=.24
vlDorsal	*F*=0.1, *p*=.97	***F*=9.9, *p*=.02**	***F*=9.0, *p*=.02**
Central	*F*=0.6, *p*=.97	*F*=0.05, *p*=.87	*F*=1.9, *p*=.24
vAnterior	*F*=1.2, *p*=.90	***F*=8.3, *p*=.02**	***F*=9.0, *p*=.02**
vlVentral	*F*=0.1, *p*=.97	*F*=0.3, *p*=.65	*F*=2.3, *p*=.24

Bold indicates statistical significance at FDR-corrected p≤0.05, whereby tests are two-tailed but patients showed increased functional connectivity (FC) compared to controls in significant results. Tests included covariates of sex and age, and additionally spatial normalisation quality for volume comparisons.

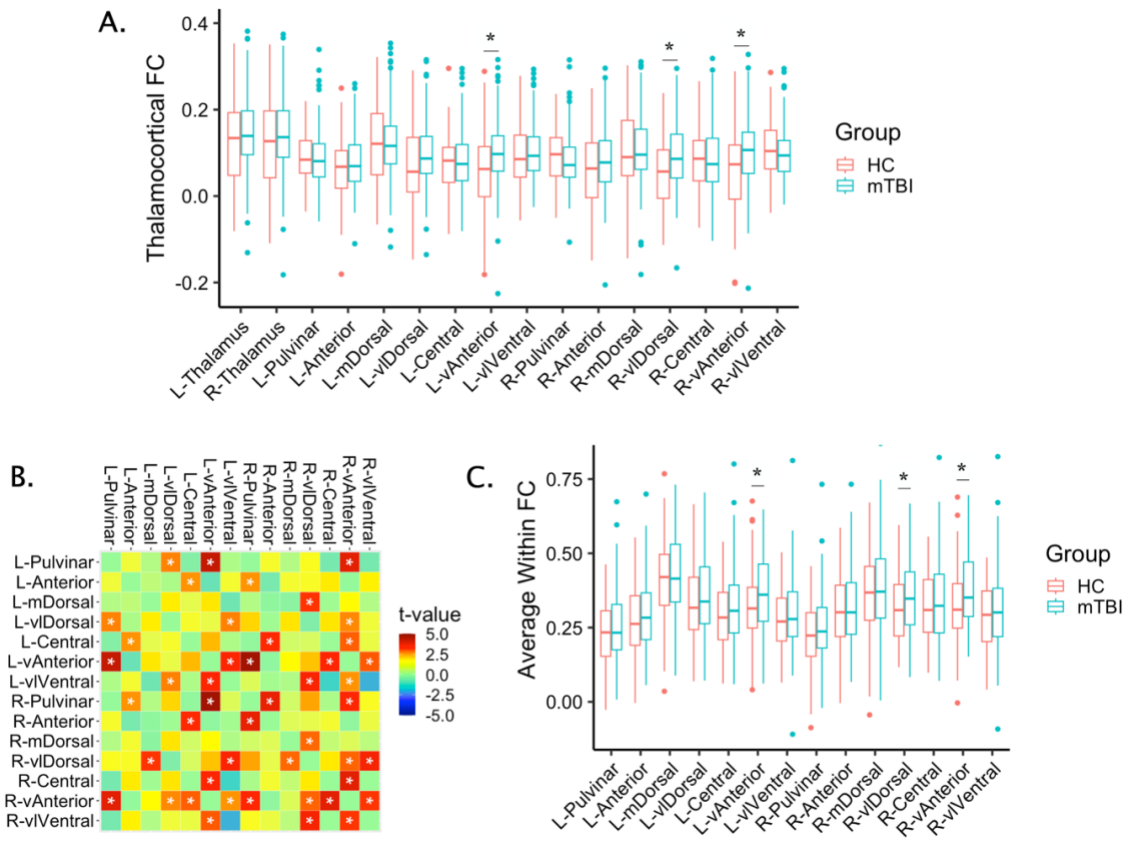


Figure 4.5. Nuclei-specific vulnerability comparing mTBI and controls. Asterisk indicates statistical significance at FDR-corrected $p \leq 0.05$, HC=controls, FC = functional connectivity. A) Thalamocortical connectivity comparisons. B) Within-thalamus connectivity adjacency matrix by t-value colour from statistical testing, where red-yellow colours indicate higher functional connectivity in mTBI compared to controls. C) Average within-thalamus connectivity values, derived from B, showing higher functional connectivity in mTBI in the same three nuclei as A.

I next looked for specific connectivity changes that underpinned the globally increased thalamocortical connectivity. The mTBI patients showed increased functional connectivity from all thalamic ROIs, except for the right-Central and right-mDorsal nuclei. In contrast, no ROI demonstrated a decrease in connectivity. This picture of acute hyperconnectivity could be split into three groups of nuclei-specific results (**figure 4.6**); at posterior cingulate cortex from more anterior thalamic nuclei (Anterior, vAnterior, mDorsal, and vlDorsal); midbrain region inferior to the left red nucleus (maximum coordinate: -4, -22, -17) from more posterior thalamic nuclei (Pulvinar, Central, vlVentral); and widespread cortical hyperconnectivity from vAnterior and vlDorsal nuclei, replicating the results of global increases in thalamocortical connectivity. These results are echoed in overarching voxelwise results from the left and right thalamus (**figure 4.6A**), but greater specificity was found by looking at the respective subdivisions (**figure 4.6B**).

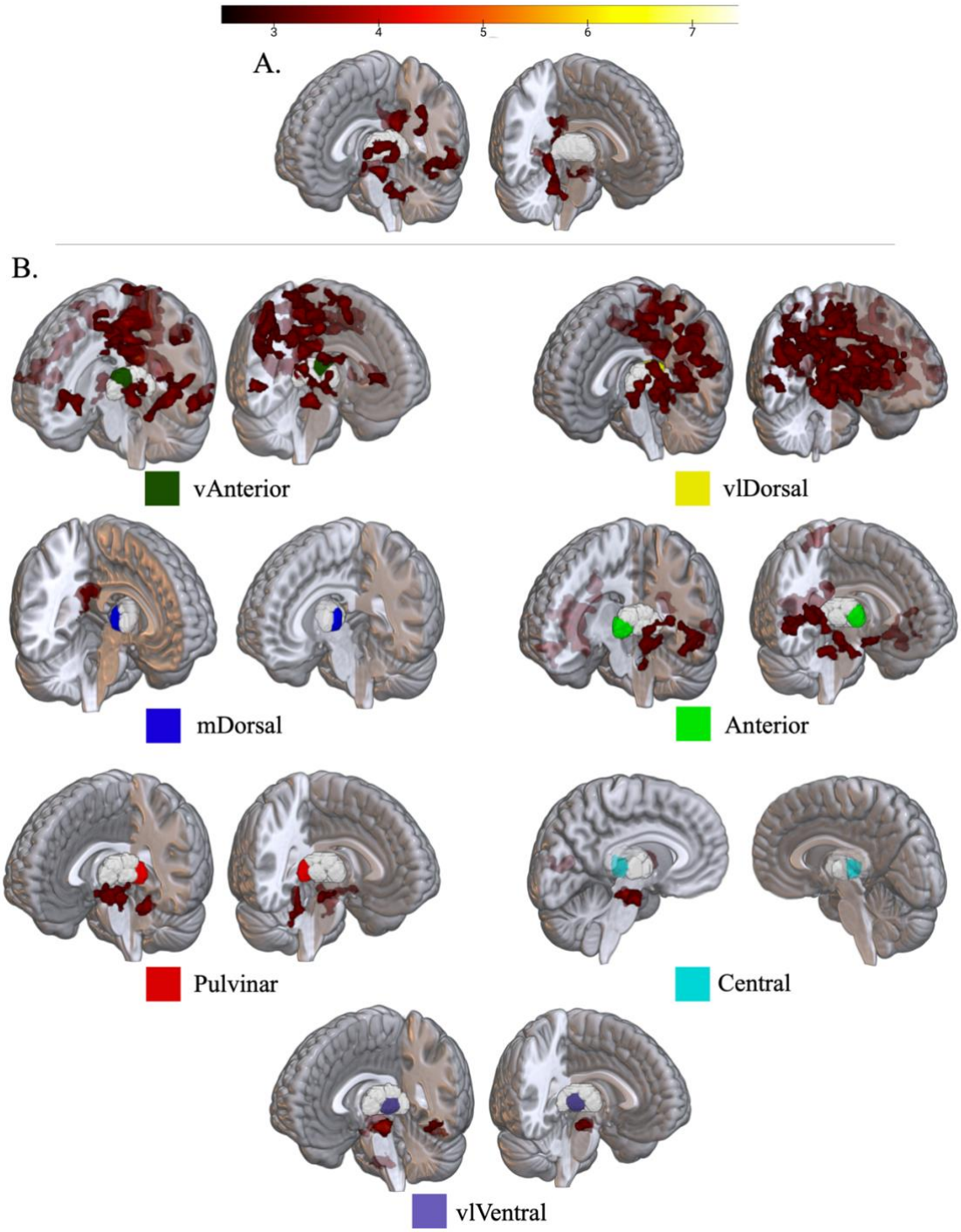


Figure 4.6. Voxelwise results of increased functional connectivity in mTBI compared to controls. All images show voxels surviving significance and cluster-level correction, using colour bar scale at top. A) Results from left and right thalamus respectively, where seed mask is presented in greyscale. B) Left and right hemisphere nuclei-specific results, where seed-nucleus is indicated by the colour legend. Images without clusters shown indicate no voxels exceeded cluster-corrected significance. Top left panel shows results seeded from vAnterior nuclei, and top right panel shows results from vlDorsal nuclei, partially obscured by hyperconnected clusters.

4.3.2 Acute thalamic hyperconnectivity is related to chronic postconcussive symptoms

Group comparisons showed greater thalamic functional connectivity at both local and global levels in patients with chronic postconcussive symptoms (PCS+) than those without such symptoms (PCS-) (**table 4.2, figure 4.7A**). The PCS+ group showed clusters of increased connectivity between R-vAnterior and middle/inferior temporal gyrus, and R-vlDorsal and inferior frontal gyrus and frontal cingulate/paracingulate (**figure 4.7B**). No connectivity differences were seen between patients with complete or incomplete recovery based on the GOSE. Further, thalamocortical connectivity was higher in those with cognitive or emotional symptoms from all three nuclei (**table 4.2, figure 4.7C,E**). Somatic symptoms were associated with significant but less prominent cortical hyperconnectivity from the right vAnterior nucleus (**table 4.2**). Hyperconnected clusters in those with cognitive symptoms mainly encompassed cortical regions associated with frontoparietal control network, with some additional increased connectivity to midbrain regions (**figure 4.7D**). Participants with long-term emotional symptoms also displayed hyperconnectivity seeded from the right vAnterior nucleus to medial temporal and medial posterior occipital regions, which have been previously associated with emotion/language and visual networks (**figure 4.7F**). Regional network relationships were identified using the ICN-Atlas toolbox in SPM[299], described in **Appendix 4.4**. No voxelwise differences were found associated with somatic symptom presentation. However fewer individuals presented somatic symptoms on average, and as such had more unequal sample sizes, which may have reduced statistical sensitivity to find an effect. No differences were found between outcome groups in within-thalamus functional connectivity comparisons (**Appendix 4.2**).

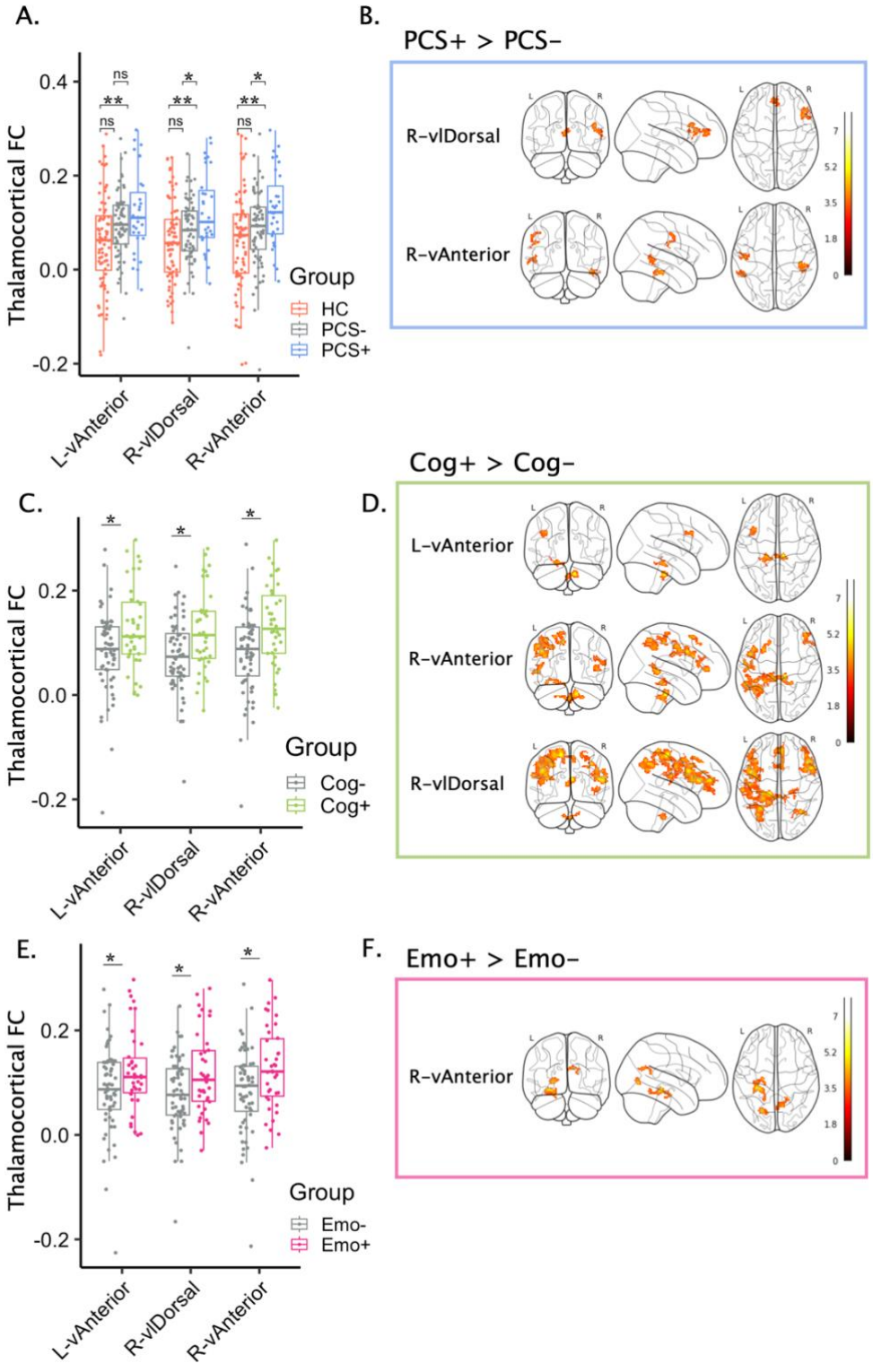


Figure 4.7. Relating thalamic hyperconnectivity to postconcussive outcomes. A, C, E compares average thalamocortical functional connectivity between outcome groups looking at the three nuclei of interest: left and right vAnterior and right vlDorsal. Asterisk indicates statistical significance at p≤0.05. B, D, F column shows voxelwise thalamic functional connectivity results seeded from these same nuclei surviving significance and cluster-level correction, compared between corresponding outcome groups. These results show higher functional connectivity in those with

postconcussive symptoms (PCS+), and cognitive/emotional symptom clusters, at the local and global level of investigation.

Table 4.2. Outcome group comparisons in thalamocortical functional connectivity.

		Acute thalamocortical connectivity		
Comparison	**Test (df)**	**Left vAnterior**	**Right vAnterior**	**Right vlDorsal**
6-Month GOSE				
GOSE≤7 > Control	F-test (*1,120*)	***F*=7.3, *p*=.02**	***F*=8.1, *p*=.02**	***F*=11.1, *p*=.01**
GOSE-8 > Control	F-test (*1,130*)	***F*=6.8, *p*=.02**	*F*=3.8, *p*=.08	*F*=4.5, *p*=.06
GOSE≤7 > GOSE-8	F-test (*1,100*)	*F*=0.01, *p*=.91	*F*=0.9, *p*=.38	*F*=1.8, p=.23
6-Month PCS				
PCS+ > Control	F-test (*1,103*)	***F*=9.5, *p*=.01**	***F*=10.7, *p*=.01**	***F*=13.2, *p*=.004**
PCS- > Control	F-test (*1,139*)	*F*=4.3, *p*=.06	*F*=2.2, *p*=.14	*F*=2.9, *p*=.12
PCS+ > PCS-	F-test (*1,92*)	*F*=2.3, *p*=.14	***F*=5.0, *p*=.050***	***F*=5.8, *p*=.04**
6-Month Rivermead Factor structure				
Cog+ > Cog-	F-test (*1,92*)	***F*=6.7, *p*=.02**	***F*=9.7, *p*=.01**	***F*=9.7, *p*=.01**
Emo+ > Emo-	F-test (*1,92*)	***F*=4.3, *p*=.052***	***F*=6.5, *p*=.02**	***F*=6.6, *p*=.02**
Som+ > Som-	F-test (*1,92*)	*F*=2.5, *p*=.12	***F*=5.1, *p*=.04**	*F*=3.4, *p*=.08

Bold indicates statistical significance at FDR-corrected p≤0.05. F-test statistics are derived from linear models comparing between-groups after accounting for covariates, equivalent to between-subjects t-test. Tests were two-tailed however the poorer outcome group (i.e., GOSE ≤7 or PCS/symptom positive), or mTBI group compared to controls, had higher FC.
* Values rounded to p=0.05.

4.3.3 Neurochemical associations of hyperconnectivity may identify treatment targets

In relating regions of injury-induced thalamic connectivity to neurotransmitter maps, regions rich in monoaminergic transmitter receptors and transporters were targets of thalamic hyperconnectivity after mTBI – with positive correlations to noradrenergic and dopaminergic targets, and negative correlations to select serotonergic transmitter system constituents. Positive associations were also found for metabotropic glutamate and vesicular acetylcholine targets. Most strikingly, a significant positive correlation between hyperconnectivity and noradrenergic transporter density was found across all three nuclei of interest for the mTBI-Control and Cog+/Cog- comparisons, and the investigated Emo+/Emo- t-map. This suggests that regions which are functionally hyperconnected after injury and associated with persistent specific symptomatology have high noradrenergic transporter density. A similar relationship was also found for lower 5-HT 2a receptor levels and Emo+/Emo- t-maps; although this latter result marginally exceeded the significance threshold in one of the tested parcellations

(Glasser360 p=.007; Schaefer100 p=.033; Schaefer200, p=.064). Results that remained significant after stringent statistical corrections are presented in **figure 4.8**. These were derived from Schaefer's local-global parcellation with 200 cortical regions[295], but are only shown if also replicated as significant using the 100-region Schafer parcellation (Schaefer-100), as well as Glasser's well-known multimodal parcellation (360 cortical regions)[296]. The remaining nonsignificant associations with PET maps are presented in **Appendix 4.5**.

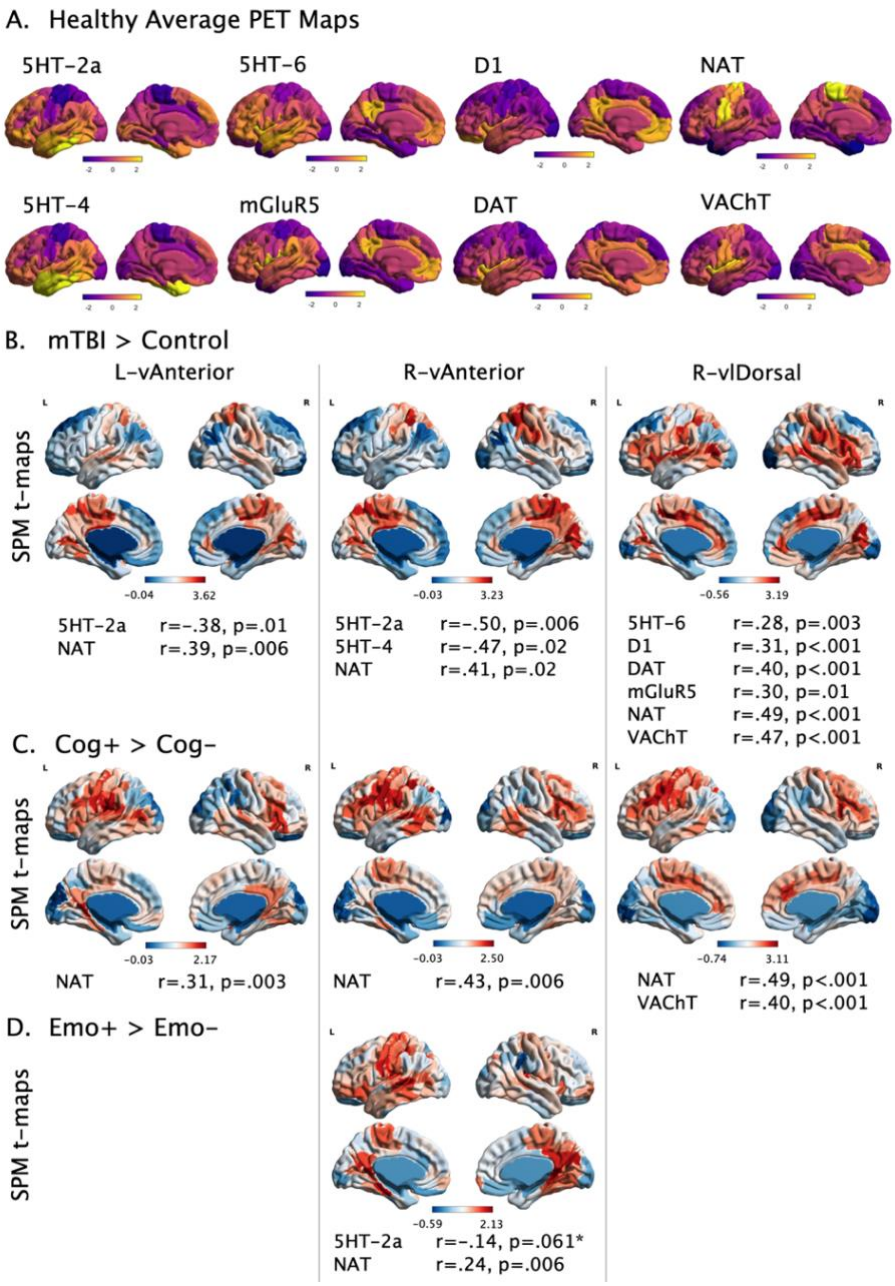


Figure 4.8. Significant correlations between averaged PET maps and voxelwise SPM-t maps from group comparisons. A. PET maps reaching significant association in one or more

comparison, each normalised within-map to show range of z-scores. Higher z-score indicates greater density of that transmitter receptor or transporter. B,C,D. Cortical SPM t-maps derived from groups comparisons of functional connectivity seeded from each respective thalamic ROI, where red regions indicate greater connectivity in one group (mTBI, Cog+, Emo+) than the second group (Control, Cog-, Emo-). These t-maps are correlated with PET maps and significant associations presented below. *Marginally non-significant when using Schaefer200 parcellation but found significant when using alternative parcellations (Glasser360 and Schaefer100).

4.3.4 Longitudinal evolution of thalamic connectivity varies with postconcussive outcome

Finally, given the potential prognostic value of thalamocortical functional connectivity, I investigated a subset of patients (n=31), in whom structural and functional imaging were available at 6- and 12-months post-injury. The serial imaging cohort did not differ in age ($X^2$1=0.8, p=.69), or baseline GCS (Fisher's exact, p=.59) to the cohort in whom serial imaging was unavailable, but had higher incomplete recovery according to GOSE at 6 and 12mo ($X^2$1=15.3, p<.001; $X^2$1=15.3, p<.001), fewer female participants ($X^2$1 =4.0, p=0.046), and were exclusively from admission stratum due to recruitment protocols in CENTER-TBI ($X^2$1=36.4, p<.001). Furthermore, n=16 of this cohort developed PCS at either/both 6/12 months versus n=15 who did not, encompassing poorer outcomes than the original cohort. This cohort therefore provides a representation of real-world follow up practice and may be less generalizable to mTBI as a whole. However, the serial imaging cohort showed acute thalamocortical hyperconnectivity compared to controls in the same three nuclei as seen in the overall mTBI group after FDR-correction (L-vAnterior $F_{1,104}$=8.46, p=.03; R-vAnterior $F_{1,104}$=7.45, p=.04; R-vlDorsal $F_{1,104}$=11.96, p=.01), and were therefore thought to be appropriately representative of the overall narrative of pathophysiology in mTBI.

When splitting this serial imaging cohort into PCS+ and PCS-, all three nuclei of interest showed significant interaction effects between PCS status and time (acute and 12 month imaging) using a two-way mixed ANOVA (L-vAnterior $F_{1,24}$=4.42, p=.046; R-vAnterior $F_{1,24}$=8.11, p=.01; R-vlDorsal $F_{1,24}$=7.94, p=.01). Post-hoc, within-subjects, tests showed that only the PCS+ cohort showed significantly decreased functional connectivity in these nuclei over time (L-vAnterior $F_{1,11}$=6.18, p=.03; R-vAnterior $F_{1,11}$=8.42, p=.01; R-vlDorsal $F_{1,11}$=10.1, p=.01), whereas the PCS- cohort showed no change (L-vAnterior $F_{1,9}$=0.28, p=.61;

R-vAnterior $F_{1,9}$=0.45, p=.52; R-vlDorsal $F_{1,9}$=1.46, p=.26). These results can be seen in **figure 4.9** and are uncorrected for multiple comparisons given the small sample sizes in this follow-up cohort. Whilst these were not explicitly compared to control groups, **figure 4.9** shows the healthy control mean and interquartile range to provide additional context. These results were reproduced when analysing only significantly hyperconnected clusters in mTBI compared to controls derived from voxelwise maps: functional connectivity in initially hyperconnected clusters decreases over time only in those with long-term PCS (**Appendix 4.7**).

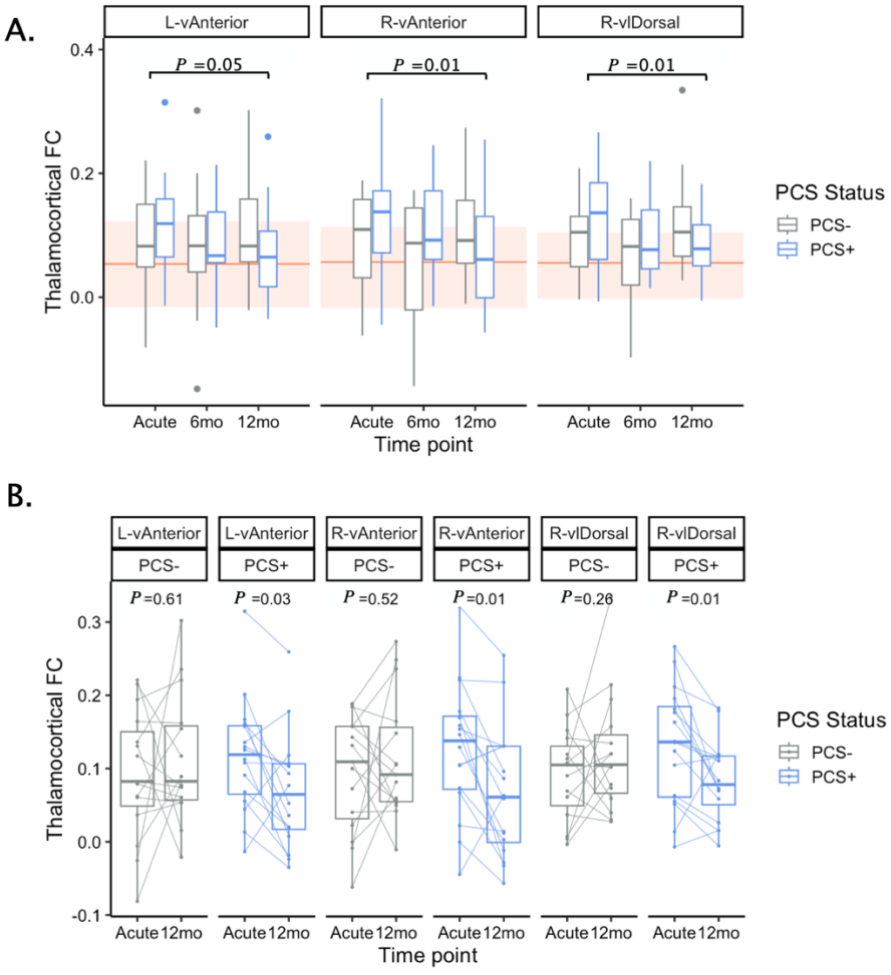


Figure 4.9. Longitudinal follow-up of thalamocortical functional connectivity in three nuclei of interest in relationship to postconcussive symptom presentation. A. Mixed ANOVA between acute and 12mo timepoints between groups, where p-values given are significant interaction effects between timepoint (acute or 12mo) and group (PCS+ or PCS-). Shaded regions give the interquartile range of controls for each nucleus, with solid line indicating the controls' mean. B. Post-hoc results within-subjects finding significant decreases in functional connectivity only in those with three or more postconcussive symptoms (PCS+). Lines join individual subjects' data at different

timepoints. All p-values in A and B are uncorrected for multiple comparisons due to small sample size, however corrected values are presented in-text.

Additionally, volume analyses were repeated at these timepoints and showed no changes to controls, or in a within-subjects ANOVA of volume change over time (acute, 6mo, 12mo), in any thalamic ROIs shown in **figure 4.10**. Specific statistical results are reported in **Appendix 4.6**. This suggests that time-dependent functional imaging changes associated with poor outcome are not reflected in routine structural imaging.

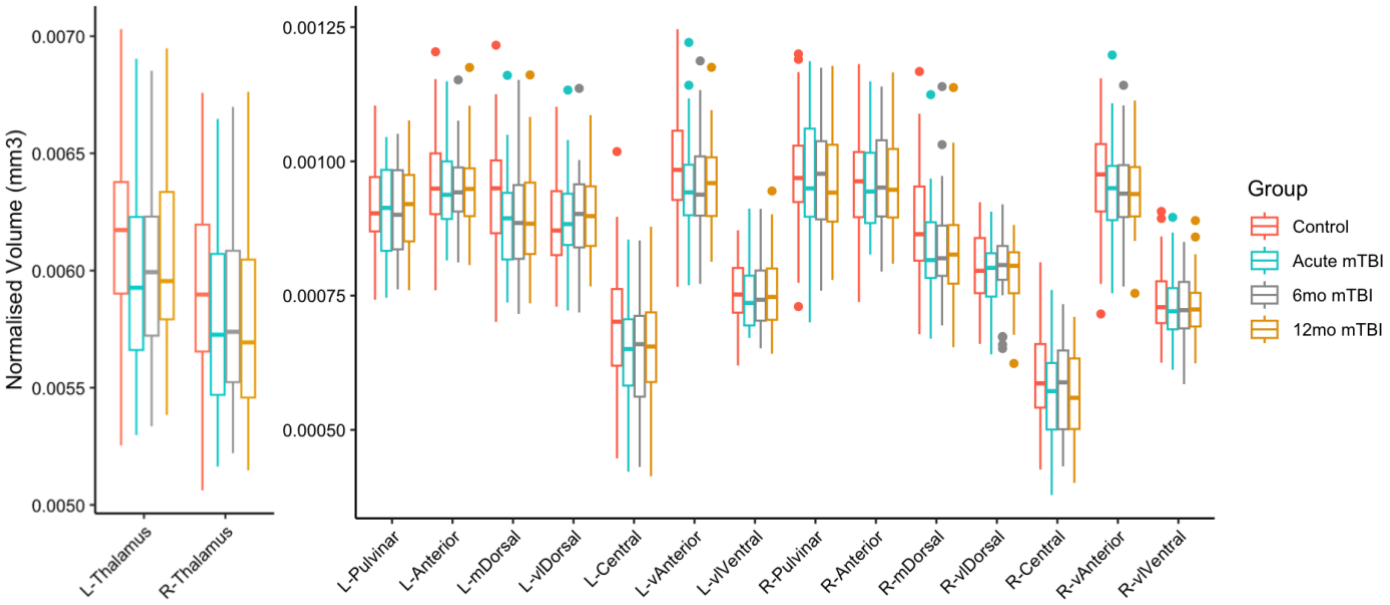


Figure 4.10. Longitudinal follow-up of thalamic volume. All volumes are normalized by total brain volume. Groups are colour-coded for the healthy control cohort (n=76) and serial imaging mTBI cohort (n=31) at each respective timepoint. No significant differences at $p<0.05$ were found between controls and acute mTBI, or within-subjects over time, including covariates of age and sex and following FDR-correction.

4.4 Discussion

In this chapter, I showed that 'mild' injury was associated with widespread increases in acute connectivity of thalamic nuclei; to cortical, subcortical, and other thalamic regions. This was in the absence of detectable structural thalamic change. Further, these changes were uniquely associated with the presence of persistent postconcussive symptoms, and not general functional outcome, with specific relationships identifiable between individual thalamic nuclei and symptom categories. Such acute thalamic hyperconnectivity evolved differentially in mTBI patients in whom symptoms persisted over time, implicating long-term functional consequences extending beyond the acute

injury event. Finally, injury-induced connectivity changes showed relationship to monoaminergic neurochemical profiles in target cortical regions.

I thus propose that acute thalamic functional connectivity has prognostic potential for enduring postconcussive symptoms, with particular importance of the vAnterior and vlDorsal nuclei groups. Crucially, functional imaging may provide earlier markers for poor outcome than routine anatomical imaging. Behaviourally relevant structural thalamic alterations have been previously found in *post-acute* mTBI[123,285] but were not found here in *acute* investigation, nor did I find thalamic structural change over time beyond this acute phase. In contrast, across all levels of investigation, increased connectivity of these vAnterior and vlDorsal nuclei were associated with postconcussive symptom presentation. These findings demonstrate the relative importance of functional investigation, in an otherwise misclassified disorder with structural imaging alone.

Thalamic hyper- as opposed to hypo- connectivity was consistent across all avenues of investigation and decreases over time were only found in those with persistent symptoms. As previously discussed in **Chapter 3,** hyperconnectivity is an increasingly common signature of acute injury[185,186], and may indicate specific neuronal damage[187] leading to less signal variability and thus increased 'connectivity', or perhaps an adaptive response aiming to overcome such injury. Indeed, several studies of moderate and severe TBI have directly tested and support this adaptive hyperconnectivity hypothesis, proposing it as a compensatory response[188–190]. However, the mild TBI literature faces greater speculation on what is adaptive or maladaptive[191]. Further work in mTBI[186] and other neurodegenerative disorders[300] posits a time-dependent change from acute hyper- to chronic hypo- connectivity as potentially adaptive mechanisms fatigue from persistent overstimulation, particularly in those with poor outcomes, whereas successful recovery is characterized by long-term recovery of connectivity to healthy levels[186]. Here, I found preliminary evidence for decreasing connectivity into healthy ranges from acute to 12-month timepoints, with significantly decreased thalamocortical connectivity only in those with chronic symptoms, partially supporting previously proposed models[186]. Such relationships were additionally found in highly connected 'hub' regions from voxelwise investigations, specifically affecting the Posterior Cingulate and the Insular cortices. These hubs have been previously identified to be relevant in mTBI thalamic connectivity[187], and are also more affected in other neurological diseases such as

Alzheimer's and Parkinson's[300]. These may be particularly vulnerable as damaged nodes lower in the connectomic hierarchy offload to higher-level hubs[300], leading to acute hyperconnectivity particularly in these regions. Thus, my findings may suggest a fatigue of initially adaptive hyperconnectivity mechanisms in those with poor outcome, particularly affecting connectivity hubs. This requires greater investigation to establish the underlying physiology of an adaptive response to injury in mTBI, and its causality in outcome.

I further explored therapeutic targets of my potential prognostic markers and found that thalamic functional connectivity was associated in symptom-specific fashion with particular neurotransmitter system profiles, converged on the importance of monoaminergic transmitter systems. More specifically, the analysis showed associations of hyperconnectivity with noradrenaline transporter and 5HT-2a receptor for cognitive and emotional symptoms, respectively. These powerful neuromodulatory systems[301] are central to the maintenance of healthy connectivity profiles in the human brain[250,251]. In the context of this cohort, it is plausible that noradrenergic and serotonergic (or broader monoaminergic) systems are involved in producing the input-output relationships required for compensatory hyperconnectivity, which is affected when these systems become/remain dysfunctional. Consequently, these data suggest that transmitter system changes might also operate in mTBI– not just in severe cases as previously suggested[252], and that these relationships represent biomarkers that have therapeutic specificity. Expressly, individuals who show noradrenaline-associated connectivity alterations might respond to drugs such as methylphenidate[302]. Similarly, the relevance of a serotonergic target for emotional symptoms after injury is intuitive in the context of pre-existing TBI and depression literature[252] and thus might represent a domain-specific therapeutic direction for future investigations. Therein, these non-invasive, easily implementable assessments could allow for precision neurotransmitter/neuromodulator therapeutic strategies to be developed in the context of mTBI.

With patient care in mind, the relevance of vAnterior and vlDorsal nuclei to injury and outcome is interesting to consider. Their specific involvement may be related to their highly GABAergic innervation[303]; which represents ~35% of total neuronal populations in the vAnterior nucleus[304]. The vAnterior forms part of the thalamic motor relay alongside

the ventrolateral nucleus, connecting GABA-rich substantia nigra pathways up to the premotor cortex, whereas vlDorsal nuclei project to the posterior cingulate[305]. There are further efferent projections from vAnterior thalamus to primary motor, supplemental motor, and possible prefrontal regions, suggesting vAnterior is important for long-range cortical modulatory loops. A previous study investigating ventrally defined thalamic nuclei overlapping these nuclei of interest indeed found both increased thalamocortical connectivity in acute mTBI and increased indicators of neuronal loss and dysfunction using magnetic resonance spectroscopy[187]. The authors speculated that these findings could be due to loss of thalamic inhibitory GABAergic interneurons reducing inhibitory control.

Indeed, excitatory-inhibitory imbalance is a known consequence of TBI[306] and has shown links to thalamocortical functional connectivity regulation[307] and fMRI-derived resting-state networks with strongest association to concurrent GABA-A binding potential[308]. GABA-related changes are also found in animal models of TBI, showing downregulation of GABA-A and GABA-B receptor subunit mRNAs related to thalamocortical relay degeneration[309] and chronically reduced GABAergic parvalbumin positive interneurons[310]. Whilst I did not find a specific association between acute functional connectivity and GABA-A in PET correlations, I only investigated cortical, rather than subcortical thalamic GABA-A binding; a limitation given that only the thalamus and not its functionally hyperconnected regions (e.g., posterior cingulate) showed these markers of neuronal loss in previous study[187]. Furthermore, given the well-defined association between TBI and GABAergic parvalbumin positive interneurons[306], it may be that such associations are clouded by the lack of neurochemical subtype specificity of GABA-A PET maps. I therefore speculate that the present results of ventral thalamic hyperconnectivity replicated across different measures may be associated with thalamic GABA-related inhibitory imbalance, which warrants further investigation.

4.4.1 Limitations

There are arguably three main limitations of this study. First, the thalamus and its subdivisions were not individually defined in each patient. While previous work has validated atlas suitability and accuracy when individual parcellation is not possible[290], individual parcellation could provide higher anatomical accuracy. There is a lack of

consensus on thalamic subdivisions' terminology[273] or a widely accepted thalamic atlas for imaging studies[311], which should be considered when comparing the present results to other studies. Secondly, prevalence rates of PCS in mTBI populations vary substantially depending on the classification method used, however the most common method in the literature aligns with ICD-10 criteria as used here[205]. There are further discrepancies in what constitutes an 'experienced' symptom. As in many previous studies, I used a less conservative definition and thus may incur some 'falsely' defined mTBI patients with PCS[204]. I additionally highlight that the cognitive and emotional groups showed overlap. Whilst postconcussive symptoms may cluster in this three-factor structure, some authors have suggested alternative symptom domains[312], and indeed individuals can concurrently present any number of symptoms. Future research should investigate cohorts uniquely presenting these symptoms for more targeted therapeutic outputs. Finally, I aimed to obtain hypothesis-setting results regarding the neurochemical associations of thalamic hyperconnectivity. However, correlating functional connectivity maps from clinical populations to averaged healthy neurochemical profiles is only the first step in this direction. Neurotransmitter systems are globally disturbed after injury and may not be best represented by these average healthy PET maps. My analysis only addressed cortical relationships- a shortcoming given that I additionally found subcortical clusters of connectivity change. Further, only a subset of all possible neurotransmitters were available for investigation such that other non-investigated neurochemical profiles may be important. Nevertheless, this recently developed method encompasses the broadest set of in-vivo neurotransmitter maps available to date for the human brain, and it begins to investigate biological systems within statistical frameworks of neuroimaging research, bridging fields with traditionally little communication; an important step in imaging-guided treatment.

4.4.2 Conclusion

The 'mild' TBI population is growing and is insufficiently supported. These results show that acute thalamic connectivity may provide an avenue to better understand, prognosticate, and potentially guide treatment of chronic postconcussive symptoms after mTBI. Despite the absence of structural changes, I found acute thalamic hyperconnectivity in mTBI, with specific vulnerabilities of individual thalamic nuclei.

Acute fMRI markers differentiated those with chronic postconcussive symptoms, with time- and outcome-dependent relationships in a sub-cohort followed longitudinally. Longitudinal studies such as this are limited and hold great power to influence clinical practice and long-term care plans, as I found symptom-relevant neurological change extends well-beyond 6-months. Moreover, emotional and cognitive symptoms were associated with changes in thalamic functional connectivity to known dopaminergic and noradrenergic targets, respectively. My findings suggest that chronic symptoms can have a basis in early thalamic pathophysiology. This may aid identification of patients at risk of chronic postconcussive symptoms following mTBI, provide a basis for development of new therapies, and could facilitate precision medicine application of these therapies.

Chapter 5

Repeat mild traumatic brain injury exacerbates acute thalamic hyperconnectivity.

5.1 Introduction

Thus far, whilst hyperconnectivity may be an adaptive response to injury in some cases- such as between-network connectivity found in **Chapter 3-** exacerbation of acute thalamic hyperconnectivity may be detrimental in the long-term. These results were presented in **Chapter 4**, in which I found that individuals with chronic postconcussive symptoms had amplified acute thalamocortical hyperconnectivity, which reduced over 12 months trending towards hypoconnectivity. This was understood as a possible overcompensation for injury in the acute phase, leading to fatigue and long-term failure of such resources. There is thus a complex relationship between mal/adaptive hyperconnectivity, time, and outcome.

Having substantiated the importance of the thalamus in both acute injury and outcome after even a single 'mild' injury, I now look to the special case of repetitive head injuries. These are often associated with sports professionals and veterans and have garnered substantial public interest in recent years.

This interest has been growing since the landmark case study of *chronic traumatic encephalopathy* (CTE) found in NFL player Mike Webster[65]. CTE is a progressive neurodegenerative disease associated with behavioural and mood problems and dementia and was initially characterised by Martland in 1928 as 'punch drunk' syndrome in professional boxers[67]. Finding evidence of mood and personality changes, marked cognitive impairment, and a post-mortem diagnosis of CTE in this case sparked public conversation surrounding the long-term safety of contact sports and combat.

Concerns regarding the safety of repetitive collision are warranted. For example, a recent post-mortem study of over 200 professional and collegiate American football players diagnosed CTE in 87% of cases, which increased in likelihood to 99% of NFL players[66]. However, some recent reviews on CTE have been sceptical about its widespread nature as high prevalence rates are not consistently reported, and have argued that more research is needed to link sports concussion and CTE[313–315]. Such variable reporting rates could be attributable to how diagnostic criteria for CTE have been applied, and indeed this issue has been noted in previous reviews of the literature[316]. In 2016, and later again in 2021, a consortium developed clear post-mortem diagnostic criteria for CTE to

distinguish from other tauopathies and signatures of neurodegeneration[317]. This defined the primary pathology of CTE, amongst additional supportive criteria, as an accumulation of abnormal hyperphosphorylated tau (p-tau) in neurons and astroglia, particularly distributed around small blood vessels at the depths of cortical sulci and in an irregular pattern. Diagnostic criteria of CTE had a variable past prior to this clear definition[318], and thus many studies may have been inaccurately diagnosing or failing to diagnose CTE in studies not accurately following these guidelines. Members of this consortium group have since evaluated independent worldwide research on CTE with more accurate diagnoses, concluding that there is a "*high likelihood of a causal relationship between RHI [*repetitive head injury*] and CTE, a conclusion that is strengthened by the absence of any evidence for plausible alternative hypotheses*"[318]. Thus, despite variable prevalence in some previous studies, CTE remains a significant concern after repetitive head injury.

Accumulating studies further demonstrate that pathological changes occurring after a mTBI can interact with the healthy aging process[319,320], to increase vulnerability to a wide range of neurodegenerative conditions[62,63], such as Alzheimer's disease and Parkinson's disease. Particularly those mTBI patients with existing genetic vulnerabilities to such disorders can experience elevated markers of neurodegeneration after injury[321]. Some studies have suggested this is particularly amplified in cases of repetitive head injury, with one finding a 56% increased risk of Parkinson's disease[64]. Whilst these effects are not consistently reported in middle-aged cohorts[322], there is a consensus that multiple impacts can have cumulative effects on the brain across the lifespan in some individuals. Finally, research has additionally identified history of multiple concussion as a risk factor for long-term cognitive impairment and mental health problems such as depression[315].

Importantly, repetitive TBI may be more detrimental than a single injury by manifesting greater behavioural and neurological changes[315,323,324]. When compared to single injury, having a history of multiple concussions can increase the number and persistence of postconcussive symptoms after a subsequent concussion[325,326], and even increasing the likelihood of subsequent injury[327]. Moreover, there are growing concerns surrounding the time required for recovery and when is deemed 'safe' to return to play. Animal models suggest a second TBI within a 'window of vulnerability' post-injury can exacerbate neuropathological cascades and long-term dysfunction[328–330]. The exact interval of this window remains under investigation, being probed between a few hours and a few days

post-injury in rodents, however shorter intervals between injuries have shown to increase neurometabolic deficits[331–333].

In humans, UK guidelines on the issue are rapidly evolving and now require a minimum of 24hrs rest after a concussion and symptom tracking thereafter, as reported in new guidelines for grassroots sports published by the UK Government in April 2023[334]. This is supported by major UK sports associations such as the Football Association, Rugby Football Union, and Association for Physical Education in schools, alongside charities such as Headway promoting an '*if in doubt, sit them out*' approach, to encourage players to avoid returning to play immediately after a head collision[68]. As with rodent models, the exact timeline in humans of when can be deemed 'safe' to return to play to avoid such a window of increased neuronal vulnerability remains under investigation.

In this Chapter, I assess the acute neurological effects of repetitive mTBI by further investigating thalamocortical functional connectivity as a marker of acute injury and long-term disease.

5.1.1 Previous literature

One way to investigate the neurological effects of multiple TBI is using neuroimaging techniques. In a unique study, Monroe and colleagues (2020)[335] measured the frequency and magnitude of head impacts *during play* in collegiate water polo players using cap-worn inertial sensors and measured resting EEG pre- and post-season. Greater head impact exposure was associated with global functional connectivity changes post-season and altered performance in information processing and inhibitory control[335]. Thus, demonstrating the detrimental behavioural and neurological impacts of greater exposure to head injury.

Importantly, neuroimaging evidence has highlighted that safe return to play may not be sufficiently assessed using symptom reporting alone. An MRI study followed concussed athletes from 1-week post-injury to 1-year after return to play and found markers of incomplete or ongoing recovery at return to play in both structural and functional imaging[336]. Namely, increased global functional connectivity and increased mean diffusivity on DWI. Those with greatest concussion severity (symptom severity and time to return to play) additionally showed lower global functional connectivity at 1 year after

return to play, substantiating long-term neurological effects which outlast symptomatic effects. This has been replicated in further studies of young athletes, which also found a delay between behavioural recovery and neural recovery[337], confirming that symptom expression is not the only determinant of safe return to play. Moreover, further studies have shown signatures of neurological change after repetitive injury even in those who do not experience postconcussive symptoms. A salient example is a study of Australian footballers, which found evidence of widespread white matter damage and cortical thinning despite participants being asymptomatic and not experiencing a concussion in the previous 6 months[338]. Thus, providing evidence of structural brain damage even in those not presenting symptoms of concussion. Return to play decisions, however, are not routinely made using neuroimaging findings.

Outside of sporting decisions of when is deemed 'safe' to return to play, many further studies have indeed found lasting neurological changes after repetitive TBI across a variety of sports and military service groups. For example, a cohort of collegiate football players demonstrated diffuse functional hyperconnectivity of a central autonomic network post-season [339]. Importantly, this was associated with reduced cognitive control in those players who had experienced the greatest number of head impacts during the season, demonstrating behaviourally relevant adverse effects of repetitive injury associated with functional hyperconnectivity. Further studies have found increases and decreases in large-scale network connectivity[180] including long-term cerebellar dysfunction in retired rugby players many years after play[340]. Chronic functional alterations furthermore have behavioural relevance, as found in a study of US veterans[341]. These authors found that when performing a task, their mTBI group showed rapid hyperconnectivity which increased with effort. However, they were unable to maintain behavioural performance and functional hyperconnectivity when effort-level was sustained at a high level over the duration of the task, demonstrating behavioural and neural fatigue at high cognitive loads[341]. Finally, years of play has been associated with greater subcortical volume loss[324], particularly within the thalamus[327], related to slower processing speed and increasing exposure to head injury[327].

Indeed, specific thalamic injury has been highlighted as a region of interest in multiple mTBI, as it has in single event mTBI[342]. At acute timepoints, collegiate footballers undergoing DTI within 36 hours of injury found decreases in axial and mean diffusivity in

the thalamic radiations[343], suggesting white matter damage of thalamocortical pathways. At chronic timepoints, retired NFL players showed a loss of anticorrelation between the supramarginal gyrus and the bilateral thalami which worsened with time since play[344]. Additionally, mouse models have shown specific thalamic vulnerability after multiple mTBI; showing greatest levels of neuroinflammation out of any brain region at 4-6 days[345], and vastly increased calcifications 4-weeks post-injury after multiple mTBI compared to those only suffering a single mTBI[329]. These few studies therefore suggest that thalamic injury may be particularly amplified after repeat mTBI at both acute and chronic timepoints.

To better understand the potential impacts of repeat mTBI, we must compare its effects to that of single mTBI. This enables us to disentangle whether the effects are truly cumulative, or if each can be treated as an isolated event. Animal models have the advantage of controlling study design in this manner, rather than chance convenience sampling. For example, Schultz and colleagues (2012)[330] compared sham, single, 3-hit, and 5-hit mTBI and found that whilst all mTBI groups showed short-term cognitive impairments, only those with repetitive injuries showed a persistence of these behavioural effects with a dose-like worsening of symptoms. Those hit 5 times additionally showed evidence of depression and anxiety, and long-term neuroinflammation, not seen in the other groups. Such dose effects of repetitive concussion have further been found in rodent TBI neuroimaging. Previous studies have found greater white matter damage in the brainstem and cerebellum and midbrain functional connectivity changes at 6-8 weeks[346], and smaller mean cortical volume associated with greater behavioural deficits of balance and neurologic outcome[347]. Thus, animal models in rodents consistently show evidence for cumulative behavioural effects[330,347,348] and cumulative neurological effects[346,349] with increasing number of concussions.

To the best of my knowledge, this has not yet been investigated in humans using functional neuroimaging, as studies have instead focussed on specific populations of professional sportspeople or military personnel, often with an unknown number of previous concussion events. Whilst human studies are unable to control number, injury interval, and severity of head injury, it is integral to our understanding of repetitive mTBI in humans, which may show differential effects to that in controlled animal models.

5.1.2 Aims & hypotheses

Here, I capitalised on the vast dataset of CENTER-TBI[43] to design a single versus multiple injury cohort. This aimed to ask whether repetitive mTBI induces amplified acute functional changes, compared to no injury (healthy controls) and a single mTBI. This is a unique design in that I am assessing the general public rather than specific sub-populations or sporting communities, and remains focussed on CT-negative mTBI. Investigations target the thalamus and thalamocortical functional connectivity, given its established vulnerability in primary injury. This enables a better understanding of the possible cumulative effects of repetitive injury and implications for public policy.

Based on previous rodent models, my hypotheses are as follows:

1) Thalamocortical connectivity is amplified after repeat injury, showing a rank-based trend of increasing acute connectivity with number of mTBIs.
2) This implicates the same thalamic nuclei found to be vulnerable after a single injury (vAnterior and vlDorsal), on a global scale.

5.2 Method

5.2.1 Cohort

Individuals were identified from CENTER-TBI, according to similar inclusion criteria as the original mTBI cohort described in **Chapter 2 section 2.1**. Namely, aged 18–70 years with no medical history of neuropsychiatric disease, sustained a mTBI [Glasgow Coma Scale (GCS) 13–15], required a head CT according to local criteria on initial presentation, showed no CT abnormalities, had both T1-weighted MRI and rsfMRI in the acute phase post-injury, and were not treated in the intensive care unit.

Additional criterion was evidence of two or more previous mTBIs. This was defined using two potential sources: i) the medical history form acquired at baseline by CENTER-TBI staff from existing health records for the individual, or ii) the self-report outcome participant questionnaire taken at 6 months post injury. Both questionnaires recorded a yes/no response explicitly for history of previous concussion/TBI, and then recorded either i) total number, or ii) total number of sports-related injuries, respectively. As only

n=10 individuals could be identified using both methods simultaneously, participants were included if they presented evidence of two or more previous head injuries from either source. This took a hierarchical approach, whereby information on self-report questionnaires was treated as the gold-standard, and only if this information was missing or non-sports related injury was reported, then data would be obtained from baseline medical history. These inclusion criteria identified n=21 participants for the repeat injury group. No information was recorded by CENTER-TBI regarding the severity or interval of these previous injuries, merely recording the number of concussion/TBIs prior to the present mTBI. Given no subjects presented abnormality on CT, we can infer these were likely to have been mild injuries consistent with a concussion/mTBI diagnosis according to GCS 13-15. This limitation of data specificity is discussed further in **Section 5.4.1.**

My first hypothesis was that there exists a rank-based trend between increasing thalamocortical connectivity and increasing number of mTBIs. The repeat group (n=21) however, had a far smaller sample size than the single mTBI group (n=108). Thus, to avoid any three-group analyses being driven by previously established effects between controls and single mTBI, I chose to reduce the single mTBI group's sample size by matching the two TBI groups on age and sex, by nearest neighbour. This resulted in n=21 single mTBI participants being matched and taken forward for analysis, while n=87 were left unmatched and removed from subsequent analyses. Success of matching is visually demonstrated in **figure 5.1**, where the light grey line represents the single mTBI group before and after matching, reaching very high similarity between groups.

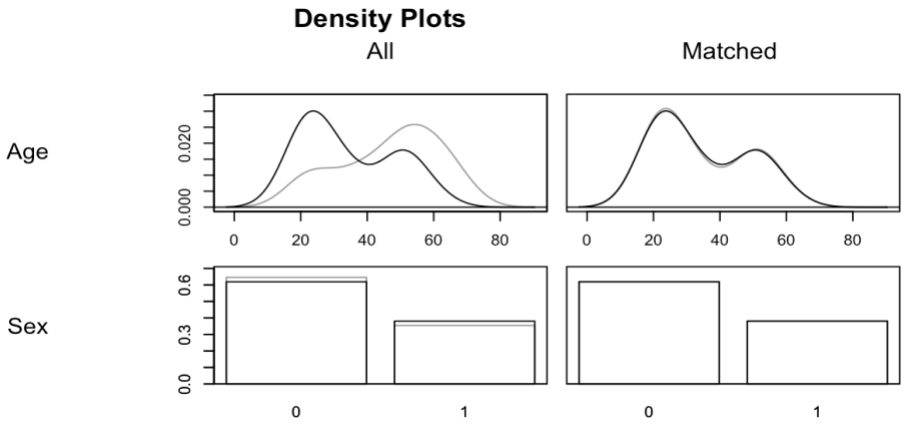


Figure 5.1. Visual representation of the success of age/sex matching. In each panel, the target group (repeat) is shown in solid black, and the pre/post matching group (single mTBI) is shown in grey. In both age and sex, matching appears to bring the matching group near identical to the target group.

5.2.2 Imaging-derived variables

Preprocessing of neuroimaging data replicated the pipeline performed on the single event mTBI group described in **Chapter 3 section 2.2,** and thus is not repeated here. Briefly, data were preprocessed using 'minimal preprocessing' of fMRIprep including steps of bias correction, segmentation of tissue classes, coregistration of T1 and rsfMRI, spatial normalisation of T1w to standard space, and motion correction of rsfMRI data prior to spatial normalisation of reference image which was applied to all volumes. Data were then denoised via signal regression of nuisance covariates, and spatially smoothed with a 6mm gaussian kernel.

All three groups were considered simultaneously in two statistical analyses: in global thalamocortical connectivity, and voxelwise thalamic connectivity. Each analysis considered the same n=16 thalamic ROIs as defined by Najdenovska and colleagues[290]. As performed in **Chapter 4**, average thalamocortical functional connectivity was calculated for each thalamic ROI (n=16) for each subject. This used previously calculated values for the healthy control and single-mTBI cohorts and calculated new values for the repeat mTBI cohort. As before, beta-maps of ROI-to-voxel functional connectivity were calculated for each subject and each ROI, using the CONN toolbox v.20.b[293]. A mean was calculated within a mask for each individual's cortical grey matter. These beta maps were investigated for voxelwise connectivity differences between groups using SPM 12[232].

5.2.3 Statistical analysis

The repeat group (n=21) was compared to the unmatched single mTBI group (n=108) and the control group (n=76) for two-tailed differences in age (Fisher's exact), and sex (chi-squared). TBI groups post-matching (n=21 per group) were also compared in baseline GCS (Fisher's exact), and overall outcome using GOSE (Fisher's exact) and PCS presentation (Fisher's exact). These outcomes were defined according to identical criteria used throughout this thesis; binarized to 'complete' (GOSE-8) versus 'incomplete' (GOSE ≤7) recovery, and postconcussive symptom (PCS) positive or negative according to ICD-10 criteria of presenting three or more prespecified symptoms on the RPQ.

Data were similarly harmonised using ComBat for site differences across the whole cohort of single mTBI, repeat, and control groups, at the relevant stages prior to group comparisons.

As performed in **Chapter 4**, average thalamocortical functional connectivity was calculated for each thalamic ROI (n=16) for each subject. These values were then compared for increasing thalamocortical connectivity across the three groups (control, single mTBI, repeat) using a one-tailed Jonckheere-Terpstra test for non-parametric rank-based trends, with N=1000 permutations. All tests were adjusted for effects of age and sex, and FDR-corrected at $p<0.05$. Significant variables were further investigated for specific differences between the single mTBI and repeat groups using a one-tailed exact Jonckheere-Terpstra test.

Thalamocortical connectivity was also compared between groups on a voxelwise level, to assess whether specific regions showed increasing thalamic connectivity with repetitive injury. As I had already established significant differences between the control and single mTBI groups, I did not perform a one-way ANOVA as is traditional in three-group analyses, as this could present redundant effects of overall group difference. Rather, I used a regression whereby controls=0, single mTBI=1, and repeat=2, to investigate a possible linear effect. These tests were conducted with covariates of age and sex, with thresholds set at $P < 0.001$ (uncorrected) at the voxel level and family-wise error-corrected $P < 0.05$ at the cluster level, repeated for all $n = 16$ thalamic ROIs. Seeds with clusters of significant change were additionally compared to maps of significant change found between mTBI and controls in **Chapter 4**. This calculated percentage overlap as the number of overlapping voxels in both maps divided by the total number of suprathreshold voxels found in regression analysis, multiplied by 100. Thalamic seeds that resulted in significant connectivity findings were further investigated using a two-sample t-test between single mTBI and repeat groups according to the same statistical criteria; $P < 0.001$ (uncorrected) at the voxel level and family-wise error-corrected $P < 0.05$ at the cluster level.

5.3 Results

Inclusion criteria identified n=21 participants with evidence for 2 or more previous mTBI, via self-report and/or medical history. This group included n= 11 with 2 previous injuries, up to 5 previous injuries in one participant. This group was not investigated by a specific number of mTBIs beyond 2, due to small sample sizes and free text allowances meaning some participants reported non-specific answers such as "3-4 times".

Repeat and control groups did not differ in age [$X^2(1) = 4.5$, $P = 0.11$] or sex [$X^2(1) = 0.01$, $P = 0.91$]. Prior to matching, the two mTBI groups did not differ in sex [$X^2(1) = 0.03$, $P = 0.86$], however the repeat group was significantly younger than the single mTBI group [$X^2(1) = 9.0$, $P = 0.01$]. Following age and sex matching of the single mTBI and repeat groups, there were no statistically significant differences in initial injury severity measured using GCS (Fisher's exact P = 1.0), injury cause (Fisher's exact P = 0.89), or 6-month outcome between the mTBI groups in GOSE or PCS presentation, respectively (Fisher's exact P = 1.0; P = 0.71). Groups further showed no significant differences in early blood-based biomarkers, as shown in **Appendix 5.1.** Of the n=21 repeat group, n=16 self-reported having a history of sports-related concussion/TBI. The sports identified in this subgroup were skiing (n=4), ice hockey/skating (n=3), horse riding (n=2), gymnastics (n=2), handball (n=2), and football (n=2). Further demographic and clinical information for all groups is presented in **Table 5.1.**

Table 5.1. Demographic and clinical characteristics. Data are presented for healthy controls, repeat, and the age+sex matched single mTBI group.

	Control (n=76) n (%)	**Single mTBI (n=21)** n (%)	**Repeat (n=21)** n (%)
Age			
18-35	26 (34.2)	12 (57.1)	12 (57.1)
36-55	36 (47.4)	8 (38.1)	8 (38.1)
55-70	14 (18.4)	1 (4.8)	1 (4.8)
Sex			
Male	46 (60.5)	13 (61.9)	13 (61.9)
Female	30 (39.5)	8 (38.1)	8 (38.1)
Glasgow Coma Score			
15	-	18 (85.7)	18 (85.7)
14	-	2 (9.5)	3 (14.3)
13	-	1 (4.8)	0 (0)
Injury Cause			
Road Traffic Incident	-	11 (52.4)	8 (38.1)
Incidental Fall	-	6 (28.6)	7 (33.3)
Other Non-intentional injury	-	2 (9.5)	2 (9.5)
Violence/Assault	-	2 (9.5)	3 (14.3)
Act of Mass Violence	-	0 (0)	0 (0)
Other	-	0 (0)	1 (4.8)
Strata			
Emergency Room	-	11 (52.4)	11 (52.4)
Admission	-	10 (47.6)	10 (47.6)
6 Month GOSE		**n=20**	**n=20**
Complete	-	12 (60.0)	13 (65.0)
Incomplete	-	8 (40.0)	7 (45.0)
6 Month PCS	-	**n=18**	**n=20**
PCS+	-	5 (27.8)	4 (20.0)
PCS-	-	13 (72.2)	16 (80.0)

5.3.1 History of multiple mTBI exacerbates thalamocortical functional connectivity

Both local and globally directed methods of investigation supported my hypothesis that increasing history of mTBIs was associated with increased thalamocortical functional connectivity. First, average global functional connectivity between the thalamic ROIs and

cortical grey matter showed a significant increase across the three groups after FDR correction shown in **figure 5.2**. Specifically, this was found in the same thalamic nuclei proposed to be vulnerable in mTBI; the bilateral ventral anterior nuclei (vAnterior; Left T_{JT} = 2399, p = .008; Right T_{JT} = 2358, p = .011) and bilateral ventral lateral dorsal nuclei (vlDorsal; Left T_{JT} = 2375, p = .008; Right T_{JT} = 2291, p = .020). There was additionally a significant increase in the whole left thalamus (T_{JT} = 2244, p = .022). However, none of these ROIs showed a significant difference between the single and repeat mTBI groups when considered in isolation (Left thalamus [T_{JT} = 265, p = .30]; L-vlDorsal [T_{JT} = 267, p = .30]; L-vAnterior [T_{JT} = 236, p = .35]; R-vlDorsal [T_{JT} = 258, p = .30]; R-vAnterior [T_{JT} = 238, p = .35]). Results for all non-significant nuclei are presented in **Appendix 5.2.**

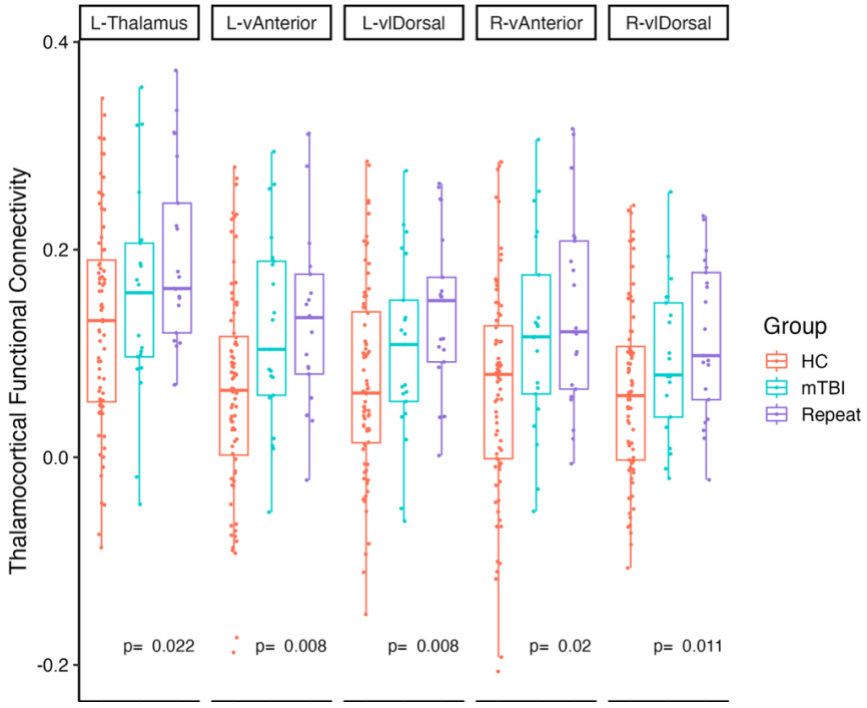


Figure 5.2. Globally increasing thalamocortical functional connectivity. Thalamic seeds showing significantly greater global connectivity with an increasing number of TBIs. Each coloured dot indicates an individual subject within that group. P-values are FDR-corrected for n=16 comparisons.

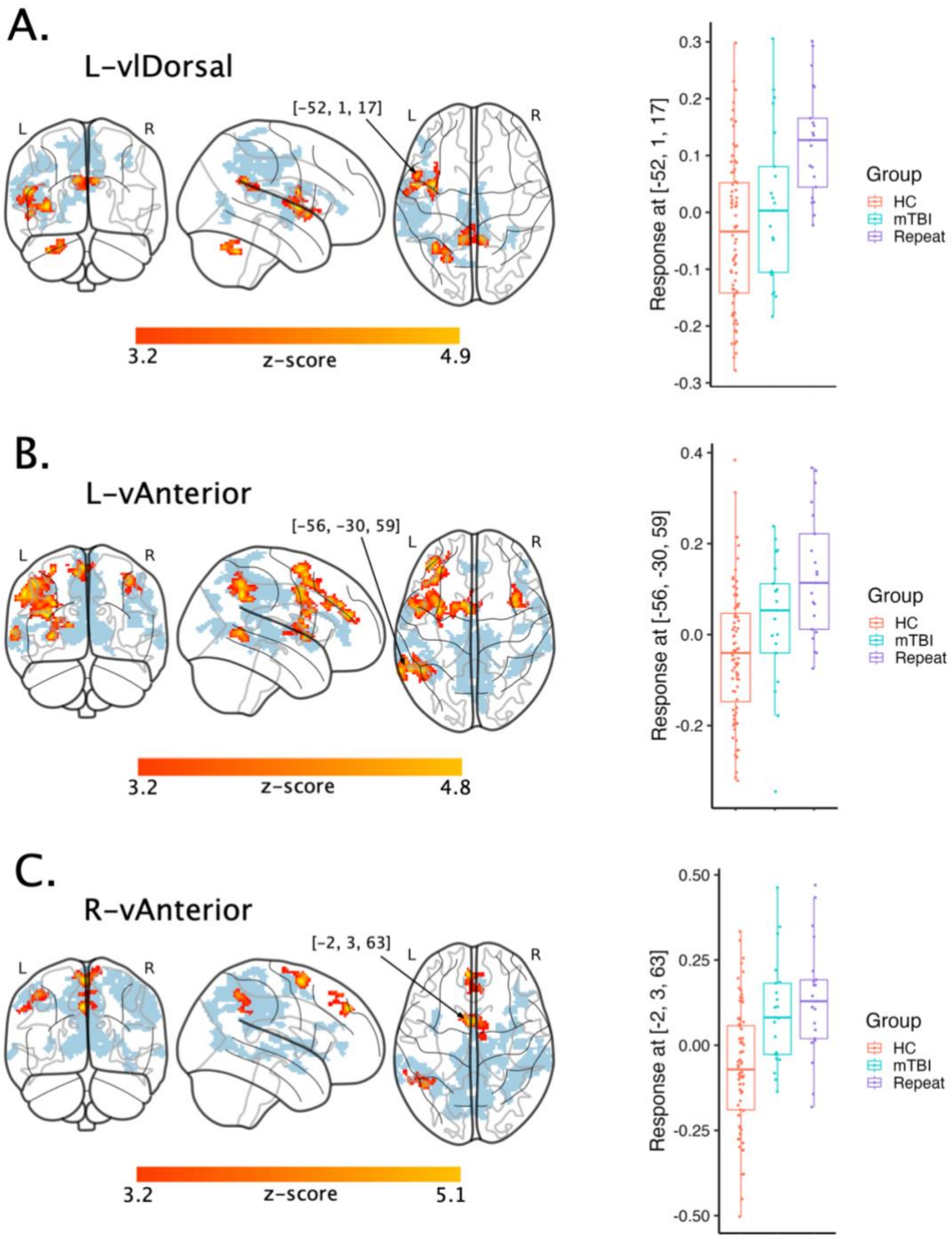


Figure 5.3. Locally increasing thalamic functional connectivity. Results from voxelwise regression analyses, showing regions of linearly increasing thalamic connectivity from controls, to single mTBI, to repeat injury (z-scores given in red-yellow). Significant regions are those surviving $P < 0.001$ (uncorrected) at the voxel level and family-wise error-corrected $P < 0.05$ at the cluster level. Regions shown in blue indicate those areas found to have significant differences between controls and single mTBI alone, as detailed in **Chapter 4**. A-C show three independent nuclei, with their global maximum z-score coordinate indicated with an arrow in the transverse slice. Individual responses of thalamic functional connectivity to this coordinate are plotted in the corresponding right hand column, for visualisation purposes only. Each coloured dot indicates an individual subject within that group.

Similar results were also found in voxelwise regression analysis, whereby clusters of significant linear increase with mTBI history were only identified in the 'vulnerable' nuclei. This included the bilateral vAnterior and right vlDorsal nuclei, as shown in **figure 5.3**. To ascertain these results were not merely a replication of the comparison between controls and mTBI found previously, I extracted thalamic connectivity values for all participants at the global maximum z-score. That is, the functional connectivity value between the thalamic ROI and its corresponding voxel coordinate displaying the strongest relationship to group membership, for each individual. These were plotted by group, to visually demonstrate the stepwise linear increase in thalamic connectivity with multiple mTBI (**figure 5.3)**. No regions of significant difference were found when explicitly comparing single mTBI and repeat groups, in any of the nuclei.

Interestingly, clusters identified as increasing acute thalamic connectivity with number of mTBIs partially, but not totally, overlapped with previous regions found to be hyperconnected in single mTBI (**Chapter 4)**. This is also shown in **figure 5.3**, with previously found regions of hyperconnectivity in blue. Namely, 46.9% overlap between clusters with the left vlDorsal seed, 13.8% overlap with the left vAnterior seed, and 33.9% overlap with the right vAnterior seed.

5.4 Discussion

This chapter aimed to directly study the potential cumulative effects of repetitive head injury, in comparison to a single mTBI and no injury. This analysis was performed through the lens of the thalamus- previously shown to indicate both injury and outcome after a 'mild' TBI. Here, I found that acute thalamic hyperconnectivity post-mTBI was amplified to an even greater extent by having a history of two or more mTBIs. This suggests the neurological effects of repeat mTBI are cumulative and may hinder adaptive injury responses and their recovery.

Cumulative neurological effects have been consistently reported in animal models of repeat TBI. These have aimed to better understand increased cerebral vulnerability post-injury which can lead to greater adverse effects and neuropathological cascades than a single injury. Such cumulative effects include neurometabolic and lipidomic dysregulation associated with decreased sensorimotor performance[328], persistent

neuroinflammation associated with cognitive impairment and depression[330], and thalamic calcium influx[329], to name a few examples. In one of the few studies to evaluate this in humans, Vagnozzi and colleagues[332] quantified cerebral N-acetylaspartate (NAA) using proton magnetic resonance spectroscopy, as an established biochemical marker of brain metabolic imbalance. In single-concussion athletes, this showed an average recovery time back to healthy control levels of 30 days post-injury. However, a second concussive event shortly after the first injury produced further decreases of NAA beyond that of single-concussed athletes, extending the recovery of NAA back to baseline levels by 15 days[332]. Whilst this double concussion group only included three individuals, it demonstrated the cumulative and extended neuropathological cascades of repetitive injury, which can prolong recovery times and exacerbate adverse effects of concussion.

To the best of my knowledge, this exacerbated neuropathology following repetitive injury compared to single injury has not been previously shown in humans using functional neuroimaging. I thus propose thalamocortical functional connectivity is a potential biomarker for a vulnerable neural environment in cases of repetitive injury. Importantly, this study included participants from the public, rather than specific sporting or military populations. Whilst research in these groups is undeniably significant, they are unable to extrapolate to the wider population of recreational sports players. Here, participants presented merely 2 or more previous mTBIs, of which the majority reported 2 prior injuries, with no evidence of structural damage resulting from this injury or previous injuries, no neuropsychiatric history, and were largely young adults. Such characteristics are associated with a positive recovery post-injury. Thus, I have shown for the first time how even a few repetitive injuries in a non-specialist and otherwise healthy population can induce a vulnerable neuronal environment, centred on the thalamus.

As discussed, there is ongoing debate on what can be considered adaptive versus maladaptive responses to injury in terms of functional connectivity. I postulate this exacerbated thalamic hyperconnectivity is an adverse environment for several reasons. My previous results in **Chapter 4** suggested that there may be a maladaptive consequence of *too much* compensatory hyperconnectivity, due to associations with later postconcussive symptoms[342]. This has recently been replicated in an independent study, finding increased thalamocortical coherence predictive of later postconcussive symptoms[350]. This study further included a translational rat model to corroborate their

results in human participants and found a replication between species of acute thalamic changes. Moreover, rats experiencing two TBIs experienced greater changes in microstructural white matter damage surrounding the thalami, greater markers of thalamic neuroinflammation, and prolonged periods of thalamic hyperconnectivity, compared to rats experiencing one mild TBI. Thus, increased acute thalamic hyperconnectivity following repeat concussion in our human cohort could suggest a predisposed neural environment for poor recovery.

I did not find poorer functional or symptomatic recovery after repetitive injury than the single injury group, however this may be a limitation of this small cohort rather than an absence of cumulative behavioural effects reported in the wider literature. Several behavioural studies have previously associated multiple sports concussion with higher symptom reporting, longer recovery, and higher rates of symptom persistence[315,325,327,336]. This association was noted as early as 1975, whereby young adults concussed twice showed significantly slower processing speeds and symptomatic recovery times than those concussed once[326]. Given existing associations between thalamic function and poor outcome reported in the present thesis[342] and in recent independent replication in both humans and rats[350], one could infer this exacerbated thalamic hyperconnectivity reflects a vulnerable neuronal environment. My results in this small sample are preliminary, however, and require further investigation in a larger sample.

Interestingly, identical nuclei were found to present exacerbated cortical hyperconnectivity after repeat mTBI, as those found in **Chapter 4**. Namely, the vAnterior and vlDorsal groups. As previously discussed, these nuclei are highly GABAergic and thus amplified hyperconnectivity may be associated with thalamic GABA-related inhibitory imbalance. In the recent replication of our previous results, authors found that injury of the thalamic reticular nucleus (TRN) was associated with increased thalamocortical coherence in rats[350]. The TRN forms an inhibitory GABAergic mesh around the largely excitatory thalami to locally inhibit thalamic activity alongside inhibitory interneurons[274], and thus further suggests a loss of inhibitory control may be integral to thalamocortical dysfunction and poor outcome. I was unable to directly study the TRN as this is a very thin layer in humans- being in the submillimetre scale. However, thalamocortical function and its relationship to excitatory-inhibitory imbalance remains an important

field in translational neuroscience for mTBI, and how this can be harnessed in future therapies.

5.4.1 Limitations

The findings presented in this chapter are a first step in understanding repetitive mTBI in humans, and thus have several limitations. Primarily, these encompass the identification of previous mTBI and limited sample sizes. As presented in the participant inclusion criteria, this cohort was defined based on hospital medical history reports *or* self-reported sports concussion. Ideally, only one source of information should be used for patient inclusion for consistency, or corroborated using both sources concurrently, but small sample sizes did not allow for this. As only half of the cohort could be identified on both questionnaire sources, this suggests that many previous head injuries were not reported or recorded on official health records. Under-reporting of milder TBI including concussive events is thought to be very common in previous literature[5], and demonstrates the possible harm of sports-related concussion when not sufficiently recorded and treated. The discrepancy between sources is also amplified as self-report questionnaires did not ask about the number of non-sports related concussion/TBI events, thereby limiting my ability to fully encompass all participants with history of multiple mTBI outside of sports settings.

Moreover, information is not given regarding the severity of these previous injuries. These are inferred to be consistent with mTBI given the lack of CT damage to suggest a more severe injury, however I am unable to say for certain whether the self-reported injuries not reported on patient medical history reached clinical diagnosis of mTBI. This however strengthens my results that even these mildest repetitive injuries can induce vulnerable neuronal environments. Additionally, no information is known about the inter-injury interval in these patients, nor their time since most recent mTBI prior to the injury at time of recruitment, limiting my ability to probe windows of vulnerability to cumulative neuropathological effects. Animal models have shown that multiple hits with 24-hour intervals showed significantly more adverse effects than multiple hits with 48-hour intervals, which instead presented similar effects to a single injury[333]. Nevertheless, this chapter presents clear adverse neurological effects of repetitive mTBI, irrespective of

whether these occurred in a short inter-injury interval, which is an important factor when discussing whether multiple concussions are ever 'safe' in sports.

A further consideration is how the mechanism of injury may impact specific thalamic burden. Whilst the repeat and single injury group did not statistically differ in mechanism of *this* injury, many of the repeat group reported previous sports-related injuries. High sheering and strain forces centred on the thalamus may be heightened during rapid acceleration-deceleration in sports-related injuries, as in road traffic incidents, which is yet to be explicitly studied. Thus far, only one study has investigated sports-related versus non-sports-related head injuries at comparable timepoints and found no significant differences in recovery or overall symptom presentation[351]. Nevertheless, injury mechanism could be considered in future investigation of the thalamus after repeat injury.

The smaller samples also limited ability to associate thalamocortical functional connectivity to outcome as only n=4 of the repeat cohort presented PCS+ at 6 months post-injury, albeit thalamic connectivity has been identified as a marker of postconcussive symptoms in work from **Chapter 4**[342] and replicated in a recent independent study[350]. I was also unable to follow this small cohort longitudinally, as only 5 participants returned for serial imaging at 6 and 12 months, and such analysis would be insufficiently powered to find an effect. Whilst these associations were not investigated here due to sample limitations, I encourage future research to fully investigate differences in single and multiple concussion in human studies, guided by these preliminary findings.

5.4.2 Conclusion

Repeat mTBI is of growing public and political interest regarding the safety of sports players, and when, if ever, can be deemed 'safe' to return to play. In this preliminary study, I have for the first time compared the acute neurological effects of repetitive injury to that of a single injury in humans using functional neuroimaging. I found that thalamocortical connectivity is amplified in the repeat group, showing a rank-based trend of increasing connectivity with number of mTBIs. This suggests that having a history of merely two previous mTBIs can induce a vulnerable neural environment of exacerbated

hyperconnectivity, even in a cohort of otherwise healthy members of the public. Moreover, these results further establish thalamocortical functional connectivity as a marker of acute injury and long-term disease following mTBI and has important implications for both public and professional sports players- substantiating no injury as 'mild', particularly in repetitive cases. It therefore stands that neuroimaging, including thalamic function, is one potential avenue for more definitive determinants of return to play decisions. These cumulative neurological effects have rarely been studied in humans, and suggests more research is required to understand if any timepoint is truly 'safe' to return to play. This will demand larger samples than the present preliminary study, including timelines of increased cerebral vulnerability to subsequent injury.

Chapter 6

Late selective thalamic neuronal loss in traumatic brain injury: outcome associations and transneuronal mechanisms

6.1 Introduction

This thesis has thus far demonstrated that mild TBI shows extensive thalamic involvement in both injury and outcome, which may perpetuate into longitudinal functional changes and accumulate in repetitive injury. I have not, however, fully explored how a TBI event can perpetuate into a lifelong *disease* many years post-injury. This can include an increased risk of developing neurodegenerative disorders[58–60] and emotional disorders such as anxiety and depression[352,353], and ongoing cognitive impairment[354]; all of which can vastly impact the TBI survivor's quality of life[56,353,355,356]. This chronic phase of injury, and lifelong injury, are thus important to fully understand the burden of injury for each individual.

For example, a recent study on quality-of-life post-TBI investigated 859 mTBI, 188 moderate/severe TBI, and 152 orthopaedic controls, for 5 years post-injury[355]. These were followed up via telephone call at least once between the 2–5-year post-injury period, up to three times each year. They found that the moderate/severe group had higher mortality and lower functional recovery than the mild group, however, both TBI groups displayed similar levels of TBI-related symptom burden and quality of life, which were lower than controls and did not change over the 5-year period. This exemplified that impacts on quality of life are persistent irrespective of injury severity and suggested that greater monitoring and rehabilitation are required for TBI.

Poor cognitive and functional outcomes following traumatic brain injury (TBI) remain a major concern for public health, and understanding the pathophysiological processes that result in poor outcome are essential[2,54,201]. In earlier chapters I have linked the thalamus to primary injury and outcome. Next, I will ask how the thalamus can help our understanding of long-term disease. Using rarely collected neuroimaging techniques, I look to better characterise the lifelong burden of thalamic injury and its relationship to outcomes and ongoing neuronal loss.

6.1.1 Previous literature

The thalamus has shown clear links to post-acute degeneration and poor long-term outcome. This was shown as early as 1996[357], whereby TBI patients with non-thalamic

lesions showed reduced chronic thalamic volume than controls, independent of injury severity. Additionally, participants with cortical lesions displayed reduced thalamic volume compared to those without cortical lesions, which was interpreted as preliminary evidence for *transneuronal degeneration* after cortical injury. The process of thalamocortical transneuronal degeneration describes an instance where primary cortical damage causes reduced structural and/or functional connectivity to that thalamic region, causing downstream degeneration of the thalamus over time. Numerous animal models have indeed demonstrated thalamic abnormalities at later timepoints than cortical damage[358–360], consistent with retrograde neuronal injury and apoptosis, even mirroring the location of cortical damage after TBI to its structurally connected thalamic regions[361]. As discussed, the thalamus has vast cortical connectivity[269], and thalamic nuclei have established structural connectivity to differential regions of the cortex[120,362]. For instance, the ventral Anterior nucleus structurally connects to the premotor cortex, ascending from the substantia nigra. These anatomical targets were even used in the delineation of nuclei in the thalamic atlas used throughout this thesis[290]. Thus, there is a *mirroring* of thalamic location to cortical location depending on connectivity targets. This suggests the thalamus may be an important region of interest in understanding chronic neuronal consequences of injury, potentially at later timepoints than cortical damage, and even in the absence of visible primary injury.

Crucially, long-term thalamic volume loss shows prognostic utility. Particularly in moderate/severe TBI, specific thalamic nuclei have shown longitudinal decreases in volume related to poor outcome[363], and this longitudinal thalamic volume loss was most powerful in outcome prediction models above all other brain areas[364]. Thus, the thalamus appears to be a link between the initial injury event and long-term disease. However, this previous study [364] identifying prognostic value of longitudinal thalamic volume loss only associated this with 'good' versus 'bad' outcome according to GOSE, and not more specific outcome measures related to emotional outcome or cognitive impairment. These outcomes are important to consider in the context of chronic TBI, as physical outcomes (GOSE) may be stable or treated with physical rehabilitation, whereas mental health and cognition could present ongoing/worsening outcomes affecting daily quality of life.

As in previous chapters, we can utilise neuroimaging to probe underlying structural and functional thalamic changes in TBI and associate these with outcome measures beyond

the GOSE. CT and MRI are commonly used to identify evidence of structural brain injury in TBI such as focal contusions, haemorrhage, evidence of traumatic axonal injury, and volume loss[96,365]. However, these modalities lack insight into the neuronal *integrity* of tissue and may lack prognostic specificity. For example, a recent study found that whilst voxel-based morphometry showed clear losses in chronic TBI in many regions, these did not relate to depressive outcomes, which could only be associated with measures of *functional* connectivity[366]. It is thus important to characterise the true neuronal burden of injury in chronic TBI, even in tissue which appears structurally 'healthy' on CT and MRI, to better understand ongoing transneuronal degeneration and its possible impacts on clinical and neuropsychological outcome.

This can be further explored using positron emission tomography (PET) imaging of the radioligand ^{11}C-flumazenil (FMZ). This binds to the central benzodiazepine/γ-aminobutyric acid (GABA) receptor and can be used as a marker of selective neuronal loss, whereby individual neuronal death remains supported by viable extracellular matrix and tissue bulk, as distinct from pan necrosis seen in MRI where there appears to be complete cellular loss[367]. This is under the assumption that as gamma-aminobutyric acid (GABA-A) is universally present throughout the brain, a greater degree of GABA-A binding indicates greater neuronal integrity within a tissue. This technique has shown great utility for understanding selective neuronal loss in cerebrovascular research, finding reduced binding potential (BP) – a metric of binding site density - indicative of global damage not identified with structural imaging in infarcted, perilesional, and even cortex which appears 'healthy'[367]. This pattern of findings is similar to that discussed in TBI literature, particularly recognising the combination of focal and diffuse injury types caused by trauma which may not present on routine imaging[2]. For example, a case report of an individual with chronic TBI experiencing ongoing memory and sleep problems could not be related to damage on MRI, but symptom-relevant alterations could be identified using FMZ PET[368]. Furthermore, such alterations in neuronal integrity have been previously related to adverse outcome after stroke[369] and other clinical populations of Alzheimer's disease[370], post-traumatic stress disorder[371], and multiple sclerosis[372].

Indeed, several studies have employed FMZ PET in TBI populations with overlapping results, albeit in small samples. In chronic TBI cohorts with no visible structural damage on MRI, studies have found widespread reductions in FMZ BP within bilateral frontal,

temporal, and thalamic regions, correlated with reduced intelligence[373] and persistent cognitive problems[374,375]. These studies demonstrate the increased sensitivity of FMZ PET to neuronal burden after injury compared to more commonly used imaging techniques such as MRI. Most recently, longitudinal analyses found broad FMZ BP decreases in sub-acute TBI which persisted chronically in frontal cortices and thalamic regions [376]. An increase in FMZ BP towards healthy levels in these regions correlated with improvement in executive attention in the chronic phase post-TBI, highlighting the utility of FMZ PET in understanding the burden of neuronal injury and predicting functional outcome. Finally, a study combining FMZ and oxygen-15 PET ($C^{15}O$, $C^{15}O_2$, $^{15}O_2$) in chronic TBI was able to determine whether regional cerebral hypometabolism could be attributed to neuronal loss rather than other causes[377]. These studies substantiate the unique clinical potential of FMZ PET in chronic TBI, highlighting widespread selective neuronal loss despite small sample sizes (n=5-11). Collectively, these studies report that selective neuronal loss in TBI is predominantly centred on the thalamus and frontal regions, is related to a variety of long-term outcomes, and can occur in the absence of damage seen on MRI. This requires further exploration in a larger sample with a greater breadth of long-term outcome measures, to improve understanding of the burden of neuronal injury seen in chronic TBI which may not present on routine imaging.

6.1.2 Aims & hypotheses

This chapter therefore aims to investigate the enduring consequences of TBI, with a particular focus on the thalamus. This study is conducted in the largest cohort to-date using FMZ PET in TBI, in a multicentre collaboration between Cambridge (UK) and Weill Cornell Medicine (WCM, USA). I aim to substantiate the unique ability of FMZ PET to characterise the long-term burden of neuronal injury after TBI and increase our understanding of long-term thalamic injury in relationship to a variety of functional, cognitive, and neuropsychological outcome measures. I will further explore whether changes in thalamic integrity are associated with their connections to cortical regions that had suffered primary damage, potentially driven by transneuronal injury mechanisms.

My hypotheses are as follows:

1) FMZ PET can be used to demonstrate chronic selective neuronal loss (> 6 months post injury) within brain regions that appear structurally healthy.
2) Chronic selective neuronal loss is particularly prevalent in the thalamus, with relationship to long-term outcomes.

With the additional exploratory hypothesis:

3) Regions of thalamic selective neuronal loss mirror regions of cortical damage.

6.2 Method

6.2.1 Cohort

In this retrospective cohort study of chronic TBI, patients were at least 6 months post-injury and compared to healthy volunteers. Participants were over 18 years of age and exclusion criteria were other neurological disease, taking benzodiazepines, or contraindication to MRI.

An initial cohort was collected in Cambridge (UK) between September 2004 – November 2007. This included n=19 TBI patients with moderate/severe TBI (GCS ≤ 12) and n=16 healthy controls. All patients with TBI experienced secondary neurological deterioration requiring management of raised intracranial pressure within the Neurosciences Critical Care Unit (NCCU), Addenbrooke's Hospital, Cambridge. Those included in this study were initially enrolled during the acute post injury phase with data collected prospectively. Patients were subject to non-consecutive recruitment to a follow-up imaging protocol limited to those that attended, and also driven by convenience and logistics which makes generalizability difficult to assess. Imaging was not possible on days where scanners or PET ligands were unavailable. As such, the data are a convenience sample of TBI patients who underwent follow-up with MRI, FMZ PET and outcome assessments. Ethical approval was obtained from the Cambridgeshire Research Ethics Committee (reference numbers 97/290 and 04/Q0108/51), and written informed consent, or written assent from next-of-kin where appropriate, was obtained in all cases.

Following the publication of the recent longitudinal study using FMZ PET in TBI[376], we formed a collaboration between Cambridge and Weill Cornell Medicine (WCM, USA) resulting in the largest cohort to-date using FMZ PET in chronic TBI. From this second centre, participants were recruited according to the same inclusion criteria but were enrolled for sub-acute imaging at 3-months post-injury, with a subset returning for chronic imaging at least 6-months post-injury. Only this chronic subset was therefore included in the cohort studied in this chapter, to explicitly study chronic TBI and better match the patient characteristics from Cambridge. From WCM, n=5 patients with TBI and n=17 healthy controls were included in my cohort. All patients with TBI presented either mTBI (GCS 13-15) with evidence of intracranial lesion verified on acute neuroimaging, or moderate/severe TBI (GCS ≤ 12). These data from WCM were collected between November 2018 – May 2021, and study participants were recruited following written consent in accordance with approval granted by the Weill Cornell Medicine's Institutional Review Board.

The final cohort thus included 24 chronic TBI patients (Cambridge 19, WCM 5) and 33 healthy volunteers (Cambridge 16, WCM 17).

6.2.2 Data acquisition & PET image reconstruction

The data acquisition and PET image reconstruction methods have been previously described for both the Cambridge[378] and WCM data[376], but to combine the data from the two sites some modifications have been made to the previously published methodology.

Cambridge

The MRI protocol included high-resolution 3D volume T1-weighted, T2-weighted, and fluid-attenuation inversion-recovery (FLAIR) sequences, acquired on a 3T whole body magnet (Medspec s300; Bruker, Ettlingen, Germany). T1-weighted images were resized to voxels of $1 \times 1 \times 1$ mm^3 and re-orientated to the AC-PC line. The FMZ PET data were acquired in 3D mode on a GE Advance PET Scanner (GE Medical Systems, Waukesha, USA). Prior to FMZ injection a 15 min transmission scan using rotating ^{68}Ge rod sources was acquired to correct for photon attenuation. FMZ was produced using a methylation process[379], providing high specific activities (370–550 GBq/mmol). FMZ was injected intravenously as a bolus (418 ± 21 MBq) and data were acquired for 75 minutes post-

injection (55 times frames: 18 × 5s, 6 × 15s, 10 × 30s, 7 × 60s, 4 × 150s and 10 × 300s). Images were reconstructed using the PROMIS 3D filtered back projection algorithm into 128 × 128 × 35 arrays with a voxel size of 2.34 × 2.34 × 4.25mm. Corrections were applied for randoms, dead time, normalisation, scatter, attenuation, and sensitivity. Given that the duration of the WCM scans was 60 minutes, only images from the first 60 minutes of the Cambridge scans were used for subsequent analysis.

WCM

A 3T Siemens Prisma scanner with a 32-channel head coil was used to collect high-resolution 3D T1-weighted images. Dynamic PET scans were performed on a Biograph mCT PET/CT scanner (Siemens Healthineers, Erlangen, Germany) over a period of 60 minutes from the injection of FMZ (407–595 MBq). For attenuation correction a low-dose CT scan was acquired. To match the Cambridge data, the list-mode PET data were histogrammed into the same time frames and were reconstructed using the same reconstruction algorithm –3D filtered backprojection - into images with the same transaxial voxel size (2.34 × 2.34 mm). Corrections were applied for randoms, dead time, normalisation, scatter, attenuation, and sensitivity. To harmonise the spatial resolution between the Cambridge (~ 6.5 mm FWHM) and WCM images (~ 5 mm FWHM), the WCM images were smoothed with a 4 mm FWHM isotropic Gaussian.

6.2.3 Image processing

Following review of MRI data to exclude evidence of injury, the reference tissue ROI in the pons was drawn using Analyze 14.0 on 10 contiguous transverse planes of the re-orientated T1w image, as previously described[380] and empirically validated in TBI[378]. The reference tissue ROI was then projected onto co-registered dynamic PET images to generate a time-activity curve. Voxel-wise BP relative to non-displaceable distribution volume (BP_{ND}) was determined with a basis function version of the simplified reference tissue model (RPM2[381]) with 100 basis functions for $0.001 \leq k_2 \leq 0.01$ sec^{-1}. A parametric map of k_2 was produced to determine a map of k_2', i.e., k_2 in the reference tissue. The median value of k_2' in voxels with $BP_{ND} \geq 0.5 \times BP_{ND}^{max}$ was used as a fixed parameter in the final estimation of voxel-wise BP_{ND}.

PET images were realigned and co-registered to the corresponding T1-weighted (T1w) MR image using SPM12[232]. For global grey matter and voxel-wise analysis of FMZ BP_{ND}, T1w images underwent SPM12 unified segmentation with light regularisation (0.001), found to be successful in normalising lesioned brains[382]. Forward-deformation fields were then applied to bias-corrected and segmented T1w images for spatial normalisation into MNI152 standard space with 4th-degree B-spline interpolation. Each BP_{ND} map was spatially normalised using the forward-deformation field of the co-registered T1w image for global analysis (SPM12) and ROI analysis (ANTs). Spatially normalised BP_{ND} maps were smoothed with an 8 mm FWHM Gaussian kernel.

For thalamic and frontal region of interest (ROI) analysis of FMZ BP, ROI volume was extracted for inclusion as a covariate in the linear model, to distinguish neuronal loss from gross volume loss. Each native T1w scan was corrected for scanner bias field inhomogeneities[235] and spatially normalised to the respective atlas template[291] via affine and non-linear registration in ANTs[236]. To determine ROI volume in native space, the inverse transformation was used to project ROIs from MNI template space to native T1w space with nearest neighbour interpolation. For each subject native T1w space ROI volumes were then normalised by total brain volume, estimated via automated brain extraction[292].

6.2.4 Contusion masks

Patients recruited in Cambridge additionally had FLAIR imaging acquired at the same time as MR and PET. These images were used to identify areas of structural injury consistent with traumatic contusions, which were manually delineated using Analyze 14.0 (AnalyzeDirect, Overland Park, USA), with reference to the other MR sequences obtained at follow up (T1-weighted, T2-weighted, and gradient echo).

6.2.5 Statistical analyses

Potential group differences in age and sex were assessed between patients and controls using a two-sample t-test and chi-square, respectively. Patients within the Cambridge and WCM TBI groups were additionally compared for differences in injury severity and time from injury to scan using two-sample t-tests. All imaging-derived variables

described below were initially compared between control and patient groups at an FDR-corrected p-value <0.05, unless otherwise stated. Additional statistical analyses were conducted using R-Studio (v.4.1.2).

To first assess global differences, for each subject a mean FMZ BP_{ND} value was calculated within each subject's spatially normalised grey matter mask (probabilistic map thresholded >0.2). This explicitly excluded contusions in the Cambridge cohort where masks were available, as these regions would display low FMZ BP_{ND} due to tissue loss, rather than selective neuronal loss of non-lesioned tissue being investigated here. Values were compared between patient and control groups using a two-sample t-test with covariates of age, sex, and acquisition site. In an attempt to differentiate neuronal loss versus gross volume loss following TBI, mask volume normalised by total brain volume was included within the linear model.

Subsequently, regional differences in FMZ BP_{ND} were explored between groups using voxel-level analyses conducted in SPM12. This utilised a two-sample t-test, excluding regions of CSF with SPM12's CSF tissue probability map (thresholded >0.75), and was conducted at a significance threshold of $p<0.001$ (uncorrected) at the voxel-level, and $p<0.05$ FWE corrected at the cluster-level to define minimum cluster size. This included covariates of age, sex, and acquisition site.

Based on voxel-wise results in group comparisons, the left and right thalamus and seven thalamic nuclei per hemisphere were further investigated to probe specific thalamic vulnerability. This used the atlas defined by Najdenovska et al[290] in a large healthy population, shown to be successful when individual thalamic parcellation using diffusion-weighted imaging data is not available. These thalamic comparisons excluded n=3 patients with visible thalamic lesions on MRI. I additionally investigated a frontal cluster of decreased FMZ BP_{ND}, identified in voxelwise analyses as differentiating patient and control groups. This overlapped with the frontal medial and paracingulate ROIs defined by the Harvard-Oxford Probabilistic Atlas. These frontal comparisons excluded n=11 patients with visible frontal contusions on MRI.

For each of the sixteen thalamic ROIs and two frontal ROIs, a mean FMZ BP_{ND} value was extracted within-mask and compared between groups using a two-sample t-test. The linear model for each ROI included covariates of age, sex, acquisition site, and separately,

with the addition of subject-specific normalised ROI volume. Similar to global comparisons, normalised ROI volume was included to distinguish effects due to gross volume loss versus selective neuronal loss not reflected in pan necrosis. These extracted normalised thalamic volumes were additionally compared between patient and control groups, with covariates of age, sex, and acquisition site.

Each imaging-derived variable found to have a significant difference between control and patient groups was then related to available outcomes, detailed below. These associations were only conducted in the TBI cohort from Cambridge (n=16; the 3 patients with visible thalamic lesions on MRI were excluded), as similar outcomes were not available for the TBI cohort from WCM (n=5). Covariates of age, sex, initial injury severity (Glasgow Coma Scale (GCS)), time from injury to scan, and normalised ROI volume were included in comparisons of mean ROI FMZ BP_{ND}.

Given the smaller sample size of our remaining patient population after frontal contusion exclusion (n=13), particularly that of the Glasgow Outcome Scale (GOS)-3 group (n=2), I postulate there may be relevance of the frontal medial cortex in larger sample sizes. For the present analysis however, I chose to solely focus on the thalamus for further investigation.

Outcome was assessed using the GOS, 36-Item Short Form Health Survey (SF-36), Animal Fluency, and cognitive assessments as part of the Cambridge Neuropsychological Test Automated Battery (CANTAB). For GOS, Kendall's Tau was used to assess correlation between outcome status and imaging-derived phenotype. The 7 dimensions of SF-36 were assessed between groups as correlations for continuous measures (bodily pain, general health, vitality, mental health), or transformed into categorical measures where participants presented either none or strong responses in these domains (role-physical, social functioning, role emotional). Measures of animal fluency (the number of unique animals named within a given period) were recorded within 60 and 90 seconds, and the number produced in the last 30 seconds (60-90) was calculated as a measure of sustained fluency. Finally, cognitive outcome (CANTAB) assessed seven tests across three domains; sustained attention (rapid visual processing task), executive function (intra-extra dimensional set shift task; spatial working memory), and memory (paired associates learning, pattern recognition memory, spatial recognition memory, spatial span). Specific

test measures were particularly chosen if found informative of TBI outcome in a recent large-scale outcome study[354]. All tests were FDR-corrected at $p<0.05$, within test.

6.2.6 Contusion mapping to thalamic damage

I next aimed to better understand whether thalamic regions with reduced FMZ BP_{ND} in TBI were related to the cortical contusions exhibited by this specific cohort. In the event of injury, I hypothesized that primary cortical damage could be associated with late thalamic damage, by finding evidence for greater neuronal loss in thalamic nuclei structurally connected to original contusion sites. Explicitly, I predicted that reduced thalamic FMZ BP_{ND} would mirror contusion location. Analyses were conducted in the TBI cohort collected in Cambridge for which contusion mapping was available.

As diffusion-weighted imaging was not available for the Cambridge cohort with contusion masks, I used a healthy average template constructed from a total of 1065 healthy adult scans[383] as a proxy for pre-injury normative structural connectivity. A deterministic fibre tracking algorithm[384] was used with augmented tracking strategies[385] to improve reproducibility. This used 1,000,000 seeds in ROI-to-ROI tractography with standardised parameters[383]; tracts with length shorter than 30mm or longer than 200 mm were discarded, anisotropy threshold was randomly selected, angular threshold was randomly selected from 15 degrees to 90 degrees, and step size was randomly selected from 0.5 - 1.5 voxels.

Using this method, a proxy for the total number of thalamic nucleus-to-contusion tracts was obtained for each patient with TBI who had a manually delineated contusion mask, who additionally, did not present any thalamic lesions (n=15). The number of tracts obtained in this manner were normalised by the respective total number of nucleus-to-whole brain cortex tracts, to produce a probability of nucleus-to-contusion structural connectivity for each subject. Patients for whom tractography algorithms found zero nucleus-to-contusion tracts were excluded for that analysis (n=2 subjects excluded from all analyses). Thalamic nuclei FMZ BP_{ND} values were correlated with nucleus-to-contusion connectivity probability using Pearson's correlation following adjustment for sex, age, and respective normalised ROI volume. Results were FDR-corrected at $p<0.05$. Analyses were repeated with an independent cohort of n=18 healthy control diffusion-

weighted data collected in Cambridge (details given in **Appendix 6.5**), for additional validation of these results.

6.3 Results

The control and patient groups did not differ in age or sex, nor did these variables differ between the patients with TBI recruited in Cambridge and WCM (**Table 6.1**). There were, however, significantly more TBI participants recruited from Cambridge, who additionally had a more severe initial injury as measured by GCS, poorer outcome measured by Glasgow Outcome Scale-Extended (GOSE), and a greater time from injury to imaging. The most common mechanism of injury across patients was road traffic collision. All patients recruited following TBI demonstrated evidence of traumatic injury on acute neuroimaging. Demographic information and statistical results for all groups are presented in **Table 6.1**.

Table 6.1 Summary of demographic and clinical characteristics

Characteristic	Controls	Patients with TBI	
n	33	24	
Age, years			
Range	22 – 71	19 – 66	*t(55)=1.8, p*=.078
Mean (SD)	45.5 (14.5)	39.2 (12.3)	
Sex			
Male n(%)	23 (60%)	18 (75%)	*X(1)<0.01, p*=1.0
Female n(%)	10 (30%)	6 (25%)	
Site			
Cambridge n(%)	16 (48%)	19 (79%)	*X(1)=4.3, p*=.038*
WCM n(%)	17 (52%)	5 (21%)	
	Patients with TBI		
	WCM	**Cambridge**	
n	5	19	
Age, years			
Range	34 - 58	19 - 66	
Mean (SD)	47.6 (20.5)	36.9 (11.9)	*t(22)=1.9, p*=.10
Sex			
Male n(%)	3 (60%)	15 (79%)	
Female n(%)	2 (40%)	4 (21%)	*X(1)<0.01, p*=.96
GCS			
Median (Range)	8 (3 - 15)		
	14 (9 - 15)	6 (3 – 12)	*Fisher's exact p*=.018*
Injury Mechanism			
Road traffic collision	3	11	
Fall	2	5	
Assault	0	1	*Fisher's exact p*=1.0
Other	0	1	
Unknown	0	1	
Time from injury to scan (months)			
Median (Range)	29 (7 – 95)		
	14 (13 - 20)	36 (7 - 95)	*t(22)=4.1, p*<.001**
GOSE			
Median (Range)	6 (3 – 8)		
	7 (7 - 8)	6 (3 – 8)	*Fisher's exact p*=.003**
GOS			
Median (Range)	4 (3 – 5)		
	5 (5)	4 (3 – 5)	*Fisher's exact p*=.12
		Missing (1)	

WCM = Weill Cornell Medicine. GOSE = Glasgow Outcome Scale-Extended (Wilson et al., 1998)); 1 = death, 2= persistent vegetative state, 3 = lower severe disability, 4 = upper severe disability, 5 = lower moderate disability, 6= upper moderate disability, 7=lower good recovery, 8= upper good recovery.

6.3.1 Local reductions in FMZ BP_{ND} are centred on the thalamus

I first assessed global changes in FMZ BP_{ND} in whole-brain grey matter. This found that whilst FMZ BP_{ND} was significantly lower in the TBI cohort compared to controls ($F_{1,52}$=5.66, p=.021), this did not survive when normalised grey matter volume was included in the model ($F_{1,51}$=3.41, p=.070). I thus explored voxel-wise changes in FMZ BP_{ND}

between groups to define local changes in FMZ BP_{ND}. This found no regions with increased FMZ BP_{ND} in the patient group, but two main clusters of decreased FMZ BP_{ND}; the thalami and frontal medial / paracingulate cortex. These are shown in **figure 6.1A**.

These regions were consistently identified, even when excluding the TBI cohort from WCM (n=5) who presented milder initial injury severities and excluding subgroups of patients with injured brain regions (frontal contusions n=11, thalamic contusions n=3), all shown in **Appendix 6.1**. A summary of patient contusion distribution is presented in **figure 6.4C.**

Based on voxel-wise findings, I next assessed FMZ BP_{ND} in the thalamus and its nuclei, and the frontal medial and paracingulate. In each case, patients with mass contusion in frontal (n=11) or thalamic (n=3) regions were excluded from that respective analysis to distinguish selective neuronal loss from pan necrosis. As shown in **figure 6.1B,** there were significant differences between patients and controls, and many of these regions remained significantly different when normalised ROI volume was included in the model (**figure 6.1C; Table 6.2**). Differences were uniquely found in the thalamic ROIs; in the left thalamus, bilateral anterior, medio dorsal, ventral-lateral dorsal, central, and ventral anterior thalamic nuclei. Thus, the thalamus and specific thalamic nuclei show chronic neuronal loss, not solely attributable to gross volume loss, and in absence of evidence for traumatic injury.

Figures of unadjusted mean FMZ BP_{ND} are presented in **Appendix 6.2.**

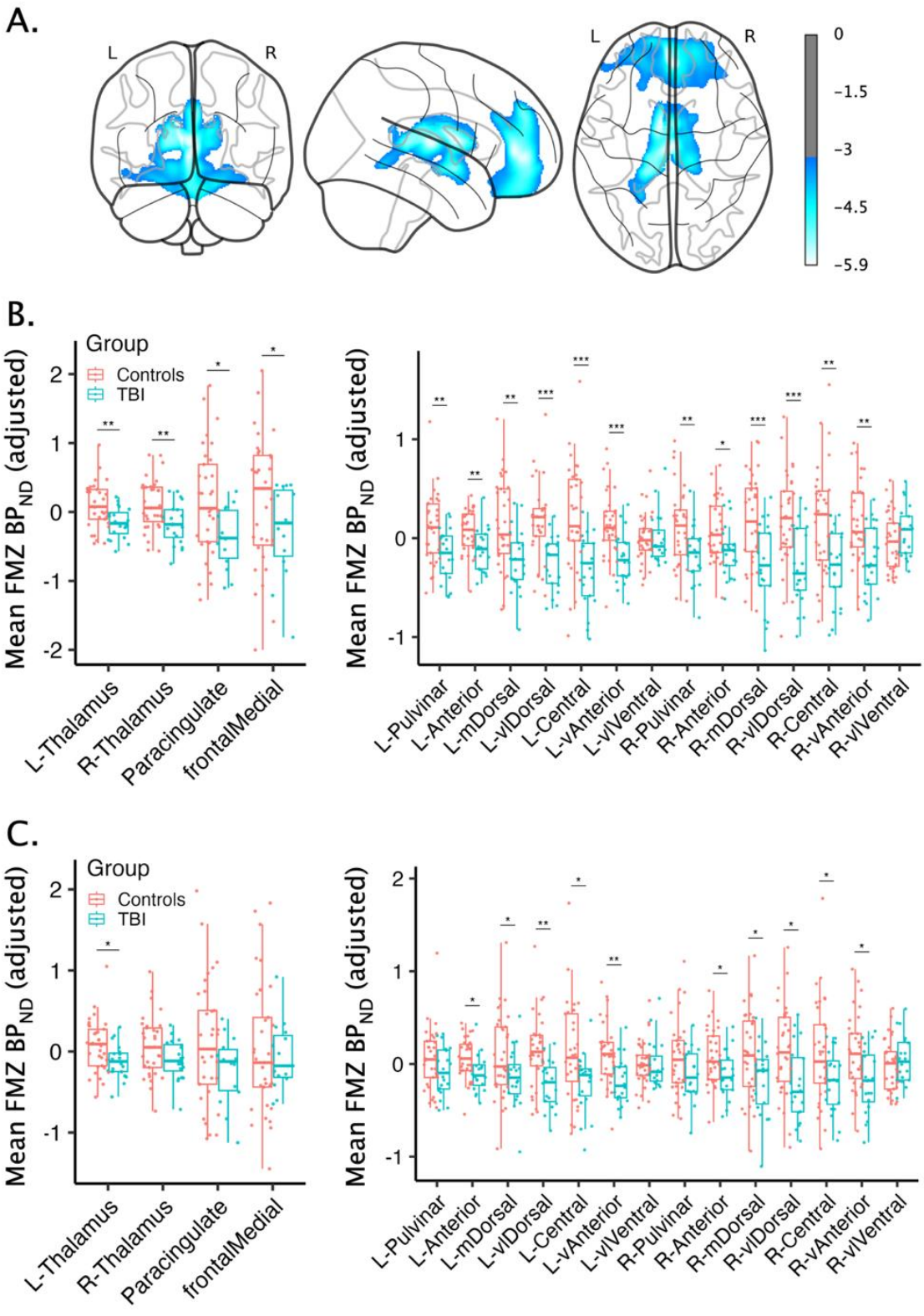


Figure 6.1 FMZ BP$_{ND}$ reduction in chronic TBI. A. Voxel-wise comparisons of FMZ BP$_{ND}$ between control and TBI groups, with all patients included (n=24). Colour bar shows t-values surviving significance thresholds of p<0.001 (uncorrected, voxel-level) p<0.05 (FWE, cluster-level). B-C. ROI-level comparisons between controls and patients, with significant differences surviving FDR-correction indicated with * p<0.05, ** p<0.01, and *** p<0.001. Y-axis of mean FMZ BP$_{ND}$ is adjusted for covariates. B shows comparisons when including covariates of age, sex, and research site. C

demonstrates the remaining significant differences when normalised ROI volume is additionally included as a covariate in the linear model. Patients with TBI were excluded from each comparison in B and C if they presented a contusion within that region; n=3 excluded for thalamic ROIs, n=11 excluded in frontal ROIs.

Table 6.2 Group comparisons of regional FMZ BP_{ND} between control and patient cohorts

Region of Interest	FMZ BP_{ND} Controls vs TBI
*Left Thalamus	$F(1,47) = 8.69$, $p = .016$
Pulvinar	$F(1,47) = 3.07$, $p = .098$
*Anterior	$F(1,47) = 7.35$, $p = .021$
*mDorsal	$F(1,47) = 6.34$, $p = .027$
**vlDorsal	$F(1,47) = 14.90$, $p = .005$
*Central	$F(1,47) = 8.22$, $p = .016$
**vAnterior	$F(1,47) = 13.61$, $p = .005$
vlVentral	$F(1,47) = 0.06$, $p = .814$
Right Thalamus	$F(1,47) = 4.56$, $p = .051$
Pulvinar	$F(1,47) = 3.98$, $p = .064$
*Anterior	$F(1,47) = 5.44$, $p = .035$
*mDorsal	$F(1,47) = 6.58$, $p = .027$
*vlDorsal	$F(1,47) = 10.09$, $p = .014$
*Central	$F(1,47) = 6.11$, $p = .027$
*vAnterior	$F(1,47) = 8.66$, $p = .016$
vlVentral	$F(1,47) = 1.09$, $p = .322$
Paracingulate	$F(1,39) = 1.34$, $p = .253$
Frontal Medial	$F(1,39) = 5.09$, $p = .051$

Included covariates of age, sex, research site, and normalised ROI volume. All p-values are FDR-corrected, and significance is indicated with *p<0.05, and **p<0.01.

6.3.2 Chronic thalamic neuronal loss is associated with outcome

FMZ BP_{ND} was subsequently compared to outcome measures within the TBI cohort collected in Cambridge (moderate/severe TBI only) and showed several significant associations (**figure 6.2**). Firstly, significant associations were found between lower GOS and decreased FMZ BP_{ND} in the left thalamus (τb = .50, p = .032), bilateral central (left τb = .52, p = .032; right τb = .57, p = .030), bilateral medio dorsal (left τb = .52, p = .032; right τb = .59, p = .030), bilateral anterior (left τb = .61, p = .030; right τb = .48, p = .037) and left ventral anterior (τb = .50, p = .032) thalamic nuclei. Analyses included patients for whom GOS was collected who presented no visible thalamic lesions (n=15), whereby groups included n=3 with GOS=3, n=7 for GOS=4, and n=5 for GOS=5.

I next found significant association between lower FMZ BP_{ND} in the right central thalamus and subscales of the SF-36; lower scores on mental health (t(13)=3.64, p=.032) and the sub-group reporting role limitations due to emotional problems (F(13) =11.8, p = .048). Additionally, a significant relationship was found between decreased performance on the animal fluency task (n=2/15 did not complete) and decreased FMZ BP_{ND} in the left thalamus (r(11)= .72, p=.029), bilateral central (left r(11)= .78, p=.021; right r(11)= .66, p=.047), and right medio dorsal (r(11)= .66, p=.047) thalamic nuclei. Interestingly, this relationship was only found with task performance in the last 30 seconds (i.e., 60-90 seconds), but not if considering the total number of animals named in either sum period (i.e., 60 or 90 seconds). No significant relationships were found with CANTAB measures after correcting for multiple comparisons. All comparisons included the covariate of normalised ROI volume, thus can be attributed to neuronal loss rather than pan necrosis.

These significant findings are summarised in **figure 6.2**, and results from all comparisons are provided in **figure 6.3**. Figures of unadjusted mean FMZ BP_{ND} are presented in **Appendix 6.2.**

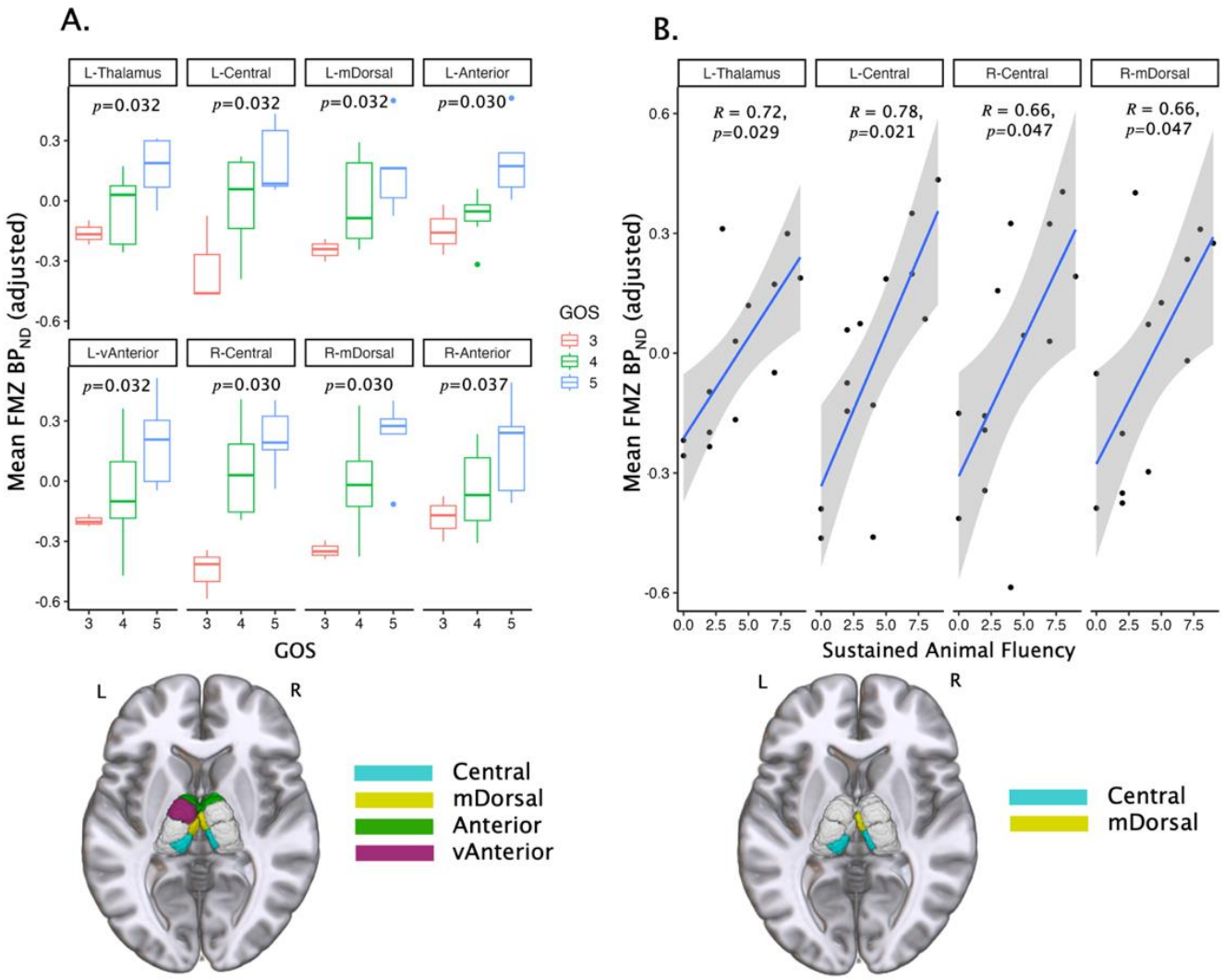


Figure 6.2 Relationship between FMZ BP_{ND} and chronic outcome. Significant correlations between mean FMZ BP_{ND} within an ROI and outcome, for Glasgow outcome score (GOS) (A), and Animal Fluency in 60-90 seconds (B). Full test results are presented in-text. Y-axis of mean FMZ BP_{ND} is adjusted for covariates of age, sex, research site, and normalised ROI volume. Each test included patients with non-thalamic contusions (n=15) with that outcome available (n=2 did not complete Animal Fluency). Corresponding thalamic render highlights significantly associated nuclei in A and B, respectively.

To demonstrate the relative value of FMZ PET above simple volume loss within the thalamus, I additionally compared thalamic volumes to outcome. Despite finding statistically significant gross volume reductions in 10/16 thalamic nuclei between TBI and control groups (**Appendix 6.3**), these changes were not significantly associated with any outcome. This contrasts with the outcome associations found with thalamic selective neuronal loss, and both are shown in **figure 6.3**. Thus, chronic neuronal loss of the

thalamus and medial/central thalamic nuclei was uniquely related to outcome and may be a more sensitive diagnostic and prognostic tool than volume loss alone.

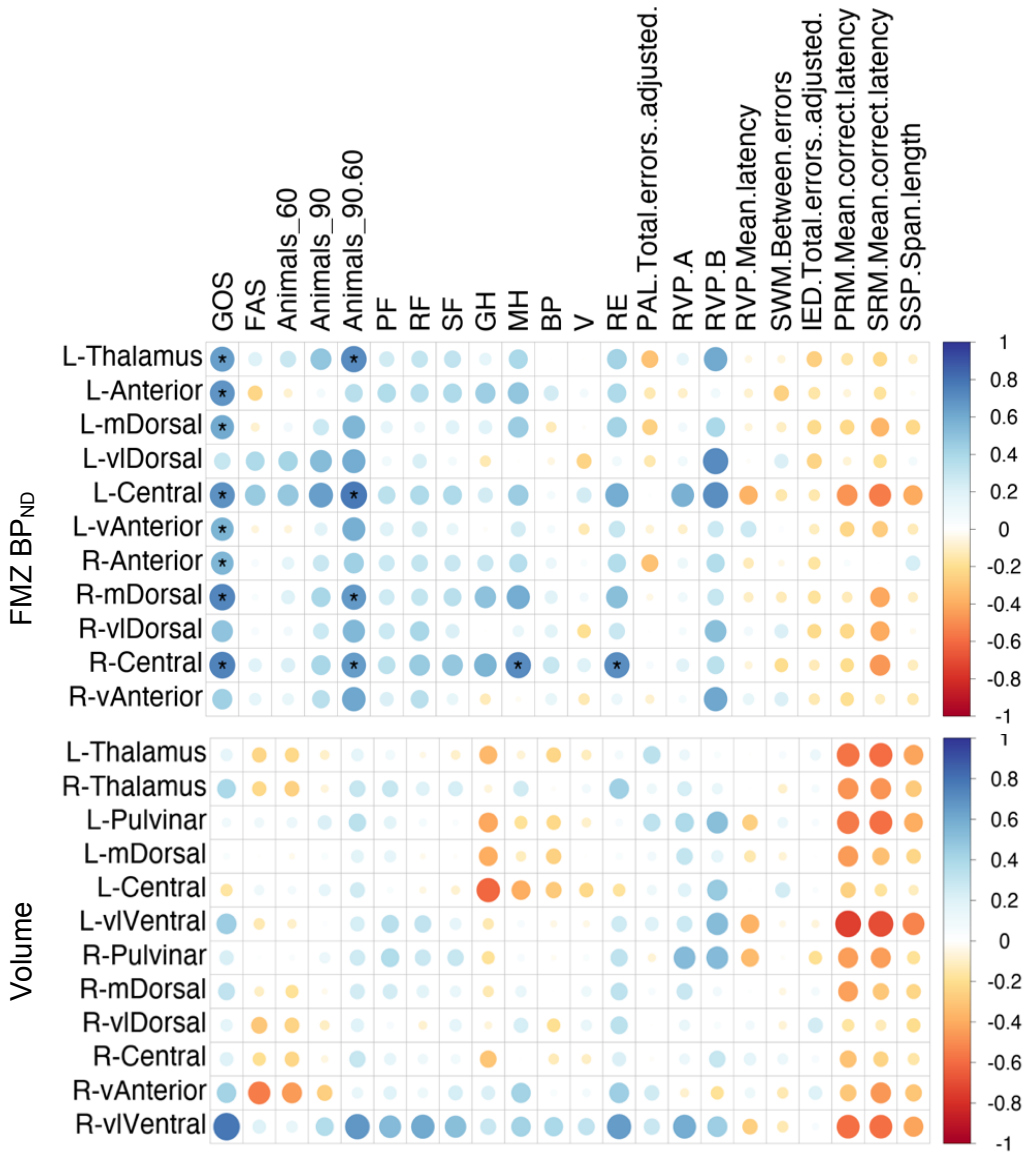


Figure 6.3. Summary of tests between thalamic FMZ BP_{ND} (top) and thalamic volume (bottom) and outcome measures. All data included covariates of age, sex, normalised ROI volume, baseline GCS, and days from injury to imaging, within the linear model. Colour bar and dot size indicates correlation statistic, with tests surviving FDR-correction ($p<0.05$) indicated with an asterisk. Outcome measures included are; Glasgow outcome score (GOS), verbal fluency (FAS), animal fluency at 60, 90, and 60-90 seconds, SF-36 subscales (physical functioning (PF), role physical (RF), social functioning (SF), general health (GH), mental health (MH), bodily pain (BP), vitality (V), role emotional (RE)), CANTAB subscales (paired-associates learning (PAL), rapid visual processing (RVP), spatial working memory (SWM), intra-extra dimensional set shift (IED), pattern recognition memory (PRM), spatial recognition memory (SRM), spatial span (SSP)).

6.3.3 Chronic thalamic damage mirrors cortical damage

I was interested to further investigate whether neuronal loss in thalamic nuclei mirrored locations of cortical damage. Indeed, frontal, and temporal cortices have established structural connectivity to the medial and central thalamic regions[120], and this cohort primarily exhibited contusions in these cortical areas (**figure 6.4A**) with outcome-relevant neuronal loss in these thalamic nuclei.

Correlations between FMZ BP_{ND} (adjusted for covariates) and nucleus-to-contusion structural connectivity probability (**figure 6.4B** illustrates this probability) found negative relationships in all thalamic nuclei, such that reduced FMZ BP_{ND} was correlated with greater likelihood of pre-injury structural connectivity of that nucleus-to-contusion. These survived FDR-correction in n=4 nuclei; the right medio dorsal ($r(9)= -.76$, $p=.048$), right central ($r(9)= -.72$, $p=.048$), right ventral anterior ($r(9)= -.78$, $p=.048$), and right ventral-lateral dorsal ($r(9)= -.71$, $p=.048$), shown in **figure 6.4C**. I did not find significant associations in the corresponding left-hemisphere nuclei, which may be due to inherent network asymmetries often associated with neurodegenerative diseases[386], or a limitation of our specific cohort. Owing to their acute contusions being solely in one hemisphere, n=2 patients consistently produced zero nucleus-to-contusion tracts in right-hemisphere thalamic nuclei, whereas n=3 patients produced zero tracts to left-hemisphere nuclei, reducing power to find an effect in the left hemisphere nuclei. Additionally, non-significant results from the bilateral ventral-latero ventral nuclei are attributable to contusion locations not presenting in structurally connected cortical regions, with n=4 and n=6 subjects producing zero tracts to the left and right nuclei respectively. Results for all nuclei are shown in **Appendix 6.4.**

Results were replicated using a healthy control diffusion tensor imaging (DTI) dataset collected locally in Cambridge, before FDR-correction (**Appendix 6.5**), whereby nuclei partially mirror those found to have lower FMZ BP_{ND} in patients versus controls, and those found to be related to GOS.

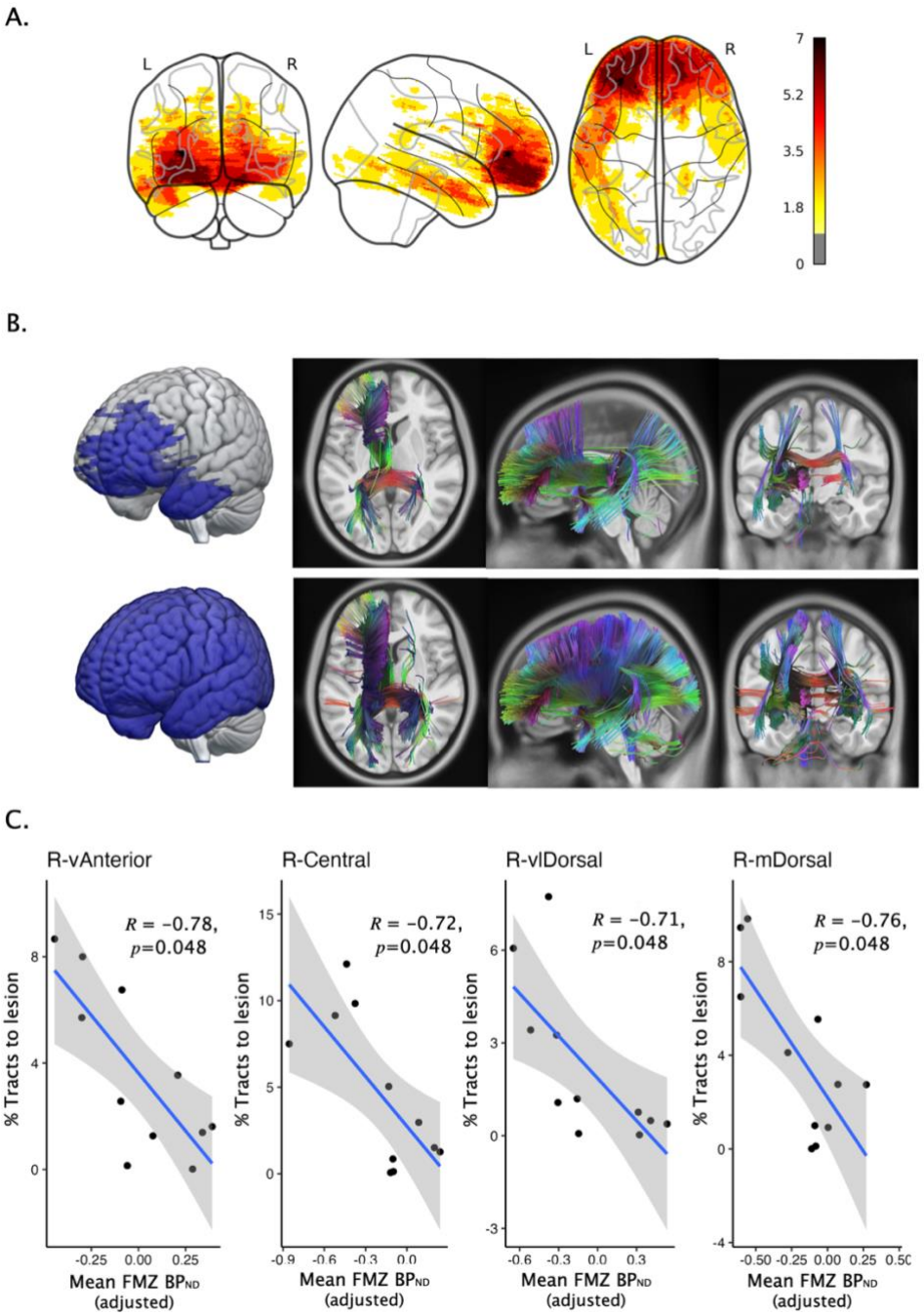


Figure 6.4 Relationship between FMZ BP_{ND} and contusion structural connectivity. A. Traumatic contusion masks summed across the TBI group. Values indicate number of subjects with a contusion in that region, i.e., value of 4 indicates n=4 patients exhibit contusions at this location. B. Top: example tractography from thalamic nucleus to individual's contusion mask (blue), bottom: example tractography from thalamic nucleus to individual's cortical mask (blue). Both images are shown for the same individual, as example. Percentage of tracts from nucleus-to-contusion is (total tracts in B(top)) / (total tracts in B(bottom)) x 100. C. Significant negative correlations between FMZ BP_{ND} and structural connectivity probability calculated from A and B, where each point is an individual subject. Subjects were included if they did not present a thalamic lesion with contusion mask available (n=15) and were successful at producing some tracts between the respective thalamic nucleus and their contusion mask. Results indicate a mirroring effect between cortical damage and chronic thalamic neuronal loss. X-axis of mean FMZ BP_{ND} is adjusted for covariates.

6.4 Discussion

This study found that chronic TBI is characterised by selective thalamic neuronal loss, identified using the binding potential of the benzodiazepine antagonist FMZ measured with PET. Particularly, this neuronal loss was present in all TBI severities extending up to 7.9 years post injury, highlighting the enduring consequences of TBI on thalamic integrity. Such neuronal loss was further related to worse functional outcome identified with GOS, worse cognitive outcome identified by lower scores on measures of sustained executive attention, and worse emotional outcome identified by mental health components of SF-36. Preliminary associations further showed mirroring of thalamic neuronal loss and cortical contusion location, which may relate to injury mechanisms of transneuronal degeneration. Thus, I propose that selective thalamic vulnerability perpetuates into lifelong neuronal consequences with relevance to long-term outcome, particularly focussed on the central and medial thalami.

FMZ PET has unique potential to improve our understanding of the ongoing neuronal burden after TBI, due to greater sensitivity and specificity to neuronal loss than routine MRI/CT imaging[368]. While other studies have shown the importance of volume loss in predicting functional outcome, particularly within the thalamus, this was not replicated here at later timepoints as thalamic volume was not related to any of our outcomes. Rather, I demonstrated the utility of FMZ PET as a specific marker of neuronal loss, over and above gross volume loss. These findings confirm and extend those of previous studies, revealing consistent reductions in thalamic and frontal midline FMZ BP_{ND} in the chronic phase after TBI with relevance to long-term outcome[373,376]. As discussed in previous chapters, the thalamus is highly vulnerable to forces experienced during primary injury[113,116,118], and importantly shows a unique perpetuation of inflammatory markers post-injury suggestive of ongoing injury processes[360,387]. Thus, the thalamus appears vulnerable to both initial damage and enduring loss exemplified here, linking the injury event to the long-term disease. This was proposed by a recent study which found thalamic volume loss in the first 6-months post-injury was most predictive of poor outcome[364]. The present results demonstrate that evidence of thalamic injury is enduring well-beyond this 6-month timepoint and is found many years after TBI. Moreover, a loss of chronic thalamic integrity has potential consequences for long-term

neuropsychological and cognitive outcomes beyond general function and is thus integral to our understanding of long-term *disease* following TBI.

As in previous chapters, specific thalamic nuclei appear especially vulnerable to injury. In this case, the central-lateral and medio dorsal nuclei showed the strongest relationship with outcome. These nuclei have consistently shown evidence of injury in histological studies of TBI[388,389], gross volume loss in chronic TBI[390], and volume change over the first 6-months after injury associated with GOSE and recovery of consciousness[363]. This central aspect of the thalamus is also thought to be important in the maintenance and recovery of consciousness in anaesthesia and disorders of consciousness[277,391], and constitutes an important region in the 'anterior forebrain mesocircuit model' of consciousness recovery after TBI[276]. Also relating to brain areas identified within this framework, we show reduced FMZ BP_{ND} in frontal regions, before including normalised ROI volume within the model. It therefore stands that these central and medial thalamic regions have clear vulnerability to injury, are an integral part of healthy brain function and consciousness, and evidence of injury in such regions is important for recovery following brain injury.

One important question to ask is *why* these medial thalamic nuclei show greater long-term neuronal loss? The central-lateral nuclei may have particular relevance due to its privileged role in in mammalian corticothalamic system as shortest point to point connections[392], providing a natural model for their sustaining proportionately greater deafferentation in multi-focal injury. Moreover, the vulnerability of these medial thalamic nuclei may be due to their structural connectivity to the frontal and temporal cortices, which were the main sites of contusion within our cohort. Using a unique methodology I developed with healthy DTI, I found empirical evidence for this explanation- mirroring of cortical damage to chronic thalamic neuronal loss- which may be related to secondary injury mechanisms. Numerous animal models have demonstrated thalamic damage at later timepoints than cortical damage[358–360], consistent with retrograde neuronal injury and apoptosis. One recent study has even demonstrated explicit mirroring of cortical damage after TBI to its structurally connected thalamic regions in a rodent TBI model[361], which is replicated here in human participants. Previous evidence in TBI has indeed suggested thalamic susceptibility of transneuronal degeneration, whereby TBI patients with cortical contusions displayed smaller thalamic volumes than those without,

independent of injury severity and ventricular volume[357]. This was also replicated in other neurological disorders; stroke patients showed greater thalamic neuronal loss in the ipsilateral thalamus to their stroke than contralateral[369], and preliminary evidence for directionality of this effect was found in early multiple sclerosis whereby integrity of thalamic tracts was predictive of subsequent thalamic atrophy, but not in the opposite direction[393]. Whilst I was unable to discern directionality or causality of this mirroring effect in this cohort, previous literature suggests secondary transneuronal thalamic degeneration may underlie selective vulnerability of medial thalamic nuclei in this cohort. Future studies should investigate such mechanisms in a large human TBI cohort, ideally longitudinally, to tease apart primary and secondary thalamic injury and to better understand key timepoints for intervention which may improve long-term outcome for patients recovering from TBI.

Finally, I showed that medial thalamic neuronal loss was indicative of functional, cognitive, and emotional outcome in chronic TBI. Namely, there were associations between greater thalamic neuronal loss and reduced GOS, reduced sustained attention, and lower mental health. Previous studies using MRI have found sub-acute thalamic impairment to be associated with poor long-term outcome across all severities of TBI[342,364], and a unique FMZ PET longitudinal study showed an association between thalamic function and improvement in executive attention[376]. This relationship to executive function was also found in the present study, in my measure of *sustained* fluency, which may hold diagnostic potential. Categorical fluency has been most applied in Alzheimer's disease and cognitive impairment, where Alzheimer's disease patients performed worse on the task compared to controls, and preclinical patients showed a greater rate of decline over time[394], related to domains of language and executive function[395]. Category fluency has also shown greater attrition over task duration in patients with mild cognitive impairment, correlated to measures of T-tau[396]. Moreover, thalamic atrophy, specifically focussed on the left ventral thalamus, has been previously identified in Alzheimer's disease and mild cognitive impairment, further implicating thalamic neuronal loss with lifelong neurodegeneration[397]. Looking to category fluency in small chronic TBI samples, patients have shown performance decline with prolonged attention and task demands in classical neuropsychological tasks, partially mediated in some patients with methylphenidate treatment[302,398]. Thus, there is potential clinical

utility of time-related performance decline on the animal fluency task for TBI and mild cognitive impairment, and its relationship to later risk of Alzheimer's disease, which can be linked to thalamic neuronal loss.

This suggests that targeting existing and novel interventions based on the thalamus may have long-term therapeutic benefit. For example, in a recent rat TBI model, acute thalamic inflammation was mediated by down-regulation of the GABA-transporter GAT-3, and enhancing GAT-3 in thalamic astrocytes resulted in improved long-term outcome[399]. This is one of the first studies to identify a clear therapeutic target for improving outcomes after TBI, albeit so far only in rodents, and explicitly proposes the thalamus as a centre of inflammation and neurological outcome. This is an exciting first step in therapeutic development, given the relatively recent surge of interest in the thalamus in TBI and other neurological conditions. Nevertheless, it is important to consider those existing patients with TBI, as well as acute interventions, and how to best care and support their chronic stage of injury. Currently, methylphenidate is one of the few long-term targets investigated for cognitive difficulties and fatigue in chronic TBI, but is yet to reach sufficient sample sizes in formal clinical trials for its widespread use[71,73]. The present chapter and previous literature suggests that injury and pathophysiological processes are uniquely persistent in the thalamus, with impact on lifelong outcomes. Thalamic targets may be one avenue of future research for chronic treatment development, yet to be explored. This could also include regional thalamic monitoring based on each individual's cortical lesion location, and indeed targeting of therapies to these vulnerable thalamic locations. To translate current findings into improved outcome and long-term benefit for patients will require improved understanding of the therapeutic window for both primary and secondary thalamic injury.

6.4.1 Limitations

There are several limitations of this study to be addressed. The data are based on two cohorts which were collected independently with differences in the outcome measures obtained, thereby limiting my sample sizes for outcome group comparisons. Cambridge subjects were recruited in the acute phase from patients admitted to ICU requiring sedation and ventilation for management of raised intracranial pressure and were subsequently enrolled to the FMZ PET imaging protocol during follow up. As such, the

Cambridge cohort are composed of patients with moderate/severe TBI whilst the WCM patients suffered a milder range of injury (mild/moderate). The groups thus differed in injury severity, and some of the imaging acquisition parameters. The PET data were harmonised across the two sites by using the same scan duration and number of time frames, the same image reconstruction algorithm and transaxial voxel size, and additional smoothing was applied to the WCM PET images to match the spatial resolution of the Cambridge images. However, some differences remain, such as the attenuation correction method, and hence to minimise any impact of such differences, acquisition site was consistently included as a covariate in all analyses.

The severity and type of brain damage resulting from TBI were heterogenous, with some patients exhibiting large cortical contusions. Whilst some imaging studies have avoided such patients due to difficulties with the accuracy of spatial normalisation within injured brains, I felt this would exclude a large and important population of TBI patients with moderate/severe injury; such patients would otherwise be under-investigated. Spatial normalisation of injured brains is an ongoing discussion in the literature, which has yet to reach consensus[400]. I chose a pre-processing pipeline consistent with heavily injured brains[382], and chose not to implement a cohort-specific normalisation template which could misrepresent some analyses and explicitly exclude injury sub-groups. The cohort, consistent with the typical pattern of injuries found following TBI, demonstrated a preponderance of frontal and temporal contusions (**figure 6.4A**). Therefore, sample sizes when investigating frontal and temporal brain regions were limited after exclusion of patients with contusions in such regions, which may have restricted my ability to replicate significantly reduced frontal FMZ BP_{ND} found in a previous study[376].

Thirdly, I can merely speculate on the mechanism responsible for late secondary thalamic neuronal loss. This would require a combined longitudinal MRI and PET study of thalamic FMZ BP_{ND} progression against cortical contusion location to fully elucidate. The connection between cortical contusion location and specific thalamic nuclei did not reach significance for all thalamic nuclei. This may be a product of this specific cohort exhibiting primarily frontal and temporal contusions, thereby reducing the power to find an association in all thalamic nuclei. By necessity I used a proxy for 'pre-injury' structural connectivity and could not compare baseline and longitudinal post-injury tractography measures within individual subjects to fully quantify thalamocortical structural loss.

While no baseline imaging is available for these TBI subjects, a future study could obtain longitudinal tractography data for comparison with FMZ BP_{ND} within the thalamic nuclei. I hope future multimodal imaging studies will work towards better understanding of thalamic neuronal loss over time, and its underlying cause, to help inform future treatment strategies.

Finally, within the context of this thesis, I have transitioned from investigating 'mildest' TBI to this cohort of chronic TBI of mixed severities. My primary motivation for this chapter was to study chronic thalamic consequences of TBI, many years post-injury. Whereas, sufficient functional data from my CENTER-TBI cohort only extended up to 12 months, which indeed was investigated in **Chapter 4**. Moreover, FMZ-PET is a unique and rarely-collected imaging modality, which warrants particular investigation. I was fortunate to have access to such data collected several years previously, however this necessarily limited my cohort to largely moderate/severe TBI. Whilst I would have been interested to probe thalamic selective neuronal loss in mild TBI, this unique cohort with cortical lesions enabled me to better investigate evidence for thalamocortical transneuronal degeneration in humans, which is an entirely novel contribution to the wider literature.

6.4.2 Conclusion

I propose that selective thalamic vulnerability perpetuates into chronic neuronal consequences after TBI with relevance to long-term outcome. Neuronal loss estimated by FMZ BP_{ND} was particularly prevalent within medial and central thalami and was found up to 7.9 years post head injury emphasizing the ongoing *disease* that results from TBI. FMZ PET showed greater sensitivity to outcome measures than thalamic volume determined from MRI and may advance our understanding of the full extent of brain injury across brain regions that initially appear to be 'healthy' and injury free. Indeed, understanding both primary and evolving secondary consequences of thalamic injury are an important step towards translating the acute injury event to the lifelong disease process that results from TBI. Fully recognising the prognostic and therapeutic value of the thalamus in TBI communities is beginning to gain academic attention and should be further investigated to promote more informed prognostic modelling and patient care.

Chapter 7

Discussion

7.1 Summary of thesis

This thesis began with a clear problem: mild traumatic brain injury is overexpressed in the population yet lacks adequate attention in current clinical practice and in neuroimaging research. In **Chapter 1,** the historical and social context of this problem was discussed. Namely, that mild TBI has been viewed as a temporary and self-resolving event, which does not have significant neural or behavioural consequences. More recent large-scale studies[50,51,54,198] were beginning to change these perceptions, by showing high rates of incomplete recovery. Yet, we remained unable to sufficiently prognosticate who might be at risk of these poor outcomes using existing biomarkers[88], compounded by little understanding of how to treat these individuals. There was, however, evidence to suggest that fMRI has great potential for improving our understanding of mTBI[2].

I therefore set four aims for this thesis; i) define mild TBI as a predominantly functional disorder; ii) identify acute correlates of chronic outcome using resting-state fMRI; iii) understand how these correlates might vary over time post-injury, and in the special case of repeat injury; and iv) begin to translate findings from acute imaging into treatment-relevant targets, thereby bridging the gap between macrostructural and microstructural investigation.

I started **Chapter 2** by investigating common markers of injury and outcome in a specifically defined cohort from CENTER-TBI- the 'mildest' form of mTBI presenting to hospital settings without pre-existing markers of poor recovery. Despite an expectation of good outcome in this cohort, I found that 47.2% were not fully functionally and/or symptomatically recovered at 6 months post-injury. Moreover, these outcomes could not be differentiated by CT or MRI (as none showed any evidence of structural damage), acute global white-matter microstructure (DWI), or common blood-based biomarkers. Thus, novel biomarkers were required using alternative neuroimaging techniques.

This was answered in part by **Chapter 3**, which investigated resting-state networks using rsfMRI, and their relationship to 6-month outcomes. Functional connectivity analyses revealed a globally disrupted functional environment across cortical, subcortical, and cerebellar domains, largely affecting between-network connectivity. Thus, characterising mTBI as a global and functional disorder. A novel measure of component

distribution complexity further demonstrated that the very definition of networks was changed acutely after mTBI, with increased brain-wide recruitment of resources after injury. I further found preliminary behavioural associations of acute imaging to 6-month outcome, including the potentially adaptive nature of between-network hyperconnectivity and monoaminergic neuromodulation, with particular relevance of default-mode and cerebellar network stability. These results underlined the value of looking beyond structural imaging, to examine functional abnormalities in mTBI and their increased prognostic potential compared to current routine imaging methods.

Further prognostic and therapeutic value, however, was found by taking a globally directed approach in my investigation of the thalamus in **Chapter 4.** This approach identified a clear biomarker of long-term outcome: acute thalamic hyperconnectivity. These acute rsfMRI markers differentiated those with versus without chronic post-concussive symptoms, identified nuclei-specific vulnerabilities within the thalamus, and showed time- and outcome-dependent relationships of thalamic functional connectivity in a sub-cohort followed longitudinally. Moreover, specific symptom categories encompassing emotional and cognitive symptoms were associated with changes in thalamocortical functional connectivity to known serotonergic and noradrenergic targets, respectively. This approach began to bridge the gap between macrostructural and microstructural investigation, translating findings from acute imaging into treatment-relevant targets. This had the ultimate aim for functional imaging to go beyond neuroimaging communities and take steps towards patient care for novel therapeutic development. **Chapter 5** additionally found that thalamocortical connectivity was exacerbated in the special interest group of repeat mTBI. These results further established thalamic pathophysiology as a marker of acute injury and outcome, which had long been missing within the mTBI literature.

My thalamic investigations finally looked beyond the acute phase post-injury, into the lifelong neuronal consequences of TBI. In **Chapter 6,** otherwise 'healthy' tissue on CT and MRI was further investigated for markers of selective neuronal loss using ^{11}C-flumazenil positron emission tomography (FMZ-PET). This chronic TBI cohort displayed selective neuronal loss specifically in the thalamus, over and above gross volume loss, consistent across injury severities. Such selective loss was further related to worse functional, cognitive, and emotional outcomes. Using structural connectivity, I further showed that

chronic thalamic neuronal loss partially mirrored the location of cortical contusions, which may indicate secondary injury mechanisms of transneuronal degeneration. Thus, I proposed that selective thalamic vulnerability perpetuates into chronic neuronal consequences with relevance to long-term outcome. The highly chronic nature of this cohort, extending up to 7.9 years post-injury, substantiated the evolving and potentially lifelong thalamic neuronal consequences of TBI.

There are several common themes throughout this thesis, speaking to each of the four main aims I intended to answer. These are now discussed in turn, drawing evidence from each experimental chapter.

7.2 Common themes

7.2.1 'Mild' TBI is not mild in nature.

Perhaps the most important public message from this thesis is that so-called 'mild' TBI is not, as its namesake, mild. This has been shown both in outcome- GOSE and postconcussive symptom reporting- and in brain function. The former of these results is not novel- multiple previous studies have demonstrated poor outcomes following mTBI[50,51,54]. Yet, public and clinical perceptions of mTBI have remained. Part of this routine dismissal of long-term consequences of mTBI has been reinforced by a lack of neurobiological underpinnings on routine measurements[54,113].

The novel contribution of my results primarily highlights that a lack of structural damage on routine imaging such as CT and MRI, does not necessitate a lack of *functional* damage nor adverse outcome. Throughout my investigations, the 'mildest' of TBI showed no acute structural changes in global microstructural metrics or thalamic volume, yet showed global acute functional changes. Crucially, these global changes were seen even in those who did not experience a 'poor' outcome or long-term symptoms, exemplifying that deviations from a typical functional environment can occur even in asymptomatic individuals. Moreover, these global functional changes began to prognosticate long-term outcome. This included global alterations in resting-state networks in **Chapter 3**, and thalamic hyperconnectivity in **Chapters 4** and **5.** These results respond to my first aim- to define mTBI as a predominantly functional disorder.

As discussed in the introduction to this thesis, the development of MRI brought greater power to abnormality identification in mTBI than CT[108,109]. Beyond this, quantitative MRI and DTI presented greater specificity to structural and microstructural changes post-mTBI[114,133]. So too, does rsfMRI compliment and extend structural neuroimaging in this cohort of mTBI, whose ongoing symptoms are otherwise unexplained by structural imaging alone. Thus, functional neuroimaging, such as rsfMRI, is an invaluable tool for further understanding and researching this growing population of mTBI.

Looking beyond scientific advancement in our understanding of TBI, these findings are significant for the patient and clinician alike. For those experiencing postconcussive symptoms after a 'mild' injury, these results provide a real and physical neurological marker of their experiences, which could otherwise have been dismissed as 'malingering' or suffering from a more general umbrella diagnosis of Functional Neurological disorder[254]. These findings are furthermore important in reframing the potential consequences of mTBI as non-trivial and, in some cases, non-temporary. In the eyes of the public, mTBI should be taken seriously and reported to medical professionals, and in the eyes of clinicians, patients should equally be believed about their experiences and treated with care and respect. In both cases, so-called 'mild' TBI may not be as mild as previously thought and should be treated as such.

One shortcoming of my assessment of 'mildest' TBI is the inherent biases of data collection. Mild TBI is commonly under-reported and thus may be vastly underestimated and under-investigated in the general population[3]. This bias is ongoing even in large-scale studies with the best of intentions. For example, participants included in this thesis from CENTER-TBI were only those who presented to hospital settings and required a head CT according to local criteria on initial presentation[43]. Thus, whilst I have been describing the present cohort as the 'mildest' TBI, I cannot be sure this is the case. Many more individuals may have self-treated, been unable to reach hospital settings, or were simply dismissed at the ER or local care centres as not needing further investigation with CT to reach the first steps of CENTER-TBI inclusion for advanced neuroimaging. This is exemplified in the small cohort of those with serial imaging in **Chapter 4;** high attrition rates of 71% substantially reduced my cohort size and produced a more 'severe' cohort. This was due to CENTER-TBI recruitment protocols[43] which only followed individuals from admission or ICU stratum, who incidentally reported higher rates of incomplete

recovery than the original cohort. This serial cohort therefore provided a representation of real-world follow up practice, exemplifying how 'milder' cases are not routinely followed.

Admittedly, long-term follow up of every individual presenting to an emergency care setting may be unfeasible in time and money for healthcare systems. However, my results suggest that a substantial proportion of mTBI cases have enduring neuronal and behavioural/cognitive/emotional consequences which may benefit from long-term care. Acute biomarkers of outcome can be beneficial in this circumstance, to stratify patients into 'at-risk' groups and target follow up to those with greatest need[43]. I have identified one such biomarker in the thalamus.

7.2.2 The thalamus is a link between injury, outcome, and disease.

The second key message from this thesis regards the thalamus as an important missing link in our understanding of TBI, particularly mTBI. The thalamus sits within the cone of vulnerability[113] thereby creating a common denominator of damage across injury types. It further has capacity to induce global change across the brain, due to extensive thalamocortical connections and its integral role in healthy brain function[269]. Previous authors had suggested thalamic links to outcome in moderate and severe TBI[364], finding that thalamic volume loss was most persistent and most predictive of long-term outcome above all other brain regions. However, recognition of the importance of the thalamus was previously missing in the wider literature of mild TBI[181], which is now substantiated within this thesis.

Throughout my investigations, the thalamus has shown key alterations at all stages of injury; in hyperconnectivity during the acute phase of mTBI and after repeat injury, longitudinal changes in connectivity which varied with postconcussive outcome, and enduring selective neuronal loss many years post-TBI. Each of these changes has shown relationships to long-term outcome and the ongoing disease post-injury. Thus, the thalamus can link the injury 'event' of TBI to both chronic outcome and lifelong disease. This answers my second aim- to identify acute correlates of chronic outcome. **Figure 7.1** highlights the key aspects underlying the integral role of the thalamus in TBI discussed

within this thesis, which have great potential to aid our understanding and prognostication tools.

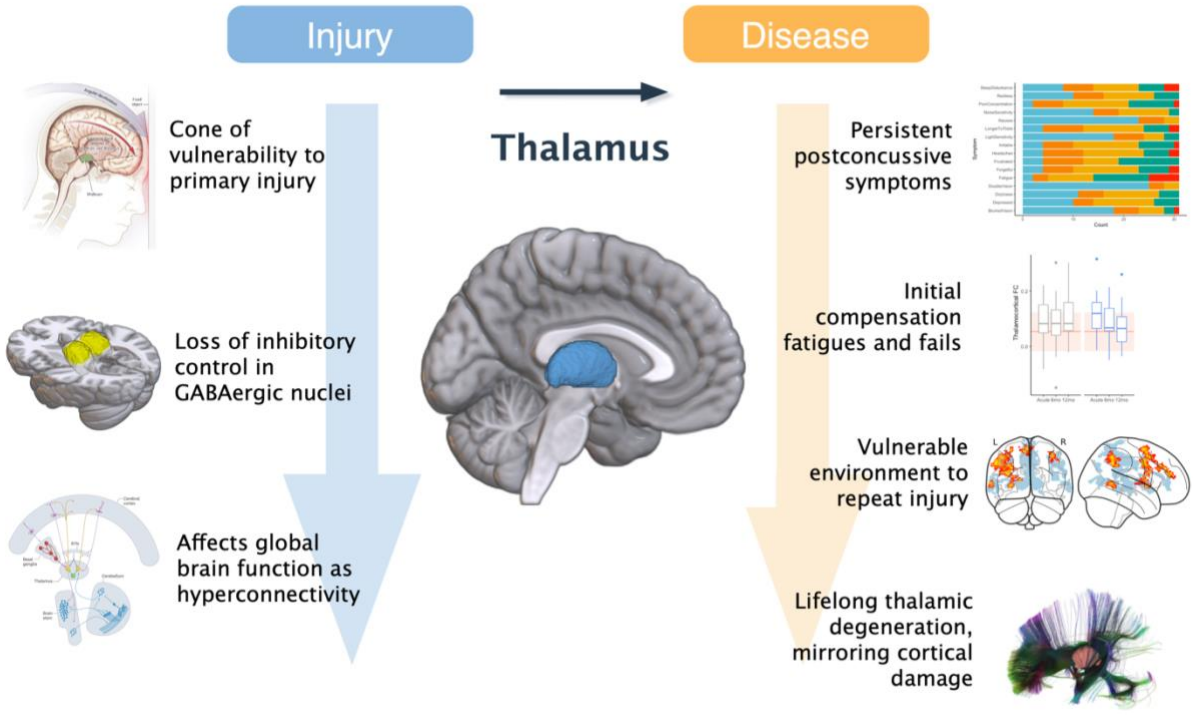


Figure 7.1. Overview of thalamic importance found in TBI. This thesis proposes the thalamus as an integral hub, uniquely affected during injury (shown in blue, left), which has clear associations to outcome and long-term disease (orange, right). Each small icon refers to specific literature or results discussed in relevant chapters. These are brought together in this schematic to highlight the continuous importance of the thalamus in almost all avenues of investigation within this thesis.

Significantly, my proposition that the thalamus is integral for mTBI prognostication has been independently replicated. Since the publication of our results detailed in **Chapter 4**[342]**,** an independent group studied thalamic functional connectivity after mild TBI, and its relationship to postconcussive symptoms[350]. Markers of within-thalamus connectivity and thalamocortical coherence (an alternative marker of functional connectivity) showed significant increases in acute mTBI compared to healthy controls, at all timepoints from 2 weeks to 2 years post-injury. Additionally, greater acute coherence was found in those with persistent postconcussive symptoms. This work did not distinguish between individual thalamic nuclei as I have, rather, authors took an average across the whole thalamus due to a presumed loss of thalamocortical multifunctionality between nuclei. In contrast, my work proposed that separating thalamic nuclei can yield greater prognostic

value, and elucidate injury mechanisms related to underlying biological properties of those nuclei. Finally, the independent study[350] additionally replicated their results in a sample of male rats, and also found more persistent increases in thalamocortical coherence after two experimental mTBIs than one hit in a mTBI model.

Thus, these results partially replicate my findings that acute thalamic hyperconnectivity is a viable acute biomarker of long-term postconcussive symptoms in mTBI (**Chapter 4)**, and further, that this can be exacerbated by multiple mTBI events (**Chapter 5)**. Independent replication is an important step in advancing our awareness of the thalamus, and its development as a viable biomarker. Indeed, this independent study further used their acute thalamic biomarkers, alongside age and sex, in a machine learning-based predictive model to successfully predict later postconcussive symptom development in a small external cohort at both 1-year and 2-year follow up. Long-term postconcussive symptoms can thus be successfully predicted by acute thalamic functional connectivity[342,350].

Having identified this prognostic marker, it is important to consider how high sheering and strain forces placed on the thalamus during primary injury[116,118], might induce this hyperconnectivity. As seen in **Figure 7.1,** I have suggested a causal directionality from primary thalamic damage to functional change, despite having purely correlational results which cannot assert causality. This is because animal models of TBI are beginning to provide support for such a directional effect. Using a rat model of TBI, a single closed-head impact induced downregulation of the GABA transporter GAT-3 in thalamic astrocytes[399]. Subsequently, this led to increased tonic thalamocortical hyperexcitability mediated by GABA-A receptors, followed by intrathalamic microcircuit hyperexcitability. A further rodent study on epileptogenesis following TBI found a permanent loss of ipsilateral thalamic GABA-A receptor subunits which preceded degeneration of thalamocortical relay nuclei, contributing to neuronal hyperexcitability[309]. These studies thus demonstrated causal steps from thalamic GABAergic damage to increased thalamic circuit excitation.

Evidence to support a directional hypothesis is also present in translational animal models and humans. In the same independent study which replicated acute prognostic thalamic hyperconnectivity, Li and colleagues[350] further capitalised on their translational

rat cohort and performed post-mortem immunohistochemistry at either 24hrs or 7 weeks post-injury. This found greater markers of injury in the thalamic reticular nucleus (TRN) at 24hrs, in the same cohort of rats who went on to display TRN damage on DTI at 1-week post-injury. Moreover, reduced tract density was correlated with increased within-thalamus coherence at 1 week. Thus, Li and colleagues[350] argue that primary TRN damage may induce thalamic hyperconnectivity due to a loss of thalamic inhibitory control, which has behavioural relevance for postconcussive symptoms.

The TRN forms an inhibitory GABAergic blanket around the largely excitatory thalami to locally inhibit thalamic activity alongside inhibitory interneurons[274]. It has also shown a key role in modulating thalamocortical synchrony and consciousness[274,401], which when dysfunctional, can cause disinhibition of thalamic relay cells and abnormal cortical synchronisation[402]. Clinically, TRN dysfunction has been linked to a variety of further neurodevelopmental disorders[403,404] including symptoms of attention deficits, sensory abnormalities, and sleep disturbances, all of which commonly present following mTBI.

My results in humans presented in **Chapter 4** also align with such a hypothesis that a loss of inhibitory control could directly induce hyperconnectivity. I consistently found hyperconnectivity seeded from thalamic nuclei with greatest inhibitory influence[303]; the vAnterior and vlDorsal, suggesting GABAergic inhibitory control of these structures and the inhibitory TRN are of great importance in understanding acute mTBI. Unfortunately, I was unable to directly study the TRN as this is a very thin submillimetre layer in humans and is thus unsuitable for investigation with functional MRI at 3T with a slice width of 2-3mm. However, excitatory-inhibitory imbalance is a well-established consequence of TBI[306], has shown an inverse relationship between local levels of GABA and strength of functional connectivity within resting-state networks[405], and demonstrates consistent links to thalamocortical functional connectivity regulation[307]. Moreover, reduced neuronal integrity of the ventral thalamus (reduced NAA/Cr identified using MRS) has been found concurrently to ventral thalamocortical hyperconnectivity after mTBI in humans[187], thereby corroborating the link between acute thalamic damage and thalamocortical hyperconnectivity. Thus, when inhibitory control centres of the thalamus are damaged, this may reduce thalamocortical control and lead to acute hyperconnectivity seen throughout this thesis. More research is needed to fully establish a causal link between thalamic GABAergic loss and thalamocortical hyperconnectivity in

humans. This will particularly require validation of translational models to understand how unique effects only available so far in rodents can apply to human TBI.

Going beyond primary injury, I have shown that ongoing thalamic damage can be integral to long-term *disease*. In **Chapter 5** I found that having a history of two or more previous concussions/TBIs can induce a vulnerable neuronal environment by exacerbating thalamic hyperconnectivity, and in **Chapter 6** I found potentially lifelong thalamic neuronal consequences of injury. Thus, the effects of TBI can be identified in the thalamus many years post injury, with direct relationships to increased vulnerability and poor outcome.

This long-term perpetuation of thalamic damage appears to be a unique feature of TBI. Whilst several structures have shown volume loss sub-acutely after TBI, such as the hippocampus, only thalamic volume loss perpetuated beyond 6 months post-injury which was uniquely associated with poor outcome in both mild[123,124] and moderate/severe TBI[364,390]. Animal models also uniquely find prolonged markers of neuroinflammation and GABA-A downregulation within the thalamus and no other brain regions[309,387], with delayed onset compared to cortical and striatal injury[359]. This was echoed in my results in **Chapter 6**, which found a mirroring effect between regions of thalamic selective neuronal loss and regions of cortical contusion. As discussed, animal models of TBI have suggested this may be a directional effect as cortical damage causes delayed transneuronal degeneration and apoptosis within the structurally connected regions of the thalamus[360,361]. I have, for the first time, demonstrated this effect may also occur in humans after TBI. Longitudinal study using concurrent FMZ-PET and diffusion imaging will enable a causal relationship to be fully established.

We can use this information to better understand previous studies, such as Anderson (1996)[357] who found that TBI patients with non-thalamic lesions had a reduced thalamic volume independent of injury severity, which was related to worse outcome. This observation was influential in proposing the thalamus as a region of interest, but lacked understanding of *why* the thalamus was affected, which can now be attributed to transneuronal degeneration. Other stereological findings, such Maxwell et al.,[388,38939] identified the greatest neuronal injury post-mortem in mediodorsal and central thalamic nuclei, related to greater injury severity. I equally found these nuclei to be most affected

by chronic selective neuronal loss, but am now able to explain this as a possible secondary effect of frontal injury and contusions. Indeed, the frontal aspect of the head is commonly implicated in blunt force primary injury as the point of contact, such as in head-on collisions and sports-related head-to-head contact, thus potentially explaining the historical pattern of findings within the thalamus and select thalamic nuclei.

The individual thalamic nuclei found to be important for understanding injury and outcome differed between **Chapters 4 & 5** (vAnterior, vlDorsal) and **Chapter 6** (Central-lateral, mDorsal), which should be discussed. This may be attributable to the distinct phase investigated post-injury when considering the theoretical frameworks discussed above. In theory, primary injury may affect GABAergic inhibitory control (vAnterior and vlDorsal nuclei, TRN) and how the thalamus coordinates with the cortex, whereas secondary injury may track back from the cortex to influence ongoing thalamic degeneration to mirror regions of cortical damage (Central and mDorsal nuclei, in cases of frontotemporal contusion). This will require a well-designed longitudinal study to fully explore such a hypothesis. However, an overarching conclusion of this work is that studying individual thalamic nuclei, rather than the thalamus as one coherent region, can yield greater explanatory power in clinical populations.

My findings thus link the thalamus, in its vulnerable location to primary injury, as a missing link between injury, outcome, and disease. In acute phases, there is growing evidence that a loss of thalamic inhibitory control can cause thalamocortical hyperconnectivity. This has been validated as predictive of post-acute postconcussive symptoms to form a novel acute biomarker. Finally, ongoing thalamic degeneration can perpetuate well-beyond the injury event, undergoing further downstream neuronal loss and inducing a vulnerable environment to subsequent injury.

7.2.3 Mal/adaptivity of functional connectivity

A third overarching theme of this thesis is the neural and behavioural purpose of functional hyperconnectivity. Two main types of acute hyperconnectivity were found in my mTBI cohort; thalamic hyperconnectivity attributed to a loss of inhibitory control **(Chapter 4),** particularly prevalent in cortical 'hub' regions**,** and potentially adaptive *between-network* hyperconnectivity found in **Chapter 3**. These findings support

propositions by previous studies in moderate and severe TBI[185,186,188] that functional hyperconnectivity is a common response to injury prevalent in cortical hubs, and importantly extends this to *mild* TBI which has been under greater speculation[191].

There are several hypotheses for why hyperconnectivity is commonly presented post-injury. From a purely statistical standpoint, functional connectivity describes the coactivation of timecourses, and thus specific neuronal damage could lead to less signal variability and thus increased 'connectivity' by timecourses of activation becoming more similar. A more favourable explanation in the moderate and severe TBI literature is that hyperconnectivity is induced by offloading neural demand to non-damaged regions, or via latent anatomical connections, to stabilise injury-induced irregularities[188]. This induces a cost-efficiency trade off; loss of neural efficiency induced by disrupted routes of functional information processing after injury, which can be compensated for with metabolic cost of rerouting information flow and/or increasing neural demand in non-damaged regions[190]. Further work within our group in mTBI[206], and replicated in the present dataset (**Appendix 3.8)**, found that an increase of global connectivity was significantly associated with a global loss of temporal complexity, i.e., greater information flow but with lower information quality. This was also suggested to further reflect potential re-routing of information flow to avoid damaged regions, as proposed in previous literature. Indeed, animal models of TBI have found more random functional connectivity acutely post-injury, such as temporary decreases in small worldness (defined as the most efficient form of neural architecture lying between complete order and disorder with highly integrated hubs and fewer long-range connections[182]) concurrently with local hyperconnectivity surrounding structurally damaged regions[406]. These studies highlight the potential relationship between local damage after TBI, compensatory hyperconnectivity, and conformational change in brain architecture.

Post-TBI hyperconnectivity is thought to be particularly present in so-called 'hub' regions, as found in explorations of thalamocortical connectivity in **Chapter 4**. Here, the greatest regional acute hyperconnectivity from the thalamus was to hub-like regions including the posterior cingulate and insula. Hub regions such as these, often termed the 'rich club'[407], are highly interconnected throughout the brain and display high metabolic efficiency[185,188]. Due to these intrinsic properties, increasing connections through these hub regions is hypothesised to combat network inefficiency whilst reducing metabolic

costs in a stressful neuronal environment, thereby mediating the cost-efficiency trade-off of compensatory changes post-injury[188]. Moreover, dense interconnections throughout the brain optimally position these regions to support neural flexibility and plasticity following injury[407].

The relative importance of hub regions after TBI is supported by both simulation and real-world evidence. In work by Hilary and colleagues[188], simulating the removal of target hub regions, or connections to those hubs, had a pronounced effect on global functional efficiency of the brain. In contrast, global efficiency was robust to random removal of a non-hub brain region/connection. Thus, hub regions are uniquely important to healthy brain function, and hyperconnectivity directed at sustaining these regions would yield the greatest benefit to information transfer across the brain to minimise behavioural deficit. In moderate and severe TBI individuals, functional hyperconnectivity is certainly disproportionately higher in these hub regions[407]. These hubs have been previously identified to be relevant in mTBI thalamic connectivity[187], and are also more affected in other neurological diseases such as Alzheimer's and Parkinson's[300].

These accounts therefore suggest hyperconnectivity is an adaptive response post-TBI, to compensate for damage of existing efficient neural pathways. Multiple studies of moderate and severe TBI have directly tested and support this adaptive hyperconnectivity hypothesis[188–190]. For example, finding greater efficiency costs of network function acutely after TBI which significantly predicted reduced cognitive performance[190]. However, the current literature faces great speculation on what can be considered adaptive or maladaptive after *mild* TBI[191]. The most recent review concluded that only hyperconnectivity of DMN and cerebellar regions could be associated with adaptive mechanisms and behaviours across studies, but even this evidence was weak[191]. Results from **Chapter 3** also support this, as increased acute between-network connectivity was only present in those with a good functional outcome (GOSE-8) compared to controls, and commonly implicated the default-mode and cerebellar networks. Moreover, increased distribution complexity was behaviourally relevant in distinguishing functional outcome in DMN and cerebellar network components. These suggest some potentially adaptive properties of increased between-network connectivity involving hub-like regions. Although, these results only pertained to functional, and not

symptomatic, outcomes, and the true mal/adaptive properties of hyperconnectivity are yet to be fully established in the wider literature of mTBI.

If acute hyperconnectivity can be considered an adaptive response, why then was amplified thalamic hyperconnectivity associated with adverse behavioural consequences? This may be due to the measures reflecting entirely different aspects of mTBI. In the case of network neuroscience (**Chapter 3**), between-network hyperconnectivity reflects a behaviourally adaptive response of increasing inter-network communication for coordinated efforts against neural damage, as discussed. In the case of thalamic function (**Chapters 4 & 5**), primary damage of GABAergic nuclei can induce inhibitory imbalance and thus global hyperconnectivity, as suggested by the present results and independent replication[350]. In this sense, each measure is reflecting a different *type* of functional connectivity. Namely, acute thalamic hyperconnectivity as a possible *consequence of* primary injury, and acute network hyperconnectivity as a possible *response to* primary injury.

Both could exist simultaneously in our cohort with the simple explanation that greater hyperconnectivity reflects greater neuronal damage, in the thalamus or in cortical networks, meaning more compensation is required. Whilst initially adaptive, persistent overstimulation of hub regions (including the thalamus) causes greater stress which is more likely to fatigue and fail over time[300], leading to chronic hypoconnectivity[186]. Indeed, hub regions appear most vulnerable to pathophysiology in abnormal aging[408] including a variety of neurological disorders[300]. Moreover, this pattern of functional hyper- to hypo-connectivity change has been found in preclinical Alzheimer's disease, whereby a transition into hypoconnectivity coincided with faster rates of neurodegeneration[409]. As such, hyperconnectivity may not necessarily be the root *cause* of adverse symptoms, but merely their correlate indicating widespread functional change which can manifest differentially across individuals. For instance, I found preliminary evidence for decreasing connectivity into healthy ranges over time, with significantly decreased thalamocortical connectivity only in those with chronic symptoms.

These results thus speak to the third aim of my thesis: to understand how acute correlates of chronic outcome (thalamic hyperconnectivity) change over time and after repeat injury. Longitudinal studies of mTBI are few, particularly with concurrent neuroimaging,

making these results valuable in assessing existing hypothesised models of connectivity change in mTBI[186]. The longitudinal model proposed by Boshra and colleagues[186] equally proposed that transitions from initial hyperconnectivity into chronic hypoconnectivity underpin adverse long-term recovery and lifelong complications of mTBI. For example, they suggested that fatigue and failure of compensatory mechanisms may be tied to a reduced neuronal reserve such as in older age, which could seek to explain some of the poor outcomes experienced by this group. A lack of neuronal reserve and neuroplasticity could mean these individuals fail to adequately adapt to a disrupted neuronal environment. These hypotheses are yet to be empirically validated, and more research is required to fully understand how connectivity changes might relate to symptomatic recovery versus perpetuation or deterioration.

A final consideration for thalamic hyperconnectivity is its phenomenological relationship to postconcussive symptoms. As thalamic hyperconnectivity was found to transition from hyper- to possible hypo-connectivity only in those with postconcussive symptoms, so too does the profile of symptom expression transition from hyper- to hypo-stimulation. Postconcussive symptoms are commonly distinguished as 'early' symptoms (e.g. dizziness, nausea, noise and light sensitivity) and 'late' symptoms (e.g. depression, fatigue, difficulty concentrating). These could each be reflecting over-stimulation in the early phase, followed by under-stimulation in the late phase. This is mere observation, but could prove an interesting avenue of research in future studies to understand why postconcussive symptoms change over time, and if the thalamus can be regulated in some way to target these symptoms.

It is clear there is a complex link between different types of functional hyperconnectivity, their root cause, and consequences for patient outcome. Some hyperconnectivity measures could reflect adaptive mechanisms of compensation, whilst others may reflect a consequence of the injury itself and inhibitory-excitatory imbalances. More work is needed to fully understand the behavioural and neuronal relevance of hyperconnectivity, and how this might develop into hypoconnectivity in a time- and outcome-dependent manner.

7.2.4 A bridge between predicting outcome and influencing outcome.

Having now found clear markers of ongoing disease by investigating thalamic function after TBI, there remains a major obstacle for translation into clinical care. Improving prognostication of chronic outcomes in mTBI may not reach its intended benefit if those algorithms or prognostic markers are not actively implemented in routine monitoring of patients or stratification for clinical trials. Hence, is it feasible for every individual with a mTBI to undergo a rsfMRI scan to quantify thalamic hyperconnectivity? Modalities such as CT and structural MRI have clear clinical benefit for surgical intervention[96], and are cost-effective and accessible in most modern care systems. This is in contrast with rsfMRI, which is largely confined to research settings due to its cost, acquisition time, lack of normative standards for individual patient comparison, and required expertise for analysis[96]. Functional neuroimaging methods such as rsfMRI were designed to seek differences between two or more populations/timepoints. These *group*-level differences may not represent the great variance seen in fMRI at the *individual*-level[197], and it is these individual measurements which are of great importance in the road to precision medicine. The neuroimaging community is beginning to define normative human brain phenotypes across the lifespan using structural MRI[410], such as regional volumes and shape, however this is currently beyond reach in functional neuroimaging. Despite this thesis demonstrating the relative value of rsfMRI above structural imaging modalities for mild TBI, it is thus unlikely that each patient reaching the ER will undergo a rsfMRI scan. At least, until we can apply rsfMRI to the individual, without requiring expert knowledge for analysis, with time- and cost-effective methods.

Hence, I wanted to explore how we can use rsfMRI as both a prognostic marker, and a tool for hypothesis-forming research across multiple fields within neuroscience. This sought to transition from predicting outcome, to influencing outcome in future patient care. Thus, the final aim of this thesis was to begin to translate findings from imaging into treatment-relevant targets, thereby bridging the gap between macrostructural and microstructural investigation.

The main translational effort from this thesis linked rsfMRI markers of outcome with neurotransmitter systems, aiming to form hypotheses regarding symptom-specific therapeutic targets. **Chapter 4** found that cortical regions displaying acute thalamic

hyperconnectivity related to noradrenergic transporter distribution and 5HT-2A receptor distribution in those with long-term cognitive and emotional symptoms, respectively. Methodologically, this allowed neurotransmitter-specific associations to be made using healthy average PET maps, which are otherwise unobtainable from rsfMRI alone, using non-invasive and easily-implementable strategies relative to performing multiple labour- and cost-intensive PET scans. Clinically, these assessments could allow for precision neurotransmitter/neuromodulator therapeutic strategies to be developed in the mTBI context by promoting translational research efforts into therapeutic targets to best *treat* these at-risk patients. Such efforts were also used on a smaller scale in **Chapter 3**, relating 'adaptive' network components with neuromodulatory brainstem nuclei. This particularly identified greater connectivity with the VTA and median raphe, known to be implicated in dopaminergic and serotonergic neuromodulation respectively. Thus, I have commonly identified *monoaminergic* neuromodulatory correlates of outcome in acute mTBI using two independent methods. It will be important that these findings are further developed through assessments of blood/salivary biomarkers of neurotransmitter metabolites, and whether integrity and/or connectivity of the brainstem sources of these transmitters to the thalamus and the rest of the brain are perturbed[251,411,412].

Monoaminergic neurotransmitters are classed as neuromodulators, which can powerfully affect the synaptic and electrochemical properties of neurons and networks[301,413]. Their functionality is necessary for the maintenance of healthy connectivity profiles [250,251], and they have shown systems-level changes in severe TBI[252] echoed here in my mild cohort. Importantly, when these systems become/remain dysfunctional, this may relate to a loss of compensatory hyperconnectivity and symptom emergence/persistence. For example, dopamine levels and dopaminergic systems are vastly altered following injury in animal models of TBI[263,264], and several therapeutic developments in dopaminergic agonists show benefits for neuropsychiatric outcomes in clinical trials[265,266]. This raises the possibility that connectivity of the thalamus – as a main target and relay station of neuromodulation[278,414] might be altered as a result of transmitter changes, and could thus constitute a preliminary biomarker to characterise transmitter-related treatment agents in mTBI. Namely, individuals who show noradrenaline-associated connectivity alterations might respond to drugs such as

methylphenidate[302,415], whereas those with serotonin-associated connectivity alterations may respond to existing targets used in the depression literature[252]. This is a clear step towards precision medicine approaches and the potential of rsfMRI to stratify patients for clinical trials of such drugs. Importantly, a rsfMRI assessment could identify correlates of outcome at earlier timepoints than CT and MRI, thus enabling earlier clinically intervention within a 'window of opportunity' to gain greatest recovery from injury. This remains speculation at present, however this further exploration of functional changes following mTBI suggests an integral link to neurotransmitter system changes, which could be harnessed for patient benefit.

A further avenue for therapeutic development considers acute thalamic hyperconnectivity. If, as discussed, this is related to adverse outcome and due to inhibitory imbalances, novel drug targets which aim to support and upregulate acute inhibitory control to avoid thalamocortical hyperexcitability could be behaviourally beneficial. Such targets are beginning to emerge, for instance, a study in rodents upregulated thalamic GAT-3 receptors ipsilaterally to injury which produced neuroprotective effects against seizures and mortality, and restored thalamic hyperexcitability back to levels seen after sham injury[399]. Some authors have further suggested neurosteroids acting through δ-containing receptors as possible strategy to enhance GABAergic transmission[309]. These suggestions are in the very early therapeutic stages, however, and will particularly require validation of translational models to understand how these effects seen in rodents can apply to human TBI, and how these can be further developed into viable treatment strategies for human patients.

The final translational application from this work surrounds the dogma that TBI patients are anticipated to have reached functional recovery at around 6-months post-injury. This is the commonly assessed timepoint for outcomes such as the GOSE, under the assumption that little functional change is likely to occur past this point as neural cascades of injury have ceased, and any neuroplastic adaptation has had time to occur and stabilise. However, I have presented several results which question this belief. In **Chapter 4**, I showed ongoing outcome-dependent changes at 12-months post injury, which have relevance for subsequent vulnerability to repeat injury in **Chapter 5.** Moreover, I used the rarely collected imaging modality FMZ-PET to better understand the long-term disease of TBI (**Chapter 6**). These results demonstrated thalamic selective

neuronal loss many *years* post-injury, potentially related to ongoing mechanisms of transneuronal degeneration, in relationship with chronic outcomes. As with rsfMRI, this is not to suggest that every individual should undergo an expensive and labour-intensive FMZ PET scan, but rather, acknowledge that changes within the individual can occur well-beyond this 6-month timepoint post-injury and long-term care could have extended benefits. For instance, a previous study using FMZ PET for TBI found persistent reductions in GABA-A receptor availability in the thalamus and anterior forebrain at 7-13 months post-injury, despite other regions increasing availability over recovery from subacute scans[376], exemplifying this ongoing impairment specific to the thalamus.

Each of the chronic functional changes found in my research of TBI were related with adverse outcome. It is thus important to further investigate these neurobehavioral changes beyond 6-months, and what can be done to help the ongoing difficulties faced by these individuals. For example, the chronic TBI cohort collected in Cambridge described in **Chapter 6** experienced severe complications of TBI requiring intracranial monitoring in the ICU. Thus, their participation and successful completion of cognitive tests such as CANTAB are arguably indicative of a good outcome given the severity of their injury. However, in a simple animal fluency task, some individuals could name few, if any, types of animals in the last 30 seconds of the task. Given the possible importance of this task for early identification of Alzheimer's disease[394,396], and known links between TBI history and increased propensity for developing neurodegenerative diseases in later life[60,61,314], chronic treatment targets are an important therapeutic avenue to investigate.

Each of these discussed looks at how we can influence outcome in the future. How can we benefit patient care right now?

The timeline of completing this thesis coincided with great public interest in concussion and regulatory change in the UK. This included new governmental guidelines being published regarding grass-roots sports[334], to encourage a minimum of 24 hours rest after a suspected concussion at any sporting level. Just a few weeks after the publication of our results (**Chapter 4**), these guidelines were discussed in an emergency debate within UK Parliament, calling for even greater action. Indeed, this inspired my subsequent work on repeat mTBI in **Chapter 5**, which found a more vulnerable neuronal environment after repeat injury. This research, and indeed these UK guidelines, mean little if they are not

adopted by the public we are seeking to help. Thus, our greatest tool to influence outcome *right now* is to engage the public with research. We were fortunate to garner international media attention from our first publication on mTBI[342], which gave my supervisor and I a platform to spread awareness of 'mild' TBI and its potential consequences. Talking to TBI experts and TBI survivors alike, almost all agreed that concussion must be taken more seriously for any real strides in patient care to be made. This includes increasing reporting rates to hospital settings, increasing recovery times following sports concussion, and increasing treatments available to those experiencing long-term symptoms. Each of these is its own unique challenge, which require further public outreach and research to fully understand what is most beneficial for long-term recovery, and how this can be achieved in a struggling healthcare system in the UK.

7.3 Remaining questions & future directions

This thesis encompasses a small facet of TBI heterogeneity and complexity, and many questions remain.

Firstly, more *causal* evidence is needed to support the hypothesis that thalamic inhibitory imbalance leads to thalamocortical hyperconnectivity. This can initially be further investigated using animal models, to selectively damage inhibitory nuclei and the TRN versus other thalamic nuclei and observe subsequent effects on thalamic hyperconnectivity and behaviour. The BOLD signal is inherently an indirect measure of neural activity, whereby changes can be attributed to several different biological properties, and thus these kinds of biophysical relationships are valuable for our interpretation for alterations after mTBI.

A related avenue for future investigation is how selective neuronal loss within the thalamus (identified with FMZ-PET) could manifest in mild TBI as well as moderate and severe TBI, and similarly, how this can interact with functional connectivity. One of the key papers influencing my work into thalamic hyperconnectivity was that by Sours and colleagues[187]. They found evidence for neuronal dysfunction/depletion uniquely in the thalamus in the same mTBI participants exhibiting thalamocortical hyperconnectivity, postulating that selective neuronal loss of GABAergic interneurons could induce a loss of inhibitory control. Such a hypothesis has already been discussed at length, with support

in recent rat models by Li and colleagues[350]. To fully identify this in *humans*, thereby combining work across the present thesis and these two previous papers, I would have liked to directly relate markers of selective neuronal loss in the thalamus (FMZ PET) with thalamic hyperconnectivity (rsfMRI).

In the ideal study design, both mild and moderate/severe TBI individuals would undergo rsfMRI, DTI, and FMZ PET, all acquired at acute, 6-month, and perhaps even later chronic timepoints, alongside postconcussive and cognitive outcome measures. This would enable us to investigate i) how structural and functional connectivity of the thalamus is associated with primary thalamic injury using FMZ PET (acute timepoints), ii) how these associations may change over time to include mechanisms of transneuronal degeneration in patients with cortical lesions (chronic timepoints), and iii) whether a causal link can be established in humans of all TBI severities between GABAergic selective neuronal loss, thalamic hyperconnectivity, and its complex changes over time in relationship to outcome. I would like to explicitly use FMZ PET in mTBI individuals, and indeed a longitudinal study design, as this unique type of imaging has never been applied in a mild TBI cohort (except n=4 individuals from the mixed TBI cohort included in Kang et al.,[376] who also presented damage on CT), and has only been used longitudinally in TBI in one recent study with merely n=7 patients[376]. I would also like to combine these multiple imaging modalities given the great scientific potential of FMZ PET to aid our understanding of functional connectivity changes and ongoing thalamic damage not identifiable with CT/MRI. To the best of my knowledge, one study did acquire both rsfMRI and FMZ PET in a cohort of patients with disorders of consciousness (DOC)[416], some of whom due to TBI. They found globally decreased FMZ binding potential and decreased between-network functional connectivity in chronic DOC compared to controls, but did not directly relate the two modalities to form significant associations between these findings, which is a missed opportunity of such a unique dataset. As mild TBI attracts greater interest in public and research communities, and more funding is made available to achieve these multimodal datasets in mTBI, I hope these questions can be answered in the future.

A second direction for future research is to further assess the mal/adaptive nature of hyperconnectivity in mTBI which remains under debate, and validate how functional connectivity changes over time with outcome-dependent trajectories as proposed by

Boshra and colleagues[186]. This will ideally require a larger sample size than my present longitudinal cohort, and larger than the comparatively small sample sizes often used in moderate and severe TBI, simply due to the smaller effects seen in a milder injury phenotype. Such a study sample requires globally coordinated efforts, perhaps by combining data across large-scale projects including CENTER-TBI[43] (Europe), TRACK-TBI[51] (US), and emerging datasets in Australia (The Australian Traumatic Brain Injury National Data[417], OzENTER-TBI[418]) and India (CINTER-TBI[43]). This project should aim to track functional connectivity change, symptom progression and recovery, and identify specific timepoints for intervention when functional trajectories diverge into good and poor outcome. Moreover, the work undertaken in **Chapter 5** on repetitive injury was in a very small sample, which would also benefit from coordinated efforts. This is important to establish clear markers of injury and outcome, and better characterise the 'window of vulnerability' in humans to allow a safe return to play after sports-related concussion. These are ongoing debates I was unable to fully answer with the data currently available on mTBI and repetitive injury, which will undoubtedly benefit from global collaboration.

There are two further features of this thesis which could be built upon in future work. As highlighted in **Section 7.2.1**, my assessment of the 'mildest' TBI was dictated by the mildest phenotype available within CENTER-TBI. Many mTBIs will go unrealised due to self-treatment and/or not reaching inclusion criteria for advanced neuroimaging. It would therefore be beneficial to understand the relative differences in outcome and functional connectivity between those who attended versus did not attend healthcare settings- both in initial injury severity, and long-term behavioural management. Moreover, much of my research on the 'mildest' TBI has negated previously established markers of poor outcome to find novel biomarkers for this misunderstood population, as was the aim of this thesis. I have not, however, considered if this acute biomarker might benefit other populations with TBI, nor its prognostic utility over and above existing markers. Future development of prognostic models may seek to understand if some markers are more beneficial to sub-types of TBI than others. For instance, damage on CT is useful for surgical intervention in moderate and severe TBI[95,97], but arguably less useful in mild TBI who do not commonly display large lesions on CT[54,88]. Yet, both groups are known to experience similarly levels of quality of life[355], demonstrating the need for biomarkers irrespective of injury severity. Perhaps, more subtle markers of thalamocortical

connectivity present prognostic value for milder TBI but may be inconsequential for more severe injuries with existing structural biomarkers[84,85,87], which is yet to be established.

Finally, an under investigated facet of TBI is the potential sex differences in injury and outcome, and the persistent ignorance of female TBI. A recent review on sex differences after TBI found that whilst limited by small sample sizes, there was a trend for worse outcome in female patients than in males[19]. This disparity appears to also apply in mild TBI, as emerging research from TRACK-TBI found significantly worse cognitive and somatic symptoms in female mTBI, which were particularly amplified in the middle-aged female group compared with young and older female patients[20]. Recent data from CENTER-TBI additionally found that women experienced less intensive and shorter hospital stays after mTBI than men, yet displayed significantly worse 6-month outcomes such as quality of life, depression, and anxiety[419]. Finally, in the growing field of sports-related concussion, a three-year observational study identified significantly greater chance of such injury in high-school females than males, but females were less likely to be removed from play and experienced differential care[420]. It is therefore necessary to increase our attention on female TBI in research and clinical practice, to reduce and better understand these poor outcomes. In the present thesis, sex was included as a covariate in all analyses, and all cohorts persistently presented a greater percentage of male patients than female, which is characteristic of TBI demographics worldwide[3]. I did not, however, consider the potential interactions of hormones, sex, and age, and their influence on specific postconcussive outcomes after TBI. For instance, sex differences may be pertinent to rsfMRI as recent work identified sex-specific differences in resting-state networks explicitly in postconcussion syndrome[421]. These questions should be fully investigated as their own research project, using both male *and female* animal models, and specific hormonal measurements, some of which have previously related to mortality[18]. Additionally, future research needs to consider the distinct area of gender expression and identity, which could influence vulnerability to violence and subsequent TBI, including social support networks and reintegration post-TBI[422]. These are emerging areas of demand for future research within the TBI community and should be reflected in future cohorts to better understand all members of the public experiencing TBI, not just the historically prevalent demographic of young males.

7.4 Conclusion

In this thesis, I have established that 'mild' TBI has vast functional and behavioural consequences in a high proportion of individuals and is currently not treated as such. In response, I have determined that functional neuroimaging is an indispensable tool for understanding and prognosticating these poor outcomes, in mTBI populations who are otherwise misrepresented by routine imaging methods. At the centre of this is the thalamus- an integral hub connecting injury, outcome, and long-term disease following TBI, including the special interest group of repetitive injury. By looking through the lens of the thalamus, I have presented a novel prognostic marker for chronic postconcussive symptoms, and unique lifelong thalamic neuronal consequences extending many years post-injury. This work has furthermore been extended to consider the application of rsfMRI, to form novel hypotheses and therapeutic targets to *treat* these patients' outcomes, aside from merely predicting them. This will require substantial development across multiple areas of neuroscience to fully realise. Nevertheless, this thesis calls for greater recognition of mTBI experience, and investigation of the thalamus, in the quest for precision medicine approaches to care for this growing population of 'mild' TBI.

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Appendices

Appendix for Chapter 2

Appendix 2.1. CENTER-TBI MRI Substudy Participants and Investigators

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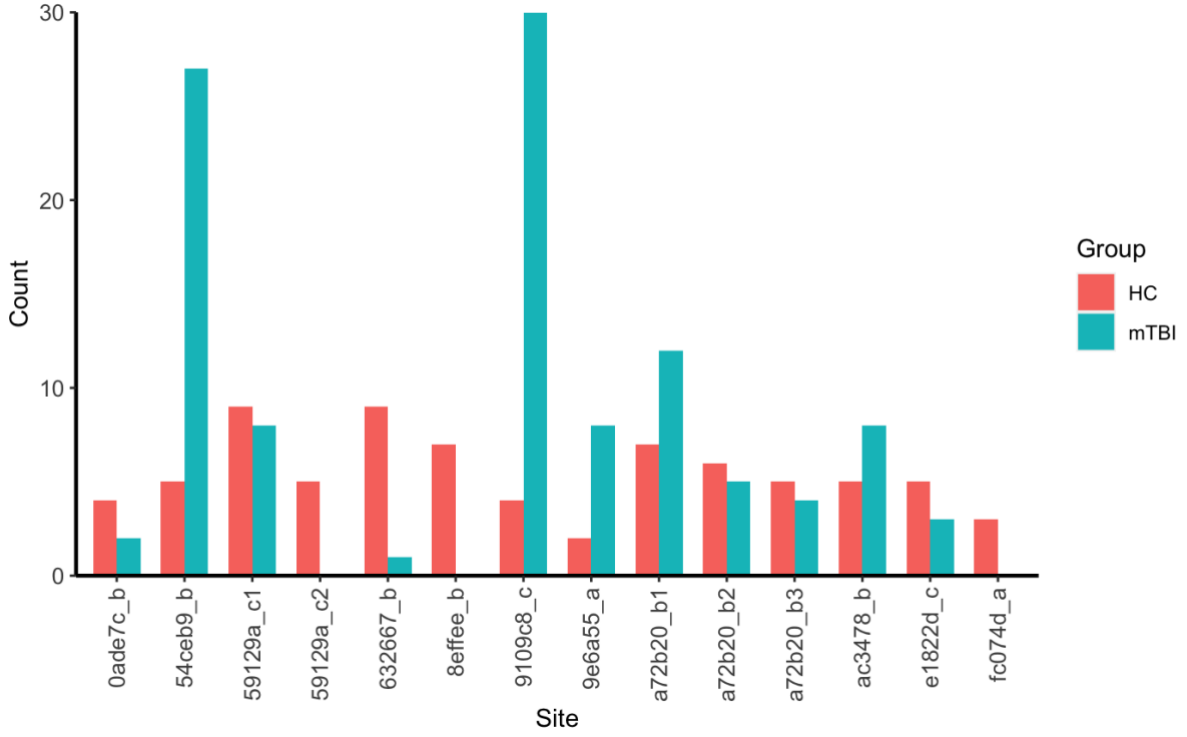
Appendix 2.2. Distribution of site/scanner across the cohort. Patient and control groups are coloured respectively, to demonstrate the variation in ratios between sites. Site is coded by its <site>_<scanner>, where a=Phillips, b=Siemens, c=GE, and further numbers indicate there are multiple of this scanner type at one location. This highlights the importance of multicentre harmonisation protocols, to avoid removing variance attributable to group differences.



Appendix 2.3. Demographic information for wider cohort of mTBI. These data are for patients meeting my specified non-imaging inclusion criteria, regardless of whether acute imaging was performed. These are to show the similarity in demographic characteristics between my patient cohort and the wider mTBI population.

	mTBI (n=975) n (%)
Age	
18-35	353 (36.2)
36-55	340 (34.9)
55-70	282 (28.9)
Sex	
Male	645 (68.2)
Female	330 (33.8)
Glasgow Coma Score	
15	836 (85.7)
14	124 (12.7)
13	15 (1.5)
Injury Cause	
Road Traffic Incident	369 (39.5)
Incidental Fall	397 (42.5)
Other Non-intentional injury	81 (8.7)
Violence/Assault	85 (9.1)
Act of Mass Violence	3 (0.3)
Unknown	44 (4.5)
Strata	
Emergency Room	466 (47.7)
Admission	509 (52.2)
6 Month GOSE	
Complete	508 (52.1)
Incomplete	467 (47.9)
6 Month PCS	**n=572**
PCS+	186 (32.5)
PCS-	386 (67.5)

Appendix for Chapter 3

Appendix 3.1. Acquisition parameters for mTBI cohort. Further details are given in the central CENTER-TBI resources at https://www.center-tbi.eu/project/mri-study-protocols . Parameters are given as specified within these resources.

T1- Weighted MRI

Scanner	GE	Phillips	Siemens
TR	6212	6,7	2300
TE	2008	3	2,98
Voxel Size	1x1x1	1x1x1	1x1x1
Flip Angle (deg)	11	9	9
N Slices	192	192	192
Scan duration (min)		5:39	5:21

rsfMRI

Scanner	GE	Phillips	Siemens (Trio / Skyra)
TR	2500	2500	2500 / 2480
TE	28	28	28
Voxel Size	3x3x3	3x3x3	3x3x3
Flip Angle (deg)	70	70	70
N Slices	32	45	41
Scan duration (min)		6:58	6:55 / 6:59
Healthy Controls (/76)	23	5	48
mTBI (/108)	41	8	59

Appendix 3.2. Boilerplate of preprocessing parameters used, via fMRIprep.

The below boilerplate text describing preprocessing pipeline was automatically generated by fMRIprep with the express intention that users should copy and paste this text into their manuscripts *unchanged*. It is released under the CC0 license. Results included in this manuscript come from preprocessing performed using *fMRIprep* 1.5.4 [200](RRID:SCR_016216), which is based on *Nipype* 1.3.1[423](RRID:SCR_002502).

The T1-weighted (T1w) image was corrected for intensity non-uniformity (INU) with N4BiasFieldCorrection[235], distributed with ANTs 2.2.0[236](RRID:SCR_004757), 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,). Volume-based spatial normalization to one standard space (MNI152NLin2009cAsym) was performed through nonlinear registration with antsRegistration (ANTs 2.2.0), using brain-extracted versions of both T1w reference and the T1w template. The following template was selected for spatial normalization: *ICBM 152 Nonlinear Asymmetrical template version 2009c* [TemplateFlow ID: MNI152NLin2009cAsym].

For each of the 1 BOLD runs found per subject (across all tasks and sessions), the following preprocessing was performed. First, a reference volume and its skull-stripped version were generated using a custom methodology of *fMRIprep*. Susceptibility distortion correction (SDC) was omitted. The BOLD reference was then co-registered to the T1w reference using flirt (FSL 5.0.9)[237] with the boundary-based registration cost-function. Co-registration was configured with nine degrees of freedom to account for distortions remaining in the BOLD reference. 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)[237]. The BOLD time-series (including slice-timing correction when applied) were resampled onto their original, native space by applying the transforms to correct for head-motion. These resampled BOLD time-series will be referred to as *preprocessed BOLD in original space*, or just *preprocessed BOLD*. The BOLD time-series were resampled into standard space, generating a *preprocessed BOLD run in ['MNI152NLin2009cAsym'] space*. First, a

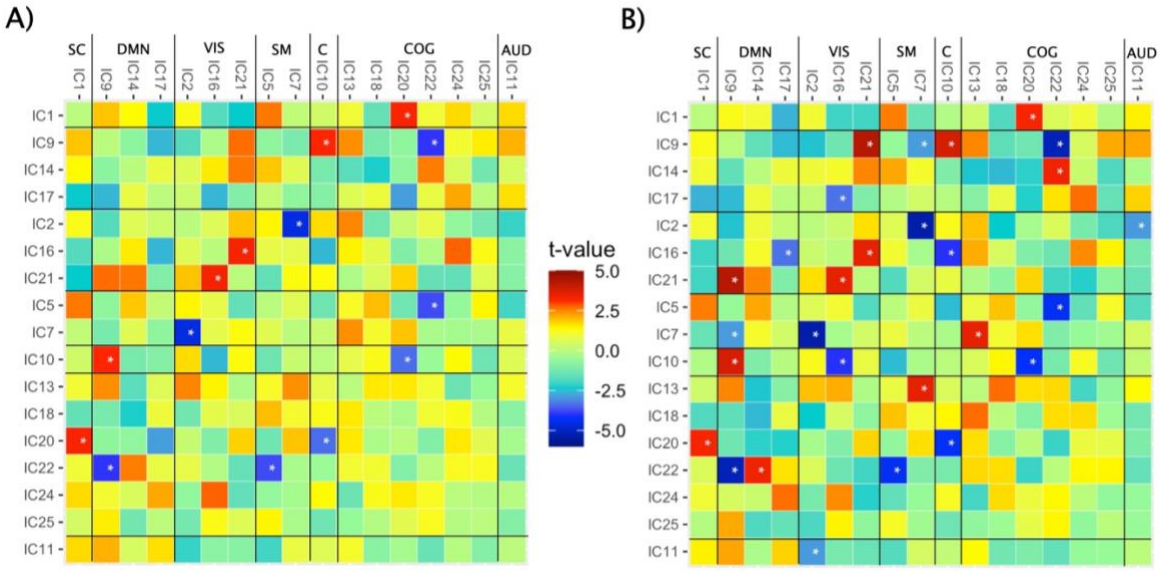
reference volume and its skull-stripped version were generated using a custom methodology of *fMRIprep*. Several confounding time-series were calculated based on the *preprocessed BOLD*: framewise displacement (FD), DVARS and three region-wise global signals. FD and DVARS are calculated for each functional run, both using their implementations in *Nipype*. The three global signals are extracted within the CSF, the WM, and the whole-brain masks. The head-motion estimates calculated in the correction step were also placed within the corresponding confounds file. 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).

Many internal operations of *fMRIprep* use *Nilearn* 0.6.0, mostly within the functional processing workflow. For more details of the pipeline, see the section corresponding to workflows in *fMRIPrep*'s documentation.

Appendix 3.3. Pre- versus post-harmonisation functional connectivity matrices. To quantify effects of harmonization, Kruskal-Wallis tests compared FC values between scanners (n=14) for every component pair before and after harmonization. These were corrected for multiple comparisons using FDR at p=0.05.

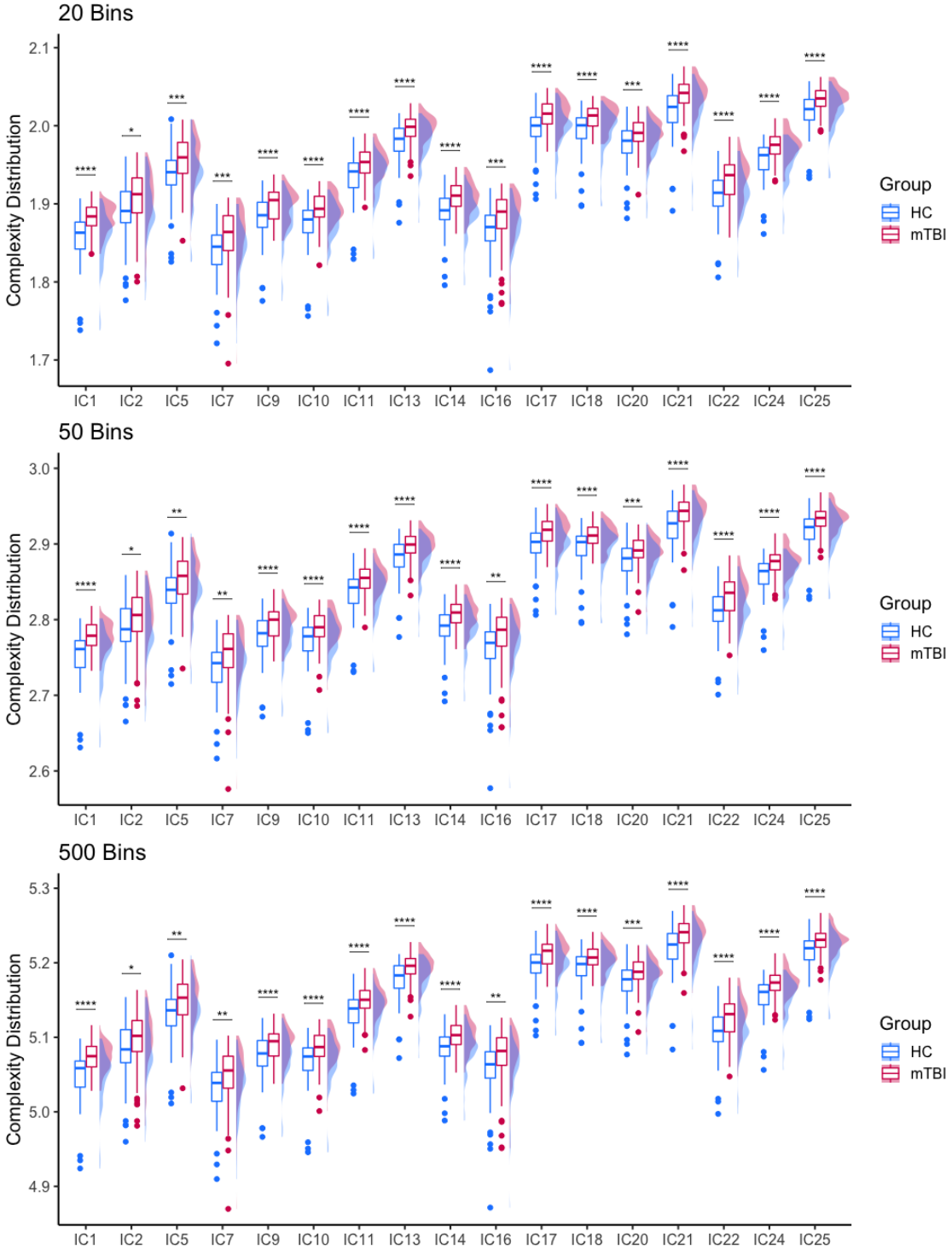
Prior to harmonization, 22.9% (n=31) of pairwise FC values were significantly different between groups, compared to 0% following harmonization, suggesting site-specific variance had been removed. To assess ComBat's retention of biologically-relevant variance, FC difference of each IC pair was calculated between controls and mTBI on both the unharmonised (A) and harmonized (B) data. Results of these are shown below, with FDR-corrected (p=0.05) significant FC pairs indicated with an asterisk. Controls<mTBI is shown in positive t-values (i.e. red/orange), and controls>mTBI is shown by negative t-values (blue).

Following harmonization, all previously significant pairs are retained plus additional group differences (unharmonized (A) = 7 significant pairings, harmonized (B) = 14 significant pairings). These results propose that harmonization has reduced site-specific variance whilst retaining biologically-relevant variance and increasing sensitivity to difference detection between groups.

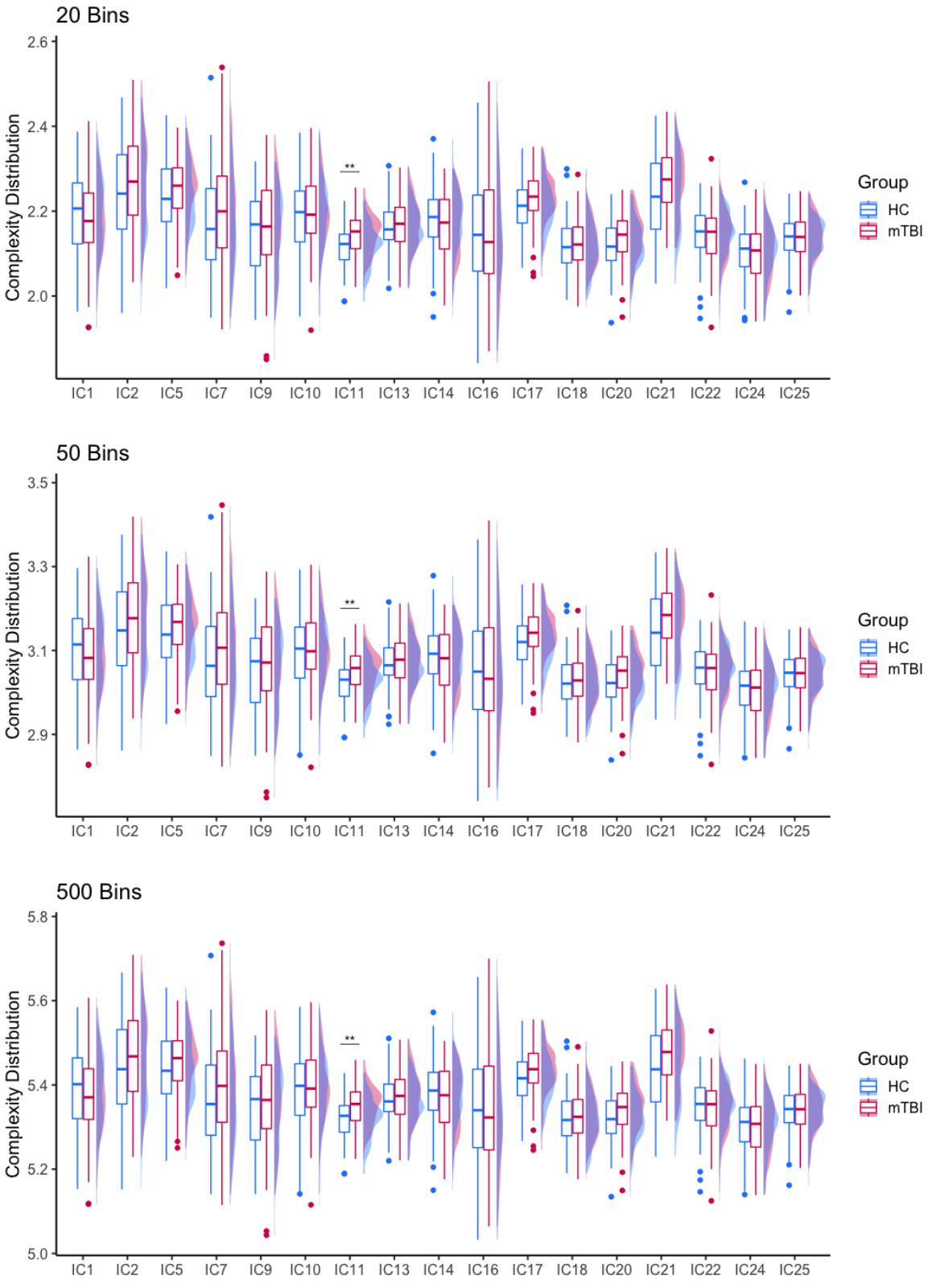


Appendix 3.4. Group comparisons (patients vs controls) in entropy measures. Repeated at n=20,50,500 bins, demonstrating stability of significant comparisons between-groups. Significantly different comparisons after FDR-correction are shown with; p<0.05 = *, p<0.01 = **, p<0.001 = ***, p<0.0001=****.

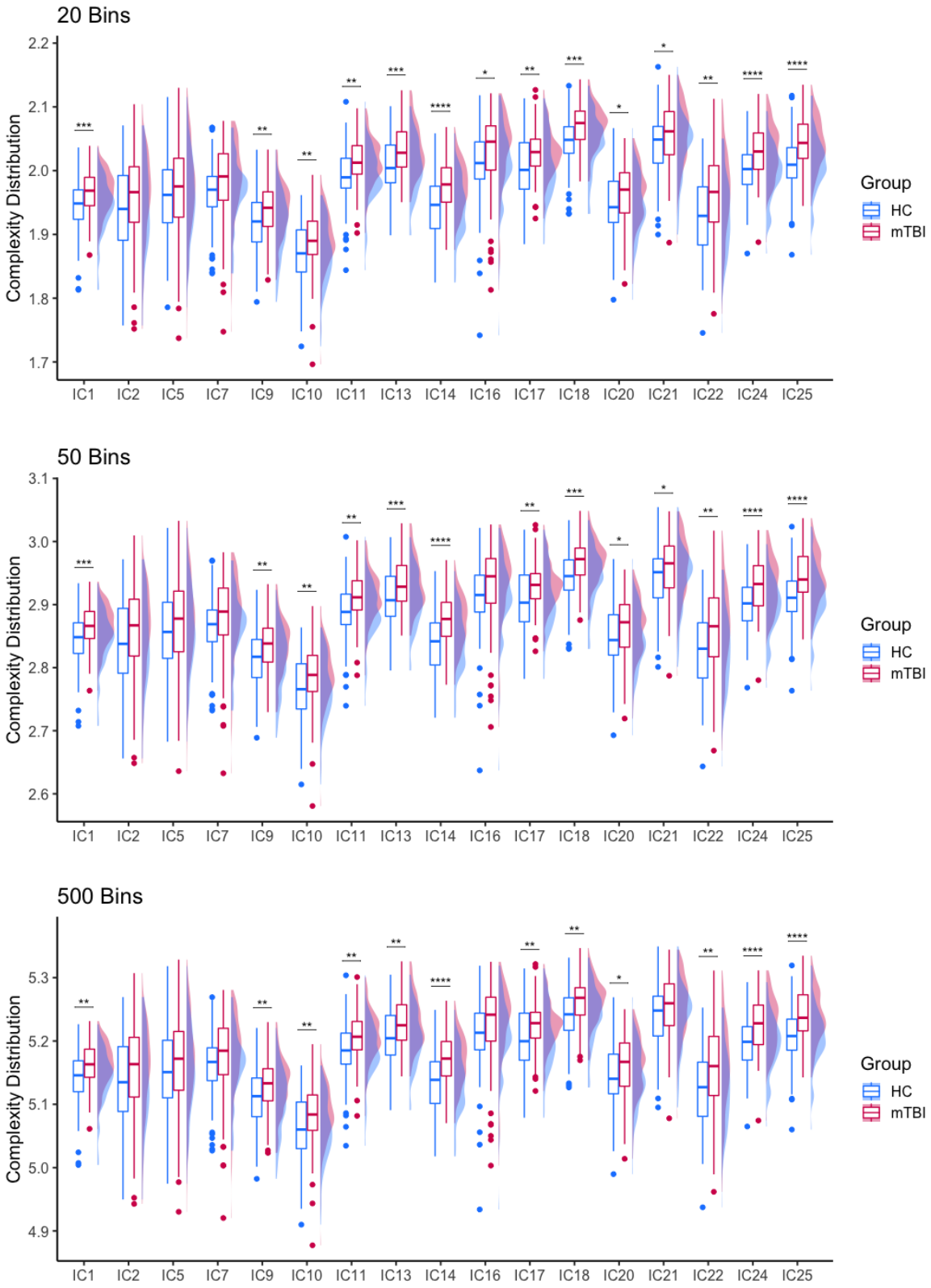
A) Whole Brain Entropy



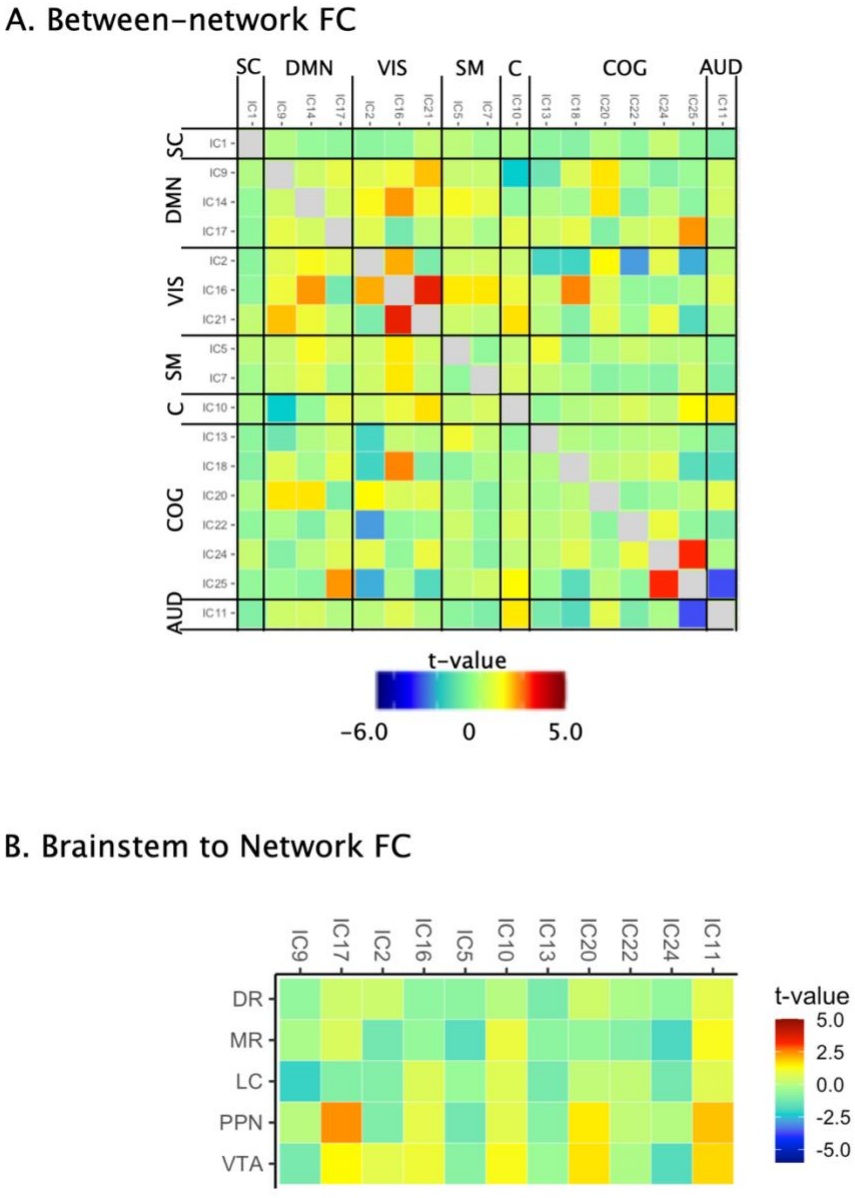
B) Outside-IC entropy



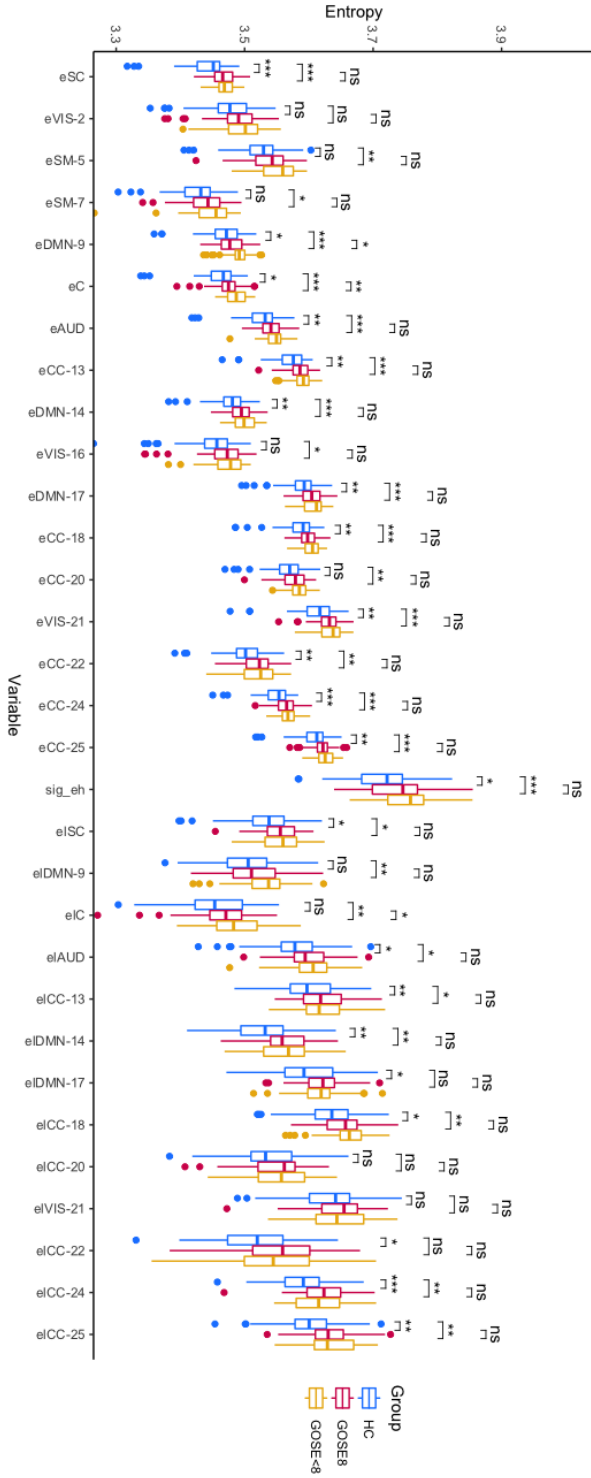
C) Within-IC entropy



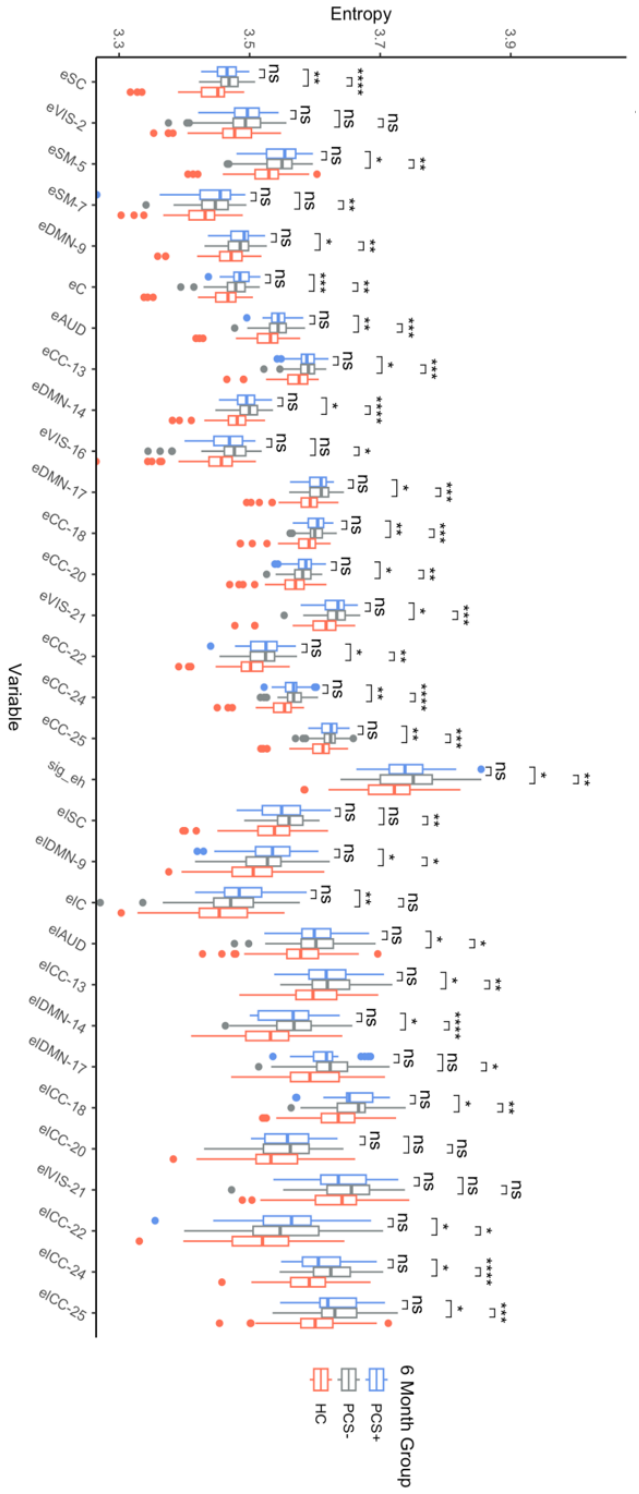
Appendix 3.5 Outcome group comparisons (GOSE) in between-component functional connectivity (A), and brainstem-to-component functional connectivity. Each figure shows matrices of t-values from pairwise statistical comparisons, whereby red indicates greater connectivity in the complete recovery group (GOSE-8) compared to the incomplete recovery group. These find no significant differences between GOSE outcome groups after correction for multiple comparisons.



Appendix 3.6. Outcome group comparisons (GOSE) across all significant entropy values. Significantly different comparisons after FDR-correction are shown with; ns = non-significant, p<0.05 = *, p<0.01 = **, p<0.001 = ***, p<0.0001=****.



Appendix 3.7. Outcome group comparisons (PCS) in entropy. Significantly different comparisons after FDR-correction are shown with; ns = non-significant, p<0.05 = *, p<0.01 = **, p<0.001 = ***, p<0.0001=****.



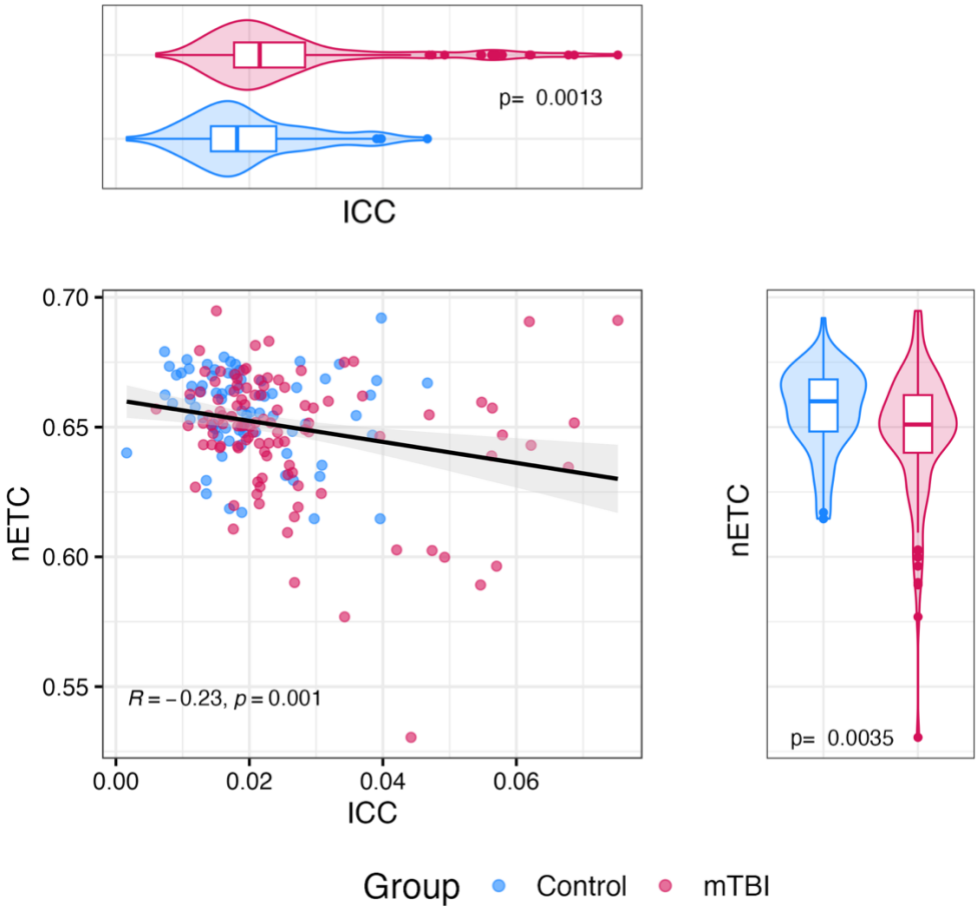
Appendix 3.8. Replication of global increases in connectivity and decreased complexity in mTBI versus controls.

Temporal complexity of brain activity was quantified as the normalised Effort-to-Compress (nETC)[424]. ETC measures complexity of a sequence (here, timeseries) in terms of compressibility: a sequence such as 01010101 is easy to summarise as "01 repeated N times" and therefore exhibits low complexity. Conversely, a more complex sequence is one that exhibits more unique patterns and is therefore harder to summarise compactly. A popular algorithm for lossless compression is the Lempel-Ziv algorithm (LZC), the basis for popular file compression applications[425], which has found applications as a complexity measure in neuroscience[426,427]. ETC is a recently developed alternative that has been shown to outperform LZC and other measures of temporal complexity such as sample entropy, for applications with short and noisy timeseries data (such as BOLD signals)[424]. For each individual, we computed nETC (ETC divided by the length of the timeseries) of each voxel's BOLD timeseries, for each voxel in their grey matter mask calculated during preprocessing. Thereafter, a single measure of nETC was obtained for each individual, as the average of all voxels' nETC values.

Global connectivity was computed as the threshold-free Intrinsic Connectivity Contrast (ICC)[428]: mean of the squared correlation between voxels' timeseries, across all pairs of voxels in the individual grey matter mask. This measure reflects the coupling (regardless of sign) between the activity of a voxel, and all other voxels. I then obtained a single measure of global ICC per person, by averaging across all grey matter voxels.

Each measure was harmonised for site/scanner differences as previously described and compared between controls and mTBI patients using a linear model with covariates of age and sex. These temporal connectivity and complexity values were additionally compared with one another across the entire cohort using a Pearson's correlation.

Following mTBI, individuals showed higher global connectivity (ICC; $F(1,180)=10.7$, $p=.001$) and lower temporal complexity (nETC; $F(1,180)=8.8$, $p=.003$) than healthy controls. Moreover, these connectivity and complexity values were significantly negatively correlated ($r(182) = -.23$, $p=.001$), suggesting a global environment of hyperconnectivity, yet less *temporal* complexity, as shown below in the figure.


p= 0.0013
ICC
0.70
0.65
0.60
0.55
nETC
R = −0.23, p = 0.001
0.00
0.02
0.04
0.06
ICC
nETC
p= 0.0035
Group
Control
mTBI

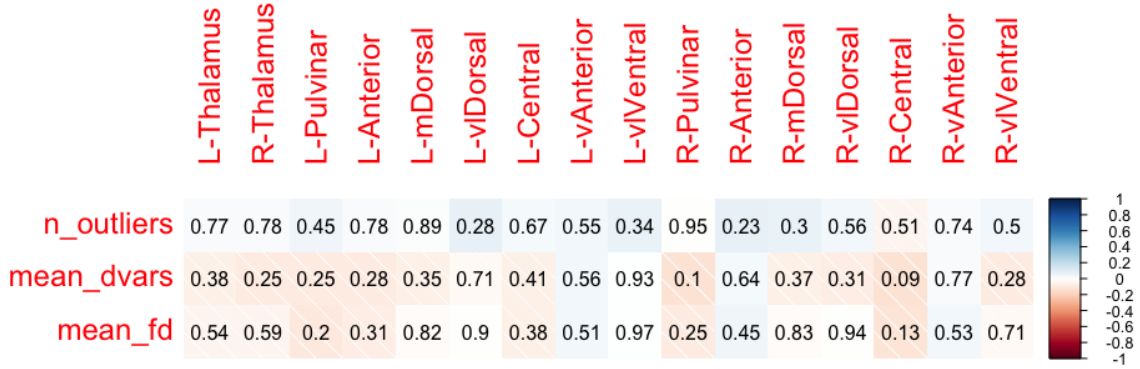
Appendix for Chapter 4

Appendix 4.1. Methods.

i) Denoising quality control – subject motion

I compared metrics of number of volumes removed, mean DVARS and mean framewise displacement (FD) between the patient and control cohorts. I additionally correlated these with thalamocortical functional connectivity, to ensure group differences in motion were not attributable to group differences in connectivity.

The mean number of volumes removed in the mTBI cohort (21.60, SD=17.97) was significantly greater than the mean removed volumes in the healthy control cohort (13.79, SD=13.87) ($F_{1,180}$=9.4, p=.004). Prior to removal of these volumes and denoising, mean DVARS was higher in the mTBI cohort (27.84, SD=7.69) versus the healthy control cohort (23.82, SD=7.65) ($F_{1,180}$=9.8, p=.004), as was mean framewise displacement higher in the mTBI cohort (21.60, SD=17.97) compared to the healthy control cohort (0.19, SD=0.10) ($F_{1,180}$=7.5, p=.007). Importantly however, I found no significant correlation between motion and connectivity estimates following denoising in any of the n=16 thalamic seeds, demonstrated in the correlation plot below (p-value as text, r as background colour).



ii) Harmonization validation

To provide some additional support for the use of Neurocombat harmonization for possible site differences in our multicentre study, we have compared site differences before and after harmonization in two imaging domains; T1w MRI thalamic volume, and rs-fMRI thalamocortical functional connectivity. Replicating methods used in the original NeuroCombat papers, I have used a Kruskal Wallis test on each variable before and after harmonization, to ascertain change in site differences.

Thalamus volume: both before and harmonization, 0/16 nuclei showed significant differences between sites.

Thalamocortical FC: before harmonization, 4/16 (25%) nuclei showed significant differences across the 11 sites. These 4 nuclei were: left pulvinar ($H(10)=38.4$, $p=.002$), left vl-ventral ($H(10)=29.2$, $p=.024$), right pulvinar ($H(10)=40.5$, $p=.002$), and right vl-ventral ($H(10)=29.8$, $p=.024$). None of these 4 nuclei were found to have significant differences between the mTBI and control groups in our manuscript and thus were not explored in further detail.

Following harmonization, 0/16 nuclei showed significant site differences, nevertheless demonstrating the success of these methods.

I am therefore confident in the ability of this method to address some of the harmonization issues arising within multicentre acquisition.

Appendix 4.2. Within-thalamus comparisons of outcome groups

		Average within-thalamus FC		
Comparison	**Test (df)**	**Left vAnterior**	**Right vAnterior**	**Right vlDorsal**
6-Month GOSE				
GOSE≤7 vs HC	F-test (*1,120*)	*F*=4.4, *p*=.17	*F*=1.7, *p*=.34	***F*=9.0, *p*=.03**
GOSE-8 vs HC	F-test (*1,130*)	*F*<0.01, *p*=.99	*F*<0.01, *p*=.99	*F*=0.9, *p*=.53
GOSE≤7 vs GOSE-8	F-test (*1,100*)	*F*=1.9, *p*=.34	*F*=0.3, *p*=.73	*F*=2.0, *p*=.34
6-Month PCS				
PCS+ vs HC	F-test (*1,103*)	*F*=2.8, *p*=.43	*F*=1.8, *p*=.46	*F*=6.7, *p*=.10
PCS- vs HC	F-test (*1,139*)	*F*=0.1, *p*=.84	*F*<0.01, *p*=.99	*F*=1.6, *p*=.46
PCS+ vs PCS-	F-test (*1,92*)	*F*=0.1, *p*=.84	*F*=0.2, *p*=.84	*F*=1.3, *p*=.46

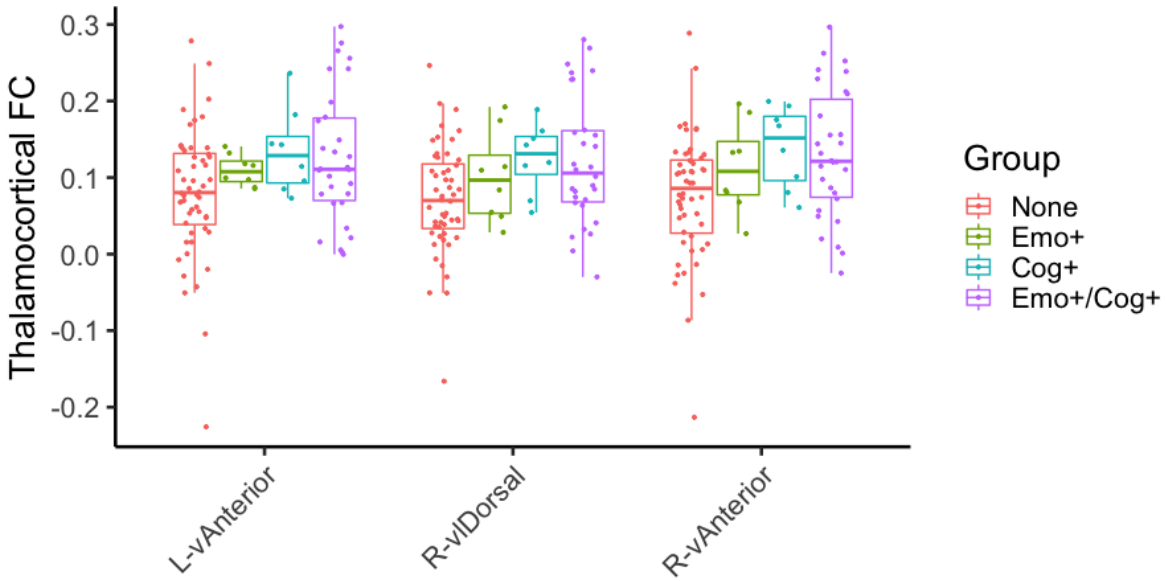
HC= Healthy Controls. All p-values shown are FDR-corrected. Bold indicates statistical significance at p<0.05, whereby GOSE≤7 group had higher FC than HC.

Appendix 4.3. Symptom-specific outcome groups' thalamocortical connectivity

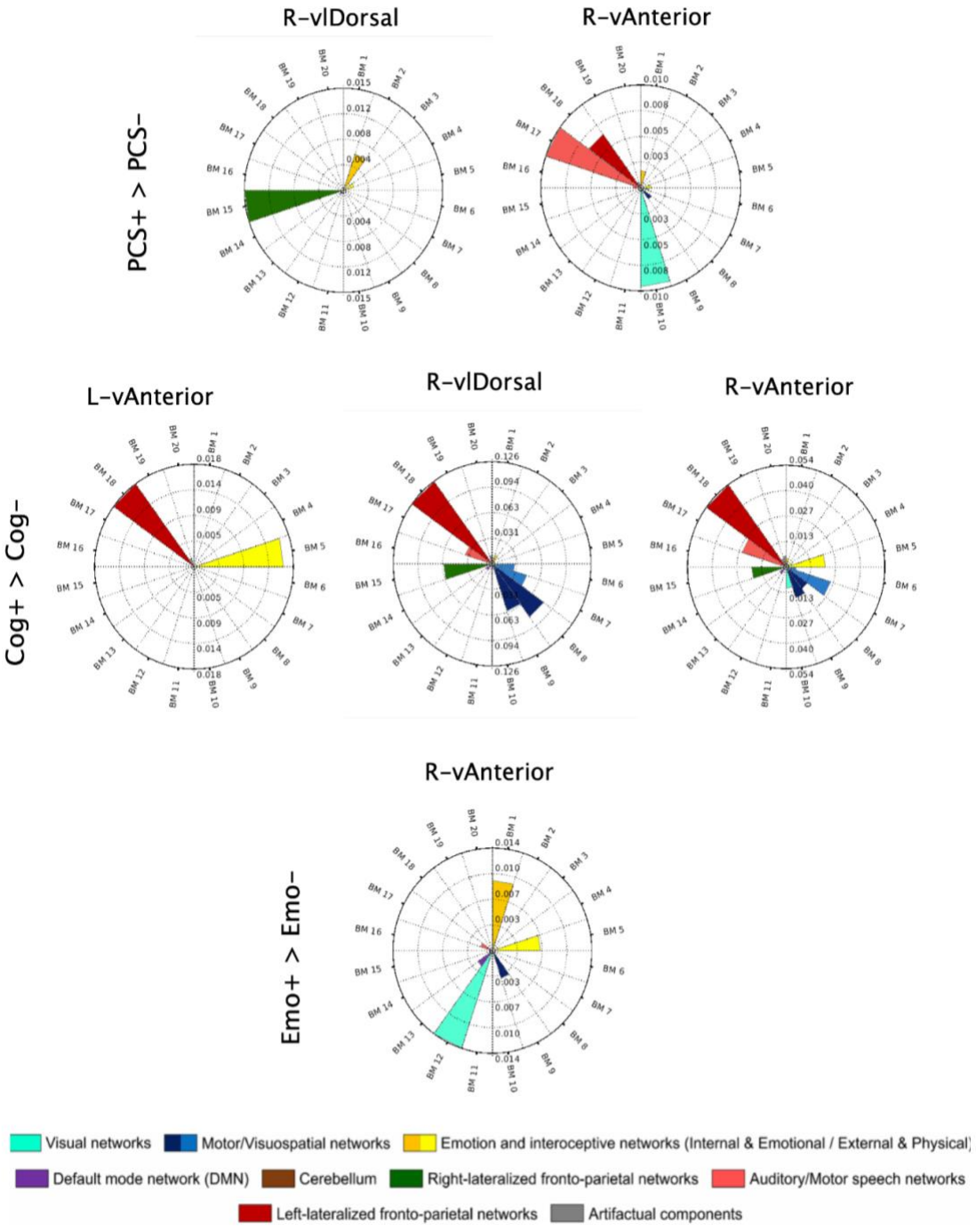
In symptom-specific outcome groups, patients were split into groups with or without cognitive (Cog+ n=38; Cog- n=60), emotional (Emo+ n=38; Emo- n=60), or somatic symptoms (Som+ n=23; Som- n=75). Although the cognitive and emotional groups have identical group numbers, they do not comprise identical patients. There is, nevertheless, a high overlap of these groups which have a significant association ($X^2 1=42$, p<.001).

To answer whether specific subgroups might be driving group effects of higher thalamocortical connectivity, I have plotted here the 4 possible groups; presenting no chronic symptoms (n=52), only cognitive (n=8), only emotional (n=8), and both cognitive and emotional symptoms (n=30).

Importantly, a between-subjects ANOVA between the three possible subject groups with symptoms shows no statistical differences in the L-vAnterior ($F_{1,2}$=0.55, p=.68), R-vlDorsal ($F_{1,2}$=0.38, p=.68), or R-vAnterior ($F_{1,2}$=0.56, p=.68). These tests additionally included covariates of sex, age, time since injury, and initial GCS, and are corrected for multiple comparisons. Thus, presenting both cognitive and emotional symptoms concurrently is not significantly different to either individually in this small cohort.



Appendix 4.4. Network involvement of voxelwise results. Wedge plots of spatial overlap between voxels surviving cluster correction in thalamic voxelwise connectivity tests between outcome groups and canonical intrinsic connectivity networks (ICN). These networks are defined by ICN_atlas toolbox as an extension to SPM using the ICN-BM atlas [299]. Colour bars indicate functional relevance. These are not statistically tested, merely a visual aid for relative functional involvement of SPM-t maps.



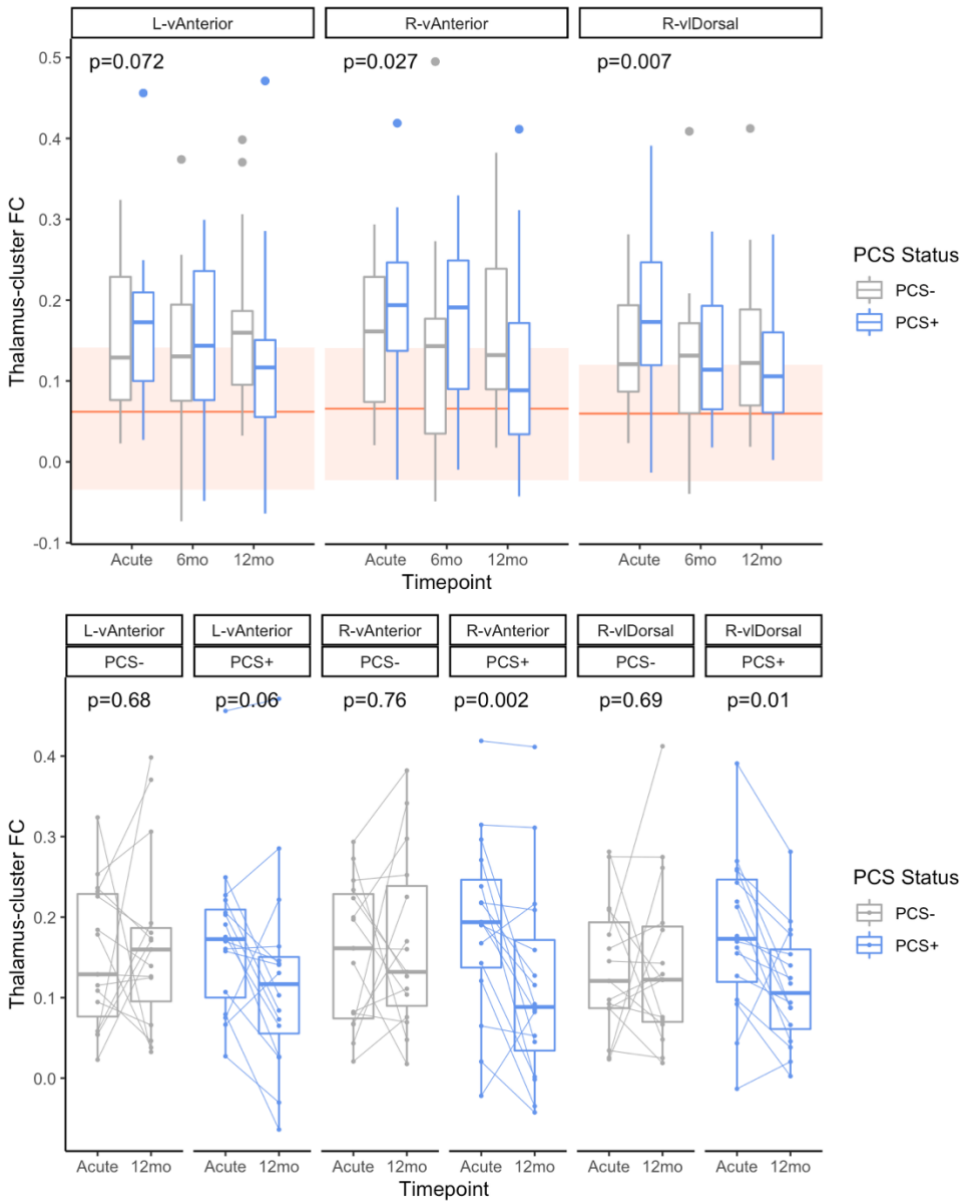
Appendix 4.5. Additional correlations to PET maps. All correlations between investigated seed-to-voxel t-maps and z-scored PET maps. Values shown are from the Schaefer 200 Parcellation after FDR correction, with significant associations in bold. Cells with no data indicate this correlation was not calculated as significance was not found at the mTBI-HC level. Asterisk indicates this correlation was significant in the Glasser360 parcellation (p=0.007).

	mTBI > HC			**Cog+ > Cog-**			**Emo+ > Emo-**
PET map	**L-vAnterior**	**R-vlDorsal**	**R-vAnterior**	**L-vAnterior**	**R-vlDorsal**	**R-vAnterior**	**R-vAnterior**
5HT-1A	r=-0.1, p=0.41	r=0.18, p=0.13	r=-0.28, p=0.24	-	-	-	-
5HT-1B	r=-0.09, p=0.4	r=0.17, p=0.14	r=0, p=0.57	-	-	-	-
5HT-2A	**r=-0.38, p=0.01**	r=-0.19, p=0.13	**r=-0.5, p=0.006**	r=-0.05, p=0.40	-	r=-0.16, p=0.31	**r=-0.14, p=0.06***
5HT-4	r=-0.31, p=0.11	r=-0.11, p=0.34	**r=-0.47, p=0.02**	-	-	r=-0.15, p=0.35	r=-0.12, p=0.07
5HT-6	r=0.01, p=0.49	**r=0.28, p=0.003**	r=-0.11, p=0.43	-	r=0.19, p=0.32	-	-
5HTT	r=0.05, p=0.45	r=0.12, p=0.25	r=-0.23, p=0.35	-	-	-	-
$\alpha 4\beta 2$	r=-0.16, p=0.34	r=0.19, p=0.12	r=-0.02, p=0.51	-	-	-	-
CB1	r=-0.31, p=0.12	r=0, p=0.56	r=-0.36, p=0.13	-	-	-	-
D1	r=0.12, p=0.39	**r=0.31, p<0.001**	r=-0.03, p=0.49	-	r=-0.10, p=0.46	-	-
D2	r=-0.13, p=0.39	r=0.17, p=0.18	r=-0.31, p=0.19	-	-	-	-
DAT	r=0.18, p=0.32	**r=0.4, p<0.001**	r=-0.01, p=0.51	-	r=0.01, p=0.47	-	-
GABA-A	r=-0.01, p=0.54	r=0.02, p=0.45	r=-0.08, p=0.43	-	-	-	-
H3	r=-0.14, p=0.37	r=0.17, p=0.14	r=-0.12, p=0.4	-	-	-	-
M1	r=-0.07, p=0.4	r=0.07, p=0.38	r=-0.11, p=0.34	-	-	-	-
mGluR5	r=-0.07, p=0.43	**r=0.30, p=0.01**	r=-0.16, p=0.35	-	r=0.12, p=0.40	-	-
MU	r=-0.33, p=0.12	r=0.09, p=0.37	r=-0.32, p=0.19	-	-	-	-
NAT	**r=0.39, p=0.006**	**r=0.49, p<0.001**	**r=0.41, p=0.02**	**r=0.31, p=0.003**	**r=0.49, p<0.001**	**r=0.43, p=0.006**	**r=0.24, p=0.006**
VAChT	r=0.23, p=0.18	**r=0.47, p<0.001**	r=0.09, p=0.41	-	**r=0.40, p<0.001**	-	-

Appendix 4.6. Longitudinal thalamic volume comparisons. FDR-corrected results from groups comparisons of thalamic nuclei volume, corrected for sex and age. HC = healthy controls. First results column details comparisons between controls and acute volumes of the longitudinal cohort (n=31). Second column details within-subjects ANOVA of volume change over time (acute, 6mo, 12mo) in this longitudinal cohort.

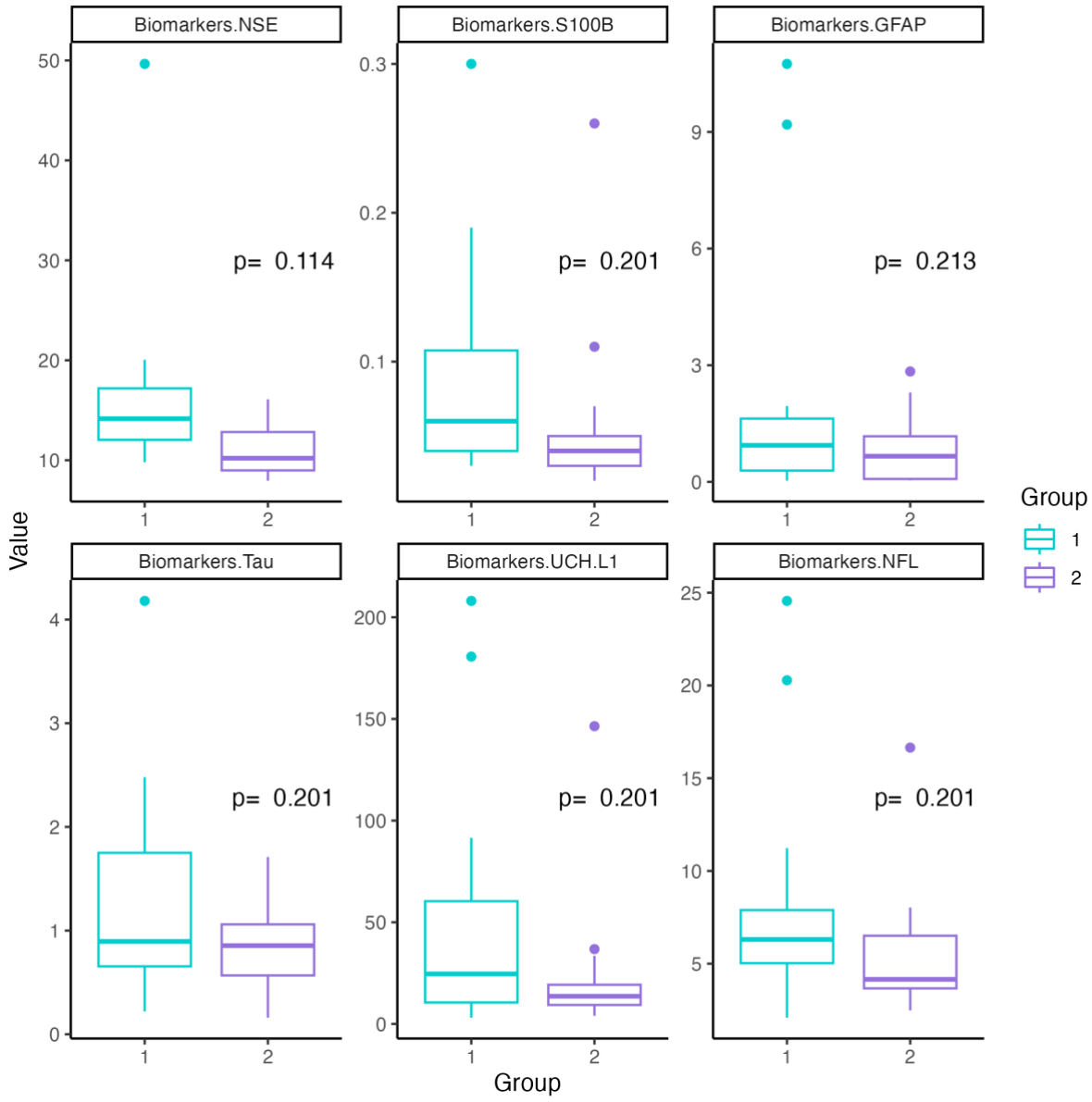
Thalamic ROI	**mTBI vs HC** **F-test (*1,104*)**	**mTBI over time** **F-test (*2,48*)**
Left Thalamus	*F*=1.3, *p*=.68	*F*=2.7, *p*=.52
Right Thalamus	*F*=1.5, *p*=.68	*F*=1.9, *p*=.52
Left-hemisphere nuclei		
Pulvinar	*F*=0.04, *p*=.87	*F*=1.9, *p*=.52
Anterior	*F*=2.2, *p*=.68	*F*=0.2, *p*=.86
mDorsal	*F*=0.5, *p*=.73	*F*=1.6, *p*=.54
vlDorsal	*F*=0.7, *p*=.71	*F*=0.3, *p*=.86
Central	*F*=0.6, *p*=.71	*F*=0.3, *p*=.86
vAnterior	*F*=5.9, *p*=.26	*F*=0.2, *p*=.86
vlVentral	*F*=0.1, *p*=.87	*F*=1.1, *p*=.67
Right-hemisphere nuclei		
Pulvinar	*F*=0.03, *p*=.87	*F*=0.2, *p*=.86
Anterior	*F*=0.9, *p*=.71	*F*=0.5, *p*=.86
mDorsal	*F*=0.7, *p*=.71	*F*=2.0, *p*=.52
vlDorsal	*F*=1.7, *p*=.68	*F*=1.5, *p*=.54
Central	*F*=0.03, *p*=.87	*F*=2.6, *p*=.52
vAnterior	*F*=3.7, *p*=.46	*F*=0.06, *p*=.94
vlVentral	*F*=0.09, *p*=.87	*F*=0.4, *p*=.86

Appendix 4.7. Longitudinal change of hyperconnected clusters. All data show distribution of mean beta value within hyperconnected clusters defined in the mTBI-control comparison (main text figure 2). Top: Mixed ANOVA between acute and 12mo timepoints between groups, where p-values given are interaction effects between timepoint (acute or 12mo) and group (PCS+ or PCS-). Shaded regions give the IQR of controls for each nucleus, with solid line indicating the controls' mean. Bottom: Post-hoc results within-subjects finding significant decreases in FC only in those with PCS. Lines join individual subjects' data.



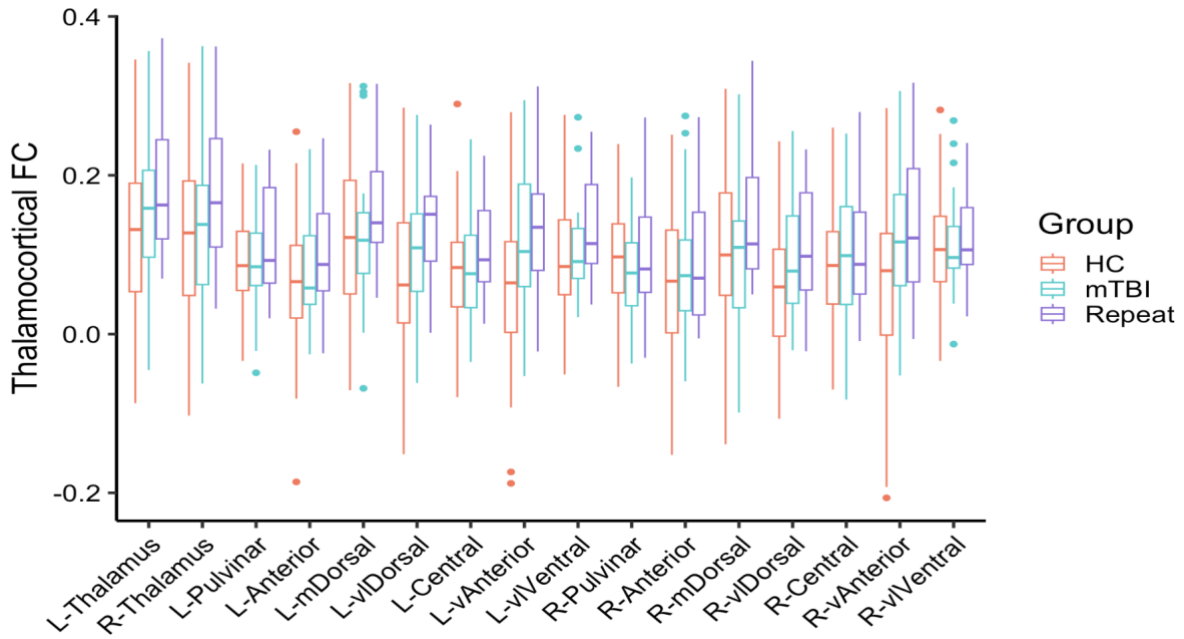
Appendix for Chapter 5

Appendix 5.1. Blood-based biomarkers compared between single mTBI and repeat mTBI groups. Values were obtained from CENTER-TBI (CORE v3.0) for six markers, and were all collected within 48hrs post-injury in this cohort. Specifically, neuron-specific enolase (NSE), S-100 calcium-binding protein B (S100B), glial fibrillary acidic protein (GFAP), Tau, ubiquitin C-terminal hydrolase -L1 (UCH-L1), and neurofilament light chain (NFL). Groups compared are single mTBI (group = 1), and repeat mTBI (group = 2), using a linear model including covariates of age and sex. Reported p-values are FDR-corrected for multiple comparisons.



Appendix 5.2. Thalamocortical functional connectivity between groups. Values were compared for increasing thalamocortical connectivity across the three groups (control, single mTBI, repeat mTBI) using a one-tailed Jonckheere-Terpstra test for non-parametric rank-based trends, with N=1000 permutations. All tests were adjusted for effects of age and sex, and FDR-corrected at p<0.05.

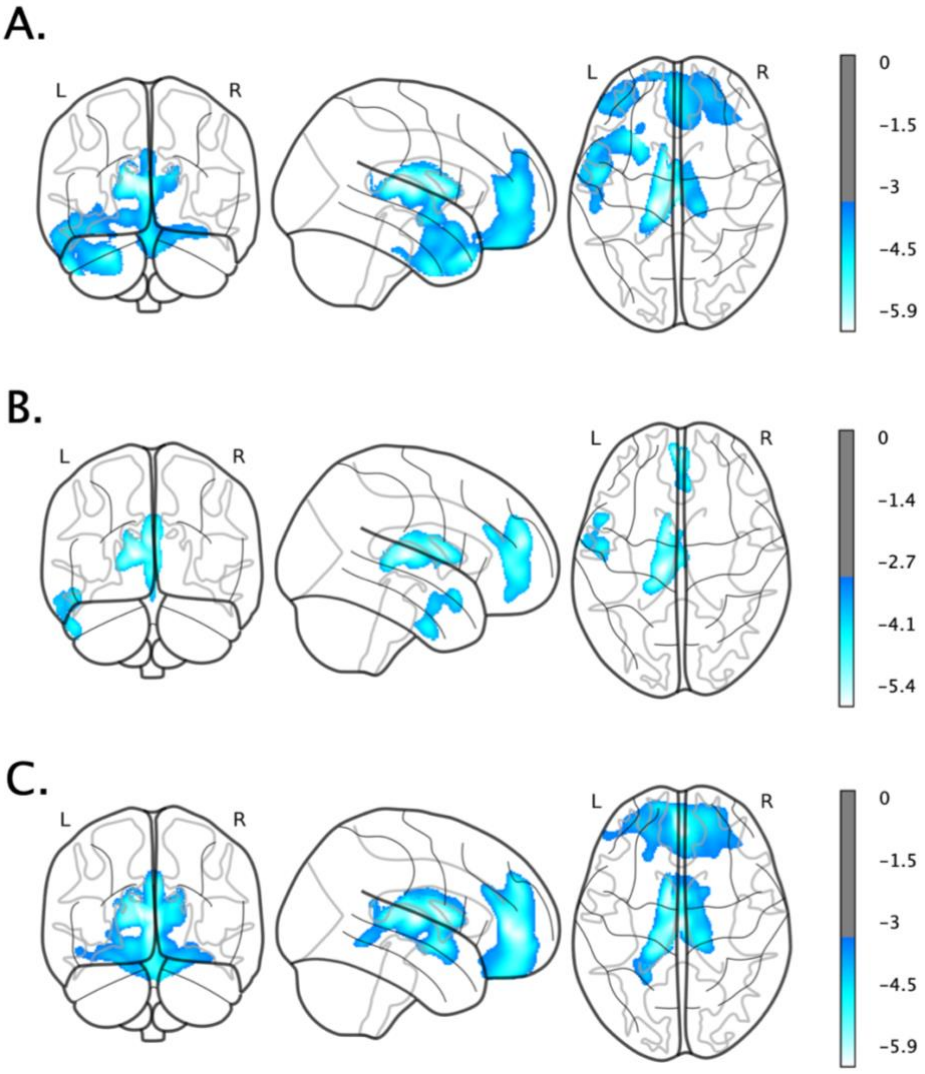
Thalamic ROI	Jonckheere-Terpstra
Left Thalamus	***T_{JT}* = 2244, *p* = .022**
Right Thalamus	T_{JT} = 2111, *p* = .12
Left Hemisphere Nuclei	
Pulvinar	T_{JT} = 1896, *p* = .36
Anterior	T_{JT} = 2120, *p* = .12
mDorsal	T_{JT} = 2030, *p* = .18
vlDorsal	***T_{JT}* = 2375, *p* = .008**
Central	T_{JT} = 2003, *p* = .23
vAnterior	***T_{JT}* = 2399, *p* = .008**
vlVentral	T_{JT} = 2135, *p* = .10
Right Hemisphere Nuclei	
Pulvinar	T_{JT} = 1703, *p* = .72
Anterior	T_{JT} = 2068, *p* = .13
mDorsal	T_{JT} = 2023, *p* = .18
vlDorsal	***T_{JT}* = 2291, *p* = .020**
Central	T_{JT} = 1910, *p* = .35
vAnterior	***T_{JT}* = 2358, *p* = .011**
vlVentral	T_{JT} = 1856, *p* = .42



Appendix for Chapter 6

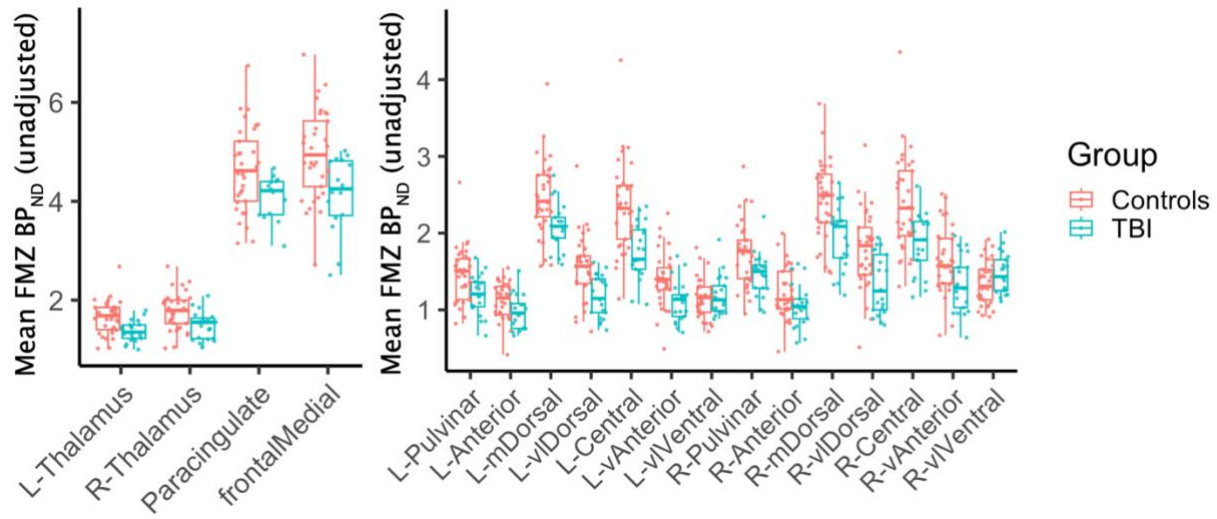
Appendix 6.1, Voxel-wise comparisons of FMZ BP_{ND} in patient sub-groups.

Voxel-wise comparisons of FMZ BP_{ND} were repeated between controls and TBI sub-groups to assess the stability of the results. A) Moderate/severe TBI (n=19), B) TBI excluding those with frontal contusions (n=13), C) TBI excluding those with thalamic lesions (n=21). Colour bar indicates t-values surviving voxel-level and cluster-level thresholding for significance. These demonstrate the relative stability of frontal and thalamic regions of reduced FMZ BP_{ND} in chronic TBI, when compared to healthy controls. Smaller clusters of significant change in B may be attributed to smaller sample size and thus power to find an effect.

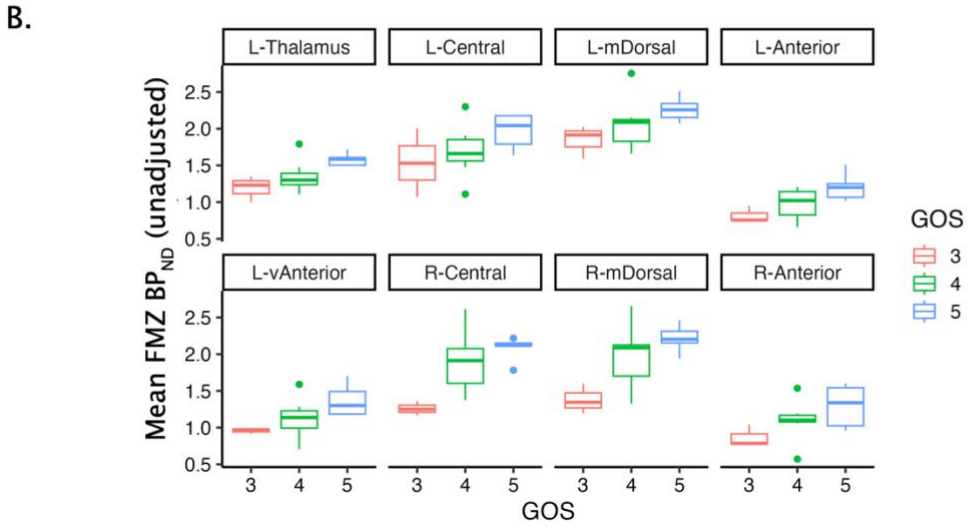


Appendix 6.2. Plots of unadjusted mean FMZ BP_{ND}. Values are prior to inclusion of age, sex, research site, and ROI volume in the linear model. A shows comparison of healthy controls and patients. B shows comparisons of outcome in patients based on GOS.

A.

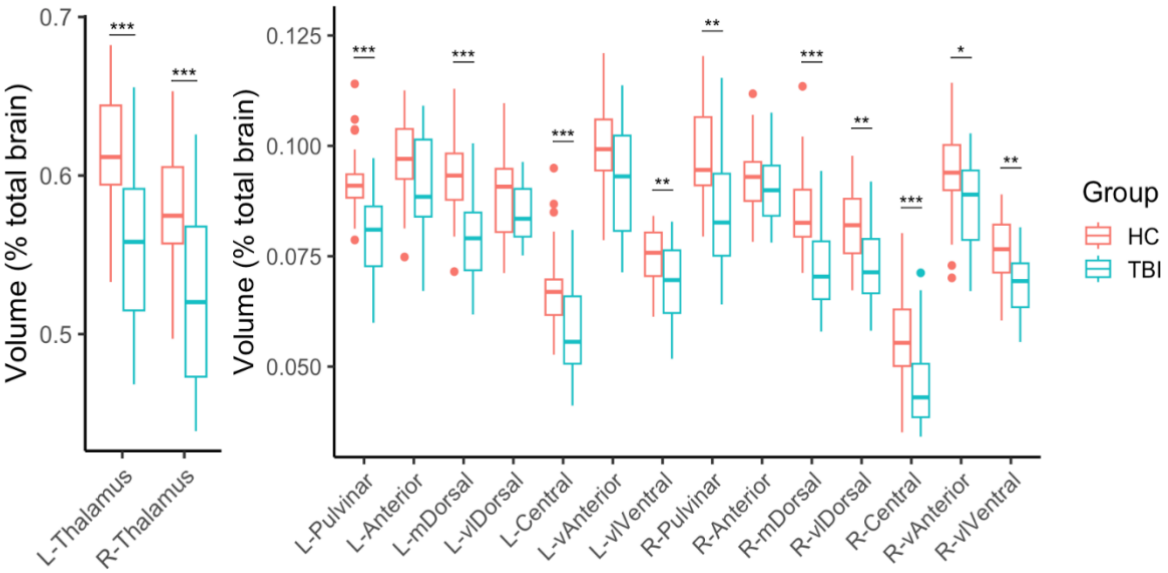


B.



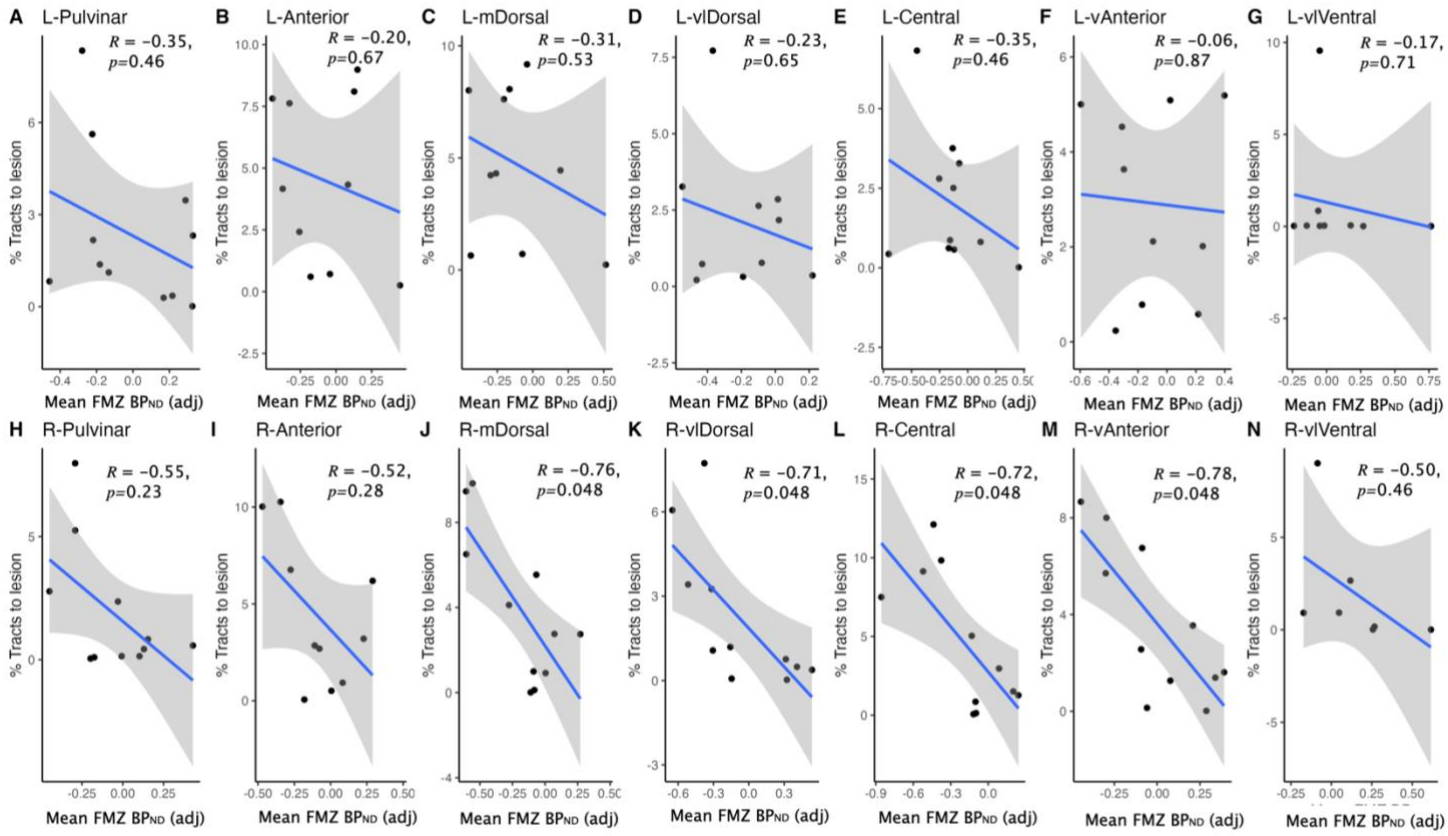
Appendix 6.3. Comparisons of thalamic volume.

A. Comparisons between control and TBI groups. Thalamic volumes are normalised by total brain volume, and comparisons include age, sex, and research site, in the linear model. Significant differences are shown at FDR-corrected p<0.05, where *= <0.05, **= <0.01, ***= <0.001.



Appendix 6.4. Correlation between thalamic nucleus FMZ BP_{ND} and nucleus-to-contusion structural connectivity probability for all thalamic nuclei.

Pearson's correlation between FMZ BP_{ND} and structural connectivity probability for all thalamic nuclei, where each point is an individual subject. All p-values are FDR-corrected. X-axes are adjusted for covariates.



Appendix 6.5. Methods- Relationship between thalamic nucleus FMZ BP_{ND} and nucleus-to-contusion structural connectivity using a local healthy control diffusion tensor imaging dataset.

Calculations of healthy control structural connectivity between thalamic nuclei and contusion masks were performed using a healthy average dataset (n=1065) in the main text. This methodology was further repeated with a locally collected dataset of n=18 healthy controls with diffusion tensor imaging (DTI).

Data acquisition & preprocessing

A Siemens Trio 3T MR system (Siemens Healthineers, Erlangen, Germany) was used to acquire the MRI data. For each subject, localiser images and 3D high resolution MPRAGE images (Relaxation Time (TR) 2300ms, Echo Time (TE) 2.98ms, Flip Angle 9°, field of view (FOV) 256mm^2×256mm^2) were acquired for use during pre-processing of diffusion MRI scans to assist spatial normalisation to Montreal Neurological Institute (MNI) space (https://www.mcgill.ca/neuro/). The diffusion MRI data (63 non-collinear directions, b=1000 s/mm^2 with one volume acquired without diffusion weighting (b=0), echo time 106ms, repetition time 1700ms, FOV 192mm^2×92mm^2, 2mm^3 isotropic voxels) were acquired to investigate white matter tissue integrity.

The diffusion-weighted imaging (DWI) scans were pre-processed using the MRtrix3 package (https://www.mrtrix.org/). Prior to the main sequence pre-processing, the data were denoised and residuals calculated. These indicate artifacts or distortions that may affect certain brain regions disproportionately. Data were unwarped and corrected for distortions, motion, and eddy currents. Following this, field inhomogeneities were corrected with the Advanced Normalisation Tools (ANTs) package (https://stnava.github.io/ANTs/). Brain masks were used to restrict analyses to only brain voxels.

DTI data were reconstructed from pre-processed DWIs using the MRtrix3 package. First, a basis function was constructed for each tissue type: grey matter (GM), white matter (WM), and cerebrospinal fluid (CSF). These basis functions were used to deconvolve and concatenate the fibre orientation distributions (FODs) for each tissue type. Finally, these were normalised for effects of intensity inhomogeneities.

Analysis

Each control subject was analysed in the same manner as in-text; a deterministic fibre tracking algorithm[384] was used with augmented tracking strategies[385] to improve reproducibility. This used 1,000,000 seeds in ROI-to-ROI tractography, whereby tracts with length shorter than 30 or longer than 200 mm were discarded. The anisotropy threshold was randomly selected, angular threshold randomly selected from 15 degrees to 90 degrees, and step size randomly selected from 0.5 voxel to 1.5 voxels. Using this method, we obtained the total number of nucleus-to-contusion tracts for each TBI subject with contusion mask available who also did not demonstrate evidence of a thalamic lesion (n=15), for each control (n=18). A mean value was then taken across the n=18 controls, and normalised by the respective total number of nucleus-to-whole brain cortex tracts, to produce a probability of nucleus-to-contusion structural connectivity. Subjects were excluded from analysis if no tracts were successfully produced between the contusion and thalamic nucleus. Probabilities calculated with the controls' data from local (n=18) and openly-available n=1065 datasets were compared with a Pearson's correlation, and were found to have high correspondence (R=0.91, p<0.001).

Mean structural connectivity probabilities from the local control dataset were then correlated, as before, to the corresponding covariate-corrected thalamic nucleus FMZ BP_{ND}. Results are presented below, prior to FDR-correction due to the smaller sample size of the local healthy control cohort (n=18) compared to the original healthy average dataset (n=1065). These results replicate those presented in-text, finding that all nuclei show a negative relationship whereby the same n=4 right-hemisphere nuclei show a significant mirroring effect between chronic thalamic neuronal loss and cortical damage, and additionally the right anterior nucleus. Thus, calculations from the two datasets show a high degree of correspondence and replication of results.

Correlation between thalamic nucleus FMZ BP_{ND} and nucleus-to-contusion structural connectivity probability determined using a locally acquired DTI dataset for all thalamic nuclei.

Pearson's correlation between FMZ BP_{ND} and structural connectivity probability for all thalamic nuclei, where each point is an individual subject. All p-values are FDR-corrected. X-axes are adjusted for covariates.