

EXPLORATION OF POST-BLAST
NEUROPATHOLOGY AND THE USE OF A
PROMISING FLUID BIOMARKER IN A RAT MODEL
OF BLAST TRAUMATIC BRAIN INJURY

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Statement of originality

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Abstract

Blast traumatic brain injury (bTBI) poses significant challenges for military and civilian populations, leading to a wide range of short and long-term morbidities. Detecting brain damage in mild bTBI is challenging and understanding the causes of the morbidities remains unclear, requiring further investigation into bTBI pathology and the development of more sensitive diagnostic tools.

This thesis investigates the underlying neuropathological alterations behind electrophysiological abnormalities observed in bTBI and the sensitivity of a promising fluid biomarker for TBI that detects diffuse axonal injury (DAI). To achieve this, I optimised a rodent model of mild primary bTBI to conduct electrophysiological, histological, and fluid biomarker analyses at different times post-injury. Electrophysiological analysis revealed a hallmark power increases in the lower frequency bands at 1 and 3 months after injury. To understand the association between electrophysiological changes and bTBI, inhibitory GABAergic interneuronal densities were examined. Results revealed a decrease in interneuronal densities at 7 days, 1 month, and 3 months post-injury, indicating a connection between excitation, inhibition, and the observed electrophysiological alterations. These alterations were paralleled with increases in microglial density at early points post-injury and elevations in microglia size at 3 months post-injury. Additionally, DAI in the white matter, determined by increased immunofluorescent intensity staining of neurofilament light (NFL) in the corpus callosum at 7 days and 1 month post-injury, may have also contributed to these electrophysiological changes.

Interestingly, measurements of the fluid biomarker NFL in cerebral spinal fluid (CSF) revealed a peak at 6 hours post-injury, remaining elevated for up to 3 months, while Plasma NFL peaked at 3 days and returned to baseline levels at 7 days post-injury. Similar NFL analysis was conducted in a focal injury model, which also exhibited pronounced elevated concentrations of NFL in the CSF and plasma particularly at early times post-injury, along with white matter thinning and histological NFL loss. These findings imply a potential role of NFL as a biomarker for brain damage in these models.

Overall, this thesis demonstrates the impact of a single mild bTBI on the brain, showcasing chronic electrophysiological alterations, reduced interneuronal density, and indications of DAI and white matter changes. The study also highlights the sensitivity of NFL as a biomarker for TBI, capable of detecting damage from both blast and focal injuries. These findings significantly contribute to our understanding of bTBI pathology and present promising avenues for future research and the search for sensitive diagnostic tools in this field.

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Acronyms

5HT3aR	Ionotropic Serotonin Receptor 5HT3a
AD	Alzheimer's Disease
ANTS	Advanced Normalisation Tools
APP	Amyloid Precursor Protein
ATP	Adenosine Triphosphate
Au1	Primary Auditory Cortex
A β	Amyloid Beta Peptide
BBB	Blood Brain Barrier
BIC	Biological Imaging Centre
BKY	Benjamini, Krieger and Yekutieli
bTBI	Blast Traumatic Brain Injury
CBIS	Centra Of Blast Injury Studies
CC	Corpus Callosum
CCI	Controlled Cortical Impact
CD	Cluster Of Differentiation
CNS	Central Nervous System
CSF	Cerebral Spinal Fluid
DAI	Diffuse Axonal Injury
DPI	Days Post-Injury
DWPLI	Debiased Weighted Phase Lag Index
EEG	Electroencephalography
ELISA	Enzyme-Linked Immunosorbent Assay
FA	Fractional Anisotropy
FC	Functional Connectivity
FDA	Food and Drug Administration
FDR	False Discovery Rate
FSL	Fmrib Software Library
GABA	γ -Aminobutyric Acid
GFAP	Glial Fibrillary Acidic Protein

HFO	High Frequency Oscillations
I.P	Intraperitoneally
IBA1	Ionized Calcium-Binding Adapter Molecule 1
IED	Improvised Explosive Device
IF	Immunofluorescent
IL	Interleukin
IN	Interneuron
kPa	Kilopascal
Kv1	Potassium Voltage Gated Channel 1
LFB	Luxol Fast Blue
MBP	Myelin Basic Protein
MD	Mean Diffusivity
MEG	Magnetoencephalography
MOG	Myelin Oligodendrocyte Glycoprotein
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
mTBI	Mild Traumatic Brain Injury
NDI	Neurite Density Index
NF	Neurofilament
NFH	Neurofilament Heavy
NFL	Neurofilament Light
NFM	Neurofilament Medium
NODDI	Neurite Orientation Dispersion and Density Imaging
NSE	Neuron Specific Enolase
ODI	Orientation Dispersion Index
P.O	Oral Administration
PAG	Periaqueductal Gray
PBS	Phosphate Buffered Saline
PC	Principle Cell
PCS	Post Concussion Syndrome
PD	Parkinson's Disease

PFA	Paraformaldehyde
PLI	Phase Lag Index
PNS	Peripheral Nervous System
PSP	Progressive Supranuclear Palsy
PTSD	Post-Traumatic Stress Disorder
PV	Parvalbumin
RF	Radiofrequency
ROI	Region Of Interest
ROS	Reactive Oxygen Species
RSC	Retrosplenial Cortex
S.C	Subcutaneously
S100B	S100 Calcium-Binding Protein B
SCI	Spinal Cord Injury
SIMAO	Single Molecule Array
SRC	Sports Related Concussion
SST	Somatostatin
TBI	Traumatic Brain Injury
TNF- α	Tumour Necrosis Factor Alpha
UCH-L1	Ubiquitin C-Terminal Hydrolase
V1	Primary Visual Cortex
VIP	Vasoactive Intestinal Polypeptide

1

Introduction

1.1 Traumatic brain injury (TBI)

Approximately 50-60 million people of the world's population will experience a traumatic brain injury (TBI) in their lifetime, and many will experience multiple TBI injuries (Maas, Menon et al. 2017). TBI is the biggest cause of death and disability in those under 40 (Silver, Kramer et al. 2001, Hammell and Henning 2009, Menon, Schwab et al. 2010, Maas, Menon et al. 2017, Young and Hughes 2020) and has strong correlation with neurodegenerative diseases (Tehse and Taghibiglou 2018). It can occur in the general public at any age, as well as among military service personnel (McKee and Robinson 2014, McKee and Daneshvar 2015). TBI is defined as "an alteration in brain function, or other evidence of brain pathology, caused by an external force" (Menon, Schwab et al. 2010). Such an external force can come from sports, falling, traffic accidents, a jolt or a bump. There is also an increasing number of TBIs in the military due to explosives (Hoge, McGurk et al. 2008).

There are different severities of TBI that range from mild to severe. This thesis will focus on mild TBI as it is the most relevant to the model and work presented. In humans one of the ways of categorising these severities is by using the Glasgow Coma Score (GCS) (Table 1.1), which utilises the symptoms to diagnose the severity that should indicate the prognosis (Andriessen, Jacobs et al. 2010). There is an issue with this index however, in that it is broad and risks inaccuracy as it doesn't necessarily consider previous head injuries that could cause the symptoms to show later and chronically. TBI is a very heterogeneous progressive injury and therefore what is defined within the GCS is not necessarily accurate of someone's mild condition.

DOMAIN	RESPONSE	SCORE
EYE OPENING	Spontaneous	4
	To speech	3
	To pain	2
	None	1
BEST VERBAL RESPONSE	Oriented	5
	Confused	4

BEST MOTOR RESPONSE	Inappropriate	3
	Incomprehensible	2
	None	1
	Obeying	6
	Localizing	5
	Withdrawal	4
	Flexing	3
	Extending	2
	None	1
TOTAL SCORE	Deep coma or death	3
	Full alert and orientated	15

Table 1.1 Glasgow Coma Score (GCS) table

This GCS table states the score someone should get if they suffer a TBI and are tested for their eye, verbal and motor skills. Mild is 13-15, anything lower than that is considered not mild (NICE 2014)

1.1.1.1 Mild TBI

In general, TBI can cause tearing and shearing of axons and blood vessels in the brain that results in short- and long-term damage (McKee and Daneshvar 2015). However, for milder cases of TBI, there may not be as profound initial symptoms as in severe or moderate cases, and mild TBI (mTBI) can therefore go undetected (Madhok, Rodriguez et al. 2022). mTBI, the focus of this thesis, is defined when a patient has less than 24hr of amnesia, less than 30 mins loss of consciousness post-injury and a GCS of 13-15. It is the most prominent type of TBI; approximately 77% of TBI cases seen within the US military have a mTBI (Marshall, Holland et al. 2012). It can also be defined as a TBI that has no detectable differences upon early assessment nor changes seen in standard neuroimaging tests (Elder, Mitsis et al. 2010, Magnuson, Leonessa et al. 2012, Elder, Stone et al. 2014, Sundaramurthy and Chandra 2014). Therefore, mTBI can go undetected due to having non-visible symptoms for days or weeks after injury, potentially resulting in a lack of clinical care post-injury (Ghaith, Nawar et al.

2022). Consequently, it can lead to a cascade of delayed neurodegenerative events and a myriad of issues. These may include diffuse axonal injury, blood brain barrier (BBB) disruption, neuroinflammation, excitotoxicity and altered brain connectivity among many other symptoms (Mckee and Daneshvar 2015). The plethora of issues listed can then lead to chronic problems that result in significant cognitive and sensory alterations and neurodegeneration. It should be noted that not everyone has the same symptoms, which can add to the difficulty of the diagnosis. Furthermore, as our understanding of TBI expands, the threshold for what can be considered a TBI is changing (Menon, Schwab et al. 2010). Innocuous sporting acts, such as heading a football, have been found to be not so innocuous. There is evidence that the repeated heading of a football, something that rarely causes concussive or mTBI symptomology, can cause TBI (Mackay, Russell et al. 2019). It was found that in a professional football career, the professionals were 3.5 times more likely to die with dementia than the general public (Mackay, Russell et al. 2019). This emphasises the importance of discovering and understanding the potential biomarkers of mTBI to facilitate recovery and/or take preventive measures against further injury. This will help avoid cumulative injuries that can eventually result in neurodegenerative diseases.

1.2 Classifications of pathological injury

In TBI, there are two types of pathological events that lead to brain damage. The initial irreversible insult is called primary injury, which is related to the biomechanical forces from either an external impact or resulting acceleration/deceleration and it occurs at the time of injury (Ng and Lee 2019). It can cause instant cell death (Loane and Kumar 2016), lesions to the brain and result in axonal shearing, haematoma, intracranial pressure and haemorrhage (Ng and Lee 2019). Secondary injury occurs after the injury, and it is related to the biochemical, cellular, inflammatory and physiological response following the impact. This can occur minutes to years after the injury. It can include some of the symptoms listed above, but also cause raised intracranial pressure, subdural haematomas, oedema, bleeding, endocrine damage, cellular dysfunction, and necrosis (Mckee and Daneshvar 2015, Ng and Lee 2019). Understanding both primary and secondary pathological injury can aid diagnosis, prognosis, and treatment and inform preventative measures.

Another type of classification of brain injury arranges the types of TBI into focal or diffuse (Mckee and Daneshvar 2015). Focal injury occurs in a specific location of the brain, while diffuse injury occurs over a more widespread area. It is common for both focal and diffuse damage to occur as a result of the same event; many traumatic brain injuries have aspects of both focal and diffuse injury (Ng and Lee 2019).

1.2.1 Focal TBI

Focal TBI involves initial damage localised to the area of impact. The onset of effects can spread more widely but that is dependent on the injury severity. It is due to a direct impact to the skull from a blunt or penetrative event causing compression and, in some cases, breaking of the skull. Pathology of this type of injury includes immediate cell death and Wallerian degeneration, macro/micro bleeds, contusions, and haematomas (Andriessen, Jacobs et al. 2010). These pathological outcomes are not unique to focal injury and are also seen in diffuse TBI. Focal TBI is easier to investigate using neuroimaging techniques like computational topography (CT) and magnetic resonance imaging (MRI), (concepts that are introduced later) as they are more easily detected using these tools.

1.2.2 Diffuse TBI

Diffuse TBI is a non-focal impact on the brain, it is an injury that does not affect just one area, with potential for affecting the whole brain. It can occur from a blunt force, mechanical movement such as acceleration/deceleration, or result from internal dysfunction e.g hypoxia. The pathology that is aligned with diffuse injury is the damage to axons, diffuse axonal injury (DAI), blood brain barrier (BBB) damage (especially since cerebral vasculature is susceptible to blast), oedema, vascular injury and in some cases hypoxia/ischemia (Mckee and Daneshvar 2015). A good example of diffuse injury would be those experienced in a road traffic collision (Andriessen, Jacobs et al. 2010) or blast TBI (Hicks, Fertig et al. 2010).

The brain is not made up of uniform tissue, it is made up of white and grey matter and areas of clustered nuclei with different densities. This renders the brain vulnerable to damage between the interfaces of these different densities (Andriessen, Jacobs et al. 2010). The acceleration and deceleration experienced by diffuse TBI results in different tissues moving at different rates from each other and therefore leading to axonal tearing, shearing and compression. This happens as the maximum elasticity that the axon can bear is exceeded (Andriessen, Jacobs et al. 2010, Johnson, Stewart et al. 2013, Mckee and Daneshvar 2015). Axonal damage can also depend on the direction of the movement and if the acceleration/deceleration is linear, rotational or both. The brain can have different thresholds for each type of acceleration, and this can alter severity and type of an injury. The differences in brain tolerances for directionality of acceleration can influence the making of protective head gear (Kleiven 2013, Siegkas, Sharp et al. 2019).

In humans, diffuse and focal injury can be caused by car accidents, sports injuries, falls, blast exposure (either with blast wave, displacement and/or flying debris) and domestic violence (Ahmed, Venigalla et al. 2017). These causes are not exhaustive but are among the most common. Diffuse TBI is less easy to identify via common neuroimaging techniques and it is possible to go undetected as the imaging isn't sensitive enough to detect the injury (Huang, Theilmann et al. 2009). Instead, the diffuse injury can lead to progressive cellular changes, oxidative stress, Wallerian degeneration and chronic inflammation which if left undiagnosed can lead to long-term damage (Burda, Bernstein et al. 2016). It should be noted that not all TBIs can be dichotomised into these two groups, there is usually a mixture of the two (Mckee and Daneshvar 2015).

1.3 Animal models of TBI

Animal models are a vital part of understanding traumatic brain injury. They allow us to investigate under controlled conditions the minute aspects of pathophysiology, mechanistic investigations and reproducibility. They also allow us to investigate questions that would otherwise be unethical to be performed on humans (Wojnarowicz, Fisher et al. 2017). Given that mice and rats are cheaper, they are more likely to be chosen for studies that can be

translated to humans rather than their genetically and physiologically closer alternative, primates. However, mice and rats present differences in brain anatomy and have lissencephalic (smooth) brains, while primates have gyrencephalic (in which the cerebral cortex has convolutions) brains (Hicks, Fertig et al. 2010). Primates are ideal candidates for studies, however they are more expensive, harder to deal with and house, and there are ethical considerations limiting their use (Bryden, Tilghman et al. 2019). Cats (Ball, Gloor et al. 1977, Carey, Sarna et al. 1990), ferrets (Lighthall 1988, Schwerin, Hutchinson et al. 2017, Goodfellow, Medina et al. 2022), rabbits (Härtl, Medary et al. 1997), pigs (Bauman, Ling et al. 2009, Friess, Ichord et al. 2009, De Lanerolle, Bandak et al. 2011), dogs and sheep (Millen, Glauser et al. 1985) have been used in the past for TBI models, however there are ethical and logistical complications to using them.

There are numerous TBI models in rodents, which aim to replicate different aspects of a TBI. They span from the historical and well established controlled cortical impact model (CCI) that generates a focal injury directly on the dura rather than the skull (like some uses of the weight drop) (Morales, Marklund et al. 2005), to those that can give more diffuse injury; fluid percussion injury (FPI) and blast injury models (Clemmedson, Frankenberg et al. 1969, Bauman, Ling et al. 2009, Risling, Plantman et al. 2011, Risling and Davidsson 2012, Risling 2019) (Figure 1.1). There is also the rotational/acceleration model that is less used. It should be noted that the suitability of the inertial acceleration model is limited to large animals such as swine. Brain mass affects the required acceleration to simulate clinically relevant neuropathology, with lower brain mass leading to higher required acceleration. As a result, the device is incapable of producing such acceleration levels in smaller animals such as rodents (Hicks, Fertig et al. 2010). Of the numerous models used to investigate TBI I will introduce blast, as it is the model most relevant to this thesis.

1.3.1 Animal models of blast injury

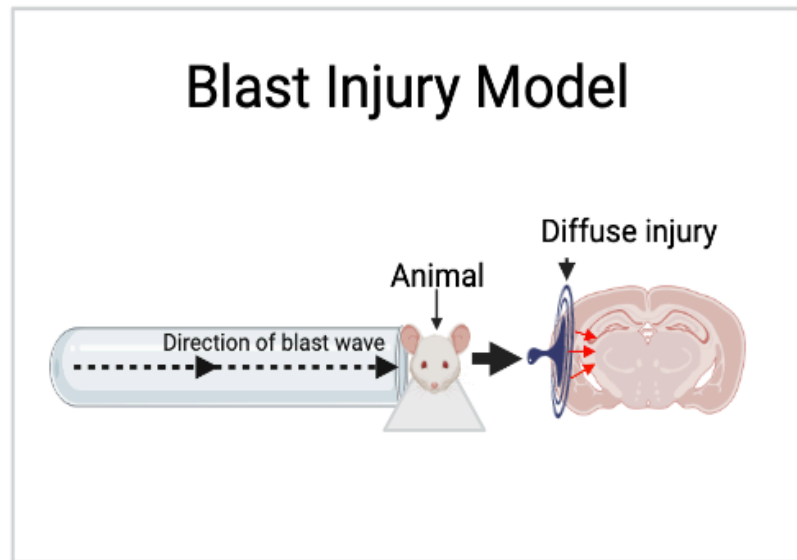


Figure 1.1 Example of blast traumatic brain model of TBI.

Blast injury model whereby the pressure wave is generated either from explosions or pressured gas in a blast shock tube and the animal can be inside or outside the shock tube. It is used to model a diffuse model of TBI. Figure was adapted from ((Shah, Gurdziel et al. 2019) Figure made in Biorender.com

Blast injury models have become more popular within the literature due to the increased use of explosive devices in warfare (Elder, Mitsis et al. 2010, Su and Bell 2016). Blast injury models can vary depending on which element of blast they are trying to emulate; vehicle/enclosed spaces, debris, bone injury from being inside a vehicle during an explosion, or open field (Axelsson, Hjelmqvist et al. 2000, Risling and Davidsson 2012, Ladlow, Bennett et al. 2019, Nguyen, Pearce et al. 2019). Each type comes with its own set of limitations; for example, an open field design commonly uses a blast shock tube which is a simulation of a real-world scenario. It utilises mylar sheets that are compressed in a compression chamber within the shock tube with gas, until they break at a certain pressure (Figure 1.1). The pressure wave propagates down the expansion chamber (rest of the shock tube) and out the tube exit. The animal can be placed inside or outside the shock tube can be full body, partial body exposure or head only.

The model does not replicate the heat and toxicity of explosive devices; however, that depends on what elements are required to answer the scientific question (Su and Bell 2016,

Ma, Aravind et al. 2019). On the other hand, it can allow for disentanglement of the polytrauma seen in war zones. Furthermore, it allows for the suspension of biological samples and pre-set determined parameters of duration and intensity (Nguyen, Wilgeroth et al. 2014). Advantages of blast models are the variability of choice to emulate scenarios, and the ability to alter severity levels by changing blast parameters (e.g. pressure). Limitations of the model type are the inconsistencies of model parameters in the literature and the potential variability between experiments depending on numerous parameters. More on the controversies and variabilities of the blast model is explored in Chapter 3.

1.4 Blast Traumatic brain injury

Blast traumatic brain injury (bTBI) is caused by a blast wave, which is generated by an explosion. There are various explosive types; mortar shells, rocket propelled grenades, land mines and improvised explosive devices (IEDs). Blast TBI has increased in occurrence because of the improvement of body armour against injury (Hicks, Fertig et al. 2010). The thorax is now better protected, so what once was a lethal blast exposure, now leaves personnel with a TBI and in other cases with other polytrauma (Warden 2006, Hicks, Fertig et al. 2010, Wood, Panzer et al. 2013). As previously stated, there is an increased incidence of blast TBI and, in particular, an increase in IED usage in warfare. It was published that IEDs are the most common cause of blast injury in the Iraq and Afghanistan wars, and estimated to be responsible for 40% of the deaths of the coalition and a similar percentage of TBIs (Elder, Mitsis et al. 2010, Gollob and O'Hanlon 2020, Kumar and Nedungadi 2020).

Blast TBI has been called a signature injury from the Iraq and Afghanistan wars (Hicks, Fertig et al. 2010). It has also been seen that 10-20% of those returning from Iraq and Afghanistan had a TBI (Elder, Mitsis et al. 2010, Mckee and Robinson 2014). However, these percentages differ as it should be noted that mTBI and TBI in a combat scenario can go unreported. This was demonstrated in a survey by Hoge and colleagues, who found that despite soldiers not stating they had a TBI, they had been exposed to a blast exposure at least twice, thus indicating the number could be substantially higher (Hoge, McGurk et al. 2008). Therefore, research into understanding its mechanisms and pathology has increased (Sundaramurthy

and Chandra 2014). However, there is still much controversy over blast mechanisms and heterogeneity in the literature and thus enlightenment needed within the field.

There are five well-established categories of blast mechanisms, primary, secondary, tertiary, quaternary and quinary (Hicks, Fertig et al. 2010, Bryden, Tilghman et al. 2019) (Figure 1.2).

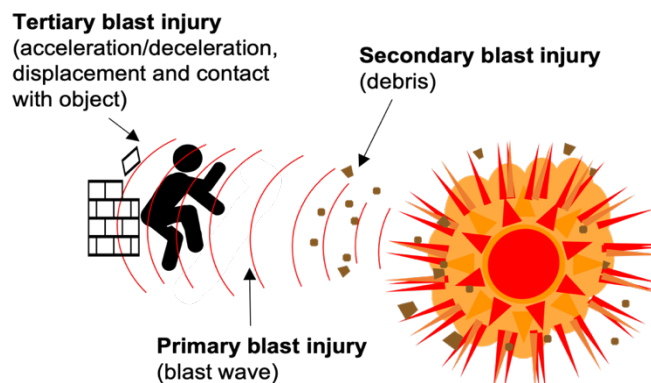


Figure 1.2 Categories of blast injury mechanisms

- **Primary:** Entails only the shockwave and thought to cause damage as it transverses through the brain and body. This mechanism of injury is debated within the literature as to whether it causes injury to the brain. It is thought to cause brain injury as the different brain tissues moves at different rates. Primary blast can cause injury and death by affecting large gaseous organs in the body by causing the gas to expand. Blood vessels and tissue damage can also occur.
- **Secondary:** Usually, a blunt or penetrative injury. It involves brain and body injury from projectiles from shrapnel or fragments contained in/from the bomb.
- **Tertiary:** Acceleration/deceleration can cause damage with coup contrecoup within the brain with compression and stretching/shearing. Acceleration can be linear and rotational. Injury can also be caused by the displacement of the body, and impact against a wall/ground.
- **Quaternary:** Heat and toxic chemicals that cause injury from burns and inhalation

- Quinary: Gases, radiation and biological material which can cause asphyxiation, burns, radiation sickness and poisoning.

In this thesis I focus mainly on primary and tertiary as they can cause mild TBI rather than a focal penetrative injury from secondary blast mechanisms.

1.5 TBI and blast TBI pathophysiology: Neuroinflammation and common biomarkers of injury

The ultimate goal of any injury research is to develop greater understanding that can elucidate the timings for therapeutic intervention, both for treatment and prevention. After TBI, there is a large injury cascade that can last from days to years and the pathology is multifaceted. The fact that short term symptoms can lead to longer term problems, like brain atrophy, neurodegenerative disorder vulnerability and sensitivity to further injury, shows that TBI is a problem worth investigating as well as its prevalence in the population (Omalu, DeKosky et al. 2005, Mez, Daneshvar et al. 2017, Lennon, Brooker et al. 2023). The mechanisms that cause such outcomes are complex and therefore it is impossible to cover them all and address every aspect. I will focus on those relevant to this thesis and introduce any differences or similarities between blast and non-blast TBI.

1.5.1 Diffuse axonal injury

Diffuse axonal injury (DAI) also known as traumatic axonal injury (TAI) is a main hallmark pathology of TBI (Adams, Doyle et al. 1989). Axonal injury refers to damage to the axons, which are long, thin fibres that transmit electrical impulses between neurons. Axonal injury occurs from several causes, such as through trauma or disease, for example TBI, hypoxia ischemia (Adams, Doyle et al. 1989, Davceva, Janevska et al. 2012) or neurological diseases like multiple sclerosis (MS) or amyotrophic lateral sclerosis (ALS) (Bitsch, Schuchardt et al. 2000, Yu, Chen et al. 2021), and Alzheimer's disease (AD) (Johnson, Stewart et al. 2013).

A DAI is a type of axonal injury that occurs when there is widespread damage to axons throughout the brain (Chen, Chen et al. 2022). Typically, when caused by a TBI, such as from a car accident or a fall, DAI can be caused by rotational forces, or a sudden jerk back and forth (Braun, Vaibhav et al. 2017). DAI can occur with rotational forces; even low levels are able to cause a DAI (Bruggeman, Haitsma et al. 2021) which is important for forensic pathology and when creating animal models of TBI with restricted movements. This is why DAI is so common in TBI injuries and likely to occur in all ranges of severities as there does not need to be fracturing of the skull nor evident lesions for there to be damage within the brain.

In DAI, the axons can be damaged by shearing or tearing from tensile force, leading to disruptions in the communication and transport between neurons (Hill, Coleman et al. 2016). Furthermore, DAI can result in transport interruptions, swelling, proteolysis, and secondary injury cascades (Johnson, Stewart et al. 2013). Axonal bulbs can occur at tearing point. Other more common pathology is presented in periodic arrangements along the axon called varicosities which can occur just hours after the injury (Adams, Doyle et al. 1989, Povlishock 1992, Povlishock and Christman 1995, Gu 2021). DAI was reported to be very common in blunt TBI, which seems to exemplify the vulnerability of the white matter in the brain due to the mechanical loading from the rapid accelerations. This could be due to its highly constricted and anisotropic (unilateral directionality) structure (Johnson, Stewart et al. 2013, Braun, Vaibhav et al. 2017). However, experimental rodent studies have shown that besides white matter, grey matter can also be affected (Bramlett and Dietrich 2002, Vascak, Jin et al. 2018). Furthermore, the grey matter is also an area that contains the oligodendrocytes that are part of the myelination of axons (Braun, Vaibhav et al. 2017). Nonetheless, DAI damage can lead to a variety of symptoms, depending on the severity of the injury and the areas of the brain that are affected (Adams, Doyle et al. 1989). Symptoms may include cognitive impairment, memory loss, motor dysfunction, and changes in mood and behaviour. DAI can be a serious condition that can have long-lasting effects on a person's life (Johnson, Stewart et al. 2013).

Primary axotomy was thought to be derived from the stretching of axons and, as the stretch became too much from the acceleration/deceleration, there was retraction of the axon and

bulbs would appear from the tears. This was evidenced under pathological investigations and suggests the direct process of tearing. This mechanism of axotomy is considered to be rare and restricted to the TBIs that have a lot of widespread damage, which can alter the consciousness of an individual (Johnson, Stewart et al. 2013, Hill, Coleman et al. 2016). Traditionally, DAI symptoms were thought to occur during acute and sub-acute periods following the trauma. However, it has been shown that the DAI can occur for a long time after the trauma (Johnson, Stewart et al. 2013), showing the impact of the secondary pathological injury mechanisms, the neurodegenerative process and how it precipitated the injury throughout the lifetime of the TBI sufferer (Chen, Johnson et al. 2009).

Secondary axotomy, refers to the ongoing symptoms from primary axotomy. It is hypothesised that axons damaged by TBI may undergo Wallerian Degeneration, mitochondrial dysfunction, oxidative stress, lipid peroxidation, ionic imbalance (e.g., elevated intra-axonal calcium), and demyelination (Johnson, Stewart et al. 2013). Demyelination is less commonly observed, but studies have found evidence of demyelination and progressive white matter atrophy up to one year following FPI TBI in rats (Bramlett and Dietrich 2015). There is also evidence that neuroinflammation in white matter can contribute to cellular damage, including microglial contributions. However, microglia can also aid in tract reparation by promoting remyelination, since the myelination has been reported to be affected even in mTBI experimental models, impaired by DAI and causing loss of conductance (Baker, Phan et al. 2002, Loane and Byrnes 2010, Johnson, Stewart et al. 2013, Miron, Boyd et al. 2013, Lloyd and Miron 2019). There is also evidence that axonal repair can occur for axons with swellings. This repair may happen through various mechanisms, such as direct replacement of the damaged cytoskeleton, regulation of ionic homeostasis, turnover and production of neurofilaments, or the influence of mitochondrial density (Chen, Meaney et al. 1999, Tang-Schomer, Johnson et al. 2012). By enhancing the transport of healthy mitochondria, the density of mitochondria can be increased, providing sufficient energy for axonal regrowth and ultimately leading to axonal regeneration following TBI (Wang, Huang et al. 2021).

1.5.2 White matter damage

White matter is primarily composed of densely packed axons that form fibre tracts, with the main fibre tracts in the human brain, being the corpus callosum, fornix, internal capsules (Rutgers, Toulgoat et al. 2008). However, the rodent brain only has one main white matter tract, which is the corpus callosum.

Damage to the white matter, specifically DAI, can result in cognitive, sensory, motor, and behavioural deficits. White matter damage has also been associated with long-term diseases such as Alzheimer's disease (AD). When the white matter is affected, it alters not just the area and one type of axon, but a cascade of related problems can occur as a result. For example, cognitive abilities can be significantly affected by white matter damage, due to the modulatory neurotransmitter acetylcholine, which is widely distributed in the cerebral white matter tracts. Acetylcholine plays a role in modulating neuronal responses and other neurotransmitters, and alteration of cerebral cholinergic projections due to TBI can result in acetylcholine chronic reduction, leading to long-term cognitive problems, even if other neurotransmitters return to normal levels (Filley and Kelly 2018).

White matter is known to undergo a natural decline with age, rendering it vulnerable to the aging process. It is essential to bear this fact in mind when comparing the brains of TBI-affected older patients with healthy control subjects. However, specific demographic groups, such as military personnel and high-impact sportspersons, face the risk of accelerated white matter decline and brain atrophy. These individuals are predisposed to chronic white matter injury resulting from conditions such as Chronic Traumatic Encephalopathy (CTE), early onset Alzheimer's disease, and dementia. (Goldstein, Fisher et al. 2012, Tagge, Fisher et al. 2018, Bouchard, Sun et al. 2022, Filley 2022).

Detection of DAI in the white matter can be a major challenge for milder cases of TBI as they can present as numerous microtears rather than obvious lesions. Post-mortem analysis can be informative for detecting DAI in the white matter as there are histological markers to identify it, one of which proved to be a very sensitive marker, amyloid precursor protein (APP)

(Gentleman, Nash et al. 1993, Sherriff, Bridges et al. 1994, Bruggeman, Haitsma et al. 2021). APP was reported to accumulate in the axons that were damaged within 2 hours of the TBI and proved to be more extensive than other methods of DAI detection, including silver staining (Gentleman, Roberts et al. 1995, Bruggeman, Haitsma et al. 2021). However, these are detection and confirmation methods of post-mortem samples, and DAI has proved hard to diagnose in the living brain. Alternatively, neuroimaging techniques such as diffuse tensor imaging have been able to prove useful in the detection of DAI in mild cases of TBI (Bruggeman, Haitsma et al. 2021). However, MRI machines are expensive to use, differ in availability and quality between hospitals and, therefore, there is a need to find a different way to diagnose DAI and more broadly TBI. There remains a need to be able to detect DAI in a living being that may be provided by the use of fluid biomarkers.

1.5.3 Inflammatory response in TBI

The central nervous system (CNS) response to injury is multifaceted and the microglia and astrocytes in the CNS are a large component of the immune response. These involve the release of pro-inflammatory mediators, including cytokines, chemokines, as well as growth factors and other functional aspects such as phagocytosis of debris (Karve, Taylor et al. 2016).

1.5.3.1. Microglia

Microglia are one of the most ubiquitous cells in the brain and are a specialised population of macrophage-like cell that, depending on region, can make up to 16% of total cell population in humans (Lawson, Perry et al. 1992, Bachiller, Jiménez-Ferrer et al. 2018). Microglial cells serve numerous functions in the normal brain and are highly dynamic. They are constantly screening for damage or local changes, clearing debris, altering and pruning synapses and neural circuits. By responding to extracellular signals, they clear noxious substances via phagocytosis and therefore contribute to the brain's homeostasis (Loane and Byrnes 2010, Fernández-Arjona, Grondona et al. 2017, Paolicelli, Sierra et al. 2022). Microglia also play a crucial role in the functioning and maintenance of neurons and are involved in

neurotransmitter signalling during development, homeostasis and trauma/disease states (Colonna and Butovsky 2017, Witcher, Bray et al. 2018, Witcher, Bray et al. 2021).

What was once a dichotomy of microglia nomenclature, of M1 and M2 pro- and anti-inflammatory microglia, is now understood to be much more complex (Amor, McNamara et al. 2022, Paolicelli, Sierra et al. 2022). There are many ways to identify microglia and numerous phenotypes present in the brain depending on the conditions that can be identified using the appropriate markers (Jurga, Paleczna et al. 2020). However, they have their limitations. It should be noted that no microglial marker is expressed by all types of microglia (Kenkhuis, Somarakis et al. 2022). Cytoplasmic markers such as ionized calcium-binding adapter molecule 1 (IBA1) can be used to identify microglia and macrophages in resting homeostasis and microglia in trauma or disease states. IBA1 is upregulated in disease and trauma states, and it is probably one of the most widely used marker. Another advantage of IBA1 is that it allows the full visualisation of the cells, as it stains the body and the processes very well (Paolicelli, Sierra et al. 2022). Other markers used, such as cluster of differentiation 68 (CD68) are employed to identify phagocytic microglia, but some non-specific binding may occur, as other cells express CD68, such as infiltrating macrophages (Jurga, Paleczna et al. 2020). There are other markers for microglia, such as CD11, CD45 and CD115, which can bind to microglia despite their state of activation. However, according to Jurga et al., the most specific general microglia markers are transmembrane protein 119 (TMEM119) and purinergic receptor (P2Y12R) (Butovsky et al., 2014). Despite that, it was found by Kenkhuis, 2022 that microglia surrounding A β plaques exhibit a specific loss of P2RY12 expression, while the expression of TMEM119, which is not consistently present in microglia, is generally lost in AD. On the other hand, subsets of microglia in AD show an increased expression of IBA1. Therefore, this shows that the state does depend on which marker(s) is used as to the outcomes (Kenkhuis, Somarakis et al. 2022), suggesting that more than one marker could be more informative on the microglial state.

Translocator protein 18 kDa (TSPO) can be a useful marker for activated microglia. However, TSPO is not only found in microglia/macrophages, but has been detected in low levels in endothelial cells and in some cases in astrocytes (Yao, Pan et al. 2020).

Other markers that are not associated with the surface expression, but are secreted by microglia can aid with phenotyping the different activation states. Such secretions including the pro-inflammatory cytokines such as interleukin 1beta (IL-1 β), Tumour necrosis factor - alpha (TNF- α) and IL-6 and anti-inflammatory cytokines IL-10, IL-12 and others, are markers for either the old fashioned terms of M1 and M2 types of microglia (Jurga, Paleczna et al. 2020). However, due to the complexity of the cells, many phenotypes were not consistent in their markers and therefore could not be well defined (Jurga, Paleczna et al. 2020, Witcher, Bray et al. 2021). Moreover, the traditional core markers used to identify the cellular identity of microglia may not be sufficient to determine the actual microglial “state”.

Another way to identify microglia is by investigating markers in transcriptomics. However, microglia are highly responsive to their surroundings, including the cellular architecture, and their behaviour is dynamic. Therefore, investigating the vast heterogeneity of microglia through transcriptomics has its limitations, as certain markers may be altered or downregulated at a specific timepoint, making it challenging to determine their functional implications (Paolicelli, Sierra et al. 2022). In addition, analysing the mRNA and protein expression of microglia through proteomics does not always correlate with the actual function. Even combining various techniques such as gene expression, protein expression, post-translational modifications, mRNA profiling, morphology, and ultrastructure analysis could lead to inaccurate conclusions if factors such as conditions, sex, age, space, and time are not taken into consideration (Paolicelli, Sierra et al. 2022). Thus, Paolicelli states that the field is at a "cross-roads," highlighting the need for more work in identifying the functionality of microglia and relating it to the expression of applicable markers, both in disease states and homeostatic conditions.

1.5.3.2. Microglia in TBI and bTBI

The homeostatic state of microglia changes after an injury. They respond to extracellular secretions and become activated, whereby they can proliferate, migrate to the injury site, phagocytose, produce and release pro-inflammatory, anti-inflammatory cytokines, chemokines, ROS and neurotrophic factors (Loane and Byrnes 2010, Liu, Tang et al. 2011,

Loane, Kumar et al. 2014, Fernández-Arjona, Grondona et al. 2017). In this “activated” state they can take on a new morphology. Typically, when activated, their cell body increases in size and the processes become shorter and thicker, taking on an ameboid morphology (Loane and Byrnes 2010, Fernández-Arjona, Grondona et al. 2017), although microglia can display other features when activated, such as a rod-like morphology (Ziebell, Taylor et al. 2012, Fernández-Arjona, Grondona et al. 2017). Since microglia are always surveying their microenvironment and surroundings, and are able to do so due to highly mobile processes, activated microglia can also move to the area of damage. They can either contribute to further cell damage or remove damaged cells via phagocytosis and repair tissue by secreting growth factors (Liu, Tang et al. 2011, Shao, Wang et al. 2022, Yu, Wang et al. 2022).

Activation can also be characterised and measured by cell density, which can reflect the proliferation and/or the migration of microglia to the area of damage or infection, and increased expression of IBA1 and other markers mentioned previously of activated microglia (Hovens, Nyakas et al. 2014, Fernández-Arjona, Grondona et al. 2017, Jurga, Paleczna et al. 2020, Paolicelli, Sierra et al. 2022, Wittekindt, Kaddatz et al. 2022).

The neuroinflammatory response is considered a secondary injury factor, which may increase in intensity from acute time points (Xu, Schaefer et al. 2016, Donat, Yanez Lopez et al. 2021). Depending on whether the injury is very serious, this can result in chronic neuroinflammation, and microglia activation can last longer. For example, a human study showed that after a single TBI there was persistent inflammation and white matter damage that lasted for years (Johnson, Stewart et al. 2013). The study looked at those who had died 10 hours – 47 years post-injury, reporting large areas of immunopositively stained microglia in the corpus callosum (CC), even in the later timepoints, indicating prolonged neuroinflammation and white matter loss. However, this study did not show whether this activation of microglia was either responding to the white matter loss or was promoting it (Johnson, Stewart et al. 2013). In milder experimental models, the microglial activation can be attenuated after the initial peak (Xu, Schaefer et al. 2016, Hernandez, Tan et al. 2018). This difference could be due to the severity of injury or mechanism of injury, blast vs blunt.

Another study investigated the activation of microglia in the post-mortem brains of US military veterans exposed to blast and concussive injury, as well as in a mouse model of blast TBI. The mice exhibited chronic microglial activation 2 weeks post-injury throughout the brain and, in particular, the cerebellum, whereas the human post-mortem pathology displayed the microglia staining in subcortical white matter adjacent to tau pathology (Goldstein, Fisher et al. 2012). Once again, the increased microglial staining finding was present in the white matter and brain stem, as shown in human non-blast post-mortem studies (Tagge, Fisher et al. 2018), indicating that in humans the white matter is vulnerable to injury and shows activated microglia, independent on the type of injury. However, another study reported microglial activation in both grey and white matter in 18 fatal cases of TBI, without specifying the area, although they did state that microglia were perivascular and near the contusion site (Engel, Schluesener et al. 2000). This indicates that microglia for focal injuries are present in the site of injury, being that white or grey matter. In cases of acceleration/deceleration and diffuse injuries, like those suffered in sports or blast exposures, the microglial activation is found more in the white matter and brain stem (Goldstein, Fisher et al. 2012, Tagge, Fisher et al. 2018).

Studies of microglia in blast models have been carried out in different animal species. Microglia in a mild single blast model conducted in pigs, where the blast was administered via a shock tube, showed activated microglia post-blast at 14 days post-injury. It should be noted that this was the only time point they looked at. Nevertheless, they had enlarged soma and nuclei along with increased processes and there is mention of rod-like structures in the corpus callosum (De Lanerolle, Bandak et al. 2011). These morphological findings are similar to the literature already mentioned in non-blast TBI (Ziebell, Taylor et al. 2012, Xu, Schaefer et al. 2016). In this swine study, they stated that in the hippocampus, there was no discernible increase and change in microglia morphology, which was in line with the fact they also had no neuronal loss (De Lanerolle, Bandak et al. 2011). These results are in contrast to a single mild blast study conducted in rats (Hernandez, Tan et al. 2018), in which they found that microglia and astrocyte numbers were elevated in the hippocampus, cortex and striatum in the first 1-7 days post-injury, but not at 21 days despite no major macroscopic changes, e.g. haemorrhage or contusion (Hernandez, Tan et al. 2018). Similar findings were reported in

another blast TBI model in rats, whereby microglial activation was observed at early time-points 1-7 days post-injury, but at 28 days, the microglial morphology and number returned to control sham group (Kaur, Singh et al. 1995). These differences could be due to a number of reasons, including blast strength, brain and head geometry and physics, thickness of the skull and whether the brain is lissencephalic or gyrencephalic, e.g when comparing swine with murine models or any blast model (Xiong, Mahmood et al. 2013).

The lack of macroscopic neuronal loss and lack of extreme changes in neuroinflammatory morphology response can be expected from a mild blast-TBI (Hernandez, Tan et al. 2018). It is part of the reason why it is hard to explain the behavioural changes, brain sensitivity to further injury and difficulty in diagnosing a mild TBI (Menon, Schwab et al. 2010), whereas more injurious ‘mild’ blast injuries and moderate-severe blasts are more likely to have the aforementioned changes and are easier to clinically diagnose (Menon, Schwab et al. 2010, Papa, Zonfrillo et al. 2019).

1.5.3.3. Astrocytes

Astrocytes are the most abundant cell type in the brain, and makeup the largest proportion of cells in the mammalian central nervous system (Tabata 2015, Haim and Rowitch 2017). Astrocytes, like microglia, have a great deal of heterogeneity and received increased importance in the literature (Haim and Rowitch 2017, Mirzaei, Davis et al. 2022).

They can be classified according to their morphology and function, which also is related to their location in the brain and can be region specific. Classification and morphology can also be species-dependent, as human astrocytes are larger and have many more processes and are more functionally complex than that of the rodent (Sofroniew and Vinters 2010, Karve, Taylor et al. 2016, Mirzaei, Davis et al. 2022). Originally, the morphological classification was based on fibrous and protoplasmic morphologies, but more types of astrocytes have been defined recently. In the white matter, the typical astrocyte is the fibrous astrocyte, while in the grey matter it is the protoplasmic and radial around the ventricles (Chen and Swanson 2003, Privat and Rataboul 2012, Haim and Rowitch 2017). The fibrous morphology has long

processes with fewer branches that follow the white matter and intertwine with the myelin and provide support with their end feet (Chen and Swanson 2003, Tabata 2015) and have close contact with oligodendrocytes (Haim and Rowitch 2017). In contrast, the protoplasmic grey matter astrocytes have a highly bushy ramified stellate structure that are a vital part of the BBB and neurovascular unit (Chen and Swanson 2003, Tabata 2015). Another morphology, for radial astrocytes, is found perpendicular to the ventricle and has unbranched processes (Chen and Swanson 2003, Privat and Rataboul 2012). However, there are more specialised types of astrocytes that have been described in the cerebellum, such as Bergmann cells and velate astrocytes (Haim and Rowitch 2017). Velate astrocytes are protoplasmic astrocytes that are located in the molecular layer and express common markers of astrocytes such as Glial Fibrillary Acidic Protein (GFAP) and glutamate transporter 1 (GLT1).

Astrocytes are very heterogenous, and much like microglia, they have numerous functions that range from providing structural, trophic and metabolic support to neurons, to affecting neuronal survival with their release of calcium, and glutamate release and uptake (Chen and Swanson 2003, Liu, Tang et al. 2011, Zhou, Shao et al. 2020). Astrocytes can influence water transport, cytokine production and release neurotrophic factors involved in neurite growth and neuron plasticity; therefore, they can play a role in the recovery and long-term effects of injury. Astrocytes have long processes, which connect cells within the brain together and form networks. They are also part of the neurovascular unit of the BBB (Liu, Tang et al. 2011). They can regulate neurotransmitters around synapses, which affect their activities and metabolic functions (Liu, Tang et al. 2011, Zhou, Shao et al. 2020). This is important since they can play a vital role in neuronal survival post-injury.

As a result of trauma, the environment changes and damaged cells release intracellular components that trigger an activation and recruitment of glial cells in the brain. Post-injury astrocytes change morphology by increasing their size, by shortening or lengthening their processes and relinquish their stabilising roles to neurons. However, they can release neurotrophic factors that can help reparation, and also generate a glial scar to protect an injured area of the brain (Liu, Tang et al. 2011, Burda, Bernstein et al. 2016, Yuan and Wu 2022). Astrocytes change their gene expression, morphology and prolific capability as a result

of injury. These changes are varied and depend on type and severity of injury (Burda, Bernstein et al. 2016). Astrocytes can react within the first 24 hours of injury and their peak of activation can last for prolonged periods beyond microglial (Zhou, Shao et al. 2020). Excitotoxicity is another common outcome of TBI, as glutamate is released from damaged or dying cells and can damage glutamate sensitive neurons. Astrocytes can help prevent the glutamate excitotoxicity that can result from trauma, as they have a large glutamate re-uptake ability, unless damaged themselves from the initial injury (Karve, Taylor et al. 2016, Miller, Shah et al. 2017).

GFAP is an intermediate filament protein in astrocytes and is a commonly used immunohistochemical marker for astrocytes (Sofroniew and Vinters 2010). Fibrous astrocytes contain higher levels of GFAP than protoplasmic astrocytes (Sofroniew and Vinters 2010, Tabata 2015). GFAP is found mainly in the end feet of protoplasmic astrocytes and therefore is expressed less in the cortex of the mammalian brain (Tabata 2015). However, GFAP is an essential part of reactive astrogliosis (proliferation and activation as a reaction to injury) and, therefore, if the protoplasmic subtype becomes activated then it will be detected using GFAP as a marker (Sofroniew and Vinters 2010, Yuan and Wu 2022).

1.5.3.4. Astrocytes in TBI and bTBI

Astrocytes can respond to mechanical strain, such as the one resulting from diffuse TBI. The deformation from the strain can alter the structure and activate an ion influx in mechanosensitive channels. This can result in a cascade of effects that result in release of glutamate, adenosine triphosphate (ATP) and GFAP among other secretions, which initiate further cascade of immune response (Burda, Bernstein et al. 2016). ATP is also released by other damaged or reactive cells in the brain, and this increase in ATP can cause an elevation in cytoplasmic calcium in the reactive astrocyte network (Burda, Bernstein et al. 2016). This astrocytic calcium release that is ATP dependent, can then activate and recruit microglia and leukocytes to the injury site within minutes of damage, which can be seen as neuroprotective, at least in the first instance (Burda, Bernstein et al. 2016, Chiu, Liao et al. 2016). This also shows that the cell types can work together and not in isolation when reacting to injury.

Astrocytes have the ability to proliferate, undergo hypertrophy and metabolic changes in response to trauma or pathological damage (Yuan and Wu 2022). This can occur due to the infiltration of immune cells from the peripheral system due to BBB compromise and participate in the inflammatory response (Needham, Helmy et al. 2019). Studies in animal models of brain injury have reported a peak in GFAP at 3 days post injury due to proliferation rather than migration, with an appearance of extended processes (Susarla, Villapol et al. 2014). Similarly, another report in a CCI animal model showed that astrocyte proliferation was found to peak at 3 days post-injury (Loane and Byrnes 2010).

There is evidence of astrocyte proliferation and reactive astrogliosis at the interface of the grey and white matter, around the ventricles, and the sub-pial plate in chronic severe bTBI human cases (Shively, Horkayne-Szakaly et al. 2016). Astrocyte pathology can vary depending on injury severity; the higher activation has been found usually in more profound pathology related to higher severity (Shandra, Winemiller et al. 2019, Babcock, Abdolmohammadi et al. 2022).

Regarding *single* mild blast-TBI, the outcomes can be different, as it was reported in the swine model of mild blast-TBI previously mentioned. Specifically, it was observed that astrocytes presented unusual patterns of distribution, such as their absence from certain areas or poor expression of GFAP (De Lanerolle, Bandak et al. 2011). However, there were some similarities with human TBI, particularly in the hippocampus, an area known for its role in memory and sensitivity to injury, due to its unique neuronal architecture. In another study, using a mild blast-TBI rodent model, there was activation of astrocytes in the striatum, cerebral cortex and hippocampus following bTBI at 1, 3 and 7 days post-injury. The reactive astrocytes receded by 21 days post-injury (Hernandez, Tan et al. 2018), thus showing that chronic astrocyte reactivity can occur in milder forms of TBI.

1.5.4 TBI and Blast TBI and its Effect on Neurons

Both non-blastTBI and blast TBI can cause neuronal loss, but due its high variability and unpredictable nature as an injury type, rarely are two cases the same, making it hard to implement preventative measures (Tehse and Taghibiglou 2018). Neuronal loss refers to the death of neurons in the brain, which can occur as a result of the direct mechanical impact of the injury or as a result of secondary mechanisms, such as inflammation, glutamate excitotoxicity, oxidative stress, and apoptosis. Although primary insult can initiate neuronal loss, it is the secondary injury that can cause the multitude of damage and future issues such as neuronal death, necrosis and apoptosis (Tehse and Taghibiglou 2018). The extent of neuronal loss can vary depending on several factors, including the severity of the injury, location of the injury, and age of the individual. In severe cases, neuronal loss can result in long-term functional impairments, such as cognitive and motor deficits, memory loss, and changes in mood and behaviour (Taber, Warden et al. 2006, Hoge, McGurk et al. 2008, Mckee and Daneshvar 2015).

While mild traumatic brain injury (TBI) may result in modest neuronal loss, the survival of neurons following an injury does not necessarily indicate their proper functionality. Gao et al., investigated the effect of mild TBI on neuronal degeneration in the cerebral cortex in mice. They found that a blunt model of mTBI caused great morphological changes to the surviving neurons, such as dendrite and spine degeneration and synapse reduction. This can cause disruption to the proper functionality, because dendrites are critical for receiving input and providing a large surface area for spine formation. Additionally, a decrease in dendritic spine density implies a reduction in synapses, which may result in altered functionality (Gao and Chen 2011).

Conversely, in a study that looked at mTBI using a blast model, researchers found no macroscopic neuropathological changes, an absence of neuronal loss, no haematoma or contusion, but instead microvascular damage, axonal injury and neuroinflammation. In this study, they also found that the functionality of the neurons was affected, and neurons were firing differently, although no changes in the long-term potentiation were observed

(Hernandez, Tan et al. 2018). Another blast study also found dendrite changes post-injury, but it was a very different type of blast model (free field 500g of TNT usage). The investigation of the effects of mild bTBI on the morphology of neuronal dendrites in the parietal cortex and hippocampus of a mouse model, using sholl analysis on the dendritic arbour, showed that the number of dendritic intersections of pyramidal neurons in the 5th and 6th layer of the parietal cortex and hippocampus was reduced relative to sham control animals. In the same study, it was reported that the neurons in the middle sections of the parietal cortex were the only ones who had been morphologically affected by the blast, suggesting that the middle part of the cortex is sensitive to injury despite the whole brain being subjected to blast (Ratliff, Mervis et al. 2020). These middle regions refer to approximately the 5th layer of the cortex. The 5th layer has important roles within the brain with regards to networking. This is also a layer that has been found to show changes in interneuronal cell types and these morphological changes could affect the normal networking and regulation of the excitation and inhibition balance of the brain after TBI (Cantu, Walker et al. 2015). This therefore suggests that milder TBI can cause damage in the axon without mass neuronal density changes (due to necrosis or apoptosis) (Povlishock 1992) and when assessing a mild TBI it should be considered that network changes could be affected.

1.5.5 Excitation/Inhibition Balance in TBI

The E/I balance, or the balance between excitation and inhibition, refers to the regulation of the activity of neurons in the brain. Excitatory neurons release neurotransmitters that increase the activity of other neurons, like glutamate, while inhibitory neurons release neurotransmitters that decrease the activity of other neurons, like γ -Aminobutyric acid (GABA). The balance between these two types of neurons is critical for normal brain function. Since the excitatory neurons can receive a great number of excitatory inputs, if left unchecked they could all fire at once and the cortex would be overwhelmed and information processing become impossible. Therefore, the brain needs a way to prevent/inhibit excitatory activity and for there to be a homeostasis where not all excitatory neurons are firing constantly or all at once (hyperexcitability). However, there cannot be too much inhibition, as it is necessary to have sufficient depolarising neurons that are firing from distinct neurons or groups of

neurons to respond to the excitatory inputs, in order for brain function and information processing to occur (Ferguson and Gao 2018). It is known that highly interconnected microcircuits within the cerebral cortex perform dynamic computations to allow this homeostasis within the brain to be a reality. However, any changes in neuronal morphology, receptor organisation, membrane potential or potential neuronal losses in the brain can lead to network impairment (Brickley and Mody 2012, Almeida-Suhett, Prager et al. 2014, Cantu, Walker et al. 2015, Filice, Vörckel et al. 2016, Ferguson and Gao 2018, Frankowski, Kim et al. 2019, Frankowski, Tierno et al. 2022).

The brain maintains this crucial balance between excitation and inhibition with the variety of neuron types and their different properties that they possess (Ferguson and Gao 2018). The cell types that uphold this balance are composed of two categories; first is the glutamatergic excitatory neurons (~80% of all neurons), also known as principle cells (PCs), and second is the GABAergic inhibitory neurons (INs) (~20% of all neurons) (Tremblay, Lee et al. 2016).

INs play a crucial role in information processing, and the brain has a flexible use of them due to the diverse subtypes available. These subtypes have varying characteristics such as firing patterns, morphology, and connectivity, as well as responses to neuromodulators and expression of different molecules (Tremblay, Lee et al. 2016). All of these factors contribute to the IN's ability to perform their role in the brain (Rudy, Fishell et al. 2011). This is because the balance is dynamic and it is required that the inhibition is flexible in its responsiveness to the fluctuations of excitatory input (Shu, Hasenstaub et al. 2003, Fröhlich 2016, Ferguson and Gao 2018). Despite the INs relatively low presence in the brain, INs are highly ramified with many targets for their GABA release so as to be specific but wide-reaching in their inhibitory function (Tremblay, Lee et al. 2016). One IN can have an effect on thousands of PCs (Packer and Yuste 2011, Vascak, Jin et al. 2018).

GABAergic INs play significant roles in cortical functions. These roles include dynamic range modulation, plasticity, temporal precision of pyramidal cell firing, regulation of firing rates and bursting, synchronisation, and generation of cortical rhythms. These functions help maintain the necessary balance between excitation and inhibition for controlled activation and information transfer (Fröhlich 2016, Tremblay, Lee et al. 2016). It has been shown that if

there is a malfunction in the inhibition of these critical neurons from TBI or another cause, it can lead to the disruption of neuronal firing and can cause development of epilepsy post-TBI, as well as contributing towards brain disorders (e.g schizophrenia, autism and other psychiatric disorders) (Cossart, Dinocourt et al. 2001, Sohal, Zhang et al. 2009, Carron, Yan et al. 2016, Filice, Vörckel et al. 2016, Ferguson and Gao 2018, Vascak, Jin et al. 2018). Less is known about *blast* TBI and its effect on GABAergic inhibitory neuron populations over a longitudinal timeframe and that is what this thesis hopes to address.

1.5.5.1 Types of GABAergic Interneurons

The brain contains a diverse range of GABAergic IN subtypes, with parvalbumin (PV), somatostatin (SST) and ionotropic serotonin receptor 5HT3a (5HT3aR) being the most abundant subtypes, which make up nearly 100% of the INs in the neocortex (Rudy, Fishell et al. 2011, Kepecs and Fishell 2014, Tremblay, Lee et al. 2016). The 5HT3aR receptor exhibits considerable heterogeneity, encompassing a diverse population of neurons expressing the neuropeptide (vasoactive intestinal polypeptide) VIP, as well as a comparably numerous subgroup of non-VIP expressing neurons, which includes neuroglia from cells (Rudy, Fishell et al. 2011). The VIPs, can play an important role as they disinhibit pyramidal neurons via inhibition of SST interneurons (Tremblay, Lee et al. 2016, Millman, Ocker et al. 2020). While these subtypes display variability in size, distribution, electrophysiological patterns, and function, and it is known they are vital role in brain functionality, they are still not fully understood. Although there exist rarer types of INs, this thesis will primarily focus on the PV and SST subtypes, as they are the most abundant and their markers are useful and reliable to define non-overlapping IN groups (Kawaguchi and Kubota 1997, Rudy, Fishell et al. 2011).

1.5.5.2 Parvalbumin Interneurons

Parvalbumin INs, so named due to the calcium-binding protein PV they express (~40% of IN population) are fast spiking and highly interconnected (Rudy, Fishell et al. 2011, Ruden, Dugan et al. 2021). They have many chemical and electrical synapses that enable this (Rudy, Fishell et al. 2011, Borkowska, Millar et al. 2016, Tremblay, Lee et al. 2016) and receive both

excitatory and inhibitory inputs from a high number of neurons. They provide perisomatic and somatic inhibition in both feedback and feedforward circuits, and regulate the overall output of principal neurons/cells (PC)s (e.g. pyramidal) (Cardin, Carlén et al. 2009, Chandrasekar, Heuvel et al. 2019). Inhibitory inputs mainly come from other INs (Borkowska, Millar et al. 2016). PV INs consist of two morphological types; the first is the chandelier cell, it is also known as axo-axonic cells since they target the initial segment of excitatory pyramidal cells. These cells are fast spiking, but not as fast as the basket cells, which are the second type of PV IN. The fast-spiking basket cells make peri-somatic “basket” terminals on PCs and other PV INs, and are the most abundant of the two PV morphological subtypes (Rudy, Fishell et al. 2011, Tremblay, Lee et al. 2016). PV INs are positioned strategically to target the soma or axon initial segment, enabling them to control spiking. This control is achieved because of their high interconnectivity, which allows for high synchronicity among them (Kepecs and Fishell 2014). The morphological structure and electrophysiological properties of these cells differ depending on their laminar and columnar location of their soma (DeFelipe 2002, Douglas and Martin 2004).

The majority of PV INs are short axoned and will inhibit either pyramidal neurons or each other (Pfeffer, Xue et al. 2013), such as those in the 4th cortical layer of the somatosensory cortex. However, there are longer ranged basket cells that are perisomatic and thus translaminar in their reach and interactions that cause neighbouring columns to be inhibited, such as those found in the infra- (5th-6th layer) and supragranular layers (layer 1 to 3) (Tremblay, Lee et al. 2016). This is also true for some SST-expressing Martinotti cells (Caputi, Melzer et al. 2013).

Long-range PV neurons have been shown to exist; however, their functionality has not been extensively researched in the literature. Zurita et al, (Zurita, Feyen et al. 2018) have shown that the PV GABAergic neurons form a portion of the long-range GABAergic projections (Rock and Apicella 2015, Rock, Zurita et al. 2018). These projections come from all layers of the 2/3 through till the 6th layer of the cortex and project to the CC, and connect the two hemispheres of some of the sensory cortices; auditory and visual cortex (Zurita, Feyen et al. 2018). As well as their long-range reach, CC parvalbumin neurons can locally inhibit pyramidal cells as well

as long-range inhibition. In the dearth of literature on long-range IN, the study of Zurita et al. (Zurita, Feyen et al. 2018) focused on layer 5 long-range CC PV INs and found they were morphologically and electrophysiological different. Morphologically, they had extensive dendritic arborisation compared to other PV INs in the 5th layer. They also fire differently by having delayed action potential response near-threshold and a lower instantaneous frequency (Zurita, Feyen et al. 2018). This showed the electrophysiological difference, as this was due to the presence of potassium voltage gated channel 1 (Kv1) channels (D'Adamo, Liantonio et al. 2020). These channels can dampen neural excitability that are the main source of repolarisation; therefore, they control electrophysiological behaviours of these PV IN. These potassium channels allow these PV INs to filter out slow or weak signals as well as play a role in spike delayed response and firing rate and thus they serve as a separate component of PV inhibition within the circuit (Zurita, Feyen et al. 2018). Furthermore, since they are INs that travel through the CC, they are vulnerable to DAI or any compromise to the integrity of the white matter tract, which is common in TBI.

Due to their highly connected microcircuits, consisting of long and short range neurons, a single PV IN can significantly impact the functioning of the cells around it (Rudy, Fishell et al. 2011). The distribution of the PV cells in the cortex varies but they are most abundant in the 4th layer of the cortex followed by the 5th and less abundant in the 2/3rd and 6th layer of the somatosensory cortex (Xu, Roby et al. 2010, Tremblay, Lee et al. 2016). This distribution may vary in other neocortical regions.

As previously mentioned, the PV cells are fast-spiking. This is due to their ability to rapidly sequester calcium and therefore, they can suppress the calcium-activated potassium conductance, which is responsible for post-spike hyperpolarisation. This is one of the reasons why PV-expressing inhibitory neurons are known to repolarise faster and fire more rapidly than other neurons. This characteristic phenotype of PV INs is characterised by action potentials that have the aforementioned fast-spiking pattern, but it comes at a high energetic cost to the cell (Ruden, Dugan et al. 2021).

PV cells have more excitatory inputs than inhibitory (Rudy, Fishell et al. 2011), therefore, they are very energy intensive and vulnerable to energy deficits, like those experienced post-TBI (Inan, Zhao et al. 2016, Ruden, Dugan et al. 2021). The fact that they have more excitatory inputs than inhibitory could also be the reason why they have strong depressing excitatory inputs that can mean a lot of energy is needed to make a PV cell fire repeatedly. Depressing excitatory inputs occur when the release of a neurotransmitter from a presynaptic neuron leads to the gradual decrease in strength of the post-synaptic response, therefore making it is harder for the post-synaptic neuron to depolarise.

1.5.5.3 Somatostatin interneurons

Somatostatin INs (~30% of IN population) (Tremblay, Lee et al. 2016, Urban-Ciecko and Barth 2016), so named due to the neuropeptide it expresses, are different to the PV INs as they are dendritic targeting (Kepecs and Fishell 2014). Overall, they are most abundant in the 5th cortical layer with lower densities in the other layers in the somatosensory cortex (Kawaguchi and Kubota 1997, Tremblay, Lee et al. 2016).

There are two subtypes; the Martinotti and non-Martinotti, which have a different cortical distribution relative to the PV cells (Kawaguchi and Kubota 1997, Rudy, Fishell et al. 2011, Tremblay, 2016 #235). Martinotti cells, which are regular-spiking (Urban-Ciecko and Barth 2016), are in every cortical layer other than the fourth. Martinotti cells appear to arborise to layer 1, which is assumed to be the cells contacting dendrites of other neurons. They can also inhibit deep-layer pyramidal cells by connecting to the tufts of the dendrites and providing di-synaptic inhibition to some PCs (Kapfer, Glickfeld et al. 2007, Berger, Perin et al. 2009, Kepecs and Fishell 2014). In contrast, non-Martinotti cells are present in layer 4 of the cortex. Some SST axons stay within that layer and target mainly fast-spiking INs (Xu, Jeong et al. 2013), while only some project to layer 2/3, having a local effect there (Tremblay, Lee et al. 2016). There are SST long-range cells that have also been shown to be present in the brain and be present in the white matter and layers 2/3 and 6 (Tomioka, Okamoto et al. 2005, Tamamaki and Tomioka 2010, Scheyltjens and Arckens 2016), which may leave them vulnerable to DAI.

There are connectivity differences between the SST morphological subtypes; the non-Martinotti SST INs also exhibit connectivity patterns, where they mainly inhibit any other cell populations other than themselves (Pfeffer, Xue et al. 2013). Thus, they inhibit local PV INs, which may result in disinhibition (prevent the inhibitory transmission of the IN) of layer 4 pyramidal cells. It is currently unclear whether SST INs with similar characteristics to those found in primary somatosensory cortex (S1) exist in other cortical regions. Martinotti neurons are a type of interneuron that have axons extending upwards and branching out into layer 1 of the cortex, as well as horizontally to nearby columns. These axons form connections with the dendritic tufts of pyramidal neurons and also have collateral branches that innervate the basal and apical dendrites. These connections between Martinotti interneurons and pyramidal neurons are mainly located on the dendritic shafts and spines of the pyramidal neurons (Markram, Toledo-Rodriguez et al. 2004, Rudy, Fishell et al. 2011). The SST cells of the infragranular layers (layers 1-3) do not appear to project up to layer 1, instead they project to layer 4.

SST neurons function differently from PV cells in the way they react to incoming signals from other neurons. Most INs, like those of PV synapses have moderate to strong depressing excitatory synapses that weaken over time, whereas SST INs have synapses that get stronger over time (potentiating) making them better able to receive and process signals from other neurons (Tremblay, Lee et al. 2016). This property is determined by the structure of the SST INs themselves and is not due to differences in the neurons that are sending signals to them (Tremblay, Lee et al. 2016). There are some SST neurons which are specialised in inhibiting local PCs (Kepecs and Fishell 2014), whereas other are connected to PV INs, or both. SST neurons are capable of eliciting feedback inhibition from a single presynaptic burst (Rudy, Fishell et al. 2011), in contrast to PV cells that necessitate synchronised firing from multiple presynaptic cells due to their strong depressing excitatory synapses and rapid membrane time constant (Tremblay, Lee et al. 2016).

1.5.5.4 Perturbation to E/I Balance.

There are fewer studies in TBI than those in other fields that investigate these GABAergic cells and what occurs in the brain when they are damaged or perturbed. Therefore, I will briefly introduce these cell types in contexts other than TBI, as it aids the understanding of their functionality. In the context of TBI their behaviour may be similar.

An imbalance of the E/I balance due to IN dysfunction can lead to psychiatric disorders and epilepsy as PV and SST cells are crucial for networking and information processing, according to psychiatric literature. Borkowska et al (Borkowska, Millar et al. 2016) found that reduced expression of the calcium-binding protein PV in Schizophrenia can affect the function of INs. In this study, two loss-of-function point mutations in a gene linked to Schizophrenia indirectly were found to reduce PV expression during development in a rodent model, by affecting gene expression in pyramidal cells. This resulted in abnormal firing in the cortex from the pyramidal cells, subsequently indirectly reducing PV expression during the development of the PV neurons, which rely heavily on "normal" cortical firing to develop. This ultimately affected the E/I balance (Borkowska, Millar et al. 2016). In addition, it was shown that the PV expression was mainly reduced in the 5th layer of sensory cortical areas, resulting in decreased functionality of PV INs and a loss of neural circuitry fine-tuning. The reduction in PV histological immunoreactivity has been seen in human post-mortem of schizophrenia subjects in the prefrontal cortex relative to controls, thus indicating that expression of PV can affect the functionality of the IN and can be related to the psychiatric disorders (Reynolds and Beasley 2001, Hashimoto, Volk et al. 2003). An animal model of schizophrenia supports these findings and shows how a deficit in the expression of the interneuronal proteins such as PV results in aberrant behavioural outcomes and poor task performance (Lodge, Behrens et al. 2009).

Perturbation to SST expression has also been shown to be a part of other brain disorders, like Alzheimer's disease. It was reported that a decrease in SST was likely to be involved in amyloid accumulation (Song, Yoon et al. 2021). In a different study looking at SST and Parkinson's disease (PD) (Iwasawa, Kuzumaki et al. 2019), they found there was a decline in SST INs and

SST mRNA levels found in PARK2-specific induced pluripotent stem cells taken from individuals with Parkinson's disease. The mutation of PARK2 gene in SST INs could lead to a decrease in SST transcripts and dysfunction in the mitochondria, ultimately resulting in an imbalance between excitatory and inhibitory signals, which could contribute to symptoms observed in Parkinson's disease (Iwasawa, Kuzumaki et al. 2019).

1.5.5.5 E/I Balance and TBI

The findings discussed in the previous sections are all in the developing brain and relating to brain disorders rather than in a post-TBI situation. Investigation into the IN populations after TBI has been explored for blunt-TBI models and it has been found that the IN population and expression is affected by TBI.

For TBI, there are different factors to be considered, such as the primary and second injury pathology that may affect the function of INs. This was shown in the study of Vascak et al. (Vascak, Jin et al. 2018), where the effect of the FPI model on the population of PV interneurons was investigated. DAI within the PV IN axons resulted in their functional and structural disconnection from the local inhibitory neocortical networks. They showed that DAI caused transport dysfunction in the axons of PV INs and displayed PV INs to be sensitive to injury (Vascak, Jin et al. 2018). The model that Vascak and colleagues were using was a milder model than those used in other studies. In models where there was a higher severity, epileptiform activity following TBI was found, where there was higher excitatory activity and lower inhibitory activity. The authors stated that the increase in glutamatergic signalling was due to the decrease of GABAergic control due to the decrease in IN density. The study also found that there was a greater loss of PV INs in the infragranular layers of the cortex (Cantu, Walker et al. 2015). These findings were supported by Lowenstein et al., (Lowenstein, Thomas et al. 1992), who investigated in a CCI model the impact on INs and found that SST IN density was reduced due to the injury, whereas pyramidal cell population was unaffected in the hippocampus (Lowenstein, Thomas et al. 1992).

The TBI literature seems to suggest that certain cortical locations and GABAergic INs are sensitive to injury. Whilst this research is useful in understanding the implications of TBI on the brain, it is hard to know whether the crucial subset of GABAergic interneurons have been affected in humans, as there is no previous research on this topic. Therefore, it is important to find a proxy measure to identify them in live humans. For instance, if the E/I balance is disturbed, then investigating the electrical signals of a brain should help indicate this. However, it should be noted that these TBI findings were made in non-blast injury studies. There have been only two papers published about blast TBI and the effect on the IN population and the effects of that change (Wang, Zinsmaier et al. 2018, Masri, Deng et al. 2022). The mentioned studies indicate a decrease in only PV neuron density but not SST, and only cover a maximum of 10 days post-injury. It transpires that most TBI and bTBI studies on this topic are short-term, and there is limited research on chronic time points. This is where some of the novelty of this thesis lies.

1.6 Fluid Biomarkers

As previously stated, diffuse injuries like that of mild blast TBI can be hard to detect. A way to aid a diagnosis is to use a fluid biomarker as part of the diagnosis procedure. A fluid biomarker is a biological molecule that is found in bodily fluids such as blood, urine, or cerebrospinal fluid (CSF), and can be used to indicate the presence or severity of a disease or condition. Fluid biomarkers can be measured using a variety of techniques such as enzyme linked immunosorbent assay (ELISA), Western blotting, and Mass spectrometry. Biomarkers are important in early diagnosis, treatment and monitoring the progression of disease and injury. Some examples of fluid biomarkers include proteins such as amyloid beta ($A\beta$), tau, and neurofilaments (discussed later) that are associated with Alzheimer's disease and other neurodegenerative disorders and TBI (Svetlov, Larner et al. 2009, Zetterberg, Smith et al. 2013, Khalil, Teunissen et al. 2018, Wang, Yang et al. 2018).

Tau is a protein in the CNS that is found in neurons in the microtubule rich axon to facilitate interneuronal transport and maintain structural integrity (Holper, Watson et al. 2022). It has been shown that tau is present in some glial cells, such as oligodendrocytes and astrocytes in

certain neurodegenerative disorders, such as progressive supranuclear palsy (PSP) (Mckenzie, Wang et al. 2018). There is a tau presence in the peripheral nervous system, but it has a higher molecular weight (Couchie, Mavilia et al. 1992). Tau concentrations in the CSF are thought to reflect the severity of neuronal damage in the brain. Interestingly, a study showed that CSF tau levels measured 2-3 days post-TBI was able to predict the clinical outcomes for the next year. Higher CSF tau levels meant worse outcomes, thought to be due to greater axonal damage (Ost, Nylén et al. 2006). Therefore, CSF-Tau can indicate axonal and neuronal injury and can detect changes in mild TBI (Zetterberg, Smith et al. 2013). However, plasma phosphorylated tau's (p-tau) convergent validity with other measures of AD pathology has raised concerns, as studies have shown small or non-significant correlations with CSF p-tau (Zetterberg, Smith et al. 2013, Mattsson, Zetterberg et al. , Fossati, Ramos Cejudo et al. 2019). Therefore, the requirement for a well correlated CSF-plasma biomarker that is sensitive to milder injuries is required.

Amyloid beta peptide ($A\beta$) is a type of protein fragment that occurs via the proteolytic processing of amyloid precursor protein (APP), a transmembrane protein, by β - and γ -secretases. The accumulation of $A\beta$ in the brain is thought to be an initial toxic event in the development of Alzheimer's disease (AD), which is the most prevalent type of dementia characterised by plaques and tangles in the brain. Therefore, if there are high levels in the brain of a TBI patient, it would indicate AD. In AD, however, low levels of $A\beta_{42}$ in CSF are used as a sign of positive diagnosis (Spies, Verbeek et al. 2012). Despite this, in some cases there are no elevated levels and therefore even for severe TBI, it is sometimes not suitable (Zetterberg, Hietala et al. 2006, Zetterberg, Smith et al. 2013).

Other markers such as neuron specific enolase (NSE), S100 calcium-binding protein B (S-100B), ubiquitin C-terminal hydrolase (UCH-L1) and GFAP are associated with traumatic brain injury. In 2018 the Food and Drug administration (FDA) approved GFAP and UCH-L1 for moderate TBI diagnosis, but not mild TBI usage or for children (Papa, Zonfrillo et al. 2019). This supposed lack of sensitivity of GFAP is also shown by Zetterberg et al (Zetterberg, Smith et al. 2013) as they display that there were elevated levels but not as sensitive as neurofilament biomarkers in the CSF, however it is still being investigated.

There can be issues with some of the fluid biomarkers being appropriate for blast TBI diagnosis and prognosis; for example, S-100B is not only found in the brain. Therefore, if a military person suffered a polytrauma (Svetlov, Larner et al. 2009) it would be hard to know if increases in S-100B came from the brain or was a result of the polytrauma. NSE, is also found in red blood cells as well as neurons and neuroendocrine cells (Wang, Yang et al. 2018) and therefore suffers the same limitations as S-100B. There is also the issue on whether the biomarker of choice is sensitive enough to pick up a mild bTBI. There is still a need to investigate the pathology of milder TBI like mild single bTBI and a promising fluid biomarker is Neurofilament light.

1.6.1 Neurofilament Light

Neurofilament light (NFL) (10 nm diameter, 68 kDa) is a structural protein that is found only in the neuronal cytoplasm of neurons in the nervous system. It is a component of the neurofilament triplet, which includes neurofilament medium (NFM) (150 kDa) and neurofilament heavy (NFH) (200 kDa) and an α -internexin and or peripherin. Together, these proteins are heteropolymers that form the cytoskeleton of neurons and provide support and stability to the cells. It is present in the dendrites, cell body and most abundantly in the axons (Gaetani, Blennow et al. 2019). Since DAI is a central pathology of TBI, it can be considered a promising biomarker for diagnosis (Shahim, Politis et al. 2020).

1.6.1.1. NFL Structure

NFL is part of the intermediate filament family (Figure 1.3). Proteins in this family have a similar tripartite structure that consists of a globular head (N terminal), a central α -helical “rod” region, and a globular tail (C terminal). The α -helical rod region is crucial to the formation of the 10nm diameter NF, which is a key feature of this protein family (Khalil, Teunissen et al. 2018, Shaw, Madorsky et al. 2022).

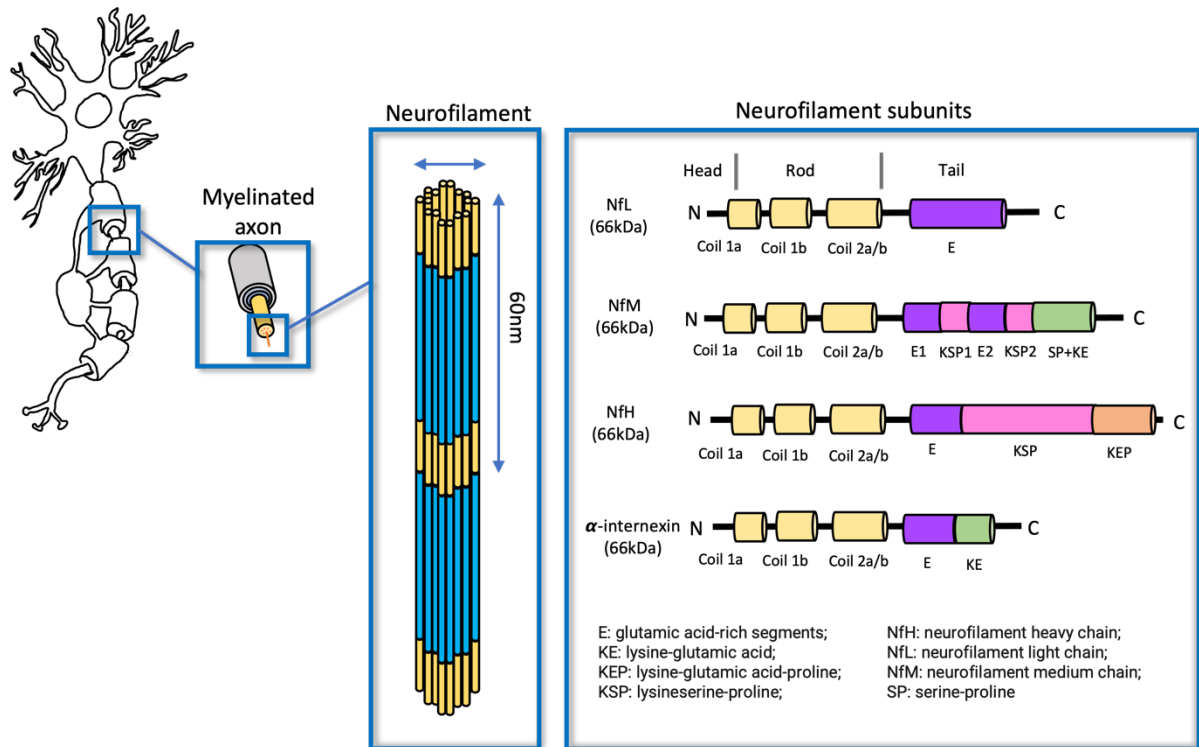


Figure 1.3 Neurofilament structural overview.

This shows a myelinated neuron which is abundant with neurofilaments (NFs) which are exclusive to neurons. NFs are part of the intermediate filament family. NFs are made up of Neurofilament heavy, medium and light and α -internexin. All of these have a globular head, a α -helical rod region that is well conserved followed by a globular tail region. Adapted from (Gaetani, Blennow et al. 2019)

The α -helical rod region is composed of hydrophobic heptad repeats, which are sets of seven amino acids that repeat along the length of the protein (Shaw, Madorsky et al. 2022). The correct arrangement of these amino acids allows for the formation of parallel dimers through interactions between similar polypeptides. These dimers form an α -helical coiled coil structure, with the hydrophobic amino acids from one α -helix interacting with those from another α -helix. This interaction results in the wrapping of the two α -helices around each other, which is crucial for the formation of the 10nm diameter NF (Shaw, Madorsky et al. 2022). In addition, the α -helical rod region contains two conserved "stutters" that break down the heptad repeat pattern, which can be considered as the insertion of extra amino acids into the pattern (Shaw, Madorsky et al. 2022). The coiled coil dimers then associate laterally and end-to-end to form higher order structures, leading to the formation of the 10nm diameter NF (Shaw, Madorsky et al. 2022). This structure is essential for the proper functioning of the

proteins in this family, and its formation is based on the interactions between the hydrophobic heptad repeats in the α -helical rod region.

NFL is the most abundant of the subunits and most soluble, and therefore useful and more reliable in detection assays (Khalil, Teunissen et al. 2018, Gaetani, Blennow et al. 2019). As stated, NFL is a protein found in the axons of neurons, and it is transported through the axon by a process known as axonal transport. This process involves the movement of NFL along microtubules, which are the structural scaffolds of axons. The NFL is moved in both directions along the axon, with slow transport moving towards the cell body and fast transport moving towards the axon terminals (Gaetani, Blennow et al. 2019). When there is injury or disease, the axonal integrity may be compromised and the NFL is then released into the extracellular space, the interstitial fluid (Khalil, Teunissen et al. 2018, Gaetani, Blennow et al. 2019). The NFL released into the extracellular space can diffuse into the CSF and the bloodstream (Gaetani, Blennow et al. 2019) (Figure 1.4).

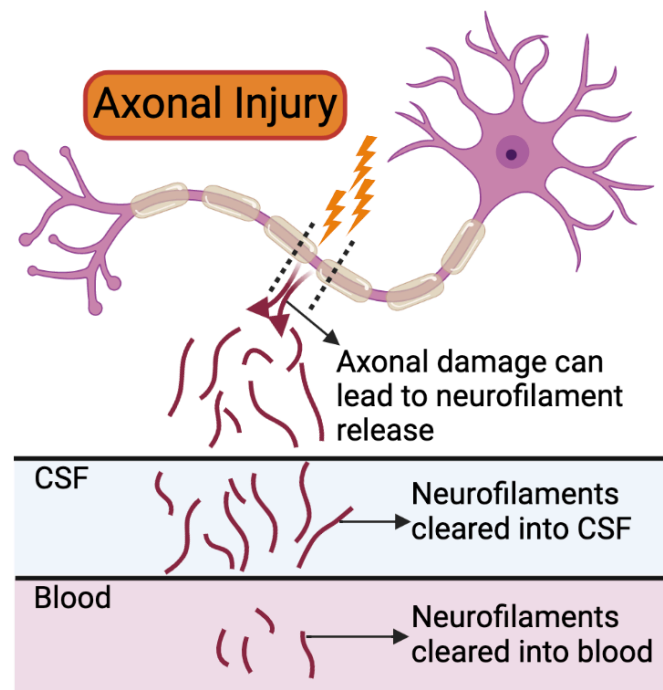


Figure 1.4 Schematic of axon releasing neurofilaments into the CSF and blood stream

(Made in Biorender.com)

Once NFL is present in the CSF, it can be transported to the blood stream. NFL can then be measured in the CSF and blood as a biomarker of axonal injury and neurodegeneration. Blood levels of NFL are lower than those in the CSF (Gaetani, Blennow et al. 2019, Barro, Chitnis et al. 2020). It is of great benefit that the NFL blood levels seem to correlate well with the CSF levels (Churchill, Caverzasi et al. 2019, Graham, Zimmerman et al. 2021) as in a clinical setting to get CSF samples it requires an invasive lumbar puncture, and therefore has been rarely performed longitudinally or systematically (Khalil, Teunissen et al. 2018). However, the exact process of how CSF levels affect the blood is still unclear. Nevertheless, due to the positive correlation between CSF and blood levels, it can be preferable to sample blood to quantify NFL as this is much less invasive. Therefore, it makes blood sampling very appealing for TBI and other neurological disease diagnosis as NFL levels can be obtained longitudinally and quickly (Barro, Chitnis et al. 2020, Shahim, Politis et al. 2020, Graham, Blissitt et al. 2023).

The detection of NFL can be performed using an enzyme-linked immunosorbent assay (ELISA) but it is not always sensitive enough to pick up the lowest levels of NFL. A breakthrough in detection was reported by Blennow and Zetterberg, who adapted and enabled an ultrasensitive method of detection (Barro, Chitnis et al. 2020). The single molecule array (SIMOA®) technology enabled the detection of the longitudinal changes in the blood and CSF at a group and individual level at very low concentration levels (Gaetani, Blennow et al. 2019, Barro, Chitnis et al. 2020).

1.6.1.2. NFL as a Biomarker for TBI and bTBI

NFL and NFH have been reported to be reliable markers for other neurological diseases, such as multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), and AD (Gaetani, Blennow et al. 2019, Jakimovski, Kuhle et al. 2019, Barro, Chitnis et al. 2020, Nielsen, Soares et al. 2020, Thouvenot, Demattei et al. 2020). In addition, NFL has displayed promise to be a biomarker for TBI, as it has been shown in preclinical and human TBI studies that it can be a good indicator for diagnosis and injury severity (Graham, Zimmerman et al. 2021). These previous studies tested NFL in moderate-severe injury severity cases rather than mild ones, although NFL levels have also been proven useful in mTBI. William T O'Brien et al (O'Brien, Pham et al.

2021) showed in a mild rat closed head injury model that NFL serum levels were increased 1-14 days post-injury relative to pre-injury levels, with a peak at 1 day. In a model of repeated TBI the resulting NFL elevation was once again significantly high relative to baseline. NFL showed a dose-dependent response such that the repeated injury produced much higher NFL levels than those of single mTBI. In addition, rats with the worst sensorimotor impairment had highest NFL levels, indicating that NFL could be used to identify injury severity (O'Brien, Pham et al. 2021). Such results were also observed in a human TBI study, where NFL proved to be a useful predictor of favourable outcomes and could even be used to establish complete recovery from TBI (Hossain, Mohammadian et al. 2019). In a report from Shahim et al. (Shahim, Zetterberg et al. 2017), it was also shown that NFL was capable of distinguishing between athletes that had resolving post-concussion symptoms and prolonged symptoms (Shahim, Zetterberg et al. 2017).

With this surge in NFL biomarker study in recent years, the results suggest that NFL is proving to be a sensitive biomarker for TBI, however there is very little published research on NFL in blast TBI. Therefore, it is unknown whether it can be as useful or if there are differences between a non-blast and blast TBI with regards to NFL CSF and blood levels, temporal profile and prognosis ability but studies are in process (Graham, Blissitt et al. 2023).

1.7. Hypothesis and Aims

The field of mild bTBI research still faces significant knowledge gaps concerning the causes behind the acute and chronic onset of post-blast exposure symptoms. Many studies have investigated either the acute or chronic phase of blast TBI but rarely both. This thesis sets out to investigate the neuropathology of blast TBI over a longitudinal timeframe. However, there is no shock tube set up to investigate blast TBI in England, and therefore there is a need to optimise a model to fulfil that role.

Once the blast model was optimised for investigating mild blast TBI, this thesis set out to investigate an under-researched topic in the blast literature; inhibitory GABAergic interneurons (which contribute to maintaining the balance between excitatory and inhibitory signalling (E/I balance)) and the potential effects of mild bTBI on their populations. Furthermore, it is not known to what extent these interneurons are affected by mild bTBI over the acute and chronic time points, and their potential role in causing the observed electrophysiological alterations as found by collaborators.

Additionally, the degree of axonal damage occurring in white matter following a diffuse mild blast injury is still not well-defined. It is uncertain whether axonal damage in the white matter occurs to a significant extent after a blast, and whether white matter is the main source of neurofilament light found in the cerebral spinal fluid and blood. Moreover, the sensitivity of the promising biomarker neurofilament light in detecting axonal injury in a mild blast model remains uncertain. Previous literature has reported spurious results in the context of blast injuries, highlighting the need for further investigation into the suitability of this biomarker in detecting axonal injury specifically in mild bTBI scenarios.

1.7.1 Hypotheses

- The optimised blast model produces a mild diffuse injury resulting in a mortality rate of 0%, an increase in loss of righting reflex and no change of welfare scores (Chapter 3).

- Blast TBI causes the parvalbumin but not the somatostatin interneuronal density to decrease at subacute timepoints (Chapter 4).
- There will be a neuroinflammatory response following blast TBI, manifested by increases in microglia/macrophage and astrocytic density after mild blast TBI at acute time points (Chapter 4).
- Loss of GABAergic interneurons and inflammatory response will be seen in a lateralised presentation; more damage will be in the hemisphere closest the blast wave (Chapter 4).
- Fluid biomarker neurofilament light will increase in both the plasma and the cerebral spinal fluid post-blast and peak at sub-acute timepoints and return to normal at 3 months (Chapter 5).
- Neurofilament light immunofluorescence intensity measured in the corpus callosum will decrease as a result of diffuse axonal injury and white matter damage (Chapter 5).

1.7.2 Aims and objectives

- To optimise a mild blast TBI model in rats using the Centre for blast injury studies (CBIS) shock tube, previously characterised by Dr Nguyen (Nguyen 2016).

Objective: This will be performed using the best practices set out by Dr Nguyen and the field to establish a working repeatable mild blast TBI model, using comparable parameters, such as blast over pressure, positive impulse and duration.

- To determine the longitudinal glial and neuronal pathology of the blast injury in the brain using immunohistochemistry.

Objective: This will be achieved using a general neuronal marker (antibody: NeuN), interneuronal markers (antibody: Parvalbumin and

Somatostatin) and glial (antibody: IBA1 and GFAP) markers to quantify changes in the blast model using immunohistochemistry post-blast at acute and chronic time points (6hours – 3 months).

- To analyse the extent and temporal profile of axonal injury in the corpus callosum in the blast model

Objective: Axonal damage in the white matter damage assessment will be conducted using immunostaining of neurofilament light and luxol fast blue in the corpus callosum.

- To assess a potential traumatic brain injury biomarker that could be useful for the detection, diagnosis and progression of the brain damage.

Objective: I will investigate the axonal/neuronal damage using fluid biomarker NFL quantified by the Quanterix SIMOA® Assay at acute to chronic time points in the blast model, compare the results to histological results.

2

Materials and Methods

2.1 Overview of Experiments, Analysis and Results and where to locate them within the Thesis

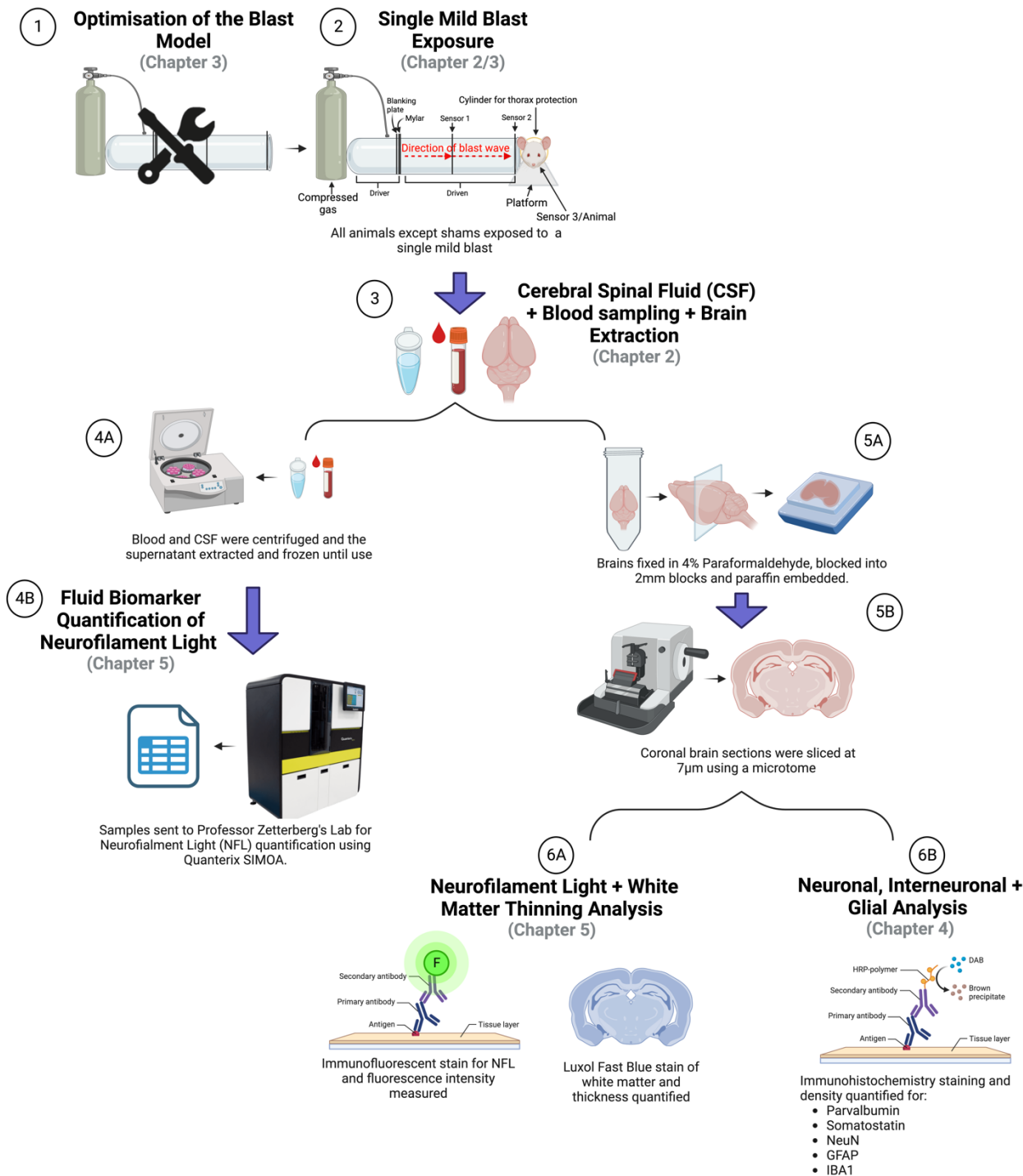


Figure 2.1 Overview schematic of the experiments and analysis performed within this thesis.

1. Depicts the optimisation of the blast model in order to achieve mild blast traumatic brain injury, this methodology and data can be found in chapter 3. **2.** Depicts the optimised model used to exposure rats to a mild blast TBI, all rats except shams were exposed to one mild TBI. Data from this is found in both chapter 2 and 3. **3.** Depicts the tissue samples from these animals; cerebral spinal fluid, blood and brain, how this was performed is found in chapter 2. The next step dichotomises and depicts how each of the samples were processed, **4A.** Blood and cerebral spinal fluid (CSF) were extracted and centrifuged. The resulting supernatant was pipetted into vials and frozen prior to being sent to Professor Zetterberg's lab at University College London. **4B.** This quantification of fluid biomarker neurofilament light (NFL) was performed using the Quanterix single molecule assay (SIMOA). The analysis and results of plasma and CSF NFL can be found in chapter 5. **5A.** The dichotomised arm depicts how the extracted brains were prepared for immunostaining and analysis. First the brains were fixed in 4% paraformaldehyde, following fixation the brains were blocked into 2mm blocks, and one of these blocks (the 4th one) was then paraffin embedded ready for slicing with a microtome. **5B.** Coronal sections were sliced using the microtome at 7µm. **6A.** Brain slices were immunofluorescently stained for NFL and the fluorescence intensity measured. Other slices were stained with Luxol fast blue to assess the white matter thickness of the corpus callosum. Results from these immunostainings can be found in Chapter 5. **6B.** Brain slices were immunohistochemically stained for neuronal, interneuronal and glial density changes in the neocortex. Quantification results are found in chapter 4. Made with Biorender.com

2.1 Animals

All animal experiments were carried out in compliance with a home office licence ([Scientific Procedures] Act 1986 and EU legislation). Male Sprague Dawley rats (Charles River, Margate, UK) were housed in individually ventilated cages of four animals. The cages included enrichment such as cardboard tunnels, wooden bite brick and paper shredding. The facility provided a 12 hour light/dark cycle with temperature and humidity control. Animals were provided with standard rodent chow (Rat and Mouse No.1 Maintenance, Special diets service, UK) and sterile water. Animals were kept for one week prior to procedures to acclimatise. All procedures and experiments were conducted during the light hours. Rats were weighed and observed for welfare checks at least a day prior to experiments and post-experiment for every day for two weeks (twice a day for the first three). Animals that lived beyond seven days were monitored once a week.

2.2 The Blast Model

2.2.1 CBIS shock tube blast wave generation

The blast model was a collaborative effort with Dr Cornelius Donat, Dr Michael Bruyns-Haylett and Konstantinos Tsikonofilos, although the main procedure was carried out by me.

The set-up of this model is explained in chapter 3. This section will explain briefly how the shockwave was generated with the shock tube once the model was established.

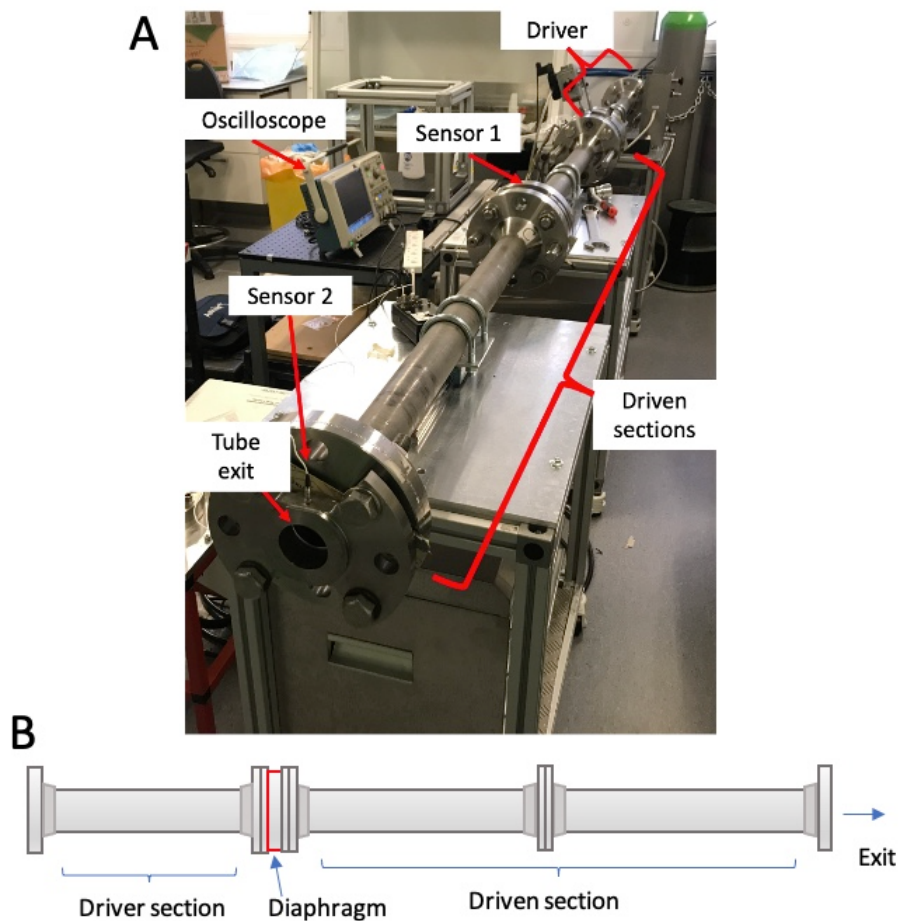


Figure 2.2 CBIS Shocktube

This shows the driver and driven section, the two sensors, oscilloscope and gas cylinder prior to the setup required for the blast experiments.

The blast wave was generated using the shock tube at Imperial College London, located at The Royal British Legion Centre for Blast Injury Studies (TRBL-CBIS) facility in the bioengineering building. As shown in Figure 2.1 and Figure 2.3 the shock tube is made up of three separate tubes of about 1.22m and a diameter of 59mm (Nguyen 2016). One tube is the driver section whilst the other two are driven section, which are joined with flanges with holes in the middle that have the same diameter as the tubes and bolted together. The flanges have pressure transducers, which are embedded in them, so that they are perpendicular to the pressure wave and flush with the internal side of the tube. The driver and driven sections are divided by a double breach diaphragm assembly so that only 7% of the driver tube was used to generate a Friedlander waveform (Nguyen 2016).

When this waveform/shock wave is simulated in this shock tube, a Mylar® diaphragm is placed between the driver and driven section. This Mylar® diaphragm seals the 7% assembly volume off from the driven section. The 7% volume is filled with natural gas from a cylinder that is released by the experimenter using a turn wheel at a control panel. As the air from the cylinder is filling the 7% volume, the pressure inside it increases. The pressure increase starts to press against the Mylar® diaphragm that divides the driver tube from the driven tubes. As a result, the Mylar® diaphragm starts to bulge. When the pressure of the gas reaches a certain threshold the Mylar® diaphragm bursts and releases the pressurised air down the driven tubes and towards the exit of the shock tube (Nguyen, Wilgeroth et al. 2014, Nguyen, Sory et al. 2018, Nguyen, Pearce et al. 2019). As the pressurised gas moves down the tube it becomes a Friedlander wave form (Nguyen, Wilgeroth et al. 2014, Nguyen 2016).

For our animal model using the CBIS shock tube, the pressure transducers (sensors 1 and 2, (Figure 2.2 and Figure 2.3) and when used, sensor 3, were connected to an oscilloscope through a current source and sampled at a rate of 25million-samples/s for 4ms, which was triggered by a rising edge in the first sensor. The waveform recordings from these sensors were converted into a pressure against time plot with the calibration factor that is provided by the manufacturer for each sensor, using a custom-made MATLAB script. The readings were converted into kilopascals (kPa).

We required 250 μm Mylar® diaphragms to produce our Friedlander waveform of choice. The bursting pressure of this tube is 18.2bar (abiding safety regulations). We burst at an average 17.55 ± 0.06 bar for all animal experiments.

To safely place an animal at the end of the shock tube to induce blast traumatic brain injury, a platform, as shown in Figure 2.3 and chapter 3, with an exit tube hole flush to that of the exit of the shock tube was bolted on. On this platform, we placed a black sorbathane cover for protection against blunt injury to the rat's head. On top of the sorbathane we placed a 1cm thick PVC tube for thoracic protection of the rat and fastened it to the platform. During the troubleshooting and development of this model, we put a third sensor where the rats head would be (as described in chapter 3), but this sensor was not present during *in vivo* experiments.

2.2.2 Blast Exposure

The optimisation of this model is described in chapter 3.

Animals were weighed and then anaesthetised with 5% isoflurane ($\sim 2.5\text{mL/min}$) and once induced the animal was moved to monitoring platform where they were maintained using 1.5-2.5% at $\sim 2\text{ mL/min}$. Animals were provided with at least one day of jelly (Hartley's strawberry, 1:2 jelly/water) prior to blast injury experiments. Analgesia was administered 30 minutes prior to blast; buprenorphine (0.05 mg/kg subcutaneously (s.c); Vetergesic, UK) as was eye lubrication. A rectal probe was used to maintain body temperature at 37°C that fed into a responsive heating pad. Breathing rate was monitored using the monitoring platform and on occasion manual counting to confirm. Blood oxygenation was monitored using pulse oximeter that was integral to the monitoring platform.

The animals breathing was allowed to stabilise under anaesthesia for 20 minutes. Animals were then placed into a modified piping bag to keep limbs from obstructing breathing or getting caught in the blast apparatus. Masking tape was then wrapped around the ears of the rat to prevent compression injury that resulted from the movement of the blast.

Blast Characteristics

Compression gas	Compressed Air
Mylar burst membranes	250 μm
Shock tube inner diameter	5.9 cm
Burst pressure of Mylar (kPa)	1753.98 $\pm$ 9.16
Sensor 2 incident peak pressure (kPa)	232.72 $\pm$ 0.91
Sensor 2 impulse (kPa ms)	109.96 $\pm$ 0.76
Sensor 2 positive duration (ms)	1.38 $\pm$ 0.01
Shock wave speed (m/s)	560.52 $\pm$ 0.84
Sensor 3 total pressure (kPa)	310 $\pm$ 10.90
Sensor 3 impulse (kPa ms)	245 $\pm$ 15.99
Sensor 3 positive duration (ms)	2.6 $\pm$ 0.34
Rat snout distance from shock tube	4.5 cm
Rat protrusion distance from PVC thoracic protection tube	5 cm

Table 2.1 Shock wave parameters from all live thesis blasts

This table displays what gas was used, the mean blast over pressures, impulses and durations of all animal blast experiments in this thesis. Positions of the rat head is displayed. Data is presented as mean $\pm$ SEM

Once the animals were stable under anaesthesia, they were moved into a protective PVC shield that was placed on the blast platform outside the end of the shock tube, off-centre to the tube exit to prevent blast lung injury. Rats were placed such that their snout was 4.5cm and their right eye was 4cm from the exit and 5cm out from the protective shield. Blood oxygenation was monitored whilst the animal was on the blast platform, and breathing was manually counted prior being fixed from the torso down in the prone position, inside the protective tube, whilst the head free to move with the shockwave. The shockwave was generated by the gas-powered shock tube and hit the right lateral side of the animal's head.

The blasting parameters (see details in Table 2.1) were averaged for all animal experiments.

After blast, the animal was removed and placed on a heat mat on their side, oxygen was provided, and levels monitored during recovery. 3mL of sodium chloride 0.18% and glucose 4% saline solution for Infusion (Baxter) was given s.c.

Loss of righting reflex (LORR) was measured from the time of the blast until the animals had all four paws touching the ground in the recovery cage. LORR was used as a proxy for loss of consciousness (LOC), which is used in humans. After blast, animals were given wet mash for food in case of difficulty eating. Once the animals had fully recovered, they were returned to single individual cages and monitored until the end of the experimental day, when they were returned to their cage of four. Analgesia (buprenorphine) was added to the jelly (0.3 mg/kg, oral administration (p.o)) for 3 days following injury, as published previously (Donat, Yanez Lopez et al. 2021).

Animals were monitored daily, using welfare scoring system based on Morton and Griffiths 1985 guidance (Morton and Griffiths 1985) and weighing once a day for two weeks, followed by once a week checks until termination.

2.2.3 Highspeed video

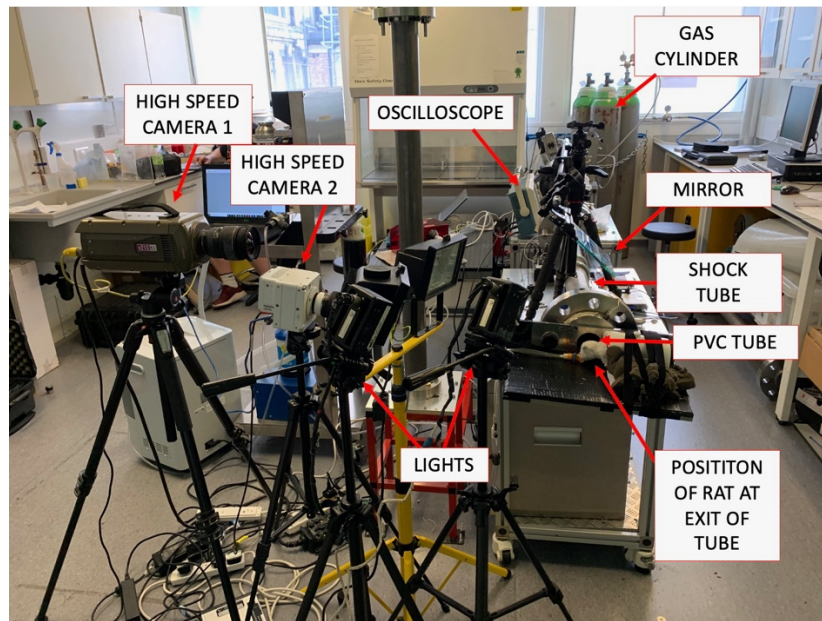


Figure 2.3 Two highspeed camera set up around the exit of the shock tube.

It can be seen that one camera facing the animal on the platform, the second is facing a mirror to get top-down plane of view. Other aspects of the shock tube are visible in the set up such as the positioning of a plush rat, the PVC tube, gas cylinder and oscilloscope.

High-speed videos were taken during the *in vivo* experiments to analyse the head kinematics of the blasted animals. These videos also allowed assessing the variability of the shock among the different animals, and whether this was different from cadavers (shown in chapter 3). Figure 2.3. Two highspeed camera set up around the exit of the shock tube.

It can be seen that one camera facing the animal on the platform, the second is facing a mirror to get top-down plane of view. This figure shows that two planes of view that were captured for some animals, although for most cases it was only one plane. One camera (Phantom v210) was directly opposite the animal and faced the animal head-on, so that it contained the x and y movement. This was the view that was captured for all blast experiments. Another camera (Phantom VEO710L camera, AMETEK, United States) was placed at a higher position and opposite the animal's face. However, it was pointing towards a mirror, which was placed above and 45 degrees to the top of the animal's head, so that it could catch the z and the x planes of motion. The cameras filmed at 10,000 fps and settings were made in the Phantom

Cine (PCC) v1.3. Data was processed and used to acquire initial displacement results using Physlets Tracker 5.1.5 (project source of: Open Source Physics www.compadre.org/osp, downloaded from <https://physlets.org/tacker>).

2.3 Blast Experimental Design

The timeline for the blast experiments is shown in Figure 2.4. It represents the experimental timeline conducted for the work presented in this thesis. There was another cohort of animals that were performed and used for electrophysiological experimentation by collaborators Dr Michael Bruyns-Haylett and Konstantinos Tsikonofilos, and their work can be found in The Supplementary Material and is not displayed in this figure.

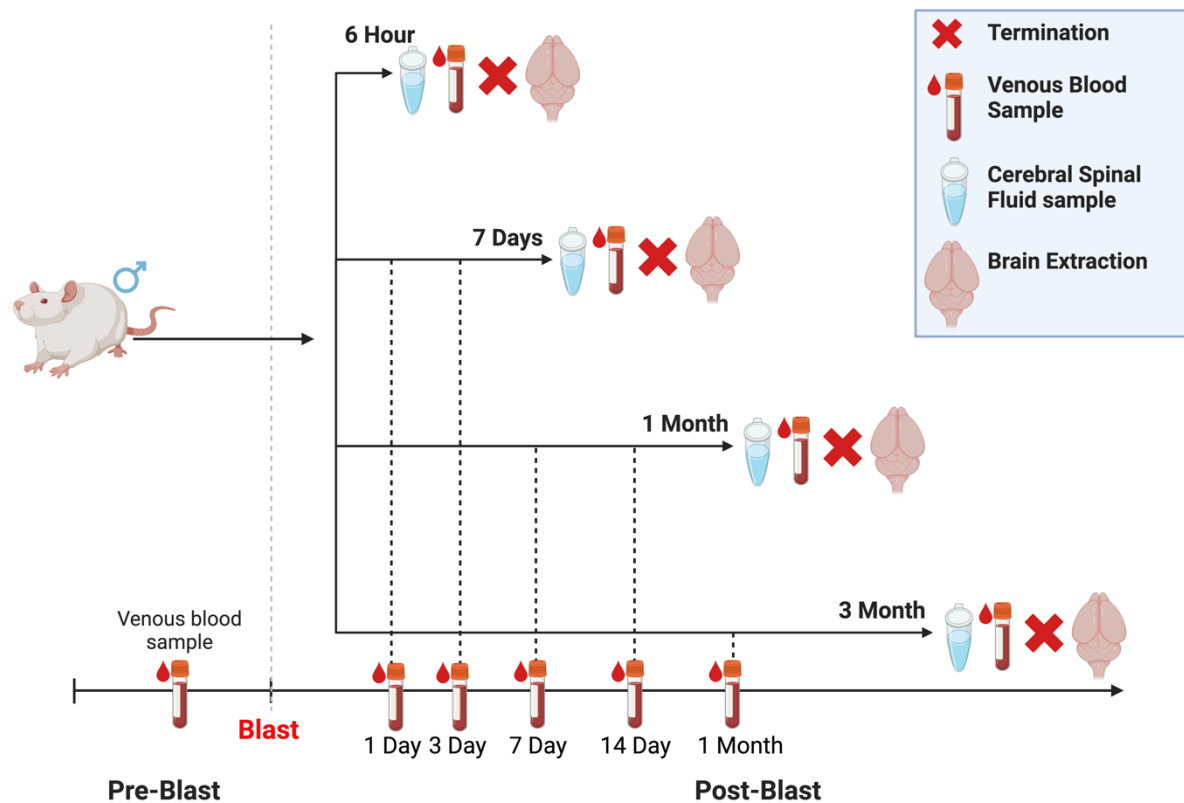


Figure 2.4 Experimental timeline of the blast experiment.

Male Sprague Dawley rats were allowed to acclimatise for at least 7 days. Baseline venous blood samples were taken pre-blast as shown by a red vial with a blood droplet. All animals except shams underwent one single mild blast exposure. Blood samples were serially taken for all groups except the 6 hours post-injury as the only other blood sample taken for this group was at time of termination. Serial blood sampling is represented by dotted lines for each post-injury time point group. Cerebral spinal fluid (CSF) and final venous blood samples were taken at time of termination for each group together with brain extraction. Made in Biorender.com

The animals that were used for this thesis were allowed to acclimatise pre-blast for at least 7 days. All animals had a pre-blast baseline venous blood sample. All animals except shams were subjected to a single mild blast and were assigned to their timepoint groups that consisted of 6 hour, 7 days post injury (DPI), 1 month and 3 months post-injury). For each group, except the 6 hour post-injury, serial blood samples were taken. The times used were pre-blast, 6hour 1 DPI 3DPI, 7 DPI, 14 DPI, 1 month and 3 months post-injury. At the time of termination (6 hour, 7 Days post injury (DPI), 1 month and 3 months post-injury) final venous blood sample and cerebral spinal fluid was sampled followed by brain extraction as depicted in Figure 2.4.

2.4 Fluid biomarker extraction and analysis for the blast

For the blast model, tail vein blood sampling took place at baseline (prior to injury), 1, 3, 7, 14, 1 month and 3 months post-injury (depending on which time point group the animal was in). Final blood sample was taken before perfusion and IP injection. The blood samples were spun in a centrifuge at 2000G for 10 minutes and then the blood plasma was extracted and placed into -20 freezer. Blood samples were taken for the histological blast cohort of animals only (Table 2.2).

Time point	Blast/Sham	Total CSF	Total Plasma	Number of plasma samples/animal
Naïve	Naïve	5	1	B
6hr	Sham	6	6	C
6hr	Blast	10	10	C
7 Day	Sham	6	6	D
7 Day	Blast	6	8	D
3 Month	Blast	5	5	E

Number of plasma sample/animal Key:

A = none

B= Naïve = 1 sample/animal

C = baseline + 6hr = 2 samples/animal

D = baseline, 1,3 and 7 day = 4 samples/animal

E = baseline, 1, 3, 7, 14, 1 month and 3 months = 7 samples/animal

Table 2.2 Samples used for analysis of NFL protein levels in the blast cohort.

CSF was collected at time of termination and therefore there was a single CSF sample per animal.

Plasma key shows how many samples were used per animal from which timepoints.

Cerebral spinal fluid (CSF) was extracted at the last time-point from the cisternae magna using a 30G needle, 28mm tubing and a 1mL syringe. Once the CSF was extracted, it was put into the centrifuge at 2000G for 10 minutes, then separated from the pellet and stored into a -20 freezer. CSF was sampled from all animals (Table 2.2).

Neurofilament Light (68kb) expression was analysed in the blood plasma and CSF of 49 of the cohort of animals used for histology (Table 2.2), using an ultra-sensitive Quanterix Simoa-HD1 platform. Samples were measured in duplicate by a technician at UCL in Dr Henrick Zetterberg's lab. Mean results were used for analysis. Blood plasmas were diluted 1-40 and CSF was diluted 1-100.

2.5 Histopathology

List of primary antibodies

Name of Primary Antibody	Catalogue #	Supplier	Species	Dilution used
Somatostatin (SST)	ZRB1042	EMDMillipore Corporation	Rabbit	1:100
Neurofilament Light (NFL) (intact and damaged)	171-002	Synaptic Systems	Rabbit	1:200
Neurofilament Light (NFL) (intact polypeptide)	RPCA-NF-L-Ct	Encor biotech	Rabbit	1:2500
Neurofilament Light (NFL) (binds to unmasked polypeptide)	MCA-1D44	Encor biotech	Mouse	1:1000
Neurofilament Light (NFL) (binds to unmasked polypeptide)	MCA-6H63	Encor biotech	Mouse	1:10,000
Neurofilament Light (NFL) (binds to unmasked polypeptide)	MCA-1B11	Encor biotech	Mouse	1:4000
Parvalbumin (PV)	PV-235	Swant	Rabbit	1:10,000
Ionized calcium-binding adapter molecule 1 (IBA1)	019-19741	Fujifilm Wako Pure Chemical Corporation	Rabbit	1:1000
Glial fibrillary acidic protein (GFAP)	Z0334	Dako`	Rabbit	1:10,000
Neuronal nuclear protein (NeuN)	MAB377	EMD Millipore Corporation	Mouse	1:1000
Fibrinogen	A0080	Dako	Rabbit	1:2000

Table 2.3 Primary antibodies used for immunostaining

List of secondary antibodies

Name of Secondary Antibody	Catalogue #	Supplier	Species	Dilution used	Application
anti-rat biotinylated	BA-9400	Vector Labs	Goat	1:250	IHC
anti-rabbit biotinylated	BA-1000	Vector Labs	Goat	1:250	IHC
anti-rabbit Alexa Fluor 647	A21245	Invitrogen	Goat	1:250	IF
anti-rabbit Alexa Fluor 546	A11010	Invitrogen	Goat	1:250	IF
anti-rabbit Mouse Fluor 647	A21245	Invitrogen	Goat	1:250	IF

Table 2.4 Secondary Antibodies used for immunostaining

Reagents used in immunostaining

Name of Reagents	Catalogue #	Supplier	Application
DAB	DK-4105	Vector Labs	IHC
DAB	AB64238	Abcam	IHC
ABC HRP-kit	32032	Thermo Scientific	IHC
ABC HRP-kit	PK-6100	Vector Labs	IHC
Prolong Diamond	P36970	Life Technologies Corporation	IF
Super Sensitive™ Polymer-HRP IHC Detection System/Mega Volume (SS kit)	QD430-XAKE	Biogenex	IHC
Luxol Fast Blue (LFB)	315390	RAL Diagnostics	IHC
Haematoxylin Mayer's	HS315-1L	Sigma Aldrich	IHC
Haematoxylin Mayer's	HEMM-35/21	Solmedia	IHC
ImmPRESS anti-rabbit IgG polymer kit, peroxidase	MP-7401	Vector Labs	IHC
Cresyl Violet	HD1285	Raymond A Lamb	IHC

Table 2.5 Reagents used in immunostaining

Immunostaining procedure

Investigating	Primary antibody or reagent used	Method
Axonal and integrity	NFL (Synaptic Systems) Blast tissue	Primary + IF secondary
Axonal and integrity	NFL (Encor)	Primary + IF secondary
White matter thinning	LFB	Overnight Incubation
Interneurons	SST	Primary + Immpress + DAB
Microglia	IBA1	Primary + Immpress + DAB
Interneurons	PV	Primary + ABC HRP-kit + DAB
Astrocytes	GFAP	Primary + SS kit + DAB
Neurons	NeuN	Primary + SS kit + DAB
Cytoarchitecture/lesions	Cresyl violet	Same day incubation

Table 2.6 Immunostaining procedure

2.6.1 Tissue Processing

For the blast exposed brains extracted for the histological analysis, the brains were incubated with 30% sucrose between 24-48h at 4°C, to process them as fixed-frozen. However, after more than six months troubleshooting this protocol, and slicing the frozen brains on a

cryostat, I observed that the brain slices were falling off during immunostaining. Therefore, I decided to process the brains in paraffin, to preserve the tissue integrity, and therefore no longer kept the blast brains in sucrose. The tissue processing was identical to the protocol reported by Dr Cornelius Donat in (Donat, Yanez Lopez et al. 2021), as follows:

The blast brain samples that were PFA-fixed brains ($n = 40$) were cut in 8 different blocks (2mm thick) using a 3D-printed brain matrix, as previously described in (Donat, Yanez Lopez et al. 2021) and shown in Table 2.5 and kept in PBS (with 0.05% NaN_3) at 4°C. The fourth block, containing the site of EEG changes for the blast model (Supplementary material: Collaborative project blast TBI electroencephalogram results), was embedded in paraffin wax. The it was the block that was closest to the blast.

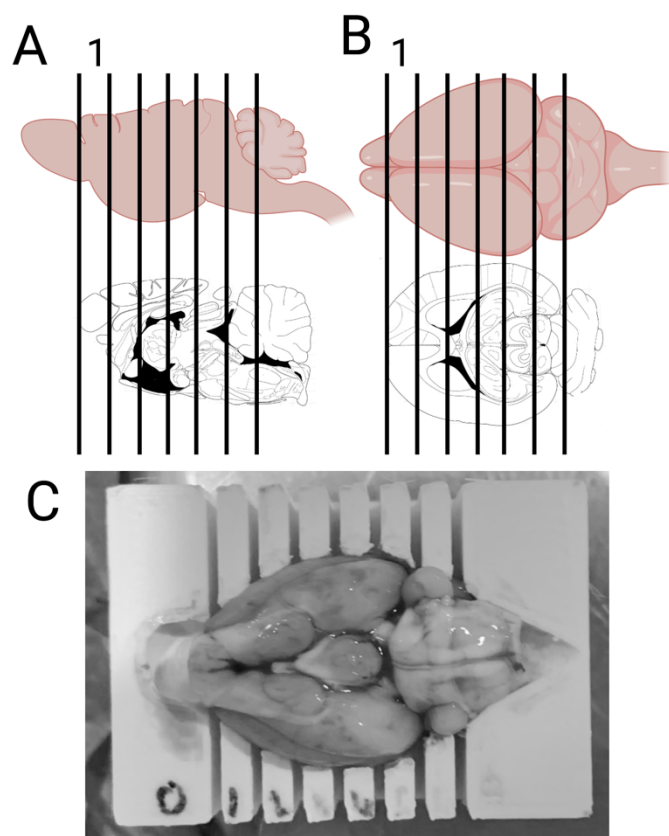


Figure 2.5 Schematic of brain blocks and 3D printed matrix with rat brain.

A) and B) show where on the brain the blocks were cut approximately (2mm blocks). C) Actual 3D brain matrix with rat brain.

Tissue was sliced using a Leica microtome (Shandon Finesse E+, Thermo Fisher Scientific, UK) at 7 μm thickness and collected on separate slides (Superfrost Plus, VWR, ref: 631-0108). Every 5th slide was selected for each stain. Thus, there was roughly 100 μm between sections. There were 3 sections per animal used for each type of staining (antibody or staining type) and analysis. For the CCI, 3 sections were mounted per slide and every 5th was collected onto Super Frost Plus slides (Thermo Fisher Scientific, UK).

2.5.2 Immunostaining

2.5.2.1 Immunofluorescence (IF)

Immunofluorescence staining was carried out for NFL as previously described (Donat, Yanez Lopez et al. 2021). Sections were deparaffinised and rehydrated and then subjected to antigen retrieval with 0.01M citrate buffer in PBS in a steamer for 20 minutes. Following this, the slides were washed and incubated with blocked with 10% FBS/10%NGS/1%BSA + 1% Triton-100-X (PBS-Tx) (w/v) for 2 hours. After this incubation, the slides were washed with PBS and incubated with the primary antibody (Table 2.3). After overnight incubation, the sections were washed and incubated with secondary fluorescent antibody in 2%FBS/2%NGS or 2% NHS/0.2%BSA 1% + 1% Triton-100-X (PBS-Tx) (w/v) (Table 2.4) and protected from light for 90 mins. Following incubation, the slides were washed and incubated with 4',6-diamidino-2-phenylindole (DAPI) 1:1000 for 5 min to visualize the nuclei, then washed and mounted with Prolong. Finally, the slides were sealed with all-purpose nail varnish and cured overnight at 4° C.

2.5.2.2 Luxol fast blue staining

White matter was stained using Luxol fast blue (LFB), which recognises myelin, as previously described (Donat, Yanez Lopez et al. 2021). Paraffin slices were dewaxed and rehydrated using xylene and decreasing concentrations of ethanol (100%, 90% and 70%) finishing with deionized water (dH_2O). The tissue was then incubated in Luxol Fast Blue (Table 2.5) at 60°C

overnight, but no more than 16 hours. The next day tissue was washed with 10 dunks in 100% ethanol followed by 10 dunks in dH₂O. The tissue was destained using saturated lithium carbonate, whereby the white matter should appear blue and the grey matter colourless (20-55 dunks), followed by a dH₂O wash and tap water wash. The slides were then counterstained using haematoxylin from Sigma Aldrich (Table 2.5) followed by tap water wash to stop and develop the blue. Finally, sections were dehydrated using increasing concentrations of ethanol and xylene and coverslipped with permount (Sigma Aldrich).

2.5.2.3 Cresyl Violet staining

The whole brain was investigated for anatomical changes, mass lesions or signs of mass neuronal loss during the blast optimisation. Cresyl violet stains stained the nuclei and Nissl bodies (rough endoplasmic reticulum). Paraffin slices were dewaxed and rehydrated using xylene and decreasing concentrations of ethanol (100%, 90% and 70%, 5 minute incubations) finishing with deionized water (dH₂O). The tissue was then stained in Cresyl Violet Acetate 0.5% for 2 minutes followed by 2 minutes in dH₂O. The tissue was then destained using 70% and 95% ethanol respectively (5 min each). A 5 minute differentiation incubation followed using 95% Acetic ethanol. Finally, sections were dehydrated using increasing concentrations of ethanol and xylene and coverslipped with permount (Sigma Aldrich).

2.5.2.4 General 3,3'-diaminobenzidine (DAB) staining

Immunohistochemical staining protocols using DAB varied slightly for each antibody as different incubation times and staining kits were used. Specific methodologies can be found in the Appendix. However, they follow a similar series of steps as described below.

- Paraffin sections were deparaffinised and rehydrated using xylene and decreasing concentration of ethanol.
- The tissue was then washed in PBS followed by permeabilisation and endogenous peroxidase quenching using 1% Hydrogen Peroxidase (H₂O₂) in 1x PBS + 1% Triton-100-X (PBS-Tx) (w/v) for 30 minutes.

- The slides were washed in PBS and underwent antigen retrieval in 0.01 M Citrate Buffer (pH 6) for 20 minutes in a steamer.
- Slides were then washed and incubated with a blocking solution containing animal serum that is the same as the secondary antibody (kit dependent, see Appendix).
- Following a kit dependent blocking step, the slides were incubated overnight at 4°C (or room temperature (IBA1)) with the primary antibody (Table 2.3).
- On the following day, the primary antibody was washed off with PBS, followed by incubation with the biotinylated secondary antibody in the appropriate diluent (diluted blocking solution, kit dependent (see Appendix)) (Table 2.4) for 90 min.
- The slides were then washed and incubated with either a avidin-biotin complex (ABC) solution, or a different visualisation kit (Impress or Super Sensitive).
- The slides were then stained with DAB (Table 2.5 and Table 2.6) and then washed with tap water.
- Some protocols were followed with a counterstain (see Appendix).
- Slides were then put through steps of dehydration and coverslipped.

2.5.2.5 Double Staining of Interneuronal Markers with NeuN to Confirm the Specific Location in Neurons

To ensure that the GABAergic interneuronal antibodies did indeed bind to neurons, and did not bind to other cells types, or had cross-reactivity and bound to all neurons, a simple test of co-localising the PV and SST antibodies with NeuN was conducted on Naïve rat brain tissue. The images in Figure 2.6 showed a clear co-localisation of both interneuronal markers on a sub-set of neurons, indicating they specifically recognised that interneuronal type (Table 2.6

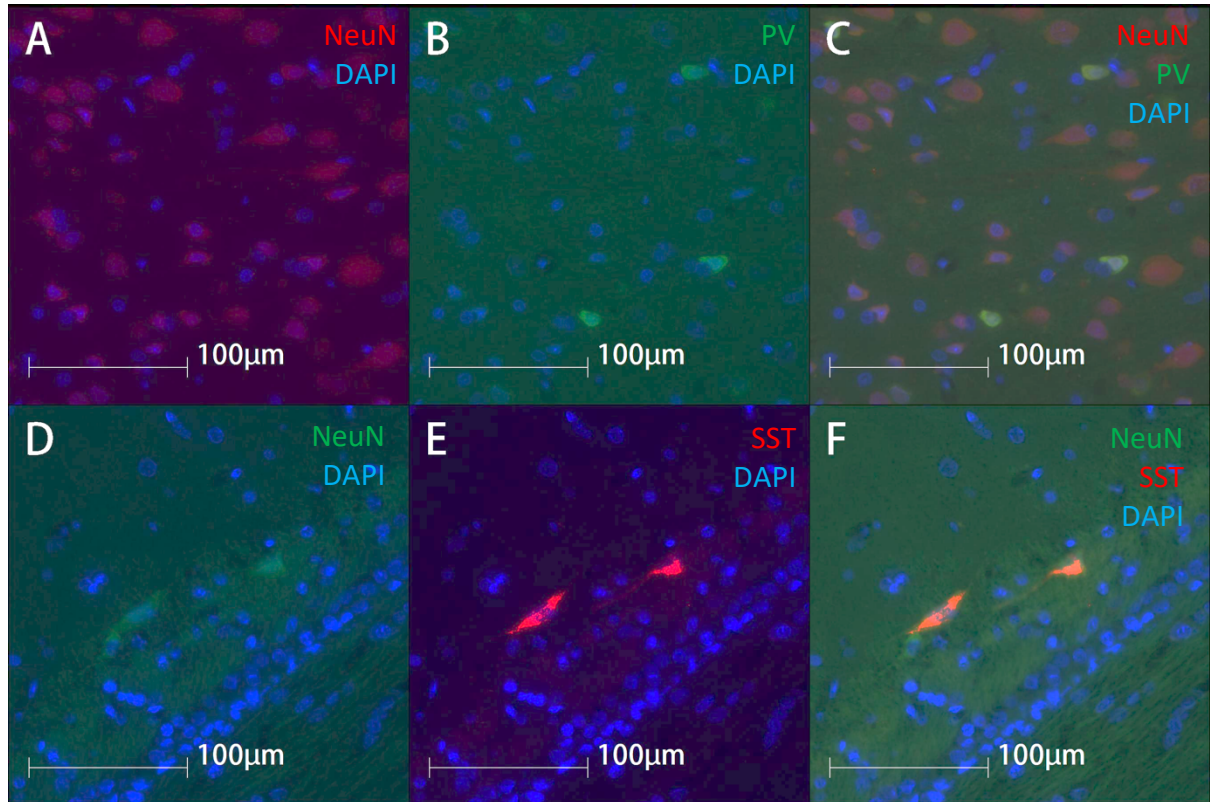


Figure 2.6 immunofluorescent staining of the brain of NeuN and Parvalbumin (PV) colocalised and NeuN and somatostatin (SST) colocalised.

A) NeuN AF647, and DAPI AF405, B) PV AF 488, DAPI 405, C) Colocalised NeuN AF647, PV AF488 and DAPI AF405 on Parvalbumin GABAergic interneurons. D) NeuN AF488, DAPI AF405 E) SST AF647, DAPI AF405, F) Colocalised NeuN AF488, SST AF647, DAPI AF405 on somatostatin GABAergic interneurons

2.5.2.6 Image Acquisition

Whole stained slices for light-microscopy (LFB, IBA1, GFAP, PV, SST and NeuN) were imaged at 20x with a slide scanner (Zeiss Axioscan Z1 with a Plan-Apochromat 20x/0.8 NA, Zeiss, Germany) as previously described (Donat, Yanez Lopez et al. 2021).

For the Immunofluorescence images, half sections of the blast IF staining, including whole corpus callosum were acquired using the tile function on the LSM 780-inverted confocal laser scanning microscope (Carl Zeiss), and Zen software (Car Zeiss) at 10x.

2.5.2.7 Image Quantification, and Analysis

2.6.2.7.1 Regions of Interest

For both TBI models, the drawing the regions of interest (ROI) in the brain were carried out using HALO® software. The primary auditory (Au1), primary visual (V1) and retrosplenial cortex (RSC) plus the corpus callosum (CC) were chosen regions of interest, as depicted in the figure below (Figure 2.7) (Paxinos and Watson 2007). The ROIs were analysed separately for both hemispheres, and later combined due to the lack of statistical differences between the hemispheres. The ipsilateral hemisphere was considered the same hemisphere as the oncoming blast wave, and the contralateral side is the opposite side from the oncoming blast wave.

These particular Regions of interest were selected because of the following reasons:

- the **Corpus Callosum** is the largest white matter tract in the rodent brain, is clinically relevant to humans and is sensitive to axonal injury. If there was to be any white matter damage, it was likely to been seen there.
- The **primary auditory cortex** was chosen because it was directly affected by the blast shockwave. In particular, only one of the hemispheres was in direct reception of the wave. It is also in the area where the invasive electrodes for electrophysiology were placed, and the area used for the functional sounds test in the electrophysiological cohort.
- The **V1** and **RSC** were selected as they served as areas that were fully under the EEG electrodes, were at a different angle to the auditory cortex and, therefore, would help to inform whether the biomechanics/directionality of the neurons during the injury were affected by the blast.

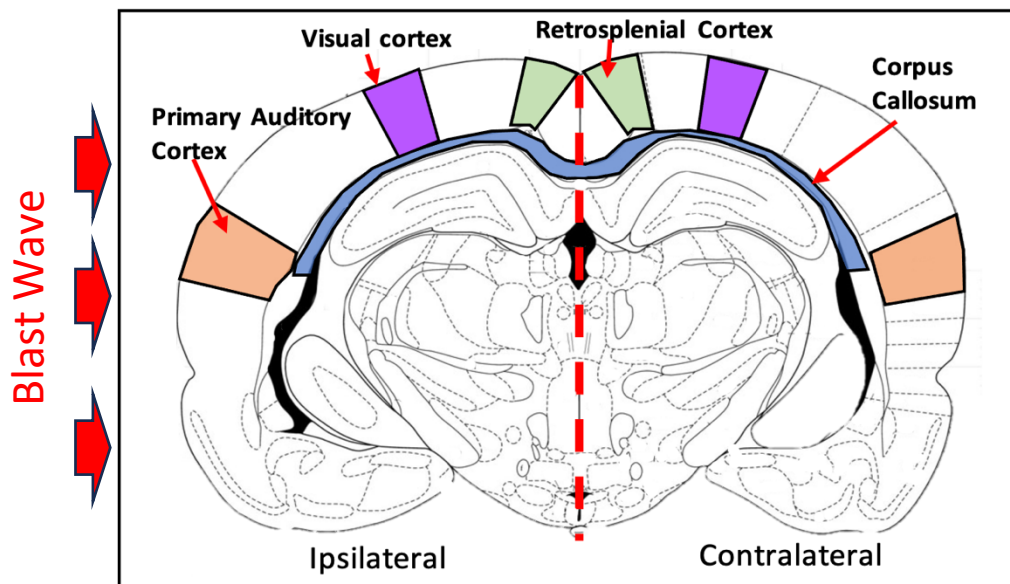


Figure 2.7 Example brain slice showing the regions of interest

A representative slice of Block 4 with all regions of interest outlined. Corpus callosum in blue. Primary auditory cortex in orange, primary visual cortex in purple and retrosplenial cortex in green. Ipsilateral and contralateral hemispheres are divided by red dashed line. Ipsilateral is considered the side of the brain where the blast wave was first received. Adapted schematic from Paxinos and Watson 2009 (Paxinos and Watson 2007)

The Au1 was identified using the hippocampus as reference and ventricle locations with the use of the atlas (Paxinos and Watson 2007). A 700µm wide box was drawn within the Au1 as the region of interest. The height of the box was drawn from the area just under layer 1 until the corpus callosum for neuronal stains and from the pia to the corpus callosum for the glial stains. This is due to the lack of neuronal cells in layer 1 of the neocortex. The box was then divided into the cortical layers similar to the literature (Rogalla and Hildebrandt 2020), which were evidenced by differing neuronal density and cell types for the PV, SST and NeuN analysis. For the GFAP and IBA1 stains, the regions were left as large boxes and not differentiated by layer, this is shown in an example drawing in Figure 2.8.

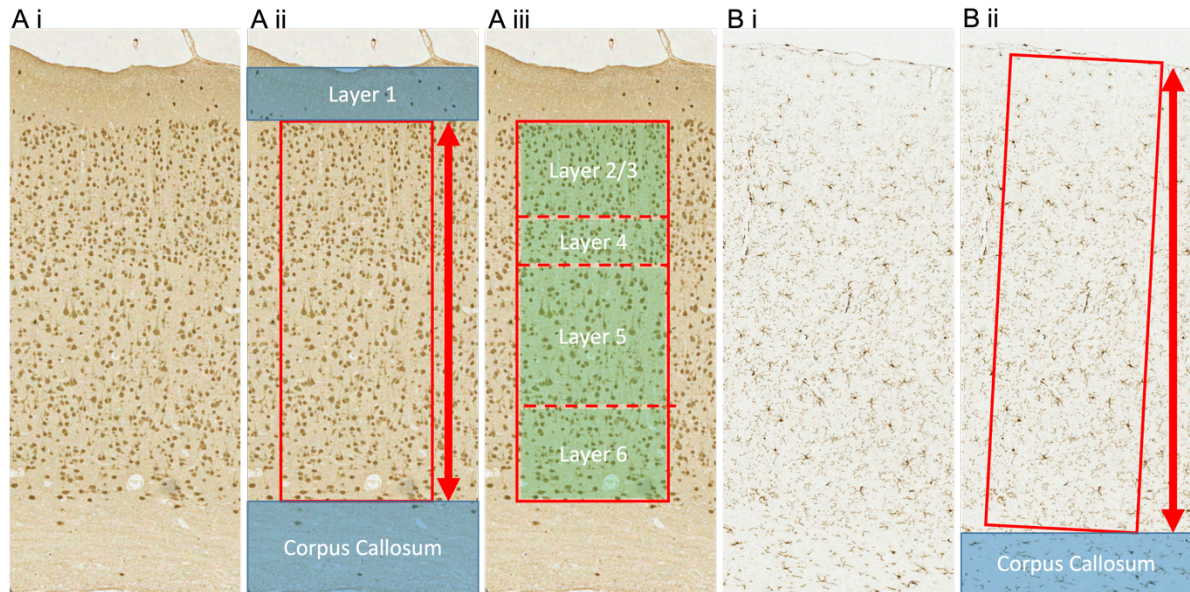


Figure 2.8 Example of region of interest in the neocortex.

Ai) Shows example neuronal staining. Aii) Shows example box that could be for Au1, V1 or RSC in the neocortex that is drawn from the bottom of layer 1 to the edge of the corpus callosum. Aiii) Shows the example laminar division where in layer 2/3 there are small stellate cells, layer 4 are high density closely packed together cells, layer 5 has larger pyramidal neurons with a few smaller neurons sparsely packed and layer 6 which has an increased density and more stellate neurons present. Bi) Example glial staining, Bii) example region of interest of the box that is from the meninges to the corpus callosum, no lamination.

The V1 was located using the Paxinos and Watson atlas, and a box of 500 μm wide was drawn (Paxinos and Watson 2007). The height of the box was drawn from the area just under layer 1 until the corpus callosum for neuronal staining and from the meninges for the glial stains. The box was then divided into the cortical layers same as stated above for the Au1 (see Figure 2.8).

The RSC was drawn using the Paxinos and Watson atlas (Paxinos and Watson 2007). A 400 μm wide box was drawn, and the height of the box was drawn from the area just under layer 1 until the corpus callosum, for neuronal stains and from the pia for the glial stains. The box was also then divided into the cortical layers, as stated above for the Au1 (see Figure 2.8).

For the blast model, the corpus callosum was analysed as a whole for the markers NFL (Synaptic System and ENCOR) as it was found that there were no differences when the corpus callosum was segmented. The whole corpus callosum was analysed for GFAP, IBA1 and LFB also.

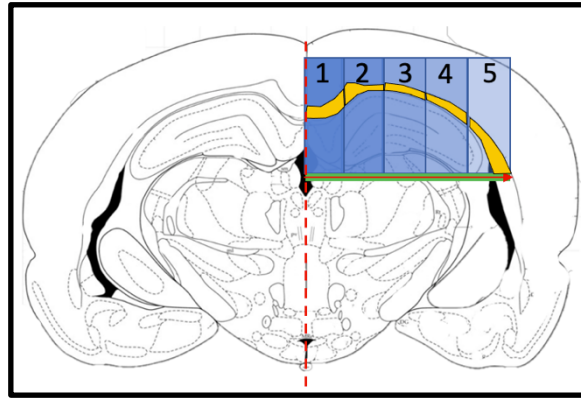


Figure 2.9 Schematic of representative brain section with the 5 segments of one hemisphere's corpus callosum .

These segments of the corpus callosum was used for analysis for one hemisphere. It was conducted for both hemispheres. The yellow segments show the outline of the white matter tract that was drawn around for analysis.

For all immunostainings and ROIs, broken or torn tissue was excluded from the analysis, as there were folds and blemishes, which would have affected the reliability of the analysis.

2.6.2.7.2 Immunostaining Analysis

Luxol Fast Blue: The CC stained with LFB was analysed whereby the CC was segmented into 5 segments per hemisphere (Figure 2.9), 7 equidistant measurements were taken per segment and averaged. Those averaged values were then averaged and displayed as a single averaged value for the CC.

IBA1: The microglial density was counted using HALO microglia algorithm, where the threshold was set to account for false positives; anything above that threshold was counted as shown in Figure 2.10. The results were normalised to their ROI area and the cell density

was shown as positive cells/mm². This density of was then normalised to the mean of the control group and presented as % from control.

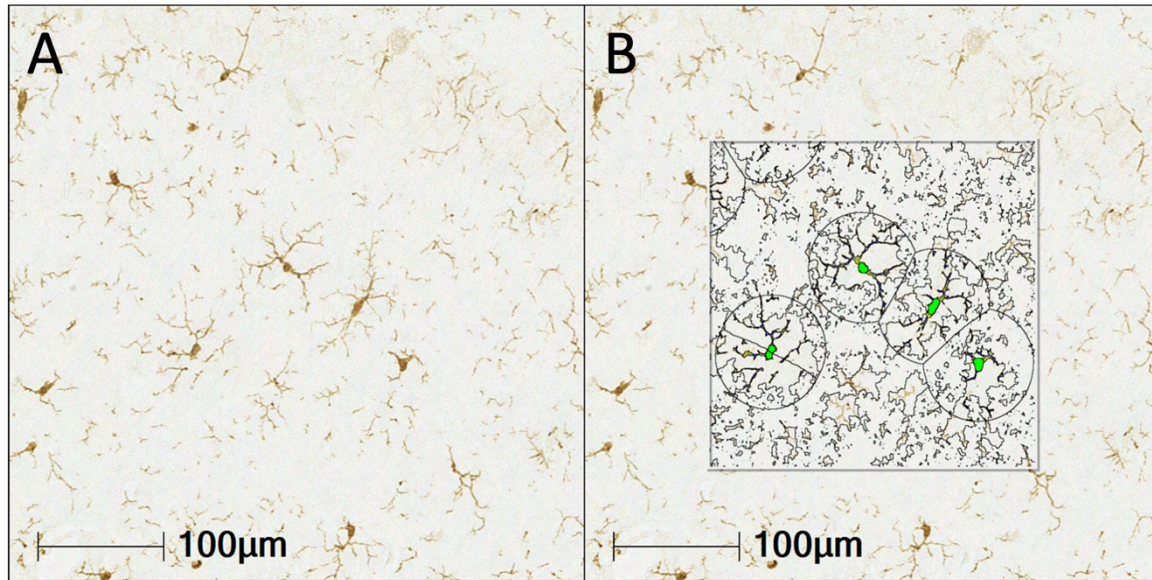


Figure 2.10 Example of microglial detection. A) Microglia. B) Microglia detected by the HALO algorithm to be counted for density.

Microglia was also analysed for changes in general morphology, like hypertrophy, by measuring the % area covered. A threshold was set on control animals (Naïve/Sham group) for each control group and then applied to their respective ROI for all animals. The % area was used because as microglia undergo activation, they can become hypertrophic and would therefore cover a greater area within their ROI, carried out in a similar way as to Figure 2.11. Results were shown as % area covered.

GFAP: The astrocyte density and morphology analysis were conducted using HALO®. The analysis of astroglial activation and hypertrophy was shown as a % area covered (Figure 2.11). A threshold was set on control animals (Naïve/Sham group) for each control group and then applied to their respective ROI for all animals. The % area was used because as astrocytes become activated, they become hypertrophic and would therefore cover a greater area within their ROI. Should there be an increase in astrocytes also, that would also be reflected in their coverage of the ROI. Furthermore, through troubleshooting, I found that the astrocytes were difficult to quantify individually and therefore I chose this methodology. The results were shown as % area covered.

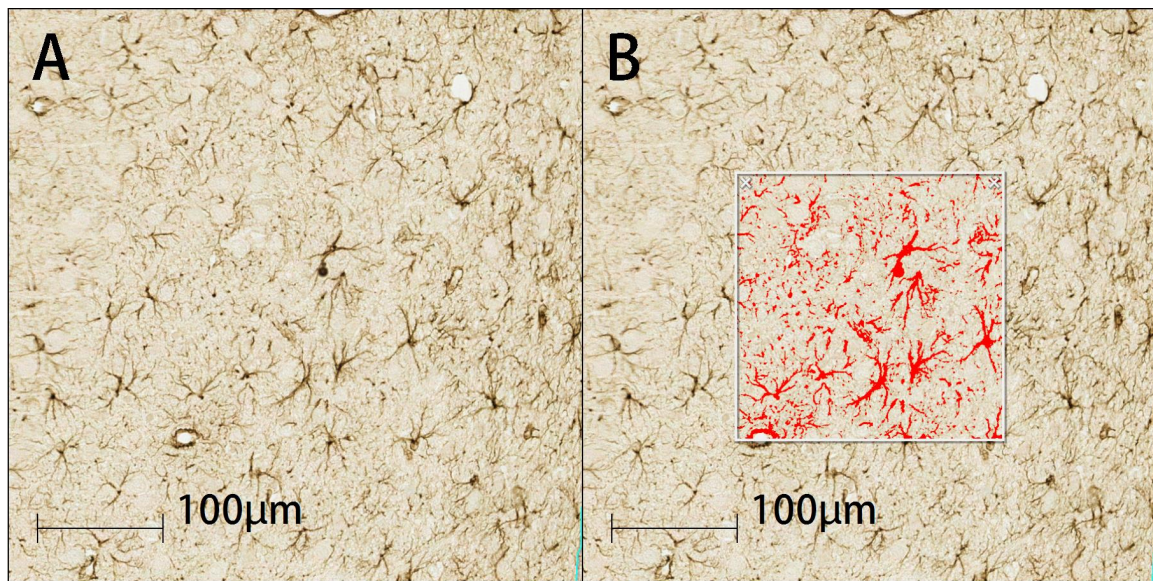


Figure 2.11 Example of astroglial detection

A) tissue segment without mask of threshold coverage that would quantify % area covered, B) example of the threshold used to analyse % area covered.

NeuN, PV and SST laminar differentiation: The laminar structure was identified fully by the NeuN staining or by the haematoxylin counterstain as shown by Figure 2.12. For layer I there was a clear lack of neurons, while for layer IV there was a clear concentration of small neurons. The height of layer IV was outlined and anything above this layer until layer I was considered layer II/III. Layer V was identified by the large uncrowded pyramidal neurons with lots of space around them. Layer VI was identified as a mixture of pyramidal neurons and smaller neurons slightly more packed together until it reached the white matter tract.

For the **NeuN** staining, the density of neurons per laminar layer and hippocampus subregion was established using an optical density threshold that separated the positively stained neurons from the background, with an idealised cell size and colour based on a representative cell (Figure 2.12). Any slight modifications to these settings were made as some slides had higher background than others. Each parameter was modified in HALO® per ROI as the neuron morphology differs slightly.

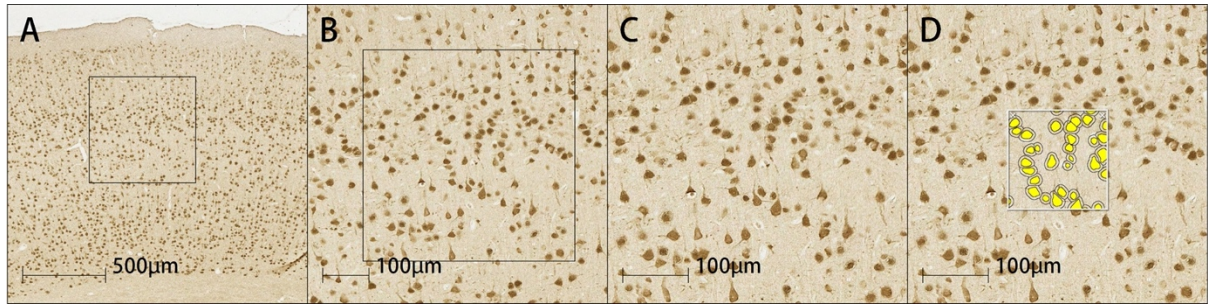


Figure 2.12 Example of NeuN cell counting and algorithm used in Halo.

A) Cortical layers evidenced, B) closer image of cortical layers, C) representative image of neurons to be selected by cytonuclear algorithm, D) example of what neurons are counted as highlighted in yellow.

In all regions and layers for the **PV** and **SST** the cells were manually counted using HALO® (Figure 2.13).

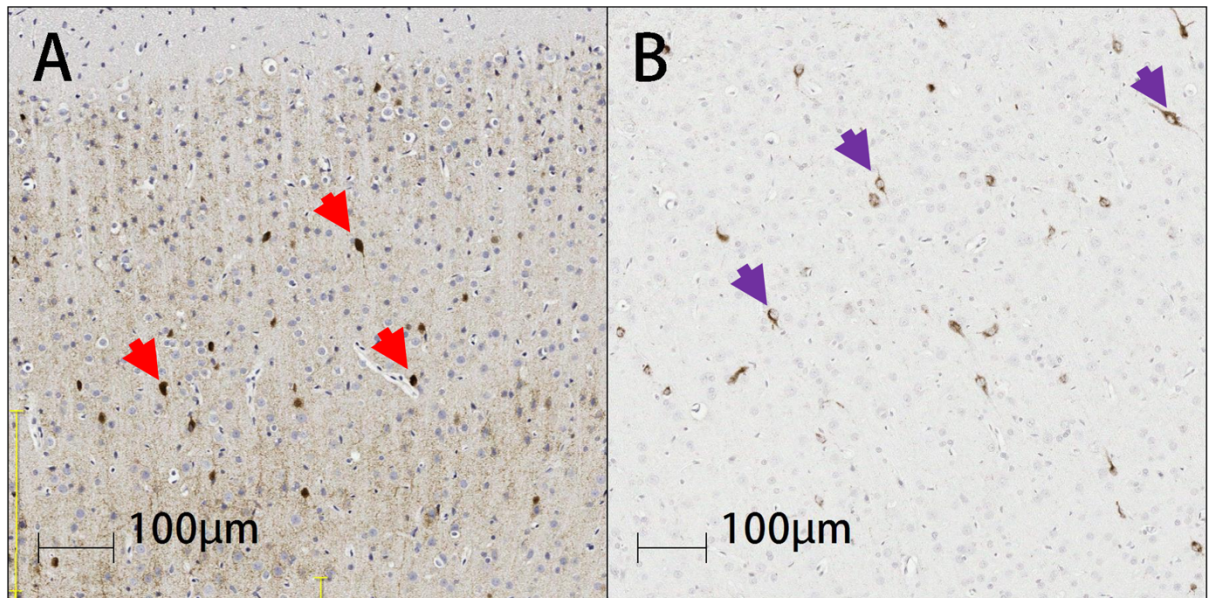


Figure 2.13. Example of PV and SST cells that would be counted.

A) Example of PV positive INs with red arrows showing example cells. B) Example SST positive INs with purple arrows showing some example cells

The results of the different types of neuronal cell counts were normalised to their region of interest area giving the cell density as positive cells/mm². This density was then normalised to the mean of the control group and presented as % from control.

NFL from Synaptic Systems (SySy): The NFL analysis was conducted using HALO® by a fluorescence intensity analysis algorithm. The pixel intensity was measured and reported for the corpus callosum that was defined as described above. For each hemisphere for each animal there was a small reference box drawn in the CA1 of the hippocampus and the periaqueductal grey area (PAG) of the midbrain. Two areas were selected and compared as there was need for an internal fluorescence control. The results were normalised to each reference area (CA1 or PAG) and presented as % change from reference area, the reference area being 100%. The region of interest that was selected for results selection was the PAG. It was selected because the CA1 is a known area of injury sensitivity in the rat brain, as well as in the human brain. Thus, the potential for higher variation in that could lead to spurious results. Therefore, PAG was chosen and the two boxes in each hemisphere were averaged and used as the internal control pixel intensity value for the internal fluorescence control. Data was then normalised to the control group (Naïve/Sham) and presented as % of that group, 100% is the same value.

NFL from Encor Biotech Inc (RPCA-NFL-CT): The analysis of the whole corpus callosum using this antibody was identical to the above one. For the blast NFL Encor biotech (RPCA-NFL-CT) staining, data was analysed as whole CC and normalised to the internal control, the PAG, then was normalised to the Naïve/Sham group. Data was presented as % of the control group (Naïve/Sham).

2.6 Statistics

2.6.1 Histological Statistical Analysis

The statistics were conducted in GraphPad Prism Version 9.4.1.

For all blast histological data, a Shapiro-Wilk test of normality was performed followed by either parametric or non-parametric analysis. If the data showed a Gaussian distribution ANOVAs were conducted (one or two-way) followed by post-hoc tests. For non-Gaussian distributed data (non-normal) either a Mann-Whitney U test or Kruskal-Wallis test. For all

analysis, Naïve and shams were combined as there was no significant differences between the groups as performed using a t-test or Mann-Whitney U test or Kruskal-Wallis test.

All statistical tests after the Naïve/Sham comparison were conducted by comparing all post-injury groups against the combined Naïve/Sham control group. Where a main factor was found not be non-significant, I then ran one-way ANOVAs followed multiple-comparison tests for each region's layers using a Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (BKY) False Discovery Rate (FDR) approach with $Q = 5\%$. If the adjusted p value (q) was below 0.05, then the one-way ANOVA was followed by post-hoc tests.

Quantitative data are represented as mean value $\pm$ standard error of the mean (SEM). A value of $p < 0.05$ was considered statistically significant. Significant values indicated on graphs unless otherwise specified correspond to the following: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

2.6.1.1 Fluid Biomarker Analysis

For the fluid biomarker analysis was conducted in stages:

1. Of the animals that had been exposed to blast, their CSF was sent off to Dr Zetterberg's lab along with the repeated blood samples from the blasted exposed 3 month post-injury group were sent off for quantification at Henrik Zetterberg's lab.
2. The results were analysed. For the repeated blood samples a Friedman test followed by post-hoc test. This analysis, shown in Table 2.7, displays a significant difference at 3 days post-injury relative to control.

Uncorrected Dunn's multiple comparisons test	Rank sum diff.	Significant?	Summary	P Value
Baseline vs. 1 Day	-14.00	Yes	*	0.0404
Baseline vs. 3 Day	-20.00	Yes	**	0.0034
Baseline vs. 7 Day	-6.000	No	ns	0.3798
Baseline vs. 14 Day	0.000	No	ns	>0.9999
Baseline vs. 1 Month	3.000	No	ns	0.6605
Baseline vs. 3 Month	-12.00	No	ns	0.0790

Table 2.7 Table 2.7. Fluid biomarker analysis stage 2 for 3 month post-injury animals

Repeated blood plasma results for the histological cohort's blast exposed 3 month post-injury animals $n=5$. Table displaying the post-hoc results that dictated further analysis on the plasma samples and resulting data.

For the CSF results, a mixed-effects analysis followed by post-hoc test I saw significant differences at 6 hour, 7 day and 1 month post-injury (displayed in Chapter 5's results).

- Therefore, the rest of the plasma samples in the histologically cohort that were sent off for quantification contained baseline, 1, 3 and 7 days post-injury only. They did not contain 14, 1 and 3 months post-injury samples. This was due to no indication from the previous analysis (Table 2.7) that blood plasma levels beyond 7 days would be significantly different from baseline.

Therefore, for the overall analysis of CSF, it was analysed with a Graphpad Mixed effects analysis which is similar to a repeated two-way ANOVA that can handle missing values. This was followed by post-hoc test. For the repeated blood samples, analysis was performed on both sham and blast exposed animals from baseline to 7 days post-injury. A mixed-effects analysis was performed followed by post-hoc test. There was also a different comparison conducted between treatment groups; sham and blast for each timepoint.

3

Blast Model Optimisation

In this chapter, I cover the optimisation of the blast model for producing a mild traumatic brain injury in rats. For this purpose, a model was developed in line with existing blast literature whilst also accommodating any parameters imposed by the CBIS shock tube. A Friedlander waveform model was established with the rodent situated both perpendicular to the blast wave and just outside the shock tube exit. This enabled the rat to experience both primary and tertiary injury. Measures were taken to ensure that the thorax was shielded from thoracic blast injury. The blast wave was measured both inside and outside the blast shock tube, and the blast wave parameters that the rodent was subjected to were determined. Additionally, the displacement and accelerations that the rodent underwent were comprehensively analysed.

I optimised the CBIS shock tube for bTBI as it had already been characterised and deemed useful for biological samples (Nguyen, Wilgeroth et al. 2014, Nguyen 2016). This was done whilst heeding previous research recommendations for a blast TBI model and reporting parameters (Needham, Ritzel et al. 2015) so that the model would be reproducible. This model incorporated both primary and tertiary blast injury mechanism.

This chapter's work was conducted in collaboration with Dr Cornelius Donat, Dr Michael Bruyns-Haylett and Konstantinos Tsikonofilos.

3.1 Introduction

3.1.1 History of Blast and Why it Has Been Researched

The use of explosives to cause physical harm is not a new phenomenon and can be traced back to the Tang dynasty (618-907AD) where gunpowder bombs were propelled with fire lances that no doubt caused numerous types of injury (Kelly 2004). Since then, there have been developments in the types of explosive and the mechanism by which they cause injury. These mechanisms can be burns, expulsion of gas or shattering. Furthermore, the materials used for some explosives are unstable and thus there is no requirement for oxygen for their decomposition and production of shockwaves (Cernak 2017). Fast-forward some years, weaponry development and subsequent offensive equipment has evolved to overcome defences such as body armour, vehicle armour or barricaded buildings. Therefore, the injuries suffered in the past like those documented in World War I (WWI) are vastly different from those of modern conflicts (Cernak 2017).

In the literature there has been a long history of bTBI research that dates back to WWI. At that time, soldiers that were exposed to explosives either survived with subsequent neuropsychiatric symptoms or died of their injuries. They also could have symptoms without external signs of injury, such as tremors, poor concentration, dizziness, hypersensitivity to noise, amnesia, headache, and tinnitus following blast exposure (Turner 1915, Kinch, Fullerton et al. 2019). It was well documented by some doctors at the frontline, and they described the condition of 'shell shock' and functional neurosis (Mott 1916, Mott 1916, Mott 1916, Mott 1917). However, there was a lot of disagreement in the field at the time, as there was no ability to discern between the emotional and physical stresses of being exposed to an explosion. During the time between the two world wars, other conflicts took place around the world and important military developments at this time occurred, which included advances in armour, bombs and aircraft design and tactics. Some physicians did "believe" in bTBI and they documented and investigated its effects with the purpose of understanding the mechanisms of blast injury (Weinstein 1917, Hooker 1924, Weddell 1939, Hickey 1945). They

found that an explosion affects the gas-filled organs because the gas-filled organs were sensitive to sudden increases in pressure, and could explode and cause death. These organs were those of the middle ear, gastrointestinal tract, and the respiratory system (lungs) (Axelsson, Hjelmqvist et al. 2000). Investigations conducted looked at how blast injury affected the whole body and importantly how it affected the brain (Celander, Clemmedson et al. 1955, Clemmedson and Nelson 1957, Clemmedson, Hartelius et al. 1957, Clemmedson, Frankenberg et al. 1969, Cernak, Savic et al. 1996, Cernak 2015).

In recent years, with the increased use of improvised explosive devices (IEDs) in conflicts that were part of the Global War on Terror, there was an increase in the number of bTBI sufferers (Warden 2006, Ling, Bandak et al. 2009). IEDs were the most common explosive weapon in Operation Enduring Freedom in Afghanistan (OEF) and Operation Iraqi Freedom (OIF) in Iraq (Shanker 2007) and it was reported that 60% of combat casualties were from blast exposure. This was supported by Okie, 2005, (Okie 2005), who stated that between January 2003 and February 2005 all patients admitted to the Walter Reed Army Medical Centre who had been exposed to blasts were evaluated for brain injury. Of those evaluations, results showed that 59% of those patients were diagnosed with TBI (moderate/severe: 56% and mild: 44%) (Okie 2005). Furthermore, due to progression in body armour and helmets successfully protecting against penetrative TBI from what was once a lethal blast, the face, neck, and limbs were still vulnerable (Okie 2005, Hicks, Fertig et al. 2010). In addition to the military personnel bTBI exposures, many of whom suffer post-bTBI symptoms to this day (Rose 2022), civilians have been exposed to high-pressure waves caused by explosions. Such instances are the Oklahoma City bombing 2005, Boston Marathon bombing 2013, New York City and New Jersey bombing 2016 and Manchester Arena 2017 (Edwards, McMenemy et al. 2016). It is hard to get accurate numbers of long-term blast symptoms for those exposed, but lessons learnt through the research on military personnel scenarios will surely help those treating a civilian population. This highlights the need to continue to research this area, as many people both military and non-military are affected and despite years of research there is still so much left unknown, and more research is required to understand bTBI.

3.1.2 An IED and its Blast Wave

An improvised explosive device (IED) is a bomb that is not commercially made and can be made from things that range from backpacks, pipe bombs, cell phones, oil trucks and other components. More recently, they have become more complicated and delivered by people wearing suicide vests, or carrying suitcases, and small packages (Nguyen 2016) as well as by fuel tankers and larger methods. They can be of any size, fuelled by numerous substances and contain extra features like shrapnel, nuts and bolts or other debris.

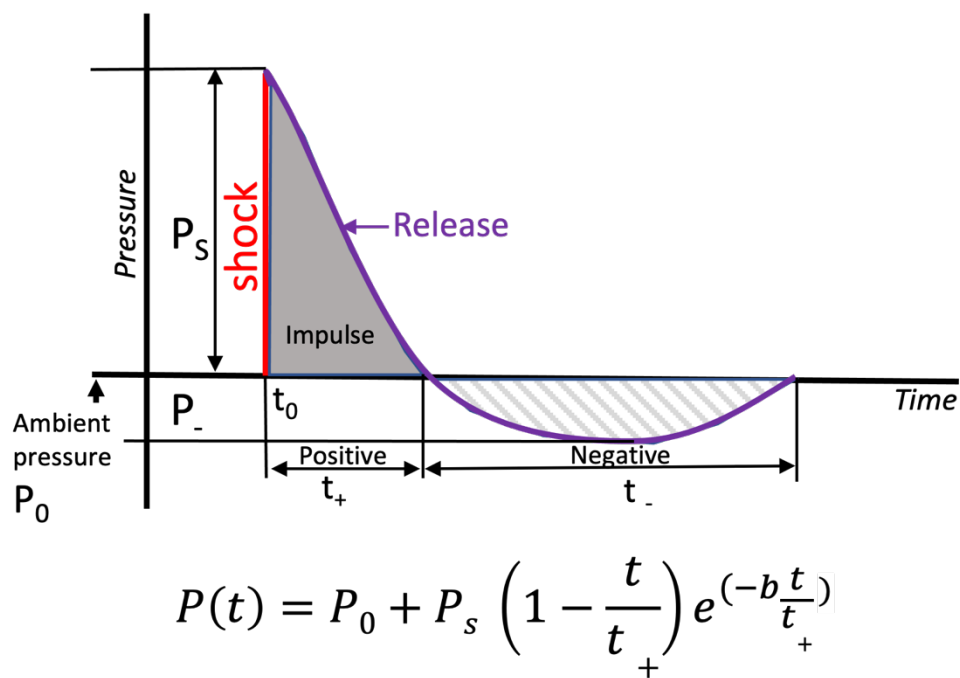


Figure 3.1 Parameters of the ideal blast wave, the Friedlander waveform.

The waveform includes the ambient pressure (P_0), typically equivalent to the atmospheric pressure, the arrival time (t_0) at a specific point of observation, the peak overpressure ($P_0 + P_s$) of the positive phase, which lasts for a duration of time (t_+) and has a positive impulse, and the minimum pressure (P_-) of the negative phase, which lasts for a duration of time (t_-) and has a negative impulse. $-b$ represents the decay constant that dictates the rate of drop in pressure. Impulse can be found by calculating the area under the curve. Underneath the graph is the Friedlander's equation for calculating the pressure of the wave. Adapted from (Weggel 2010).

The Friedlander waveform (Figure 3.1) is often used to represent the blast wave, the element of the explosion that is generated by an IED in a free field scenario that can cause both primary

and tertiary injury. An IED shockwave moves outward from the epicentre of the explosion into the non-shocked air of ambient pressure (Figure 3.1, P_0). There is a compressed region of gas with high pressure and density known as the shock front (Figure 3.1, Shock) that reaches its peak overpressure/blast overpressure (BOP) where ($P = \text{maximum value, } P_0 + P_s$). If the shock front is reflected by a large, rigid surface at the correct angle the peak pressure can be amplified up to eight times its original value (Bradley and Lindsay 1963). The shock wave will exponentially decay to atmospheric pressure (b is a decay constant). This section of the blast wave is known as the positive phase, and this process occurs over a certain time period, t_+ . As the wave travels through a material or gas, it causes the particles to move apart, leading to a decrease in pressure and density. As the expansion continues, the pressure can even drop below ambient pressure, resulting in a negative phase (Figure 3.1) (Taber, Warden et al. 2006, Weggel 2010).

The components of the blast wave are important variable parameters that can be used for simulating an explosion for experimental needs. The three main parameters are peak overpressure (Maximum pressure above P_0), positive duration (t_+), and impulse. Varying these can influence the impact of the resulting blast wave (Sundaramurthy and Chandra 2014). What the parameters are and what they entail is described here:

- **Total pressure** this is the summation of incident (static and dynamic) pressure and it is the force per unit area that an object experiences in a steady flow. It can be extracted from the equation in Figure 3.1. In some cases, reflected pressure is part of this total pressure.

Incident pressure wave:

Static pressure causes gas pressure to rise above ambient pressure, which is caused by compression or heating of the gas. It is one component of the incident pressure also known as side-on pressure or incident pressure and has a crushing action (Needham, Ritzel et al. 2015).

Dynamic pressure is the other component of incident pressure. Dynamic pressure is the differential pressure caused by the motion of the gas and can cause a drag or lift motion (Needham, Ritzel et al. 2015).

Peak overpressure or blast overpressure (BOP) is the peak pressure above ambient pressure. High BOP can cause structural damage to buildings and vehicles and can lead to injuries such as broken bones, lung contusions, and traumatic brain injury (TBI). Many studies only report the BOP (Sundaramurthy and Chandra 2014, Needham, Ritzel et al. 2015).

- **Reflected pressure** is the pressure wave that is reflected back towards its source when it encounters a boundary or obstacle and it is at its maximum when the obstacles are perpendicular to the wave (Needham, Ritzel et al. 2015). For some blast waves it can be part of the overall blast wave recorded within the total pressure.
- **Positive duration** is the time during which the blast wave is above atmospheric pressure. It is an important parameter when assessing the potential for injury from a blast wave. Long positive durations can lead to more severe injuries, as the body is exposed to the blast wave for a longer period of time (Needham, Ritzel et al. 2015).
- **Positive impulse** is the calculated area under the curve, pressure times time, and is denoted in this instance as impulse per unit area (Baker 1987) (Equation 1). If an impulse is higher, it is likely to result in greater damage and injury (Weggel 2010).

$$I_{+} = \int_{t_0}^{t_0+t_{+}} [P(t) - P_0] dt$$

*Equation 1 Impulse equation for area under the curve
(Weggel 2010)*

3.1.3 Blast TBI Field Heterogeneity

Since the invention of the shock tube in 1899, shock tubes were able to generate well-defined pressure pulses with various durations and peak pressures and were used in different fields

of research such as plasma physics, chemical kinematics and aeronautics (Ahlborn, Hertzberg et al. 1977). In blast research there is a lack of standardisation, resulting in difficulties when comparing models. Even the shock tubes themselves vary greatly in size, length and diameter. This is exacerbated by the fact that several studies fail to report the important aforementioned parameters, which are necessary for the replication of experiments.

3.1.3.1 Choice of Blast Mechanism to Model

Studies have shown a plethora of different approaches to generate a greater understanding of different components of primary blast injury and its blast wave (Long, Bentley et al. 2009, Long, Tong et al. 2010, Säljö, Mayorga et al. 2011, Goldstein, Fisher et al. 2012, Svetlov, Prima et al. 2012, Baalman, Cotton et al. 2013, Hernandez, Tan et al. 2018, Nguyen, Pearce et al. 2019, Arun, Wilder et al. 2020, Yu, Azor et al. 2020). This has come as a result of the different theories behind the mechanisms of primary blast injury: thoracic, head acceleration and direct cranial pressure changes from the blast wave (Courtney and Courtney 2011).

- One hypothesis suggests the indirect thoracic mechanism. This mechanism is via hydraulic interaction such that the blast wave can harm the body by compressing the abdomen and chest and there is kinetic energy transferred to the fluid of the body, resulting in high pressure in the circulatory system (Courtney and Courtney 2009, Courtney and Courtney 2015). This pressure results in oscillating waves that travel to the brain which can cause vascular injury (Cernak 2010, Elder, Mitsis et al. 2010). This blast-body interaction has also been suggested to cause overinflation of the lung that can result in vasovagal interruptions that cause various changes such as apnoea, bradycardia, and other outcomes (Elder and Cristian 2009).
- Another hypothesis of the acceleration/deceleration mechanism of primary injury is acceleration of the head as a result of the blast wave (Courtney and Courtney 2009). This causes a 'coup contrecoup' action from accelerations which leads to high shear stresses that can result in diffuse axonal injury (DAI) or skull flexing (Moore, Jérusalem

et al. 2009, Elder, Mitsis et al. 2010). The brain can move at different rates and cause compression, tearing and shearing in the tissue (Bryden, Tilghman et al. 2019).

- A further hypothesis is the direct cranial pressure hypothesis whereby the blast wave moves through the skull and affects the brain causing damage. This can be similar to or a confounder of the acceleration hypothesis. It can have a localised point of injury as the brain takes the bulk of the motion from the pressure wave (Courtney and Courtney 2015) and absorbs most of it. (Bryden, Tilghman et al. 2019). This can cause damage as interfaces with blood vessels, parenchyma and air-filled spaces are prone to injury (Bryden, Tilghman et al. 2019). Furthermore, the increase in blast magnitude in the skull from reflection of the blast inside the skull can be another factor (Elder, Mitsis et al. 2010).

It should be noted that these hypotheses are presented separately but this does not mean they are mutually exclusive. Thus, it is possible that in different circumstances the blast wave could involve any combination of the above. In a military setting, personnel wearing body armour leaves the limbs and head more vulnerable to blast waves whilst the thorax is somewhat protected. Therefore, the thoracic mechanism of injury is less likely to contribute to TBI in military personnel. However, in civilian settings, thoracic protection is unlikely and therefore the thoracic mechanism is more likely to contribute to TBI (Warden 2006, Hicks, Fertig et al. 2010, Wood, Panzer et al. 2013). This should be a consideration when testing blast injury for research - whether the research is applicable for both populations or just military.

With regards to modelling, these hypothesised mechanisms can be difficult to delineate. However, some try to achieve delineation by having separate head-only models of TBI and full body. Some models protect from pulmonary injury as it can complicate the brain pathology, and it is more relevant from a military perspective as many service people wear Kevlar vests, thus protecting their thorax. Other models don't protect the thorax in order to get full body exposure or to assess pulmonary injury fatality rates (Cernak, Savic et al. 1996, Cernak, Savic et al. 1999, Long, Bentley et al. 2009, Bass, Panzer et al. 2012, Sundaramurthy, Alai et al. 2012, Chen, Constantini et al. 2015). Furthermore, some studies have focused on understanding the different components of the blast wave and how it transmits through the

brain parenchyma (Shridharani, Wood et al. 2012, Yu, Azor et al. 2020). Greater understanding of blast mechanisms can also be obtained by computational modelling which is a valuable tool if combined with validation studies (Hicks, Fertig et al. 2010) as has been done for blunt injury models (Donat, Yanez Lopez et al. 2021).

3.1.3.2 Gases Used to Pressurise the Driver Section

Another source of variation in models comes from the use of different gases to pressurise the driver section of the shock tube, as gases can have different effects on the nature of shockwaves (Reneer, Hisel et al. 2011, Kumar and Nedungadi 2020). It was found by Kumar that helium was more consistent at producing a blast wave, but Reneer showed that helium can leave a hypoxic post-blast effect, and therefore compressed air was more suitable. The use of different gases can make it hard for comparison and to reproduce an exact experiment because of how the gases effect the pressure wave released. The effect is because of how the gas interacts with the material (e.g. Mylar® or Aluminium) that is used to be a barrier between the driver and driven sections of a shock tube (Nguyen, Wilgeroth et al. 2014, Nguyen 2016).

3.1.3.3. Positioning of the Blast Specimen

Another contributing factor to variation in the blast TBI field can be the positioning and orientation of the animal relative to the blast wave. Research has shown that the perpendicular placement relative to the blast wave and head-mobile design resulted in accelerations that the study deemed to be the cause of injury and behavioural sequelae rather than the blast wave itself (Goldstein, Fisher et al. 2012). However, the study didn't consider the specimen mounting of their head-mobile model. It had a "violent focal impact" during its head movement as pointed out in Needham, 2015 (Needham, Ritzel et al. 2015) which could have confounded injury pathology and their results. This is something to keep in mind when modelling TBI. The decision of which effect of blast is being modelled as well as the orientation to the oncoming shockwaves (Watts, Kirkman et al. 2019) should be considered together with choosing which of the categories of blast are incorporated (e.g primary, secondary or tertiary) so that fully informed conclusions can be made. It is worth mentioning that real world blasts

are complex, as there can be reflections from nearby objects (e.g. the ground) and buildings resulting in exacerbation of pressure. In addition, there can be tertiary elements due to acceleration/deceleration or displacement into objects or the ground (Warden, French et al. 2009). Real world blasts are not simple and are heterogeneous.

Furthermore, the size of the shock tube should be a factor in the positioning of the specimen. For instance, as shown in Hernandez, 2018 (Hernandez, Tan et al. 2018), when the shock tube is small with a small diameter, having the animal face-on may incorporate extra reflections and blockage effect (Needham, Ritzel et al. 2015). Thus, Hernandez's animals were placed perpendicular to the shock tube. Furthermore, if the model is designed to have its thorax protected from the blast wave, that should be taken into consideration for the body positioning. These factors may all affect behavioural outcomes (Watts, Kirkman et al. 2019).

The prone position is common when using rodents or pigs in blast research (Chavko, Watanabe et al. 2011, De Lanerolle, Bandak et al. 2011, Goldstein, Fisher et al. 2012, Gullotti, Panzer et al. 2014). However, some blast studies choose to not use the prone position of their blast specimen. This variation in the setup of the blast model, can be due modelling different aspects of a blast injury. This occurred in a study that had the animal with the ventral side of the rodent facing the shockwave (Cernak 2010). This is likely to increase the risk of pulmonary damage as the abdomen and pulmonary systems are exposed to the shock wave. However, they are modelling "to reproduce an in-theatre scenario where the soldier is facing the incoming shock wave with the torso turned toward the shock wave front" (Cernak, 2011 #110}. Thus, fulfilling the blast design given the mechanisms they are choosing to model.

However, despite the variation this introduces into the blast literature, the study of various blast specimen positions is useful to show commonalities or other sources of variation in blast modelling to aid useful modelling setups. Therefore, studies have been conducted to assess what degree positioning and orientation affects the outcome of blast TBI modelling in the prone position (Chavko, Watanabe et al. 2011, Dal Cengio Leonardi, Keane et al. 2012, Gullotti, Panzer et al. 2014). These reports have shown that head and body positioning/orientation during blast exposure can provide valuable insight into blast TBI and potential translational implications (Figure 3.2). Chavko et al. (Chavko, 2011 #789), conducted

a study on how body position affects the pressure in the brain of rodents, and found that the rats positioned face-on (Figure 3.2A) to the blast had higher peak pressures and faster pressure rise times (time it takes from ambient pressure to reach peak pressure) in their brains, while rats positioned perpendicular (Figure 3.2B) or facing away (Figure 3.2C) had lower pressures and slower rise times. They suggested that the “direct hit” approach of the face-on positioning can cause skull flex and subsequent damage, and that the location of the pressure probe used to assess brain pressure may also affect the results. In their discussion on the human translational implications of their findings, they talk of Moss et al.'s proposed suggestion for human helmets to have blast load deflecting capabilities to better protect the brain because their rat model showed that brains were affected by the blast regardless of orientation (Moss, King et al. 2009, Chavko, Watanabe et al. 2011). However, it should be noted that these animals from Chavko’s study were tested without thoracic protection, and therefore the injury profile in the brain tissue may have been different if their thorax was protected. This highlights the importance of deciding the type of blast mechanism and injury to be tested and how that may affect the placement of the animal and blast set up.

3.1.3.3 Placement and Head Movement

Another layer to the variation caused by different animal placements in blast modelling is whether there is movement of the head of the blast specimen. Gullotti et al., (Gullotti, 2014 #777) studied animal placement and how it may affect blast modelling outcomes similar to Chavko's study, but Gullotti had a greater number of variables such as the inclusion of thoracic protection, inside/outside the shock tube, distance from the exit of the shock tube, different blast pressures and the use of different head movements (restrained/unrestrained). Using thoracic protection, they tested these mentioned aspects in combination with a wide range of pressures from ~140-450kpa as well as impulses from 0-500 kPa.ms. They found that limiting head acceleration reduced the duration of loss of righting reflex (LORR), but the LORR was increased if the blast pressure increased whilst still restraining head movement. While head-restrained studies are useful for investigating differences between the blast wave and acceleration in laboratory settings, it may not fully represent the mechanism of pathology in a warzone where primary and tertiary blast injuries occur together (Eskridge, Macera et al.

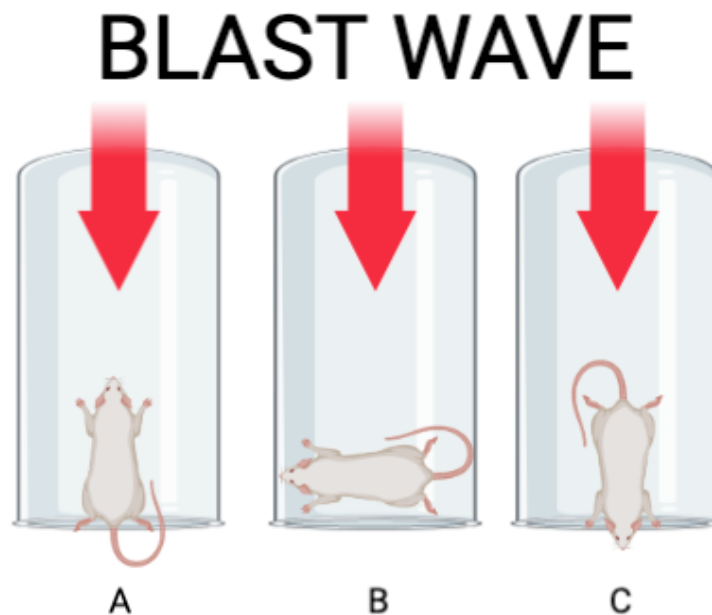


Figure 3.2 Orientation of animals exposed to blast in Chavko, 2011.

Animals were placed inside the shock tube in a) frontal, head-on position, with the head facing the blast wave; (B) lateral, side-on position with the right side of body exposed to blast; (C) backward position with the head facing away from blast. Schematic made not to scale and adapted from (Chavko, Watanabe et al. 2011). Schematic made in biorender.com

2012). Gullotti addresses the complex head movements in their studies and others (Svetlov, Prima et al. 2010, Goldstein, Fisher et al. 2012, Sundaramurthy, Alai et al. 2012) and found these to be due to the blast wind that accompanies the blast wave. They found a perpendicular orientation of rats showed the highest acceleration of $\sim 800 \text{krad/s}^2$, equivalent to a human experiencing $\sim 10 \text{krads/s}^2$, only slightly above estimated human concussion threshold as derived from field studies and computational models (Meaney, Smith et al. 1995, Pellman, Viano et al. 2003, Zhang, Yang et al. 2004). In their study, accelerations were also similar to porcine models of TBI but they state neither the porcine nor their mouse accelerations were enough to cause mass lesions or haemorrhagic damage. This only occurred in Svetlov's study where they suggest the pathology is created by a crushing motion (Svetlov, Prima et al. 2012, Gullotti, Panzer et al. 2014). This is further supported by Hick et al., 2010 who also state that it should be remembered that despite animals' usefulness as test subjects, rodents and smaller mammals are able to sustain much higher rotational forces than humans. Therefore, to look at tertiary injury only, the acceleration needs to be high enough to cause appropriate injury pathology, which for other studies were not achievable (Hicks, Fertig et al. 2010). Therefore, to investigate primary and tertiary injury as a result of the blast wave using a rodent model, the animal's head can be placed perpendicular to allow for a more natural movement of the animal's head from the oncoming blast wave. This lateral movement allows for the blast wave to hit the head (primary) and for a reasonable displacement, thus acceleration (tertiary injury) in the experimental environment, as shown by Goldstein et al, 2012 (Goldstein, Fisher et al. 2012).

3.1.3.4 Blast Severity Thresholds

Another factor that contributes to the heterogeneity of blast-induced traumatic brain injury (bTBI) literature is the threshold variability for categorising the injury severity as mild, moderate, or severe. These thresholds are often determined based on the set up and parameters of the blast waves used (Bowen, Fletcher et al. 1968, Cernak 2010, Mishra, Skotak et al. 2016:Hue, 2013 #1008). The variability in the literature of the parameters for modelling and achieving mild, moderate or severe bTBI can be attributed to several factors, such as the use of different shock tubes, different animal species, and the emphasis on adhering to the BOP measure instead of the duration and impulse of the blast. Bass et al. (Bass, Rafaels et al.

2008, Bass, Panzer et al. 2012) have demonstrated how these differences can lead to dichotomous outcomes for large and small animals and how thoracic protection can alter the thresholds established.

This variability makes it challenging to model mild bTBI accurately as it depends on the specific mechanism of blast injury being tested. Courtney and Courtney (Courtney, 2011 #793} have also acknowledged this variability, stating that injury thresholds for each mechanism are dependent on blast conditions, and that under certain conditions more than one mechanism may contribute to the injury. Therefore, a safer approach for determining the severity of bTBI may be to rather rely on these thresholds as guidelines and prioritise pathological outcomes such as loss of righting reflex, signs of pain/discomfort, weight loss, mortality rate, lesions, or macro-tissue damage to determine severity outcomes.

3.1.4 A Lack of Consensus in the Field Regarding the Blast Shock Tube Setup

Within the field there is a lack of consensus regarding the aforementioned mechanisms of injury, position of test subject, and what parameter values correspond to certain severity levels. The main differences addressed here are the positioning of the animal and the blast wave itself.

Publications from Professor Chandra's lab present evidence that the blast wave undergoes diffraction and creates turbulence once leaving the exit of the tube. Kahali et al., showed that once the blast wave is outside the shock tube it loses its planarity and a vortex ring is formed (Kahali, Townsend et al. 2020). They recommend that any experiment for primary blast injury should be conducted such that the animals do not experience the vortex ring, and this is most easily avoided by placing the animal inside the shock tube. However, they state a limitation to their findings is the oversimplification of their simulations that may call into question their fidelity, and that the post-exit turbulence may vary for all different shock tubes length and shape (Kahali, Townsend et al. 2020).

Needham (Needham, Ritzel et al. 2015) brings to light another element of blast TBI modelling that should be considered. The authors maintain that the length of the positive duration should be scaled to the species they are working with. They state that for real world IEDs the blast waves last 1-10 ms and thus when scaled to a smaller mammal like a mouse the blast should be between 0.1-1 ms based on size. This is based on energy transfer to the head of a human vs the head of a mouse, as the mouse head size Needham states is 10% of that of a human. For a rat, as it is larger, it would therefore be capable of withstanding a longer duration of blast than a mouse. Needham states that a 30 g mouse blasted for 1 ms is the same as a 70 kg person for 13 ms using Bowen blast scaling (Bowen, Fletcher et al. 1968). The authors point out that this does leave out other factors such as lung capacity, rib stiffness as well as TBI specific skull geometry thickness and porosity. Needham calculates that a mouse exposed to 10ms of blast is the same as a human undergoing 100 ms which is x10 that of a real-world IED. They also point out that the effective yield of a blast (the transfer of energy to the head) and the energy within the wave are proportional approximately to the cube of the duration. Thus, a factor of 2 differences of duration equates to a factor of 8 for the energy within the wave (Needham, Ritzel et al. 2015). Thus, when modelling bTBI, it is good to scale the blast wave according to the species in use.

Despite the controversies and variability of the field, there is a need further understand blast TBI and therefore to optimise the CBIS shock tube for blast TBI modelling. There is currently no shock tube set up for this purpose in the United Kingdom. Therefore, I set out to optimise the shock tube in collaboration with Dr Donat, Dr Bruyns-Haylett and Mr Tsikonofilos for mild blast traumatic brain injury using rats.

3.2 Aims and Objectives:

3.2.1 Aim:

To optimise the CBIS shock tube to generate a mild blast TBI rat model that has the following characteristics: a 0% mortality rate, minimal pain according to the welfare scoring and no observable structural damage using cresyl violet staining.

3.2.2 Objectives:

1. To use the CBIS blast tube at 7% driver volume with plastic mylars to produce a Friedlander waveform in a rat model, using appropriate parameters (BOP, duration and impulse).
2. To determine the most appropriate positioning of the rat with guidance from previous CBIS shock tube characterisations. To ensure protection of the rat in the animal platform and that there will be no risk of blast lung injury, by fashioning a protective shield and measuring the pressure within it during a blast.
3. To examine the head kinematics of the rat, in order to ensure the reproducibility of the model, using cadaver and live animals and high-speed imaging cameras.
4. To generate a mild TBI determined by welfare scoring indicating no pain or weight loss. To also have a loss of righting reflex increase relative to sham and an absence of lesions in the brain as shown by cresyl violet histopathology.

3.3 Description of the CBIS Shock Tube Prior to Rodent TBI Optimisation

The Royal British Legion (TRBL) Centre for Blast Injury studies (CBIS) shock tube at Imperial College London was designed by Dr Clapman 2010 and was brought into research use in 2012 (Nguyen 2016). It was characterised by Dr Nguyen during her PhD at Imperial College London (Nguyen 2016). The shock tube system consists of three 1.22m long stainless-steel segments. The total length is 4m with an internal diameter of 59mm with all joining components, like flanges, included (Figure 3.3). The initial segment acts as the driver section whilst the latter two segments act as the driven section. The driver and driven sections are divided by a diaphragm assembly and the other segments are separated by flanges with pressure sensors embedded (Figure 3.3 and Figure 3.4). The system can be pressurised to 18.2bar which abides by safety regulations. To operate the system there is a remote box that is attached to the inlet flange, a double breech in the diaphragm assembly which allows for different shock wave profiles and a compressed air canister that is attached to pressure hosing to fill the driver section. The diaphragm system is designed to allow a double-diaphragm configuration (incorporates position 1 and 2 from Figure 3.3) or single configuration (only position 1 from Figure 3.3), where only one diaphragm location is filled and ruptures under a certain pressure. Mylars[®] (polyester) were used for the experiments and were placed such that they were secured evenly over the central hollow, with o-rings used to enhance clamping and eliminate leakage. The shock tube is set up as an open tube system (where there was nothing blocking the end of the tube) (Nguyen 2016).

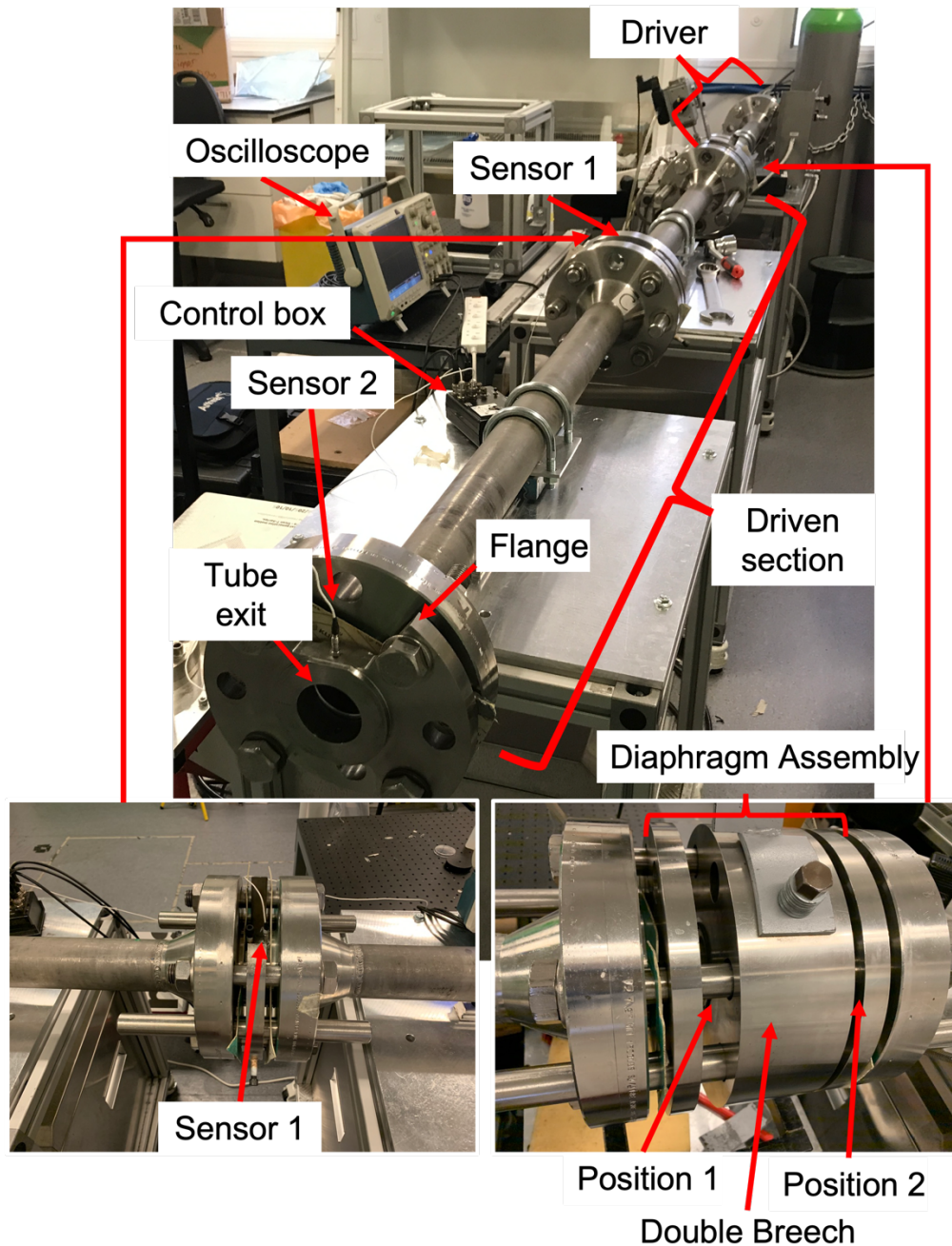


Figure 3.3 CBIS shock tube

3.3.1 Friedlander Waveform from CBIS Shock Tube

During Dr Nguyen's characterisation, she adjusted the driver section's capacities from 7-100% and investigated the type of shock wave produced. It was found that at 7% volume capacity the CBIS shock tube produced a Friedlander wave form (Nguyen, Wilgeroth et al. 2014, Nguyen 2016). This was achieved by placing a blanking flange (a flange with no hole in the

middle) where position 2 is in Figure 3.3. and thus only placed mylars at position 1. In the characterisation process, Dr Nguyen assessed the different thicknesses of Mylar[®] diaphragms and found that higher burst pressures were required to rupture thicker Mylars following a linear relationship between thickness and pressure. Furthermore, reducing the volume capacity of the driver section resulted in shorter durations of the shock wave without altering the magnitude of the wave (Nguyen, Wilgeroth et al. 2014). Thus, it was found that driving volume controlled the shape and duration of the blast wave, whereas the diaphragm material and thickness determined the blast magnitude (Nguyen 2016).

3.3.2 Sensors

In order to understand the propagation and evolution of the shock wave from the CBIS shock tube Dr Nguyen had sensors embedded laterally and flush with the inside of the shock tube (Figure 3.3) (Nguyen 2016). The sensors were connected to an oscilloscope which sampled at 25 million Hz for 4ms. The oscilloscope was triggered by the rise time of sensor 1 (Figure 3.3). Dr Nguyen states the purpose of sensor 1 is to compare the blast waves between experiments as it is far enough away from the exit of the tube to not be affected by any alterations to shock tube design. Sensor 2 and a potential sensor 3 (placed where specimens would be) are more relevant to subject or specimen placement and therefore these sensors can be used to characterise set-ups for biological samples. The sensors used were the factory calibrated Dytran[®] 2300V1 piezoelectric LIVM (Low Impedance Voltage Mode) dynamic pressure sensor, (Nguyen 2016). For the set-up of the rat blast traumatic head injury model, sensors 1 and 2 were used as they were always present and the sensor 3 was for calculating blast pressure at a rat's chest and head (Figure 3.4 and Table 3.1)

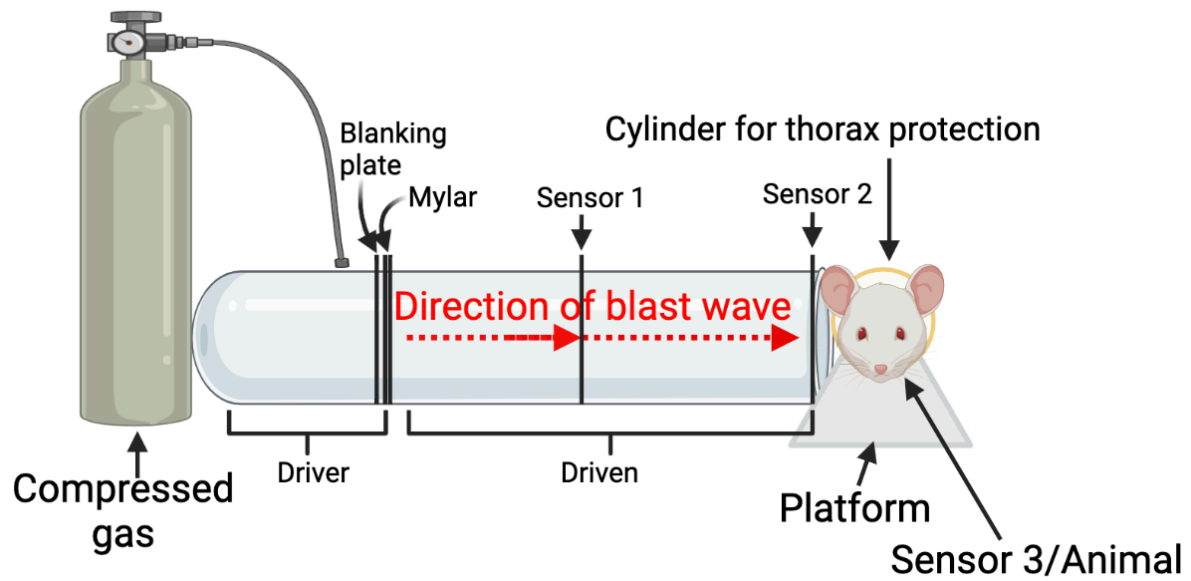


Figure 3.4 Schematic of CBIS shock tube set up

This schematic shows the placement of 3 sensors, not to scale, made in biorender.com

	Location	Purpose
Sensor1	Between driver and driven section	Internal control between experiments.
Sensor2	At the end of the shock tube flush with the inside of the shock tube	Static pressure of blast wave but also used as an internal control experiments.
Sensor3	Placed where the head or thorax would be for live experiments	Total pressure magnitude at location of specimen. This sensor is not present during live animal experiments.

Table 3.1 Sensor location and purpose on the CBIS shock tube

(Nguyen 2016)

3.4 Optimising the CBIS Shock Tube for Rodent Mild TBI

As stated in the aim of this study, we (collaborators and I) wanted to produce a mild blast TBI using the CBIS shock tube. This needed to be performed within certain parameter limitations to align the project with previous blast literature whilst also accommodating the nature of the shock tube. We selected the Friedlander waveform as we were modelling IED and the idealised waveform for IED is the Friedlander waveform. This could be produced using 7% of the driver section of the shock tube. In addition, we had a hard limit of burst pressure for the mylar of 18.2bar, as this was the maximum charging possible of the driver section without exceeding safety standards. Another restriction was the diameter of the shock tube (5.9cm). This was not wide enough for an animal to be placed inside the shock tube. Therefore, we needed to ensure that the animal was placed within a certain distance from the exit of the shock tube where the Friedlander waveform would not be compromised. In addition, given the small diameter of the shock tube, orientation of the animal would need to be perpendicular rather than face on to reduce shockwave reflections and blockage of the shock tube, and to allow for tertiary injury to be combined with primary blast injury.

To align with the blast literature we must report more than what some studies do. We found that as pointed out by Stemper, 2016, Needham, 2015, Mishra 2016 and Nguyen, 2016, despite the blast pressure being the most reported metric, it is important to also report the impulse and duration of the blast wave (Needham, Ritzel et al. 2015, Mishra, Skotak et al. 2016, Nguyen 2016, Stemper, Shah et al. 2016). The Impulse because it informs about the blast loading of the shock wave, and duration because it has a linear relationship with the impulse (as the duration increases, so does the impulse) (Mishra, Skotak et al. 2016). Duration is also an important factor to establish when considering the species of blast specimen used in the model. As stated by Needham (Needham, Ritzel et al. 2015), and as mentioned earlier, an appropriate duration must be proportional to the size of the subject. This is due to the near constant speed of the blast wave meaning that subject size (i.e. length) determines the time the subject is engulfed by the wave. For humans, Needham determines the appropriate duration to be 1-10ms and 0.1-1ms for mice at 10% size of humans. Therefore, for rats, which are approximately 250% the size of mice this gives an approximate range of ~0.25-2.5ms as a duration to be used for this blast model.

The impulse for this chapter's optimisation is harder to establish based on the literature as there is no model that is 100% comparable. Furthermore, the blast set up and parameters can determine the injury severity (Gullotti, Panzer et al. 2014). Thus, there are setup factors to consider that may alter what impulse and BOP value is chosen for this chapter; chest protection, specimen placement inside/outside the shock tube, orientation to the blast and species.

Mishra et al., (Mishra, Skotak et al. 2016) conducted their experiments on rats but inside the shock tube, face on to the oncoming blast wave, and with the chest only partially protected. They found that any blast with an impulse less than 300kPa.ms did not induce mortality. Since their threshold was based on an animal with only partial thoracic protection, and we planned to have full thoracic protection, we used this as a useful reference value. However, the corresponding BOP to this impulse was 170kPa. This is due to the fact that their blast and duration for this wave was longer (~10ms) than the optimised duration for the size of a typical

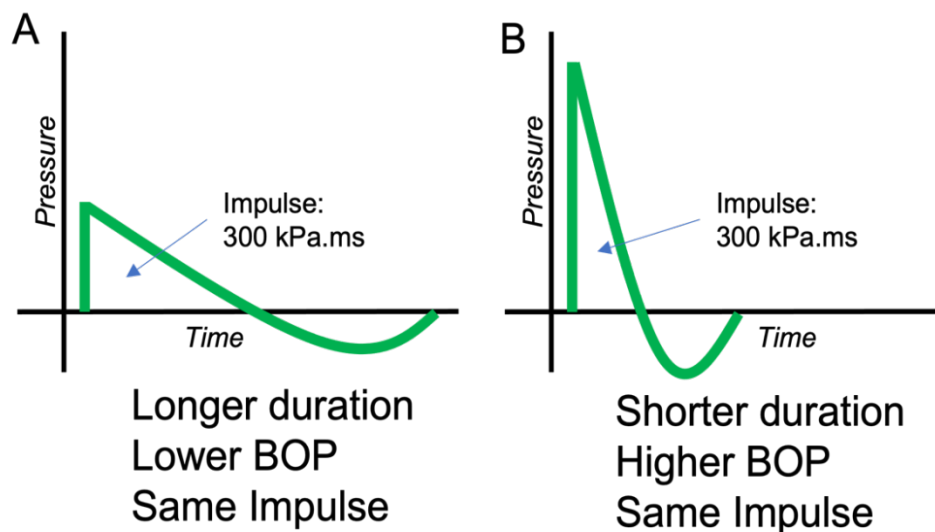


Figure 3.5 Friedlander waveform schematic of different parameters but the same impulse.

This schematic shows how the blast over pressure (BOP) and the duration of a blast wave can be altered but have the same impulse. A) Shows a Friedlander waveform that has a longer duration, a lower blast BOP and has an impulse of 300kPa.ms. B) Shows a Friedlander waveform where the duration is much shorter, but the BOP is higher than A, therefore the impulse is the same, as the area under the curve is the same. (Graphs not drawn to scale).

rat (~2.5ms). Therefore, for us to have an impulse of 300kPa.ms or below with a duration of up to ~2.5ms the BOP would need to be higher than Mishra's 170kPa (see schematic Figure 3.5).

Given these restrictions along with information gathered from the literature, we decided to first evaluate the shock tube waveform elements at the maximum burst pressure possible (~18bar). This translated into a mylar thickness of 250 μ m, and is explored below.

3.4.1 Blast Wave at 7% Capacity with 250 μ m Mylar

From the shock tube characterisation performed by Dr Nguyen, a 7% driver volume capacity would create a short duration Friedlander waveform. From previous works (Nguyen 2016, Campos-Pires, Yonis et al. 2018), it is known that the thinner the diaphragm material, the lower the BOP. Therefore 250 μ m thick Mylar[®] was used to get the maximum burst pressure possible. This was to assess whether the shock tube would be appropriate for a rat mild blast TBI model, as if the shock tube could not reach the desired impulse at maximum burst pressure then it could not be used at all. Initially we tested the resulting pressure waves at sensor 1 and 2. To do this, waves were processed with MATLAB[®] code to clean random noise from the signal. First, the voltage was converted to kPa and time was converted from seconds to milliseconds. These data were then filtered before plotting and calculating values such as the impulse and the duration of the blast wave. Figure 3.6 shows the raw data and filtered data without any other adaptations to the shock tube set up.

Blast Parameters	
BOP S1 (kPa)	297.3408374
BOP S2 (kPa)	233.6401785
Impulse S1 (kPa.ms)	303.8954082
Impulse S2 (kPa.ms)	113.3283674
Positive Duration S1 (ms)	4.21418
Positive Duration S2 (ms)	1.4106
Rise Time S1 (ms)	0.02
Rise Time S2 (ms)	0.01924

Table 3.2 Example blast wave parameters from sensor 1 (S1) and sensor 2 (S2) tests.

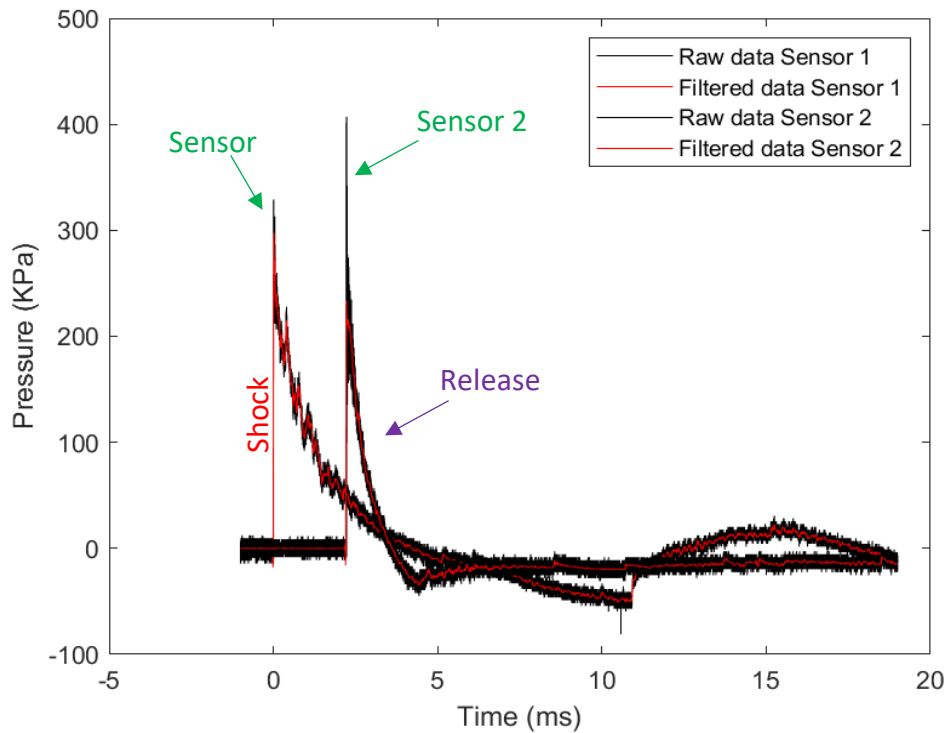


Figure 3.6 Example of blast

This shows an example wave generated using 7% driver volume and 250 μm thick Mylar[®]. The Friedlander wave is shown with raw (black lines) and filtered (red lines) data represented.

The filtered data at sensor 1 and 2 (Figure 3.6 and Table 3.2) shows the BOP of 297 kPa and 233 kPa and an impulse of ~ 303 and ~ 113 kPa.ms respectively. These values were for the maximum burst pressure that was safely achievable using the CBIS shock tube. An impulse of 113 kPa.ms at sensor 2 is below half of Mishra's threshold for mortality (up to 300 kPa) and was therefore selected for further evaluation.

We then tested repeated blasts to ensure consistency between experiments when using a 250 μm Mylar[®]. Reproducibility and consistency are shown in Figure 3.7 with little variation in shock wave dynamics.

We established a reproducible Friedlander waveform in the shock tube with these parameters. Following this, we tested the pressure wave parameters at the shock tube exit where the rat's head was to be placed. This first required us to establish a platform to

maintain the correct position for the rat to eliminate thorax issues and blockage problems and assess the blast wave pressure at the rat's head.

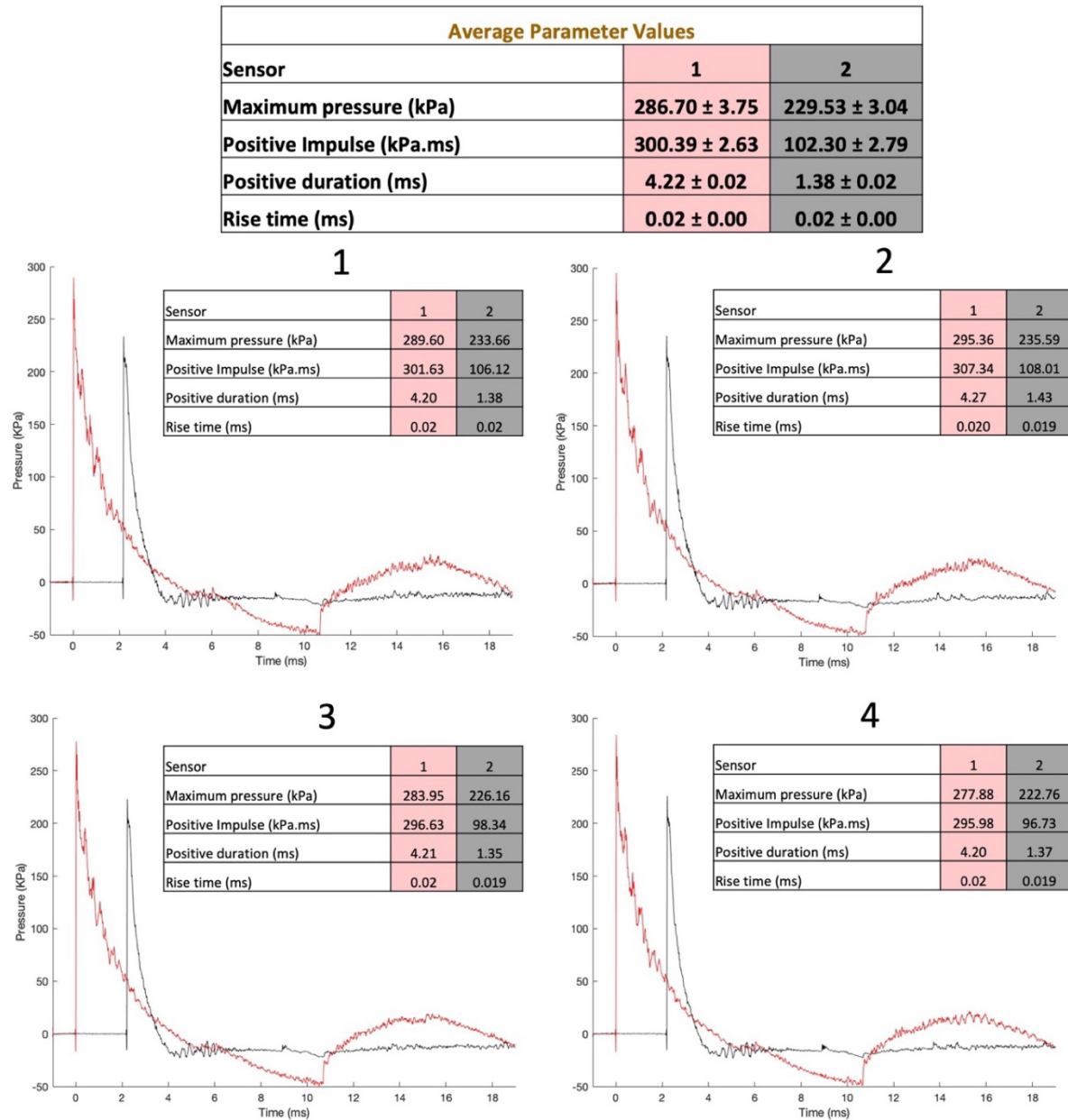


Figure 3.7 Tables and Pressure time graphs of representative blast tests of sensor1 and sensor 2.

These graphs depict the reproducibility of 7% driver capacity and 250 μm mylar. This figure shows the average values for blasting experiments and the similarities between the graphs. Data is presented in averaged table as mean $\pm$ SEM.

3.4.2 Adapting the Animal Platform for Blast Traumatic Brain Injury with Rats

3.4.2.1 The Platform

Dr. Nguyen created a purpose-made steel platform for animal use (Nguyen 2016). The aim to induce both primary and tertiary blast injury required head movement, which can be achieved most easily by positioning the rat perpendicular to the blast wave (Goldstein, Fisher et al. 2012, Gullotti, Panzer et al. 2014). To place the rat appropriately in a prone position and perpendicular to the blast, the platform was rotated 180 degrees and bolted to the shock tube (as shown in Figure 3.8). As stated, an advantage to having a perpendicular positioning (versus head-on) is that it also reduces the blockage problem by having only the head in the path of the shock front. Furthermore, it is a more natural position for the rat to move in, as having the rat face onto the oncoming wave could result in higher risk of cervical dislocation and death as an up motion that may results for the blast is unnatural for the rats relative to the side to side movement. In addition, this also protects the thorax. However, according to previous works, there can be up to a 50% mortality rate with a perpendicular model (reasoning not explained in the paper), even with the thorax placed away from the exit of the shock tube (Gullotti, Panzer et al. 2014, Beamer, Tummala et al. 2016) and therefore, precaution was taken to ensure the thorax was adequately protected to achieve 0% mortality. Logistically, the platform needed enough space for the rat to lie perpendicular to the blast wave. To ensure the rat could lie flat, an extension was made to the metal platform that allowed access to the animal's back feet for physiological recordings (as shown in the Figure 3.9C of the zip-tie extension).

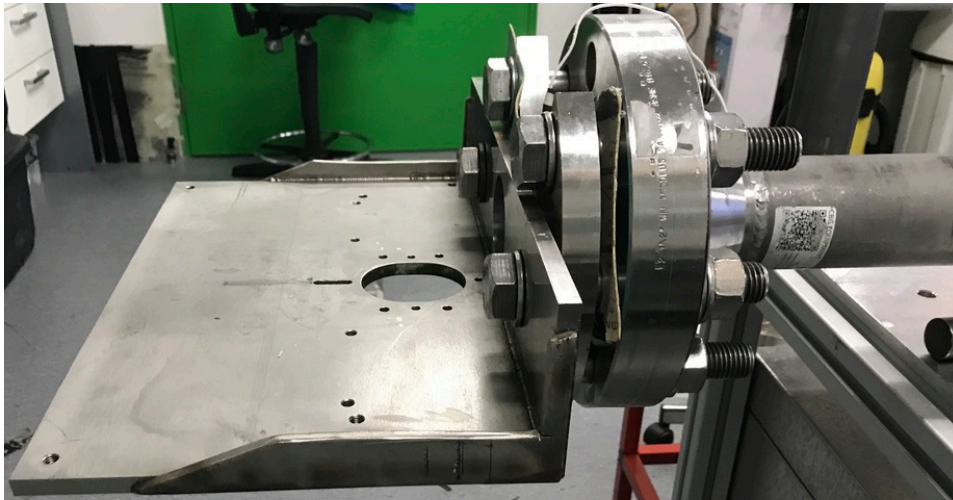


Figure 3.8 Animal platform rotated 180 degrees and bolted to the end of the shock tube

3.4.2.2 Chest Protection to Prevent Blast Lung Injury or Death

To simulate the protection provided to modern-day soldiers' torsos in the field, the model design included thorax protection which is not typically used in other animal blast studies (Cernak 2010, Budde, Shah et al. 2013, Beamer, Tummala et al. 2016, Mishra, Skotak et al. 2016). In terms of protective material, Mishra, for example, only has half chest protection with a metal plate placed underneath the animal (Mishra, Skotak et al. 2016). However, some do use thoracic protection. These studies have animals wearing kevlar vests (Long, Bentley et al. 2009, Por, Sandoval et al. 2017), pure sorbothane (Gullotti, Panzer et al. 2014), sorbothane lined aluminium (Beamer, Tummala et al. 2016), or polyvinyl chloride pipe (Hernandez, 2018 #43). Clemedson (Clemedson, Frankenberg et al. 1969) used a metal apparatus that enabled different parts of the animal to be exposed, whilst others don't specify the material used (Goldstein, 2012 #122).

In addition to minimising the risk of lung injury, the rat was secured to prevent whole body movement as a result of blast whilst ensuring unobstructed breathing. To achieve this, a 1cm thick PVC tube that could be adjusted in size with input of foam to accommodate different rat sizes was made. Furthermore, to prevent focal effects similar to those reported by Needham et al (Needham, Ritzel et al. 2015) that could have possibly occurred in Goldstein's

2012 study (Gullotti, Panzer et al. 2014), the side of the tube was cut away and replaced with inner foam padding with further padding added to both sides of the animal (Figure 3.9). The area to the side of the rat's head was cushioned with foam to secure the animal. Additionally, 1cm 50 duro sorbothane (Dexine Leyland Rubber Technology Ltd in the UK), given that as it is the most efficient available shock absorber (Windle, Gregory et al. 1999), was chosen to protect the animal against any hard contact with the platform. It was placed on top of the platform and secured with Gorilla tape to prevent its removal during the blast (Figure 3.9).

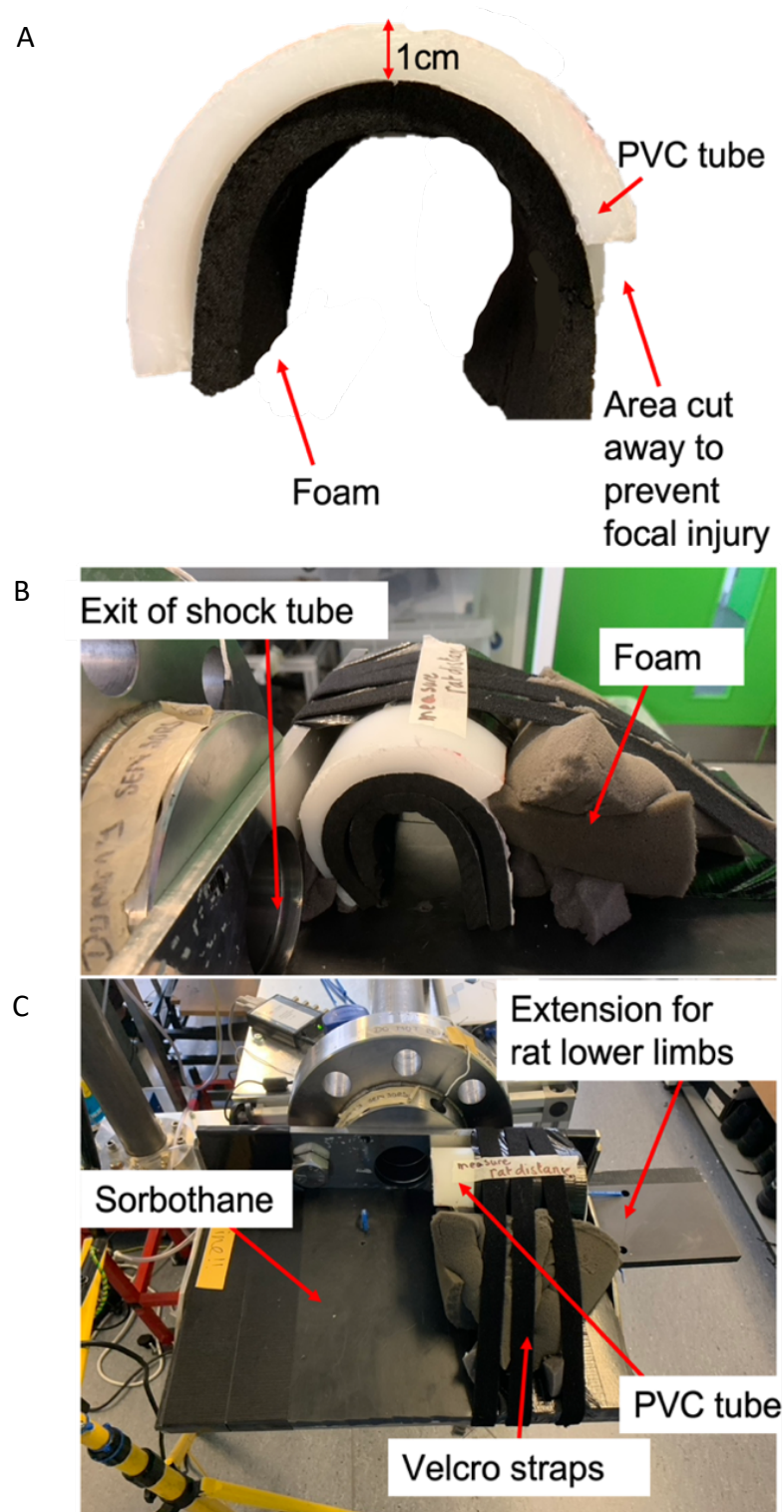


Figure 3.9 Blast experimental set up showing PVC tube and platform.

A) PVC tube used to protect thorax B) PVC on the animal platform that is strapped in with foam at the exit of the shock tube C) animal platform identifying the placement of the PVC tube, velcro straps, sorbothane and the extension for the rat's lower limbs.

3.4.2.3 Rat Head Placement

Based on Dr. Nguyen's blast model characterisation, there is a pressure drop at approximately 40-47mm from the shock tube exit, results from her characterisation are displayed in Figure 3.10.

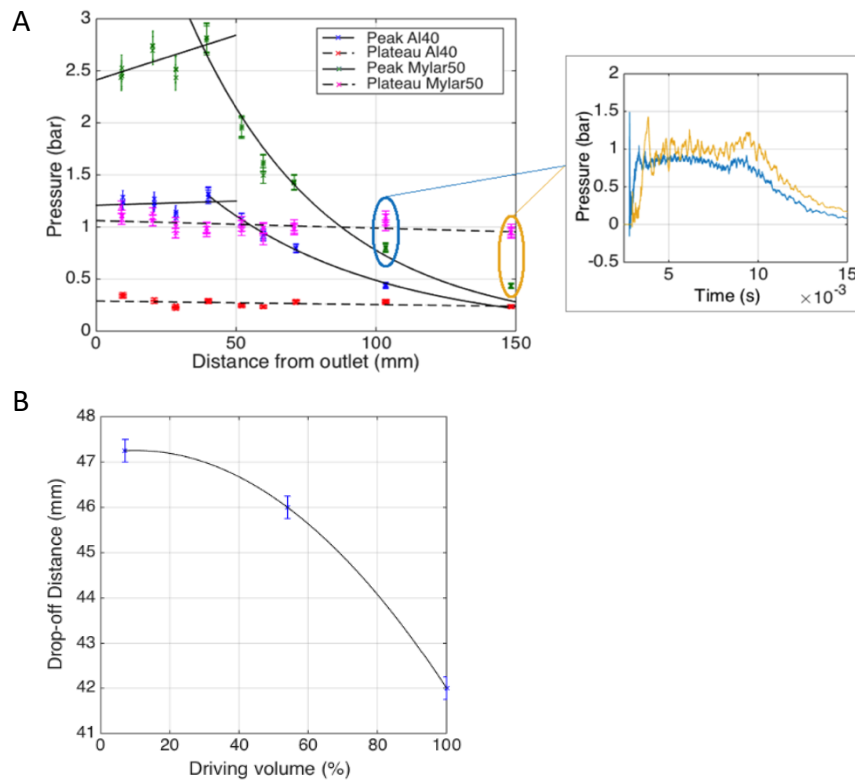


Figure 3.10 Dr Nguyen's CBIS shock tube characterisation results of the pressure drop.

This characterisation was conducted at certain distances and at certain driver volumes. A) Using 100% driver volume the drop off of pressure was established, however the blast wave was not Friedlander, but the plateaus show in the blue and yellow pressure time graph. B) the trend of pressure drop off as the driver volume was decreased. Adapted from (Nguyen 2016).

Dr. Nguyen first calculated and then demonstrated that if the rat's head is positioned between 40-47mm away from the exit the blast wave received would have the same pressure and load as inside the shock tube (Figure 3.10). To test this in our setup environment we placed a new sensor in an artificial 3D rat at the shock tube exit.

3.4.2.4 Measurement of Blast Wave at Sensor 3

To evaluate suitability of these positions for blast TBI, a third sensor was placed at two locations: inside the PVC tube to determine degree of thoracic protection and secondly at the rat's head. The PVC tube was securely fastened to the platform to prevent movement during the blast. Despite the compression from velcro straps and foam, the 1cm thick PVC tube did not deform and was unaffected by being strapped to the platform (Figure 3.9).

3.4.2.5 Assessing the PVC tube for Thoracic Protection

Another dynamic piezoelectric pressure sensor from Dytran® was connected to the control box and oscilloscope. The sensor was screwed into the PVC tube to minimise vibrations from movement that could cause interference and noise in the readings. To ensure consistency with previous experiments, the use of each of the three sensors reflected Dr. Nguyen's methodology (Nguyen 2016, Nguyen, Sory et al. 2018). Sensor 1 was positioned in the middle of the driven section for triggering and monitoring the repeatability of the testing. Sensor 2 was placed laterally to the blast wave and positioned in the shock tube adjacent to the exit to be used as measurement of incident pressure. Sensor 3 was inserted inside the PVC tube, to measure total pressure at the thorax (Bishop, Won et al. 2022). The pressure recorded from the protective PVC tube is in Table 3.3 with example graphs in Figure 3.11 showing the BOP, impulse and duration. They are shown to be much shorter than the other waves and threshold limits set by previous works (Richmond, Damon et al. 1966, Bowen, Fletcher et al. 1968, Bass, Rafaels et al. 2008) and similar to those who also tested torso peak pressures and deemed it well below threshold levels of pulmonary trauma (Gullotti, Panzer et al. 2014). These findings suggest that the PVC tube is sufficient to protect the thorax. Furthermore, the first and second sensor readings are aligned with previous blasts as shown in Figure 3.7 displaying once again the reproducibility of the shock wave.

	Trial 1	Trial 2	Trial 3	Mean $\pm$ SEM
BOP 1 (kPa)	293.81	296.22	285.10	291.71 $\pm$ 3.38
BOP 2 (kPa)	237.17	240.26	230.27	235.90 $\pm$ 2.95
BOP 3 (kPa)	18.64	16.19	23.34	19.39 $\pm$ 2.10
Impulse 1 (kPa.ms)	306.69	309.74	299.98	305.47 $\pm$ 2.88
Impulse 2 (kPa.ms)	110.02	113.05	103.92	108.99 $\pm$ 2.69
Impulse 3 (kPa.ms)	7.49	7.71	9.82	8.34 $\pm$ 0.74
Positive Duration 1 (ms)	4.23	4.23	4.18	4.21 $\pm$ 0.02
Positive Duration 2 (ms)	1.38	1.42	1.36	1.39 $\pm$ 0.02
Positive Duration 3 (ms)	0.75	0.97	0.88	0.87 $\pm$ 0.07

Table 3.3 Blast parameters for PVC tube trials

This table shows the values for sensor 1, 2, and 3 over three trials. Sensor 3 is placed in the chest protection PVC tube

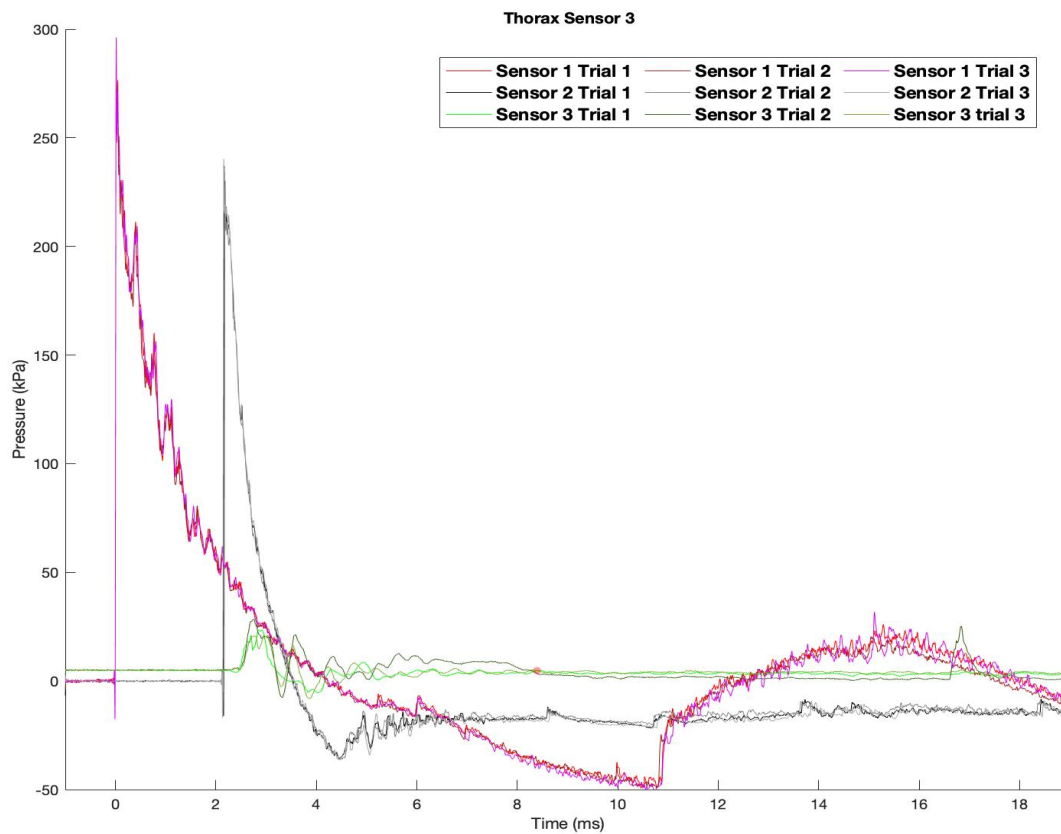


Figure 3.11 Thoracic sensor 3 pressure curves accompanied by sensor 1 and 2 over three trials.

These trials were conducted under the same blasting conditions. Red/pink are sensor 1 recordings, grey/black lines are sensor 2 and green lines are sensor 3 that were recording from inside the PVC tube.

3.4.2.6 Assessing the Rat Head using a 3D Printed Model

To accurately measure the blast loading at the rat's head approximately 40mm from the tube exit, it was necessary to use a third sensor. However, to ensure precise measurement of both pressure and blast loading, it was essential for the sensor to be placed in a fixed position and ensure that the blast wave hit the same cross-sectional area as the rat's head at that location. To achieve this, a 3D printed rat model was created and gifted by Dr Hannah Jones (Imperial College London). The 3D rat was then drilled through the head by a technician for the sensor to be screwed in flush with the side facing the blast (as shown in Figure 3.12). This replicated the spatial cross-section of the rat's head. During the platform characterisation, three sensors were used to investigate the loading on the specimen as done previously for the chest protection, and the 3D rat was fastened to the steel platform during the blast.

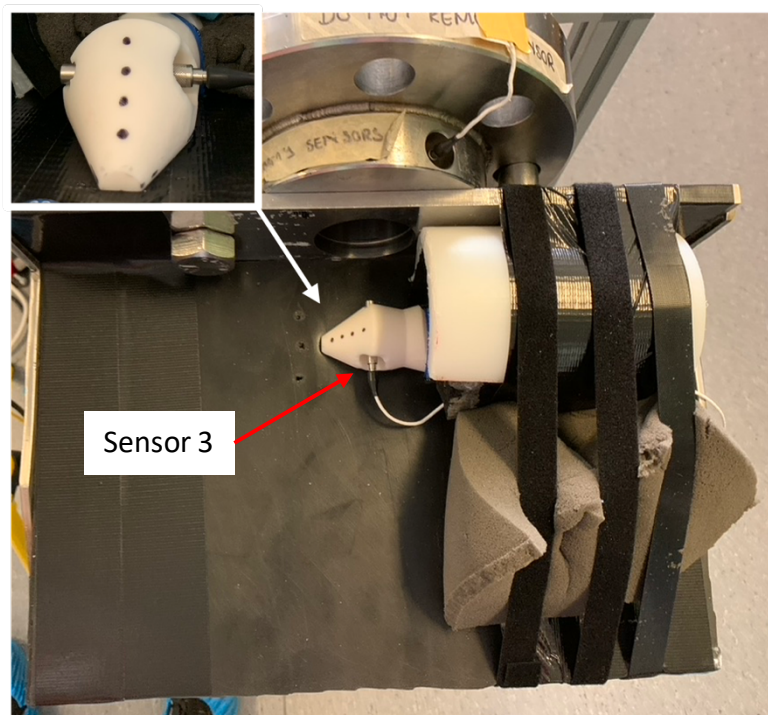


Figure 3.12 3D rat with sensor 3

Sensor 3 is embedded into the rat and the 3D rat is in the same orientation as the rat would be when receiving blast wave

The resulting blast wave parameters (Table 3.4) are the measurements of the blast wave at the rat's head. It is evident that sensor 3 is recording now both elements of incident pressure, static and dynamic pressure. There is also a possibility that there are reflected waves measured as part of the recorded blast wave, however, the blast wave does not deviate from the Friedlander waveform (Figure 3.13) and therefore is incorporated into the total pressure recorded. This is expected as similar findings of increased total pressure were recorded during characterisation of the CBIS shock tube for the same readings in Dr Nguyen's tests (Nguyen 2016) and shown in other blast studies using different shock tubes (Bishop, Won et al. 2022).

The sum of incident pressure and reflected pressure gave a total blast pressure of ~ 300 kPa, with a recorded duration of ~ 2.5 ms as shown in Table 3.4. Thus, the duration is around the target duration and is in keeping with what is recommended in the literature by Needham et al, (Needham, Ritzel et al. 2015). The BOP of this Sensor 3 in the 3D rat model study is reporting much higher blast pressures (BOP) than some studies of blast (Mishra, Skotak et al. 2016, Masri, Deng et al. 2022), but similar to Svetlov, 2010 (Svetlov, Prima et al. 2010). However, this was required in order to get the impulse of around 300 kPa.ms. The resulting blast waves had an impulse of 256 kPa.ms, which is similar to other studies (Bowen, Fletcher et al. 1968, Axelsson, Hjelmqvist et al. 2000, Chavko, Watanabe et al. 2011, Risling, Plantman et al. 2011, Mishra, Skotak et al. 2016) (despite their longer durations of 3-8 sec for some studies), and lower than others (Bishop, Won et al. 2022). Thus, the model at this point of the optimisation was in line with other studies investigating blast TBI, and in line with the injury thresholds and fatality thresholds put forward by Mishra that this study used as a guide (impulse < 300 kPa.ms for mTBI) (Mishra, Skotak et al. 2016).

Furthermore, the pressure in the PVC tube is low enough to not risk blast lung injury (Bass, Rafaels et al. 2008). Additionally, the "head" of the 3D rat where sensor 3 resided is within the ~ 40 mm recommendation from previous CBIS shock tube characterisation (Nguyen 2016). Therefore, the blast wave satisfied initial targets laid out at the beginning of this optimisation process and was deemed suitable to further investigate mild blast TBI using these blast parameters in accordance with the literature as it was expected to cause mild injury. Given that the maximum burst pressure was used and achieved a suitable blast wave to produce

mTBI, the next step was to conduct a pilot study with cadavers. With the final step to be to conduct live pilot experiments in order to investigate displacement, accelerations, post-blast welfare and histological investigation for macro-level damage to assess and confirm the severity of the blast.

	Trial 1	Trial 2	Trial 3	Mean $\pm$ SEM
BOP 1 (kPa)	293.05	291.03	300.69	294.92 $\pm$ 2.94
BOP 2 (kPa)	233.02	237.54	239.39	236.65 $\pm$ 1.89
BOP 3 (kPa)	299.34	301.36	336.20	305.70 $\pm$ 11.96
Impulse 1 (kPa.ms)	297.88	303.31	306.14	302.45 $\pm$ 2.43
Impulse 2 (kPa.ms)	104.70	109.97	111.32	108.66 $\pm$ 2.02
Impulse 3 (kPa.ms)	247.63	248.03	273.15	256.27 $\pm$ 7.03
Positive Duration 1 (ms)	4.16	4.01	4.18	4.12 $\pm$ 0.05
Positive Duration 2 (ms)	1.33	1.37	1.39	1.36 $\pm$ 0.02
Positive Duration 3 (ms)	2.20	2.92	2.88	2.67 $\pm$ 0.23

Table 3.4 Blast parameters for 3D rat trials

The 3D rat is in the same head position as the rat would be. The table shows parameters for each trial for sensor 1, 2, and 3 over three trials. Sensor 3 is placed in the 3D rat model.

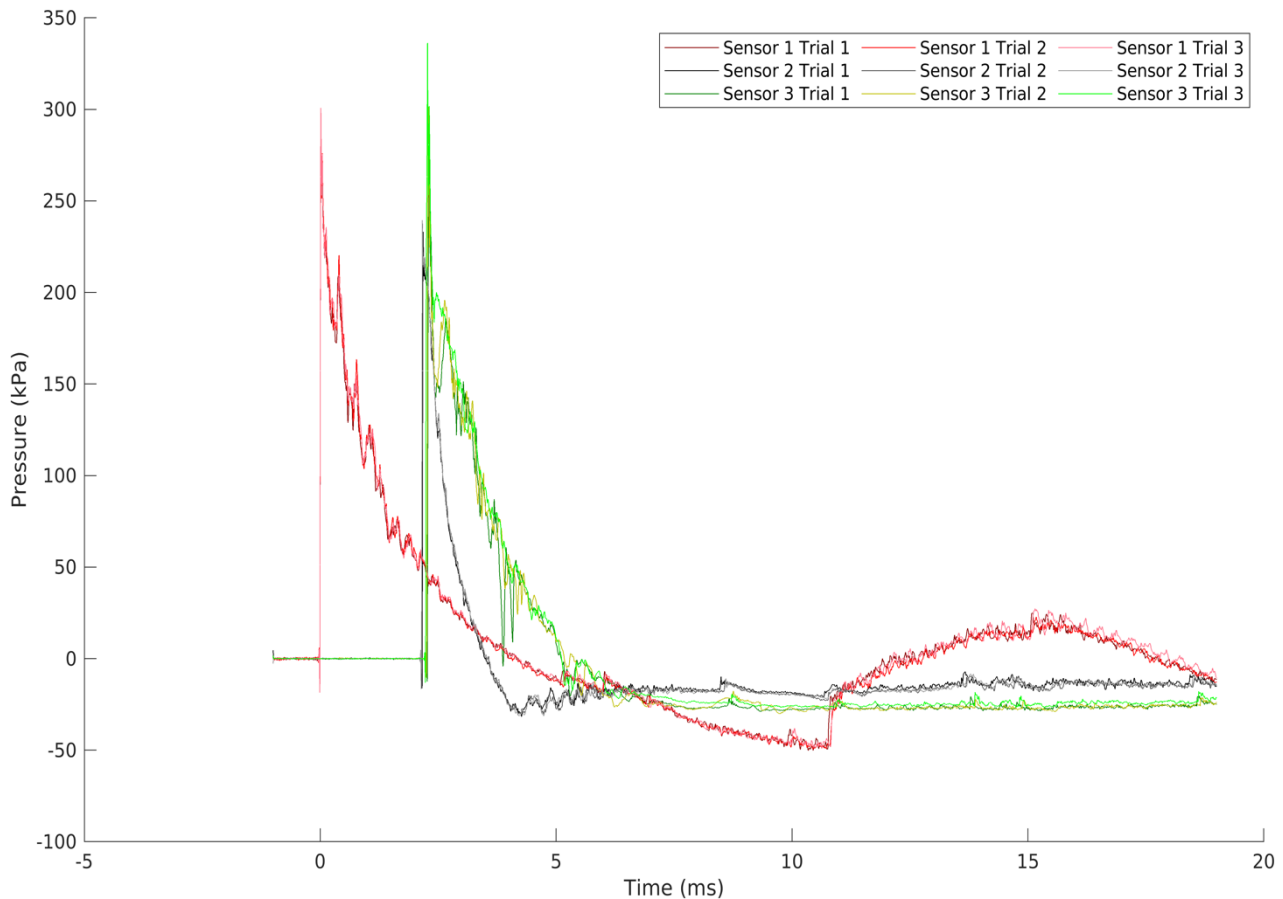


Figure 3.13 3D rat pressure curves over Head position

This time pressure curve displayed the head position recordings of Sensor 3 accompanied by sensor 1 and sensor 2 recordings. Three trials shown for the sensor 3 inside the 3D rat. The blasts were conducted under the same conditions. Representative blast wave recordings at all three sensors for the head position of sensor 3.

3.4.3 Pilot study: Testing the Blast Setup

3.4.3.1 Pilot Study Part 1: Assessments Performed in Rat Cadavers

Before testing on live animals, we tested on cadavers. To evaluate the setup, several assessments were required. Firstly, to ensure the rat inside the PVC protection tube was secure. Secondly, to assess blast wave consistency between sensor 1 and 2 during pre-cadaver and cadaver blast tests. Finally, to evaluate the head trajectory of the cadaver so that it could be compared across experiments for reproducibility.

To observe the trajectory of the head movement of the blasted cadaver, a high-speed camera was used. Similar to Dr. Nguyen's thesis and other studies (Goldstein, Fisher et al. 2012, Gullotti, Panzer et al. 2014, Bishop, Won et al. 2022), a Phantom v7.3 high-speed camera was used, along with two 500 W static floodlights to observe the displacement of the cadaver. The camera began recording when sensor 1 was triggered by an increase in pressure. Videos were recorded using Phantom Cine (PCC) v1.3 at a resolution of 512 x 364, with a sample rate of 10,000 fps, which is far lower than Goldstein, but a lot more than Bishop and Gullotti (Gullotti, Panzer et al. 2014, Bishop, Won et al. 2022) and an exposure time of 70 μ s. The videos were trimmed to one frame before the rat's head moved and until the head lay to rest on the sorbothane. The video was then exported as .mov and .cine files. Tracking was performed manually using Physlets Tracker 5.1.5 (project source: Open Source Physics).

Tracker software was used to track the movement of the animal. The nose was selected as the origin for tracking, similar to Goldstein, 2012 (Goldstein, Fisher et al. 2012), while the eye was used as a secondary tracking point as performed in other studies (Gullotti, Panzer et al. 2014, Beamer, Tummala et al. 2016). Automated tracking was not possible due to binary colouring of the videos, thus manual tracking was used on each frame. Tracking and calculations were carried out up to ~ 20 ms, as movement beyond this point was negligible. Displacement was calculated along the x and y axes over time. Different data smoothing algorithms were tested, and a 5th order low pass Butterworth filter was chosen followed by a 9-point moving average. These algorithms were selected as the root mean squared residual displacement between the filtered/smoothed and original tracked data did not vary beyond 0.5% over the sampling interval (Gullotti, Panzer et al. 2014). Velocity and acceleration were calculated using the first and second derivatives, respectively. To account for human error from manual tracking to obtain displacement data, velocity was smoothed using a 9-point moving average. Acceleration was then calculated from the smoothed velocity data for both the eye and nose.

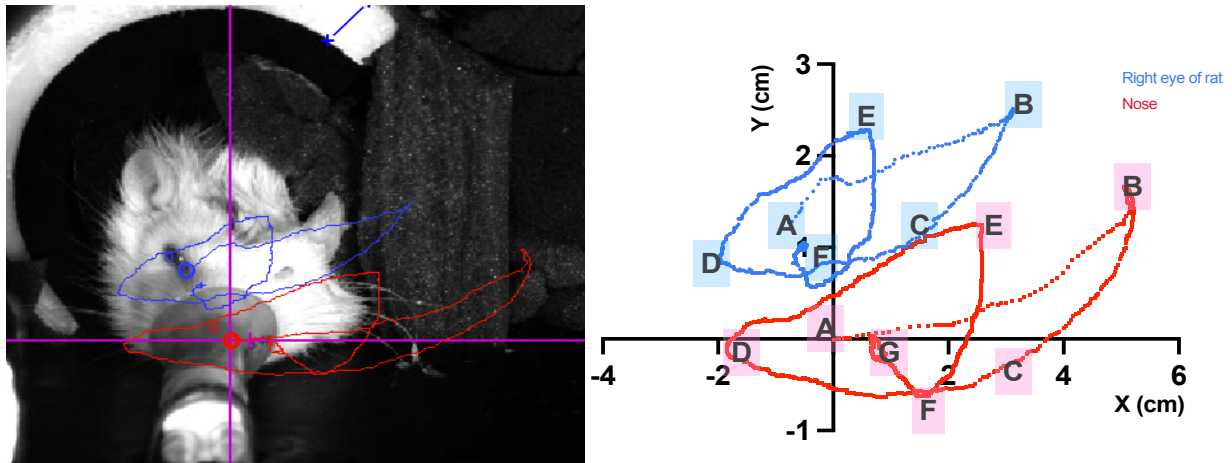


Figure 3.14 Cadaver displacement for the nose and eye in the X and Y plane.

The blue line is for the eye and the red line is for the nose displacement trajectory. Starting point for both nose and eye is denoted by A and then progresses through the alphabet to depict the movement of the head during the blast.

The trajectory of the rat cadaver during the blast wave is illustrated in Figure 3.14. The point of origin is where the nose sits at rest, inside the translucent nose cone. The blast wave meets the head of the rat and pushes the rat head away from the exit of the shock tube and it travels from **point A to point B**. (**Nose** is the **red line**, and the **eye** pink A to B is the **blue line**). **Point B** in the blue A to B graph is the maximum displacement for the eye and point B in the red line is the maximum displacement for the nose. After the maximum displacement, the head swings back towards the exit of the tube, whilst making light contact with the sorbothane at **C**, then reaches its next point of directional change, point **D**. **D-E**, this is where the head moves up slightly and away from the exit of the shock tube again to where **E** is, then moves down to rest between **F and G**. This displacement occurs over ~20ms. Overall, the shape depicts a rough figure of eight.

The degree of displacement observed in the current study has a similar trajectory to that reported in Goldstein's 2012 investigation where a bobble head motion was observed. However, the difference in magnitude is due to species difference where they used a mouse model (Goldstein, Fisher et al. 2012). The observed differences in head movement between the present study and Gullotti's perpendicular model, which was also carried out in mice, may be attributed to variations in the positioning of the animals relative to the shock tube. Specifically, in the former study (Gullotti, Panzer et al. 2014), the animals exhibited a greater degree of vertical motion (along the y-axis) than horizontal motion (along the x-axis) relative to this study.

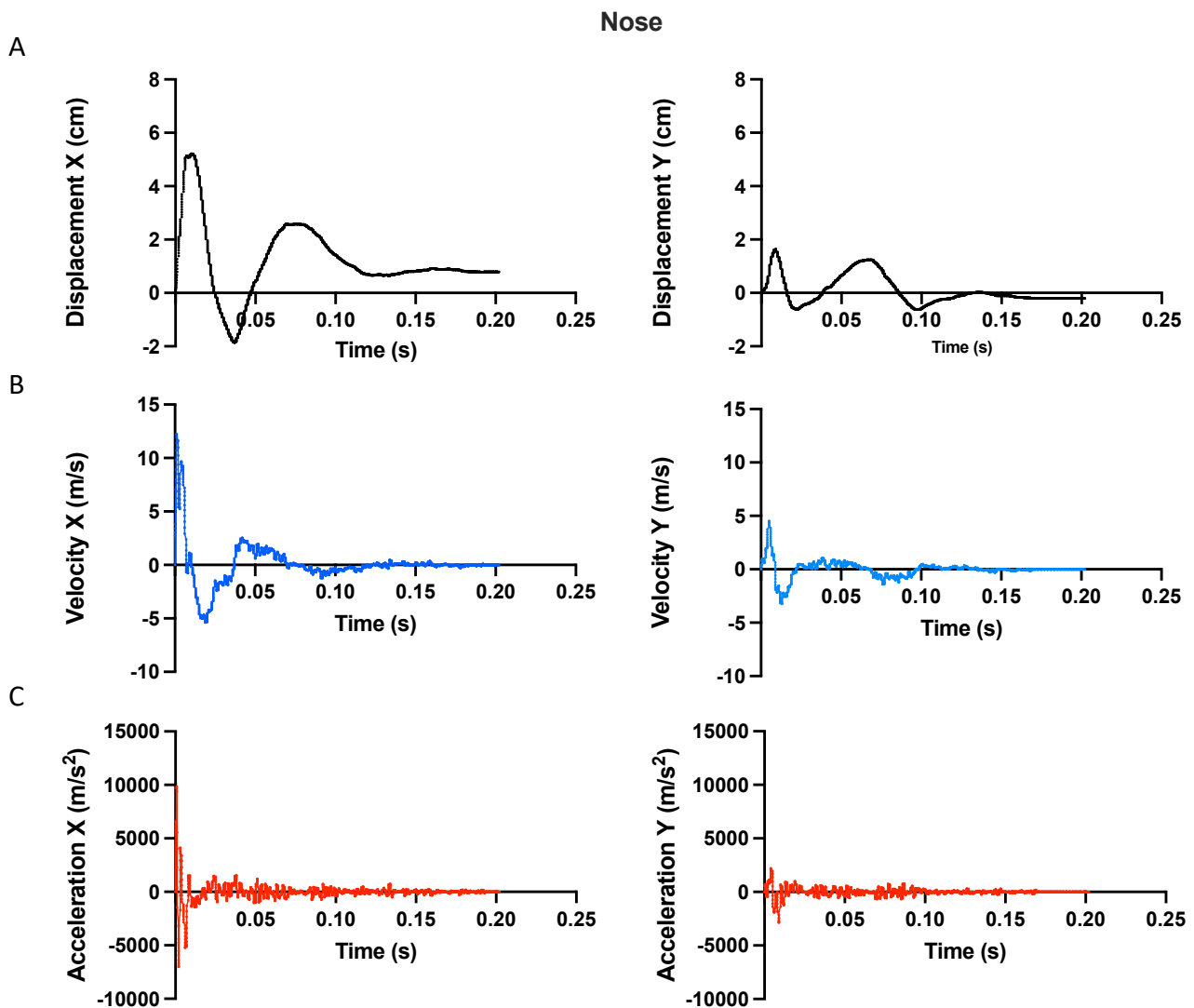


Figure 3.15 Nose displacement, velocity and acceleration for the cadaver

A) is the displacement of X and Y for the nose, B) is the velocity for X and Y for the nose. C) Acceleration for X and Y for the nose. All over time in seconds.

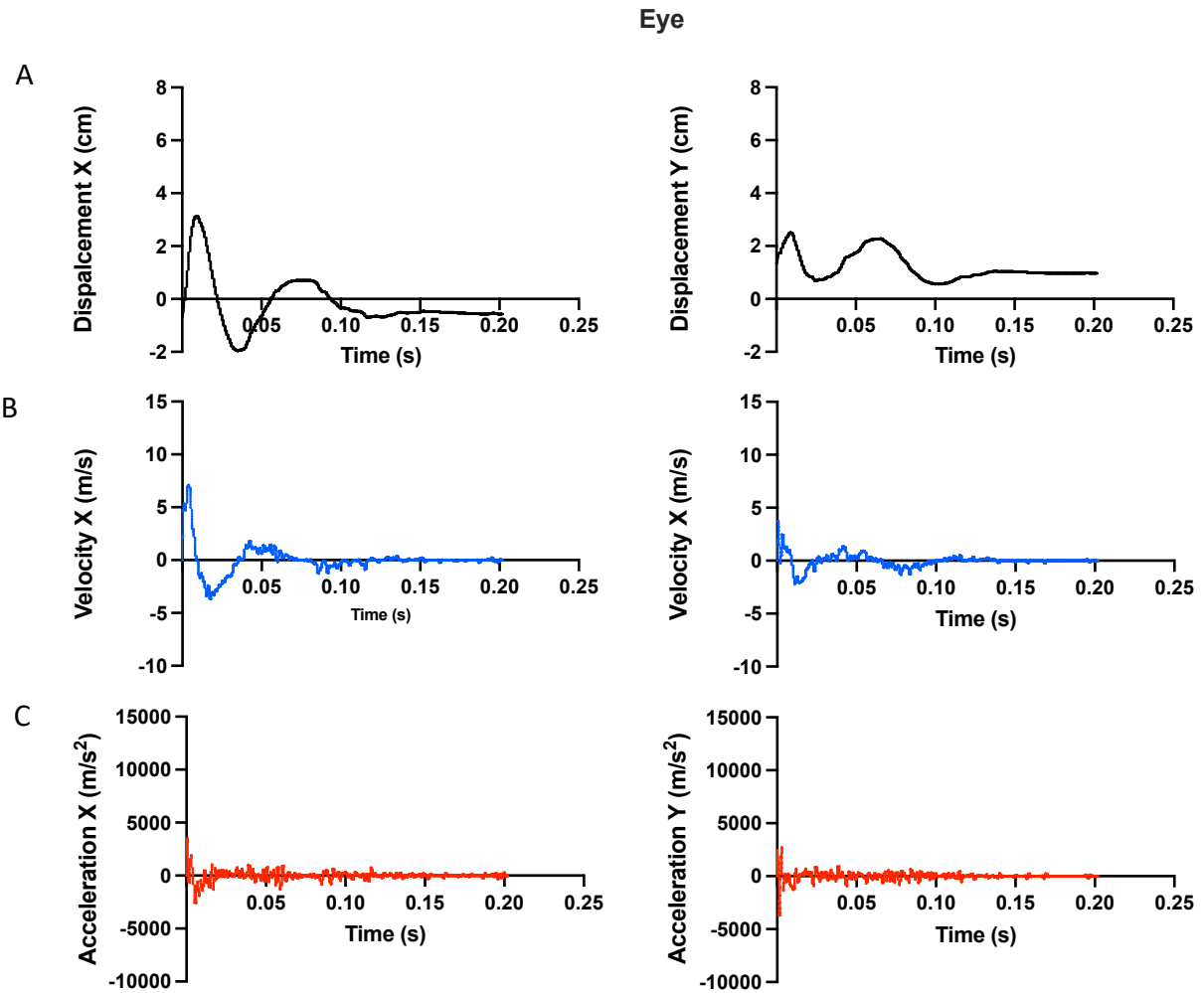


Figure 3.16 Eye displacement, velocity and acceleration for the cadaver

A) is the displacement of X and Y for the eye, B) is the velocity for X and Y for the eye. C) Acceleration for X and Y for the eye. All over time in seconds.

Figure 3.15 illustrates the displacement, velocity, and acceleration data for the nose in both the X and Y planes of the cadaver. It is important to take into account that the nose contains a greater proportion of connective tissue compared to other regions of the face when interpreting acceleration values. This can mean that the nose can move faster and further than other areas on the head. It is also the furthest from the pivot point of the neck, unlike the eye. This consideration is also relevant to Goldstein's 2012 study, which utilised the nose as a marker without a secondary marker to more accurately represent the brain. Nevertheless, the acceleration values reported in the current study are comparable to those

observed in Gullotti's perpendicular model of blast traumatic brain injury (bTBI), which employed the eye and more unspecified fiducial markers (Gullotti, Panzer et al. 2014).

The velocity data depicts the nose to have an instant increase in velocity and acceleration in the x-axis indicating that the first displacement points for the nose occur rapidly. This movement is followed by a decrease in velocity and acceleration as the nose reaches its peak displacement away from the end of the shock tube at around 1.5 ms (Figure 3.15A and Figure 3.16A). The displacement shows that the nose moves back towards the exit of the tube, which results in an increase in velocity and slight increase in acceleration at ~3-4 ms. Between 4 and 6 ms, the displacement of the nose is about to return to travelling away from the exit of the shock tube again, and this occurs at an almost constant velocity that slowly decreases and there is slight deceleration. By around 10 ms there is still slight movement from the animal but it is occurring at a much slower rate than the first 10 ms (Figure 3.16). The displacement, velocity and acceleration are lower for the y-axis, but that is due to the fact that most of the movement is away from the exit of the tube rather than swinging up, like with Gullotti (Gullotti, Panzer et al. 2014). The eye has extremely similar data but on a smaller scale, and this is more likely to depict what is happening to the brain.

The maximum accelerations experienced at the nose were $9,849 \text{ m/s}^2$ in the x axis and $2,196 \text{ m/s}^2$ in the y axis. The eye had lower maximum values; $3,523 \text{ m/s}^2$ for the x and $2,723 \text{ m/s}^2$ for the y. This is within the same order of magnitude of accelerations as Gullotti (Gullotti, Panzer et al. 2014) but much lower than Beamer 2016 ($\sim 22,000 \text{ m/s}$) (Beamer, Tummala et al. 2016). It is important to note for this study and others such as Gullotti and Beamer that these velocities are taken from 2D videos and not 3D. Therefore, these are rough estimations of the accelerations the brain is experiencing as we are assuming that the eye and the nose are moving in plane. More work would be required to achieve 3D velocity and accelerations and there is currently no study in the literature that has done this. However, these rough estimates are still useful and allow for a check of repeatability of the kinematics.

It is important to note that this is data from only one animal cadaver. To draw meaningful conclusions and make comparisons with this model against other models, it is necessary to check the repeatability of the kinematics to be similar in live anaesthetised animals.

Therefore, before the project proceeded any further, we compared these results with those from live anaesthetised rats to make any necessary adjustments.

3.4.3.2 Considerations and Choices for Testing Live Animals with this New Blast Setup

The next stage of the assessment of the set up was to perform the blast on live anaesthetised rats. This stage required more elements such as; anaesthetic choice, analgesic choice and pre and post-blast care. It also incorporated consideration of some expected outcomes from the blast, such as blast lung injury and loss of righting reflex. Furthermore, since different anaesthetics behave differently on the brain and physiology, suitable equipment was required to monitor it.

Ensuring the appropriate level of anaesthesia is crucial for the welfare of laboratory animals. However, the choice of anaesthetic should also consider both the safety of the animal and the scientific outcomes. For this blast project, a short-acting and easily reversible anaesthetic was preferred. The gaseous anaesthetic Isoflurane is commonly used in the literature, and a comparative study has shown that it provides quicker induction, anaesthetic plane, and faster recovery than Ketamine/Xylazine (Jiron, Mendieta Calle et al. 2019). However, isoflurane can depress the respiratory rate (Dárdai and Heavner 1989). Therefore, oxygen enriched air was required to be delivered along with the Isoflurane. Under veterinary guidance, the respiratory rate goal for the animals was set between 40-50 breaths per minute. This was to ensure that the animal was not too deeply anaesthetised to potentially stop breathing, but was still at a deep enough level of anaesthesia to not be conscious for the experiment. Blood oxygen was monitored throughout the anaesthetic duration to ensure animals would not be hypoxic at any point as hypoxia can also induce brain injury and therefore confound the results.

To administer the isoflurane through inhalation, a nose cone (made from flexible polyethylene) was fashioned to supply both isoflurane and oxygen and also allow scavenging for the exhaled gases from the rat. As the body can become cold from not moving and the effect of the anaesthesia, a smart heating pad with a rectal probe, pulse oximeter and respiratory measure was used (item code: 75-1501, Harvard Apparatus). Once the animal was

at a stable level of anaesthesia, it was moved to the blast platform. The tubing that delivered the Isoflurane and oxygen was secured to the platform, but the nose cone was not secured so that it would be blasted away during the blast exposure. This would allow for the LORR to be calculated from the time of blast until all four paws of the rat were touching the ground in the recovery cage.

Another consideration was animal recovery. During recovery, the animals required heat and oxygen since they would likely take some time to return to consciousness. The recovery cage was heated by a heating pad, and an extra oxygen machine enriched the area around the rat until it was on all four paws and moving around the cage. Analgesia, such as Buprenorphine, was also used for this project given that there was the possibility that the animal might experience some discomfort after regaining consciousness. It was administered 30 mins prior to blast exposure subcutaneously.

3.4.3.3 Pilot Study Part 2: Testing Live Animal with Blast Setup

To compare the cadaver and live experiment head trajectory, high-speed videos were recorded using the same camera and light setup as the cadaver experiment for the live experiment. Under veterinary supervision, a male Sprague-Dawley rat was put into an induction chamber with 5% isoflurane (~2.5 mL/min). When the animal had fallen unconscious with breathing rate of ~60 breaths per minute, it was then moved to the smart heated monitoring platform where Isoflurane was administered through a nose cone at 1.5-2.5% at ~2 mL/min. Temperature was maintained at ~37 degrees with use of a rectal probe. Respiratory rate was used as a proxy of anaesthetic depth (40-50 bpm with high blood oxygenation) along with the hind paw reflex pinch test, conducted on either foot per check. Blood oxygenation was monitored throughout to ensure no animal was hypoxic during the slow respiratory rate. The rat was injected with buprenorphine (0.05 mg/kg s.c.; Vetergesic, UK) ~30 min prior to blast exposure. When the animal had stabilised under anaesthesia, it was put into a polythene cone to ensure the forelimbs were safely tucked out of the way during the blast exposure. Following insertion in the cone, the animal was moved to the blasting platform and put into the PVC tube. Only the head and neck were exposed to the blast whilst the rest of the body was secured within the PVC tube and surrounded by foam.

The nose of the animal was placed into the isoflurane nose cone. Respiratory rate was monitored manually at this point and blood oxygenation was monitored using a pulse oximeter. The animal was exposed to a single blast and was recorded using the high-speed camera. The animal was then removed from the PVC tube, and placed on its side into a heated, oxygen rich recovery cage. In this cage, the animal was supplied with wet mash and given a subcutaneous injection of 3 mL of sodium chloride 0.18% and Glucose 4% Solution (Baxter, UK). The return of righting reflex time was recorded when the animal had placed all four feet on the surface of the recovery cage.

The rat was then monitored for signs of overt pain, and given buprenorphine every 12 hours after blast for 3 days. The rat was checked using Morton and Griffiths guide (Morton and Griffiths 1985) to assess the wellbeing of the rat by monitoring any signs of pain and distress. A score of 2 or below was considered healthy until termination.

3.4.3.4. Comparison of Blast Waves and Displacement Trajectory Between Cadaver and Anaesthetised Live Pilot Animals

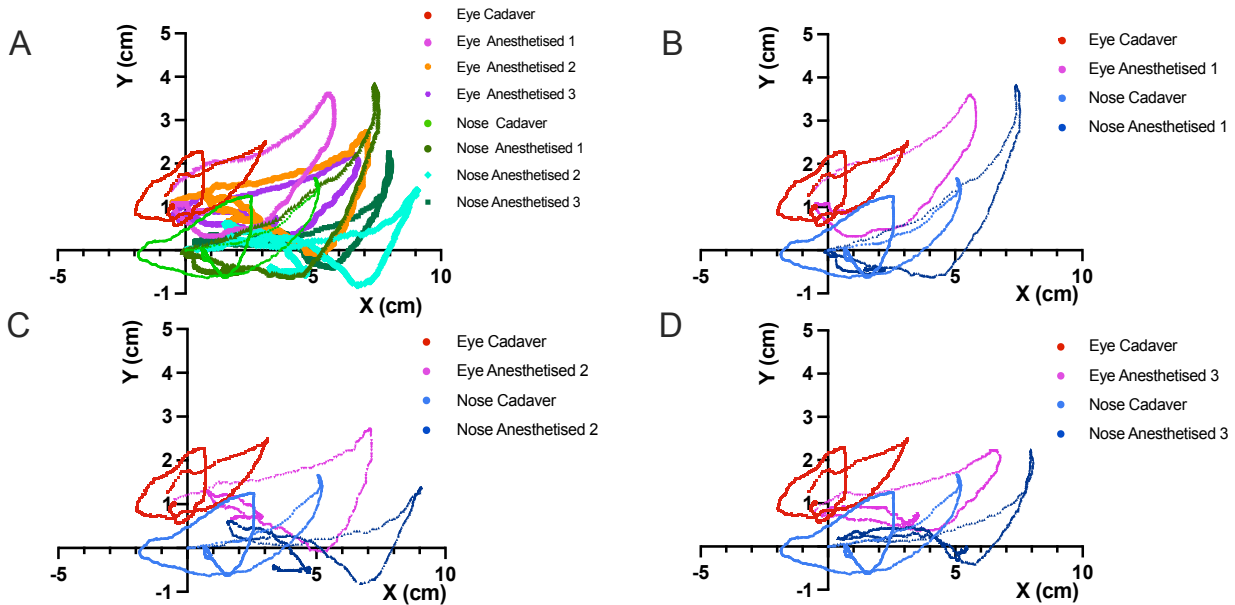


Figure 3.17 Displacement graphs of eye and nose for cadaver and three anesthetised live pilot rats.

A) All 4 animal trajectories for eye and nose, B) Separated graph of trajectory of cadaver and anesthetised animal 1, C) Separated graph of trajectory of cadaver and anesthetised animal 2, D) Separated graph of trajectory of cadaver and anesthetised animal 3. All displacement data is in cm.

Comparisons between a cadaver and three anaesthetised live animals exhibit only mildly similar trajectories as shown in Figure 3.17. The live anaesthetised test animals demonstrate greater displacement in both the x and y directions. These differences are discernible when all four rats are compared in a single graph with the cadaver (Figure 3.17B-D). Upon further scrutiny of the videos, it became apparent that the cadaver was obstructed from achieving the same x and y displacement due to the presence of foam placed to its side. Conversely, the foam was situated differently for the anaesthetised animals, affording them greater movement (~2 cm) and even causing some to be partially drawn out by the blast, particularly two out of the three subjects. Additionally, during these pilot trials, the nose cone remained intact, which impeded the rats' movements, resulting in their inability to return to the point of origin, in contrast to the cadaver's displacement.

Comparisons of displacement, velocity, and acceleration over time for the four animals of the nose and eye regions are depicted in Figure 3.18 and Figure 3.19. There is discernible variability in displacement between animals in both figures. Nevertheless, although the animals exhibit different peak displacements in terms of timing and value, their velocity and acceleration follow similar trends. Initially, there is a movement that gains momentum, resulting in higher velocity and acceleration a few frames after the initial movement (shown in Figure 3.18B, C Figure 3.19B, C) as the blast wave moves away from the tube's exit. Peak velocity and acceleration decrease as displacement reaches its peak (as demonstrated in Figure 3.18A and Figure 3.19A). After reaching peak displacement, the head moves back towards the shock tube, causing an increase in velocity and acceleration for both the eye and nose regions. This shows a similar trend to the cadaver in displacement, velocity, and acceleration, but with a 1 ms delay. This delay is most likely due to the live animals' unrestricted movement and being partially sucked out of the PVC tube. Consequently, peak displacement occurred later and at a slowed velocity. Nevertheless, despite the differences in displacement, the velocities and accelerations did not vary substantially in their trending motion, as evidenced by the lesser variation relative to previous studies (Gullotti, et al: with a variation of $\pm 5400\text{m/s}^2$) (Gullotti, Panzer et al. 2014), as depicted in Table 3.5. However, adjustments to positioning and security of the rats during the blast were needed for the blast project. These adjustments included placement of the foam inside the PVC tube and moving the zip tie that held the tubing of the nose cone to ensure that the nose cone would be the only part blasted away with the pressure wave. These adjustments would ensure similar blast loading to the head between blast experiments.

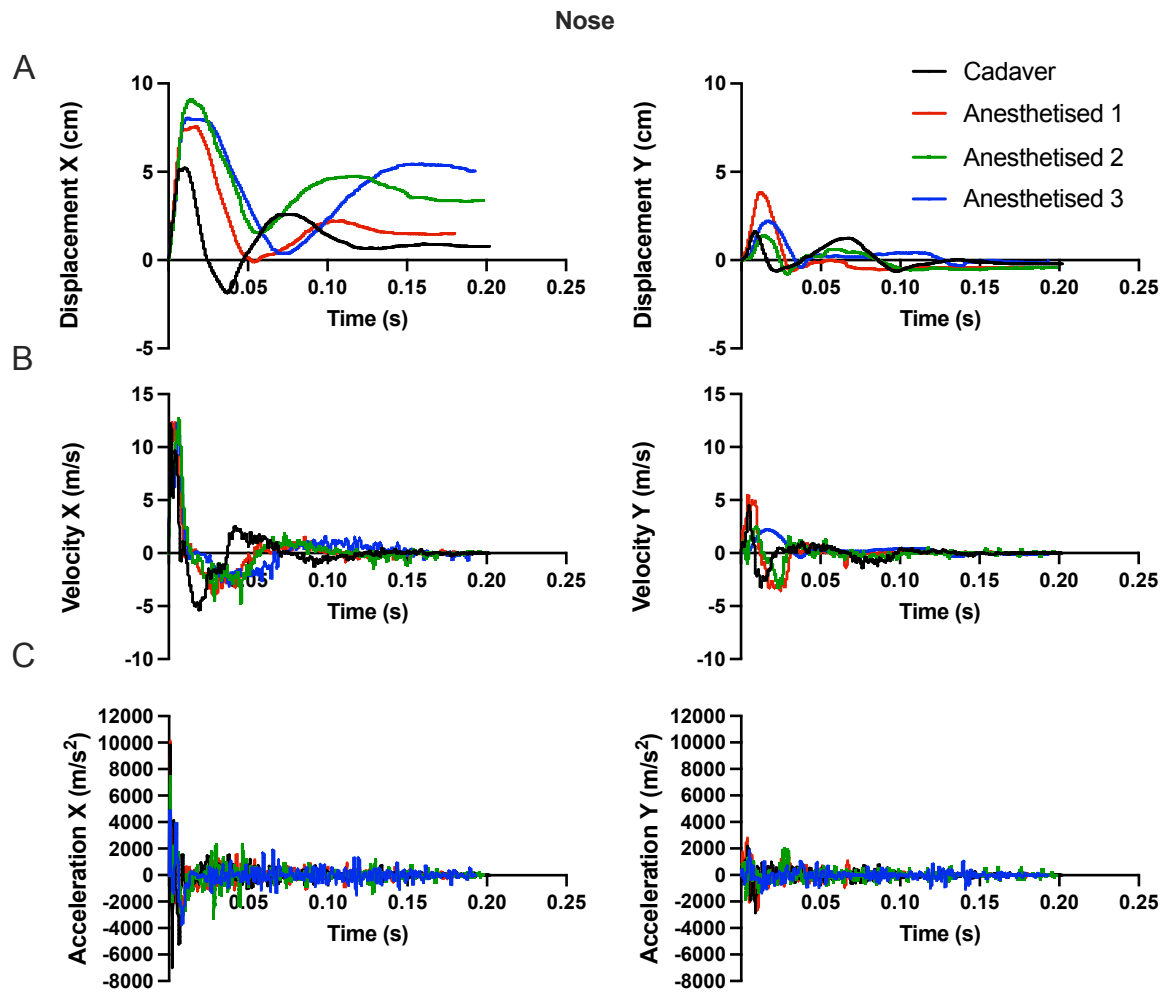


Figure 3.18 Nose displacement, Velocity and Acceleration comparison between cadaver and three anesthetised live pilot rats.

A) displacement over time B) velocity over time, C) acceleration over time.

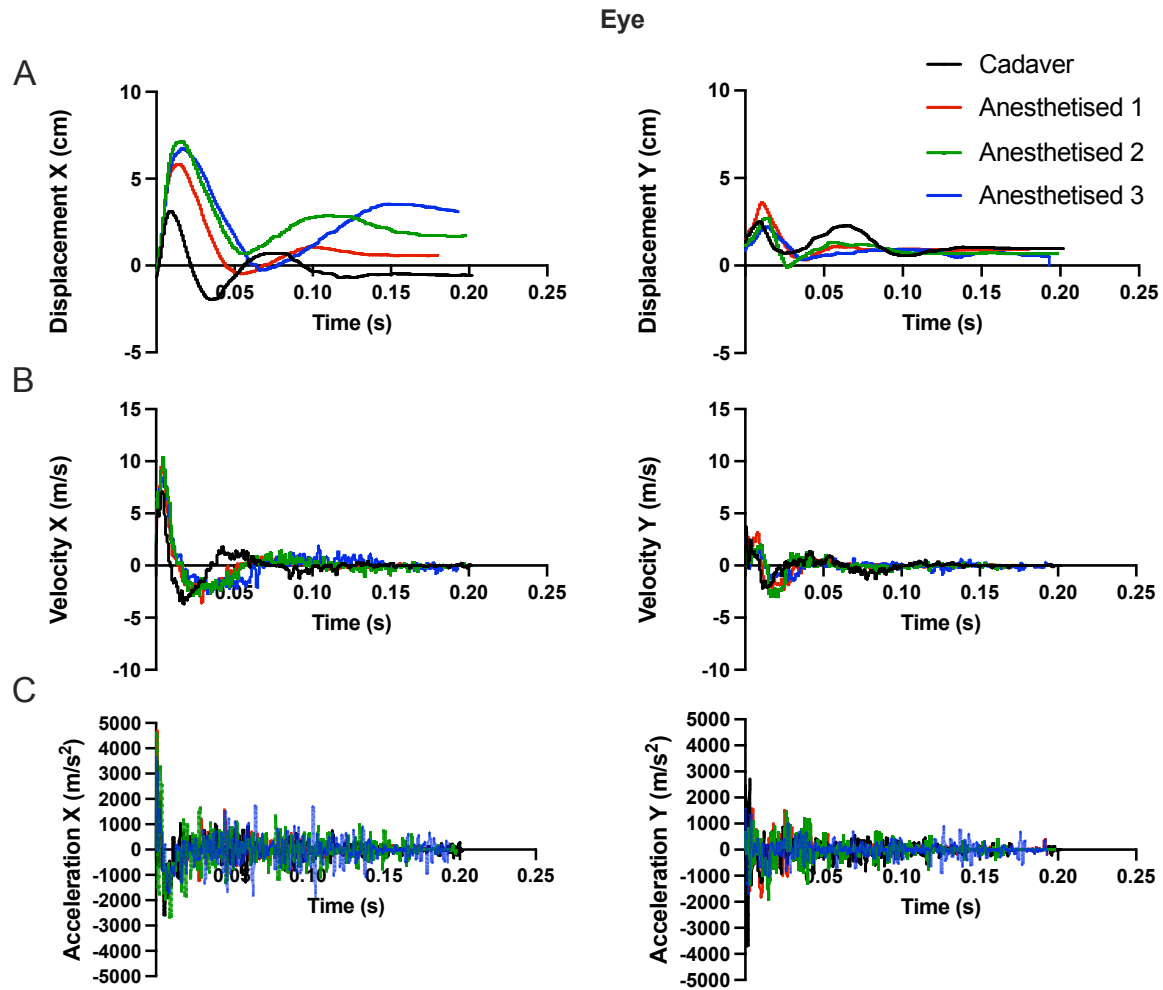


Figure 3.19 Eye displacement, Velocity and Acceleration comparison between cadaver and three anesthetised live pilot rats.

A) displacement over time B) velocity over time, C) acceleration over time.

	X NOSE m/s ²	Y NOSE m/s ²	X EYE m/s ²	Y EYE m/s ²
Cadaver	9849.12	2196.04	3523.03	2723.50
Anaesthetised animal 1	7460.41	2015.86	4637.98	1445.69
Anaesthetised animal 2	4931.68	1904.23	3671.45	1590.97
Anaesthetised animal 3	10120.02	2797.57	4692.83	1807.44
Mean ± SEM	8090.31 ± 1069.27	2228.43 ± 198.73	4131.32 ± 239.96	1891.90 ± 101.61

Table 3.5 Maximum acceleration values for the cadaver and 3 live anaesthetised rats for the X and Y axis for both eye and nose

3.4.3.5 Comparison of LORR and Tissue Structure Between Pilot Sham and Blast Animals

3.4.3.5.1 Assessment of Loss of Righting Reflex

The live anaesthetised animals had their LORR measured. They were then terminated and their brain tissue was examined for any macro changes in tissue structure. Three sham experiments were also performed, following the same procedure as the live anaesthetised blast exposure tests but without the blast for LORR comparison. The LORR was utilised as a proxy for loss of consciousness, with a threshold of <30 minutes relative to the shams to be considered mild TBI (GCS score, (NICE 2014)). Extended LORR time was utilised as indication of TBI relative to shams. It is important to note that anaesthesia and analgesia can influence LORR times, hence the significance of the sham experiments as drug controls.

There was a trend towards longer LORR times in animals exposed to the blast compared to the shams. This indicates the blast caused an injury (Figure 3.20).

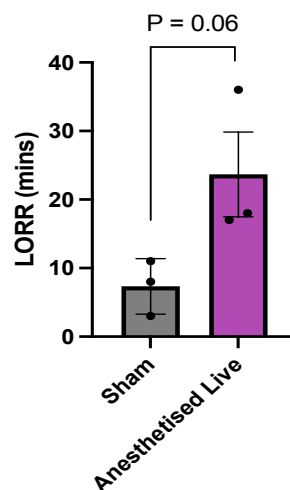


Figure 3.20 LORR between sham and anaesthetised live blast exposed animals for blast set up test

3.4.3.6 Cresyl Violet Assessment of Structural Changes in Brain Post-Blast

Cresyl violet was used to identify whether any macroscopic changes in the structure of the tissue or signs of lesions or major tissue/cell loss were present. Cresyl violet is a basic dye utilised to stain the Nissl substance, which constitutes the rough endoplasmic reticulum in all cells, including neurons and glia. It is a widely used tool in neuroscience research to visualise the cytoarchitecture of the brain. The brain tissue of the pilot blasted animals and shams were collected at 7 days post-injury (DPI). 7 DPI was used to allow for any secondary processes to occur and affect the tissue that could be visualised (lesions, tissue loss etc). There was no sign of tissue loss or lesions in the brain as shown in Figure 3.21 where the sham and blast brains look indistinguishable when stained with cresyl violet.

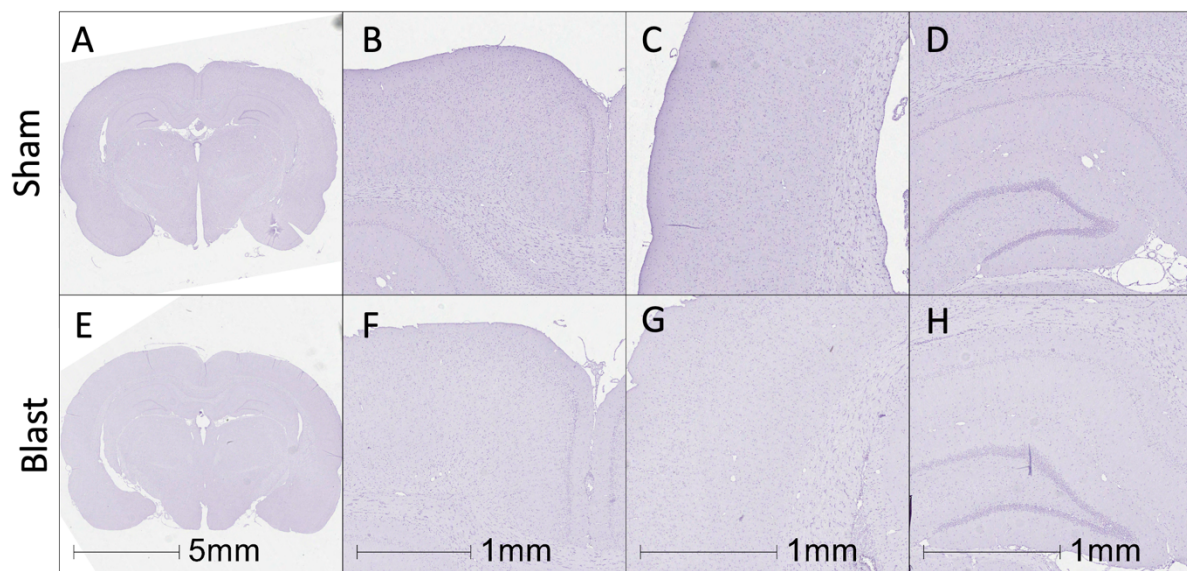


Figure 3.21 Cresyl violet staining of sham and pilot blast brain tissue coronal section.

A/E) Sham whole brain slice, B/F) Retrosplenial cortex and corpus callosum C/G) Auditory cortex D/H) Hippocampus.

3.4.3.7 Assessment of Welfare of Pilot Animals

In the live animal test, the live anaesthetised animals were subjected to blasts and their welfare scores were recorded daily after the injury. The results showed that these animals did not exhibit any signs of discomfort, stress or weight loss following the blast, as their

welfare scores remained between 0-1. This score range is considered very good according to the Morton-Griffith's welfare score sheet, as scores below 2 are indicative of good welfare (Morton and Griffiths 1985).

3.4.4 Outcome of Pilot Blast Study

Overall, the findings of the pilot study blast tests indicated the blast waves were reproducible and a blast wave was identified that was suitable for studying mild blast induced TBI with a Friedlander waveform. Moreover, there were no mortalities and no sign of blast lung injury despite the high BOP as the impulse was lower than thresholds set by others (Beamer, Tummala et al. 2016, Mishra, Skotak et al. 2016). Furthermore, the lack of mortality concluded that the PVC tube was deemed suitable for thoracic protection.

The comparison between the cadaver and pilot live anaesthetised animal blast exposures revealed the crucial role of animal placement and security. Despite acceleration similarities and a margin of error below that of previous published works, this information provided insight into the best way to secure the animals in the PVC tube. Additionally, the pilot study's LORR indicated that the model could differentiate between sham and blasted animals. This suggests that the model induced a TBI in the rats. As indicated by the absence of pain and distress in the welfare score, the TBI induced was a mild TBI. Moreover, the comparison between brain tissue of the blasted and sham experiments supported the mild nature of the TBI induced in the pilot animals. Consequently, the model and blast parameters used were considered suitable for investigating mild blast TBI and therefore no further blast pressures were investigated for this chapter. The methods used during the setup will be used for the main experimental cohort for the thesis (as found in Chapter 2).

3.5 Blast Experiments Used for this Thesis using the Blast Parameters Established in this Chapter.

Following the implementation of position adjustments, single blast exposure experiments were conducted to investigate blast pathology using the same blast conditions presented in

Table 3.4. To assess the consistency of blasts, three representative rats were selected to demonstrate the movements of the experimental cohort.

Compared to previous pilot live experiments (Figure 3.18 and Figure 3.19), the trajectory of the blast project animals showed more constrained movement, but with greater displacement than that of the cadaver, as evidenced by maximum displacement values for the nose of the cadaver (~4cm), live tests (~7-8cm), and blast project (~6cm). Importantly, the

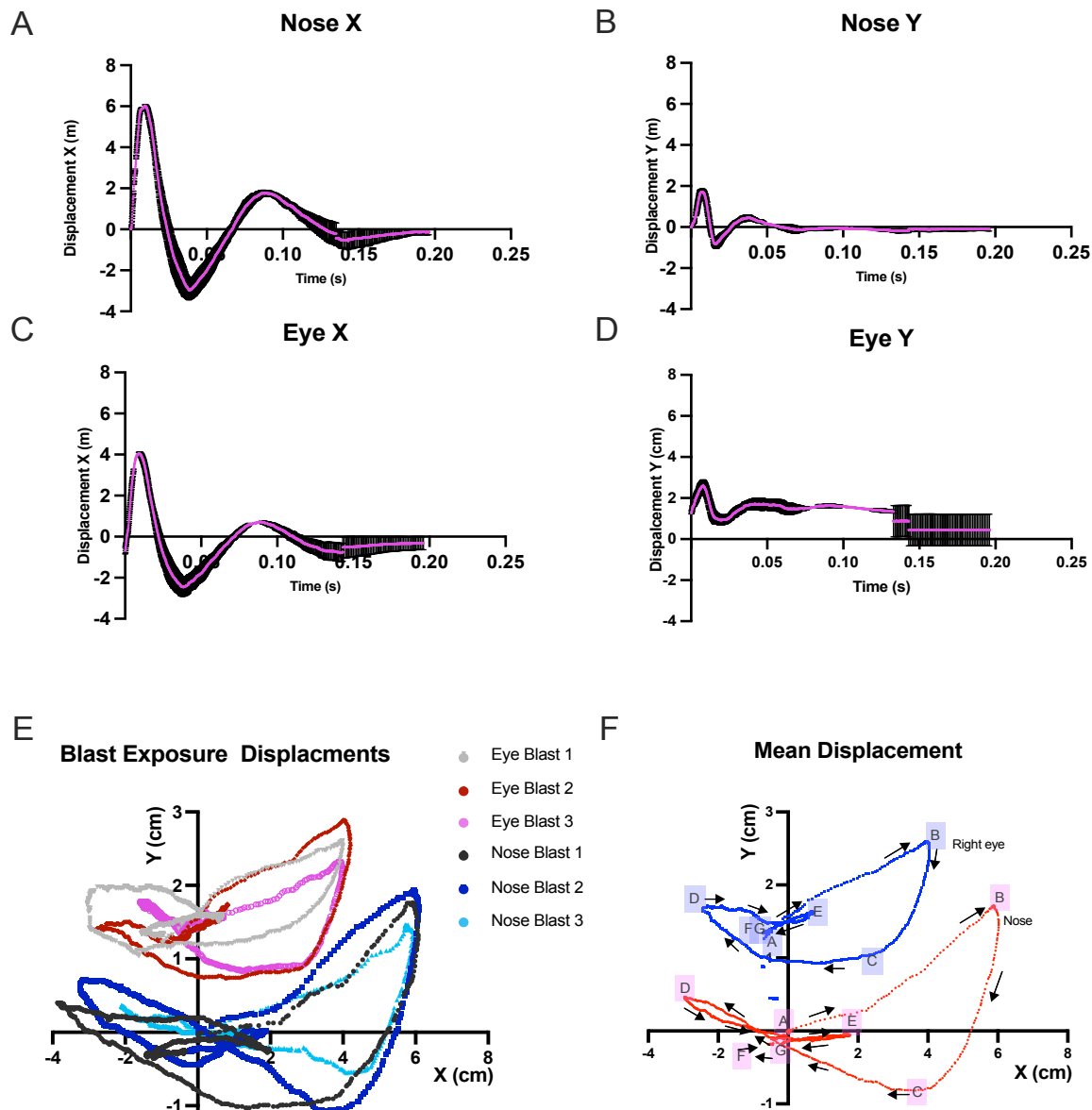


Figure 3.22 Displacements of X and Y for nose and eye for representative animals used in thesis.

(A-D) mean displacement across time with pink line as mean value and black showing the SEM for each data point. E) Three displacements for X and Y for eye and nose for the experimental cohort F) mean displacements for X and Y. This shows initial nose start point at A in pink, and the initial start point for the nose as blue A, and then the movement follows the alphabet showing a figure of eight.

movement of the blast project animals exhibited minimal variation between the animals. This consistency is particularly evident in Figure 3.22A-E, where the eye and nose traces are separate with no cross over between animal tests, unlike the traces seen in Figure 3.17A. This suggests that the positioning and movement security of the rats were consistent and reproducible, leading to similarly displaced movements and suggests similar blast loading at the animal's head.

The velocity and acceleration data were computed in a manner consistent with the previous analyses. It is noteworthy that the velocity and acceleration patterns for these animal's mirror those previously observed. Specifically, the highest velocity and acceleration occurred just after the blast wave struck the animals' head. Therefore showing primary blast injury, (the

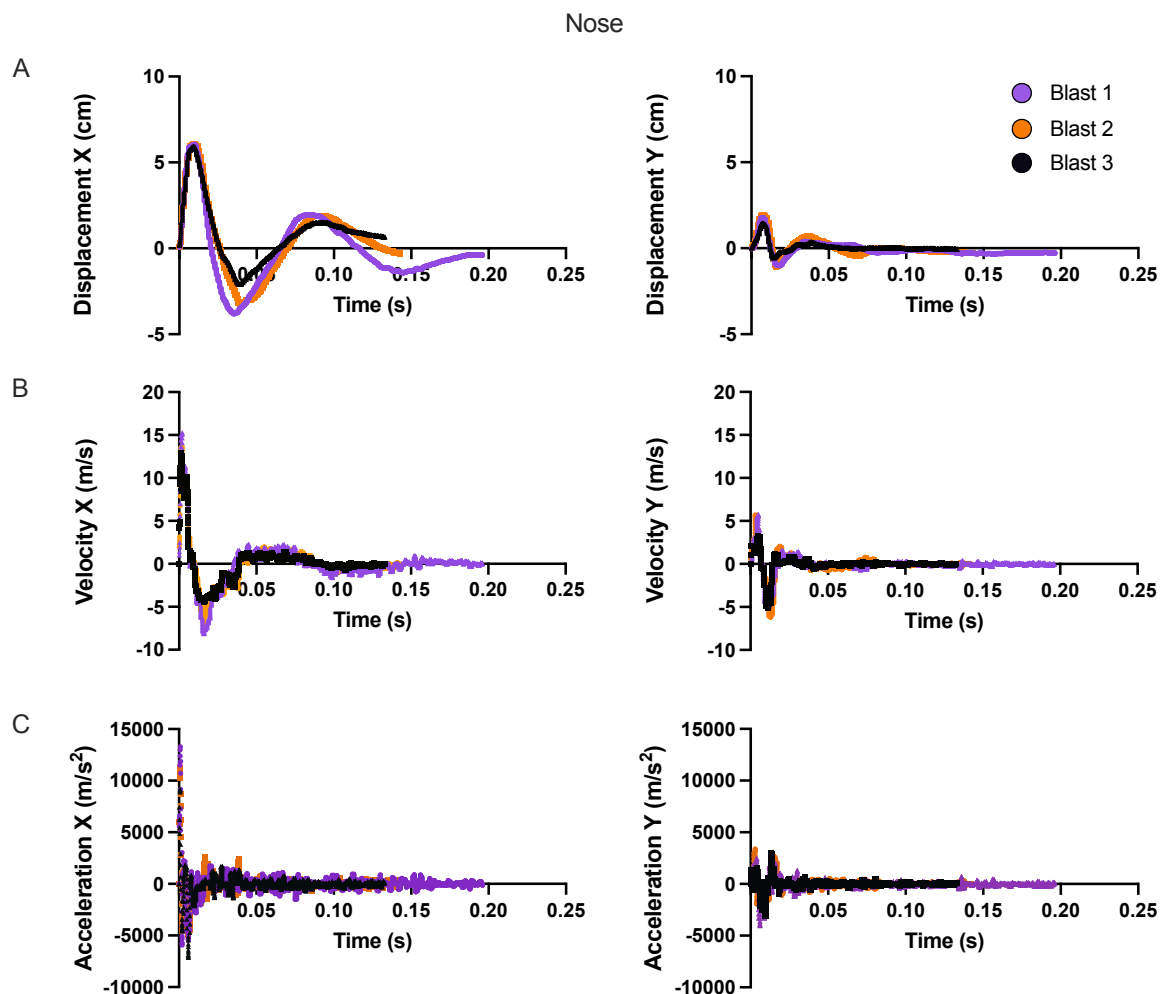


Figure 3.23 Displacement, velocity and acceleration data for three blast project (thesis) animals for the X and Y axis of the nose.

A) displacements for X and Y. B) velocity for X and Y. C) acceleration for X and Y

shockwave interacting with the head) which is then followed by movement, the tertiary component of blast injury (Figure 3.23 and Figure 3.24). The peak velocity and acceleration values were recorded within the first 10 ms of the head movement, consistent with observations made by Goldstein in 2012 (Goldstein, Fisher et al. 2012) and the previous recordings of the optimised blast model setup. What is also consistent with Goldstein's 2012 blast is how the head of the blasted rats swings away from the blast in a figure of eight type "bobble head effect" that can be depicted in Figure 3.22 and was shown in Figure 3.14. Thus, the displacement is like what is reported in Goldstein 2012 (Goldstein, Fisher et al. 2012).

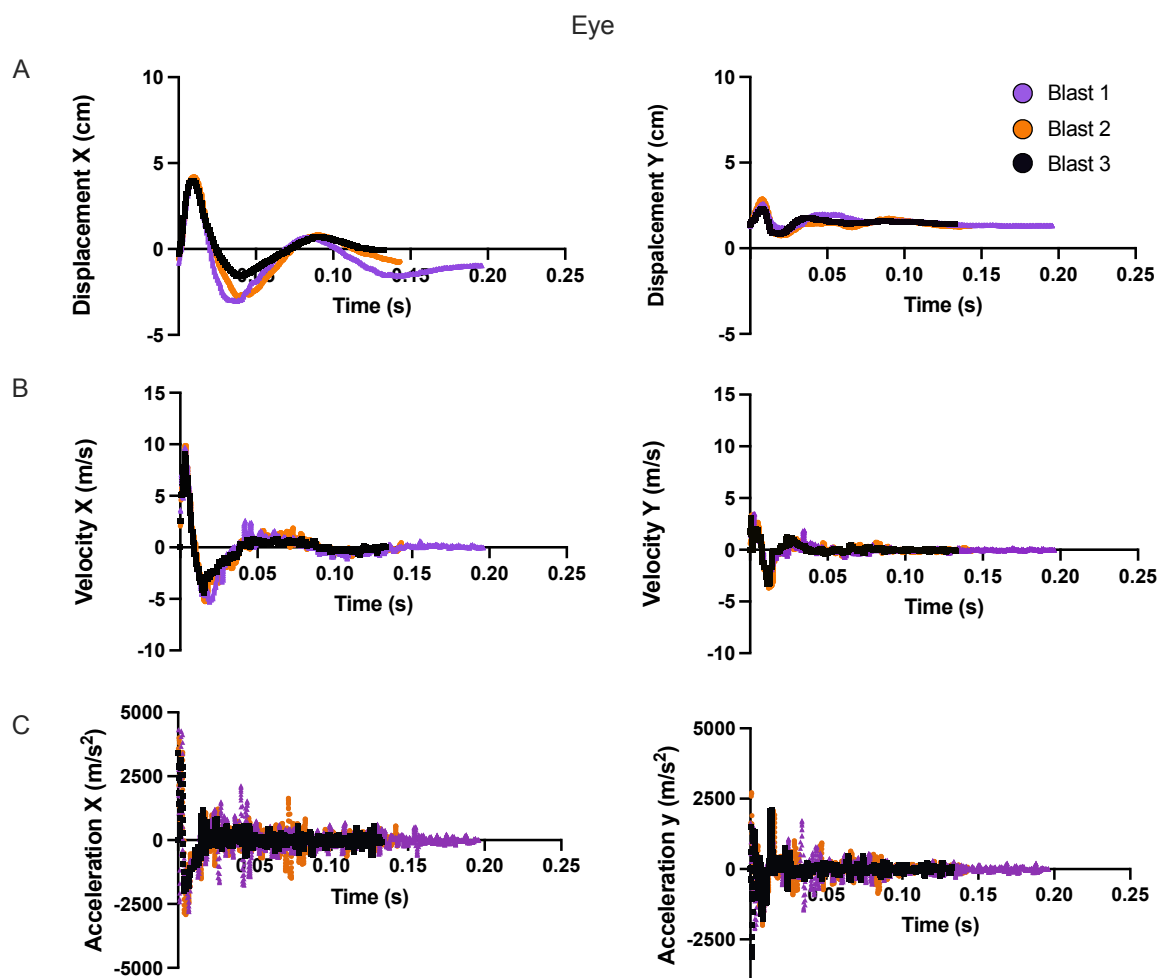


Figure 3.24 Displacement, velocity and acceleration data for three blast project (thesis) animals for the X and Y axis of the eye.

A) displacements for X and Y. B) velocity for X and Y. C) acceleration for X and Y.

	X NOSE m/s²	Y NOSE m/s²	X EYE m/s²	Y EYE m/s²
Blast 1	13285.35	2494.59	4321.03	1729.45
Blast 2	11248.11	3381.44	4001.86	2715.20
Blast 3	9151.15	2963.82	3393.50	2094.23
Mean ± SEM	11228.20 ± 1193.48	2946.61 ± 256.16	3905.46 ± 272.06	2179.63 ± 287.75

Table 3.6 Maximum acceleration values for blast experimental (thesis) cohort trials all experimented under the same blast conditions.

The maximum values obtained from the three experimental cohorts were tabulated and averaged and are presented in Table 3.6. These values were subsequently compared to the maximum acceleration values obtained from previous tests involving cadavers and live anaesthetised subjects. The results indicated that there was no significant difference between the experimental animals and the previous tests ($p > 0.05$), as shown in Figure 3.25. This finding further reinforces the notion that proper positioning of the rats is crucial in order to ensure that displacements are consistent. Consistent displacements suggest that the head was loaded in a similar manner by the blast wave, as demonstrated by the displacement graphs presented in Figure 3.22, Figure 3.23 and Figure 3.24 for the blast project cohort.

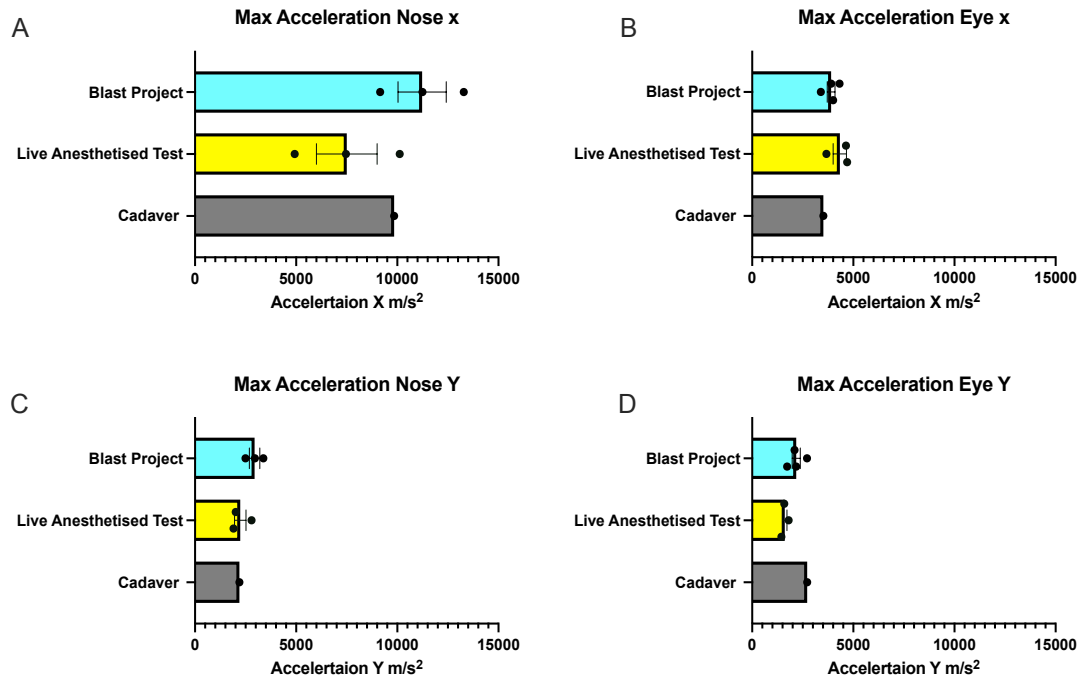


Figure 3.24 Comparison between all maximum accelerations calculated for blast project animals, pilot live anesthetised and cadaver.

These are maximum values from the testing of each group used in this chapter blast model and the values are for both eye and nose for X and Y. One-way ANOVA conducted, no significance found. Results shown as mean and SEM.

3.5.1 LORR of Experimental Cohort of this Thesis

The LORR was measured for the animals used in the experimental cohort. It was found that there was a significant difference between the drug controls, shams, and the blasted (electrophysiological cohort and the histopathological cohort) animals LORR timings. The mean time it took for animals to recover from anaesthesia in the sham cohort was 11 minutes. The mean time for the blast animals was 21 minutes. This indicated that the blast caused some neurological alteration resulting in the blasted animals returning to all four paws slower than the shams (Figure 3.26). The mean time being below 30 minutes indicates that the effect was mild (GCS score, (NICE 2014)). This also aligns with the previous LORR results during the optimisation but shows that with a larger sample size the differences are more profound.

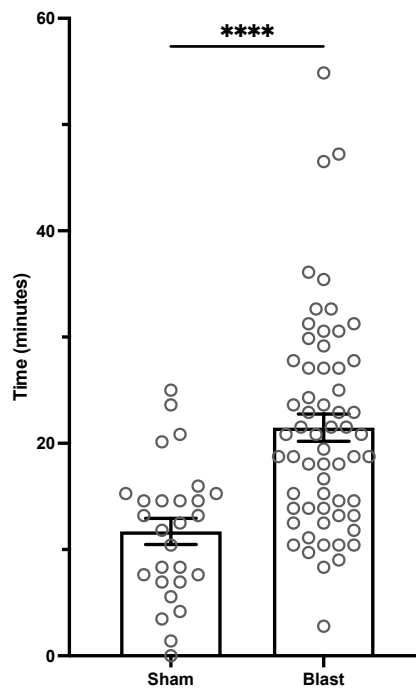


Figure 3.25 Loss of righting reflex for the blast project (thesis) animals.

The graph indicates blast exposure causes latency to return to all four paws, Mann-Whitney test, $p < 0.00001$. Sham $n = 27$, blast $n = 62$. Data is presented as mean $\pm$ SEM.

3.5.2 Outcomes of the Experimental Cohort

Investigations into blast lung injury for the 6 hour cohort were visually conducted when the animals were perfused. The lungs showed no overt signs of blast lung injury nor during the time of post-blast till termination did animals show signs of discomfort nor did they show any apnoea post-blast.

3.6 Limitations

This model has been tested throughout this chapter to produce a reproducible mild blast TBI. However, some possible limitations exist for this model. First of which is the placement of the animal head in relation to the exit of the shock tube. This resulted in a minor blockage effect. However, it was found that perpendicular to the oncoming wave was preferable to having the

animal face on to the shock tube, as there would be an even greater blockage effect (>25% of the diameter of the tube (Needham, Ritzel et al. 2015)). To determine whether this was causing a problem, the second sensor was in a position to pick up any reflections as a result of a blockage problem. It can be seen (Figure 3.13) that there are no deviations in the recording between the experiments, suggesting the blockage issue to be quite low.

Another possible limitation of the model is the approximation of the accelerations, due to the use of a 2D video. Two cameras were set up to try and achieve more of a 3D understanding of the moment, however some variables were still missing to complete the mathematical equations to understand the X, Y and Z movement within 3 planes of motion. Therefore, only one 2D video was used. Furthermore, accelerometers would be much more informative of the true accelerations of the head of the rat, and numerous accelerometers would enable 3D understanding of the head movement, radial and linear accelerations. An original aim of this thesis was to combine two high-speed video perspectives of the animal's movement to obtain a 3D understanding and collaborate with Imperial's Head lab to understand the impact of the biomechanics, similar to previously published works (Donat, Yanez Lopez et al. 2021). However, there was not enough data to complete this work. Thus, 3D models have not been achieved for blast models as of yet, and unfortunately the use of much needed accelerometers may change the weight of the animal's head, and therefore the trajectory of movement would not be true to the original non-accelerometer movement. Thus, this model is optimised using approximated values of acceleration.

Some other aspects of the model that could be considered limitations of this study are the use of anaesthesia and analgesia, although in the United Kingdom (UK) it is a legal requirement to conduct procedures under anaesthesia. When under anaesthesia, muscle tension is absent which is not translationally relevant. However, some non-UK based studies have conducted non-anaesthetised blasts in order to capture this translational aspect (Ning, Yang et al. 2013, Hansen, DeWalt et al. 2018). Furthermore, there is a chance that the analgesia may have elongated LORR times. However, having shams meant that was controlled for. Additionally, the use of isoflurane has neuroprotective effects and therefore could also confound our results by reducing the injury pathology (Statler, Alexander et al. 2006).

3.7 Conclusion

The aims of this chapter were focused on optimising the CBIS shock tube to produce a reliable and reproducible mild blast traumatic brain injury (TBI) model in rats. Through the achievement of the objectives outlined, it was possible to successfully generate the Friedlander waveform using the CBIS shock tube at a rat's head and modify the animal platform to minimise the risk of blast lung injury. Furthermore, we were able to ensure consistent positioning of the rats and obtain repeatable head kinematics. In addition, by determining that the LORR was significantly increased for blasted animals relative to shams, it was determined that the model induced a TBI. The fact that the LORR had a mean below 30 minutes relative to shams indicated that the TBI was mild. Additionally, welfare scores for the blasted animals were below 2 indicating good welfare. These two factors combined with the lack of mass structural damage as shown in the cresyl violet staining supported our hypothesis that the model would produce a mild bTBI. Furthermore, the fact that the measurements of the blast wave were recorded enables accurate blast parameter reporting for repeatability.

The achievement of this chapter's aims and objectives are significant as they contribute to the development of a reliable and reproducible mild blast TBI model in rats, which can be used in future research to further our understanding of mild blast TBI.

4

Understanding the Effects of Blast TBI on Neuronal and Glial Populations Over Time

4.1 Introduction

4.1.1 Network Changes as a Result of TBI

There is evidence of a link of traumatic brain injury (TBI) and the risk of neurodegenerative diseases, observed particularly in military personnel being affected by long-term neurological problems (Jordan 2000, Plassman, Havlik et al. 2000, Omalu, DeKosky et al. 2005, Goldstein, Fisher et al. 2012, Barnes, Kaup et al. 2014, Mckee and Robinson 2014, Stewart, McNamara et al. 2016, Mez, Daneshvar et al. 2017, Mackay, Russell et al. 2019, Zimmerman, Laverse et al. 2021). This has resulted in the need to identify early neuronal damage in milder forms of TBI, and to have an early diagnosis in these patients. Particularly, in the blast population there is a high proportion of mTBI cases, as shown by military personnel from Operation Endurance Freedom (OEF) and Operation Iraqi Freedom (OIF), who were left undiagnosed with post-concussive symptoms (PCS) for 3 months or longer (Huang, Harrington et al. 2017), and it remains unclear why some suffered PCS while others not.

Routine MRI scans and structural CTs may miss the microstructural changes in the brain that can cause these PCS symptoms, and thus left undetected and unmonitored (Yue, Upadhyayula et al. 2020). Undiagnosed mTBI can leave the brain vulnerable to further and repeated damage and cumulative effects, and this is likely to occur on the battlefield. Moreover, if TBI is not diagnosed initially, it can lead to underreporting and make it challenging to retrospectively assess for a diagnosis later on. This difficulty arises due to the subjective nature of self-reported symptoms, which may occur weeks or months after the incident, as well as the presence of other psychological, sleep, and pain conditions that often coexist with TBIs (Kiraly and Kiraly 2007, Chan, Mollayeva et al. 2017). Thus, diagnosing the TBI as soon as possible is of priority, as it was recently found that a much larger proportion of TBIs do not recover as originally thought. It was hypothesised that 90% patients would recover from a mild TBI, but instead this number is approximately 50% (Maas, Menon et al. 2022, Madhok, Rodriguez et al. 2022). This means that a large proportion of mild TBI has been

left undiagnosed, unmonitored and untreated, highlighting the need for a tool to be added to the diagnostic protocol, to be sensitive to mild forms of TBI.

Electrophysiology has demonstrated a significant potential role to identify neuronal dysfunction after injury, as evidenced by the sensitivity of both electroencephalography (EEG) and magnetoencephalography (MEG) techniques to TBI. Both methods offer temporal resolution at the millisecond scale, but with distinct approaches, one utilising an electrode cap (EEG) at detecting cortical electrical potential differences and the other employing a large magnetic machine (MEG) and detecting magnetic fields generated by the neurons (Silva 2013). Furthermore, their readings derive from the same neurological events. MEG/EEG have much more temporal resolution than other imaging modalities, such as MRI and CT, which allows for greater information on the dynamics of the brain and brain networks. MEG/EEG allow for a functional snapshot of the brain on a global brain level that can detect changes that are useful for a diagnosis of brain injury. For example, MEG was found to detect neuronal damage in mild TBI cases where MRI was negative (Huang, Nichols et al. 2012), or in cases where veterans had sought validation for their behavioural changes that were put down to psychological issues and denied treatment (Rose 2022).

Recognising electrophysiological hallmarks are the key to diagnosing TBI using MEG/EEG and understanding the temporal electrophysiological sequelae. It has been reported that in TBI cases there is a quintessential increase in the power of the low frequencies (delta and theta) (Ball, Gloor et al. 1977, Gloor, Ball et al. 1977, Lewine, Davis et al. 1999, Huang, Theilmann et al. 2009, Franke, Walker et al. 2016, Lewine, Plis et al. 2019, Buchanan, Ros et al. 2021, Mallas, Gorgoraptis et al. 2022), particularly at chronic time points (Franke, Walker et al. 2016, Mallas, Gorgoraptis et al. 2022). These changes can be distinct for those with and without psychiatric comorbidities, like post-traumatic stress disorder (PTSD), and can occur after just one concussion (Davenport, Urban et al. 2022). Identifying electrophysiological correlates of pathology in asymptomatic populations holds significant scientific importance, as it shows sensitivity to neural changes and can aid diagnosis to prevent the likelihood of developing future neurodegenerative diseases (Lennon, Brooker et al. 2023). Electrophysiology can also be used for monitoring brain function over time and for recovery, as it has shown to normalise, indicating recovery (Davenport, Urban et al. 2022).

Hallmark electrophysiological alterations like increased power changes in the low frequencies (e.g. delta and theta) are not the only changes that can occur post-brain injury. Other functional changes can also occur post-injury, which can be quantified by statistical dependency of signals from different brain regions (Sharp, Scott et al. 2014, Bowyer 2016). Alterations in functional connectivity can affect the informational processing and transmission in the brain (Wang, Ethridge et al. 2017, Lennon, Brooker et al. 2023). However, there are inconsistencies in the literature as to whether there is increased or reduced functional connectivity (FC) post-injury, and in which bands these changes occur (Sponheim, McGuire et al. 2011, Huang, Harrington et al. 2017, Wang, Costanzo et al. 2017, Lewine, Plis et al. 2019, Mallas, Gorgoraptis et al. 2022). Therefore, controlled experiments are needed to investigate the context and reasons for these changes for both blast and non-blast populations.

4.1.2 Pathological Mechanisms Underlying Electrophysiological Alterations in TBI

Understanding the cellular basis for these electrophysiological changes will help in understanding the hallmark changes and aid in using these techniques for monitoring purposes. If there was a shift in hallmark changes of a brain that suffered a TBI, that returned later lost those hallmark changes, that could be due to “recovery” from the injury.

Changes in power and FC in TBI can be attributed to lesions and axonal damage in white matter (Gloor, Ball et al. 1977, Wang, Costanzo et al. 2017) or micro-diffuse axonal damage in the grey matter (Vascak, Jin et al. 2018). This may seem logical, since FC reflects underlying structural connectivity (Hagmann, Cammoun et al. 2008, Greicius, Supekar et al. 2009). It was reported by Gloor et al, (Gloor, Ball et al. 1977) that as a result of a white matter lesion, there is an increase in delta power. This relationship has been further confirmed by other groups, showing that the increase on lower frequency power and FC changes correlate to white matter damage (Sharp, Scott et al. 2014, Wang, Costanzo et al. 2017). Furthermore, Mallas,

2022 (Mallas, Gorgoraptis et al. 2022) found a shift towards lower frequencies in post-traumatic amnesia patients who had deficits in working memory.

However, the alterations in white matter are not the only attributing factor to electrophysiological alterations. There have been limited investigations into the more nuanced structural and functional changes that arise from perturbations of the balance between excitation and inhibition (E/I). Importantly, the E/I balance plays a role in the networking's of the brain (Tremblay, Lee et al. 2016). Two main neuronal populations are fundamental parts of the E/I balance, the GABAergic parvalbumin (PV) and somatostatin (SST) interneurons (Tremblay, Lee et al. 2016). Their dense axonal arborisation allows for control over the entire cortical network (Xu, Jeong et al. 2013). They are important for the brain homeostasis and are networked to inhibit excitatory cells like pyramidal neurons, although can inhibit each other. They are the brakes of the brain and prevent runaway feed-forward excitation (Ferguson and Gao 2018). If one cell is altered, that can affect hundreds-thousands of other connections, as they are highly interconnected, despite their reasonably low density relative to other cell types (Overstreet-Wadiche and Mcbain 2015). In addition, it has been reported that the E/I imbalance may be involved in neurological disorders such as schizophrenia, epilepsy and autism (Andreou, Nolte et al. 2015, Di Lorenzo, Daverio et al. 2015, Ferguson and Gao 2018, Wang, Feng et al. 2022) as stated in Chapter 1.

E/I imbalance causing neurological and functional alterations has also been reported in the TBI literature. This is due to TBI leading to an altered interneuron population, firing pattern, or alteration in Ca^{2+} -binding protein (e.g PV) density, which can cause an E/I imbalance, resulting in behavioural as well as functional deficits (Sohal, Zhang et al. 2009, Cantu, Walker et al. 2015, Filice, Vörckel et al. 2016, Hsieh, Lee et al. 2017, Ferguson and Gao 2018, Vascak, Jin et al. 2018, Sohal and Rubenstein 2019). However, some of these changes occurred in the context of cell death, using blunt trauma or more severe models, where lesions occur (Hsieh, Lee et al. 2017), which is not the pathology of mild bTBI. However, even in milder models, it has been reported that PV and SST interneurons have been preferentially affected in non-blast injuries (Cantu, Walker et al. 2015), without general neuronal loss (a now accepted aspect of milder TBIs) (Farkas and Povlishock 2007).

There have been few studies in the blast TBI literature that have investigated the effect of blast waves on the IN populations, and of those studies, no SST changes were found (Masri 2020). Therefore, it is important to investigate the effect of blast on these two subpopulations of INs and, subsequently, the networking of the brain .

The subtypes of GABAergic interneurons are not the only cell types affected by TBI; glial cells can also be affected by blast, and they are responsible for maintenance of the homeostasis of the brains and its synapses (Perea, Sur et al. 2014, Haim and Rowitch 2017). Glial reactivity post-TBI is complex, and can result in many different phenotypes, reversible alterations in gene protein expressions that can aid or hinder the damaged brain. The phenotypic changes can depend on the different areas of the brain and be severity-dependent (Burda, Bernstein et al. 2016).

It is widely accepted that astrocytes are critically early responders to TBI and are able to aid in the glutamate increases that arises from TBI (Burda, Bernstein et al. 2016). They are important for initiating other cellular responses by secreting astrocyte-derived ATP (Burda, 2016 #232). The TBI-induced ATP, regardless of the cellular source, is able to induce cytoplasmic calcium in reactive astrocyte networks, and as a result these astrocytes may start to polarise and or recruit microglia and neutrophils to the injury site at very acute time-points (Sajja, Galloway et al. 2012, Burda, Bernstein et al. 2016). However, this may depend in some cases whether the blood brain barrier is damaged or not (Readnower, Chavko et al. 2010).

Astrocytes can also support neuronal survival after traumatic events, since they secrete anti-inflammatory cytokines and chemokines as well as neurotrophic factors. A study showed that astrocytes can extend hypertrophic processes around their neighbouring traumatised motor neurons and promote their survival and mediate their recovery of functionality (Tyack, Sitnikov et al. 2014). This is further supported by studies that prevent astrocytes from becoming activated post TBI. Myers et al. reported in a mouse controlled cortical impact (CCI) study that ablating the activated astrocytes resulted in substantial neuronal degeneration, inflammation and 60% of cortical tissue loss. This was compared to their control injury, where there was little inflammation, most neurons were intact and there was only 18% loss of

cortical tissue (Myer, Gurkoff et al. 2006). This demonstrates the reparative phenotypes of astrocytes post-TBI.

In the preclinical blast literature, it has been reported that astrocytes react and change morphology, secrete proteins/cytokines/chemokines and ROS and/or proliferate at acutely/sub-acute time points (Goldstein, Fisher et al. 2012, Svetlov, Prima et al. 2012, Budde, Shah et al. 2013, Hernandez, Tan et al. 2018). In some cases, they return to normal levels by 3-4 weeks post-injury, although in other cases this process lasts chronically (Säljö, Bao et al. 2001, Cernak, Merkle et al. 2011).

Likewise, microglia respond to blunt TBI, and have been reported to change morphology, secrete proteins and proliferate (Ziebell and Morganti-Kossmann 2010, Ziebell, Taylor et al. 2012, Witcher, Bray et al. 2021). In addition, microglia react to blast waves both acute and sub acutely (Readnower, Chavko et al. 2010, Goldstein, Fisher et al. 2012, Svetlov, Prima et al. 2012, Masri 2020, Bishop, Won et al. 2022) and like astrocytes, they can remain chronically altered by the blast (Säljö, Bao et al. 2001, Cernak, Merkle et al. 2011).

Thus, alterations in the glial populations, such as activation or changes in their density may affect the neuronal populations and, subsequently, network dynamics (Papa, De Luca et al. 2014, Zhou, Shao et al. 2020, Krukowski, Nolan et al. 2021, Yu, Wang et al. 2022).

However, it still remains unknown whether blast TBI results in the same cellular and electrophysiological changes as reported in the blunt TBI studies. Furthermore, it is unknown whether these changes can be detected at acute and/or chronic time-points. Therefore, preclinical studies can enable this investigation.

This project was a collaboration which had two elements to it, histological and electrophysiological, whereby Dr Michael Bruyns-Haylett, Konstantinos Tsikonofilios, Dr Cornelius Donat and I exposed animals to a single mild blast. Dr Michael Bruyns-Haylett and Konstantinos Tsikonofilios conducted resting-state EEG experiments on two cohorts (described in Supplementary Material 2). Analysis of this data was conducted by Konstantinos

Tsikonofilios, while I examined the histopathological changes occurring in acute and chronic time points after blast brain injury.

The electrophysiological results (Supplementary Material 2) that showed increased power and relative power (terms defined in Supplementary Material 2) in the post-blasted brains prompted me to investigate the mechanisms behind these changes and to conduct histological analysis into specific GABAergic neuronal subtypes, such as PV and SST. I focused on a specific brain block (block 4, methods section 2.7.1), where the majority of electrophysiological changes were observed (shown in Supplementary Figure 2, Supplementary Figure 4 and Supplementary Figure 6). Moreover, since glial cells play a vital role in neuronal functionality and post-injury inflammation, this chapter also investigated the changes in astrocytes and microglia in the same animals.

Since my collaborators explored the impact of blast on brain network connectivity, the aim of this chapter was to gain a deeper understanding of the potential underlying pathology of the observed electrophysiological alterations in blast TBI (bTBI). This research will be the first to investigate specific neuronal populations in a blast model across both acute and chronic time points.

4.2 Hypotheses, Aims and Objectives

4.2.1 Hypotheses

- Blast TBI causes the parvalbumin but not the somatostatin interneuronal density to decrease at subacute timepoints (Chapter 4).
- There will be a neuroinflammatory response following blast TBI, manifested by increases in microglia/macrophage and astrocytic density after mild blast TBI at acute time points (Chapter 4).
- Loss of GABAergic interneurons and inflammatory response will be seen in a lateralised presentation; more damage will be in the hemisphere closest to the blast wave (Chapter 4).

4.2.2 Aim and objective

- To determine the longitudinal glial and neuronal pathology of the blast injury in the brain using immunohistochemistry.
Objective: This will be achieved using a general neuronal marker (antibody: NeuN), interneuronal markers (antibody: Parvalbumin and Somatostatin) and glial (antibody: IBA1 and GFAP) markers to quantify changes in the blast model using immunohistochemistry post-blast at acute and chronic time points (6 hours – 3 months).
- To determine the spatial presentation of the neuronal loss and inflammatory response.

Objective: This will be achieved by assessing the hemispheres separately and establishing whether there is statistically significant lateralisation as a result of blast TBI or not.

4.3 Results

Analysis of the immunohistochemical staining for neurons was conducted in the auditory cortex (Au1), the primary visual cortex (V1) and the retrosplenial cortex (RSC). This analysis was conducted in each brain hemisphere, at 6 hours, 7 days 1 month and 3 months post-injury in order to explore potential changes of neuronal number between the ipsilateral and contralateral sides of the injury (Readnower, Chavko et al. 2010, Masri 2020, Bishop, Won et al. 2022). These specific regions were selected for analysis based on a number of considerations. Firstly, the Au1 region was the closest area to the blast wave (ipsilateral). Plus, the Au1 was chosen in accordance with previous auditory analysis, which investigated whether there was hearing loss post-injury (analysis results yet to be conducted). The RSC and V1 regions were examined to explore any potential influence of neuronal orientation on the results. These regions were also analysed because they were located beneath the collaborating lab's electroencephalogram (EEG) electrodes, providing valuable information about electrophysiological broadband changes, and ensuring comprehensive sampling of the neocortex (see Supplementary Material 2).

Quantification of neuronal cell densities were done using HALO® and statistical analysis were carried out using mixed-effects ANOVA, corrected using Benjamini, Krieger and Yekutieli False discovery Rate (BKY FDR) at 5%, where the BKY FDR adjusted p-value value is presented as q.

4.3.1 No Neuronal Density Differences are Found using NeuN as a General Neuronal Marker in the Mild Blast Injury Model

Overall neuronal density changes after blast injury were analysed by immunohistochemistry with NeuN antibody in the regions of interest mentioned above at 6hour, 7 days, 1 month and 3 months post-injury.

The assessment of the densities for each layer for each neocortical area was conducted for the ipsilateral (same side as the oncoming blast wave during blast exposure) and contralateral

hemispheres and analysed used mixed-effects ANOVA analysis using Prism 9 for macOS. The main factors were hemisphere and time post-injury (time), plus the interaction. There were 2-5 slices analysed per animal, and some slices or hemispheres were excluded due to damage or major staining artefacts (Au1: Naïve/Sham: n=13, 6 Hr: n=6, 7 Day: n=6, 1 month: n=5, 3 months: n=6. V1: Naïve/Sham: n=13, 6 Hr: n=6, 7 Day: n=5, 1 month: n=5, 3 months: n=6. RSC: Naïve/Sham: n=13, 6 Hr: n=6, 7 Day: n=5, 1 month: n=5, 3 months: n=6).

In the **Au1** mixed effects ANOVA revealed that there were no density changes in NeuN positive neurons between hemispheres or time post-injury for any layers (layer 2/3, 4, 5 and 6) , as reported in Supplementary Table 1($p>0.05$).

In the **V1**, there were also no significant changes in density of NeuN positive neurons between hemispheres for any layers in all the groups analysed ($p>0.05$), ANOVA results are reported in Supplementary Table 2.

Same results were obtained in the **RSC**, where there were no significant differences in NeuN positive neurons between hemispheres among all animal groups by ANOVA, as reported in Supplementary Table 3.

Consequently, since there were no changes in NeuN positive cells between hemispheres in all groups, the densities for each hemisphere were combined within each layer. This resulted in a single measurement for each layer within the region of interest. The decision not to pool the layers (2 - 6) was based on the considerable variation in densities across the layers. Pooling the layers would have diluted any subtle changes in the density analysis.

Results of the analysis of NeuN staining in each region of interest (**Au1**, **V1** or **RSC**) revealed no significant differences in number of NeuN positive cells among the different times post-injury any of the layers as analysed by one-way ANOVA)(Figure 4.1).

		NeuN		
		F (DFn, DFd)	p-val	adjusted p-val (q-val)
Au1	layer 2/3	F (4, 31) = 1.139	p=0.3565	q=0.8559
	layer 4	F (4, 31) = 0.3886	p=0.8151	q=0.8559
	layer 5	F (4, 31) = 0.6348	p=0.6415	q=0.8559
	layer 6	F (4, 31) = 0.8498	p=0.5047	q=0.8559
V1	layer 2/3	F (4, 30) = 0.4660	p=0.7601	q=0.7981
	layer 4	F (4, 30) = 1.04	p=0.4030	q=0.5642
	layer 5	F (4, 30) = 1.114	p=0.3683	q=0.5642
	layer 6	F (4, 30) = 1.306	p=0.2901	q=0.5642
RSC	layer 2/3	F (4, 31) = 1.741	p=0.1663	q=0.1296
	layer 4	F (4, 31) = 1.195	p=0.3328	q=0.4815
	layer 5	F (4, 31) = 2.581	p=0.0566	q=0.1296
	layer 6	F (4, 31) = 0.9998	p=0.4225	q=0.5538

Table 4.1 One-way ANOVA results for NeuN density for the layers of cortical regions of interest.

Results are shown in the primary auditory cortex (Au1), primary visual cortex (V1) and the Retrosplenial cortex (RSC) displaying no significant differences after Benjamini, Krieger and Yekutieli False Discovery Rate multiple comparison.

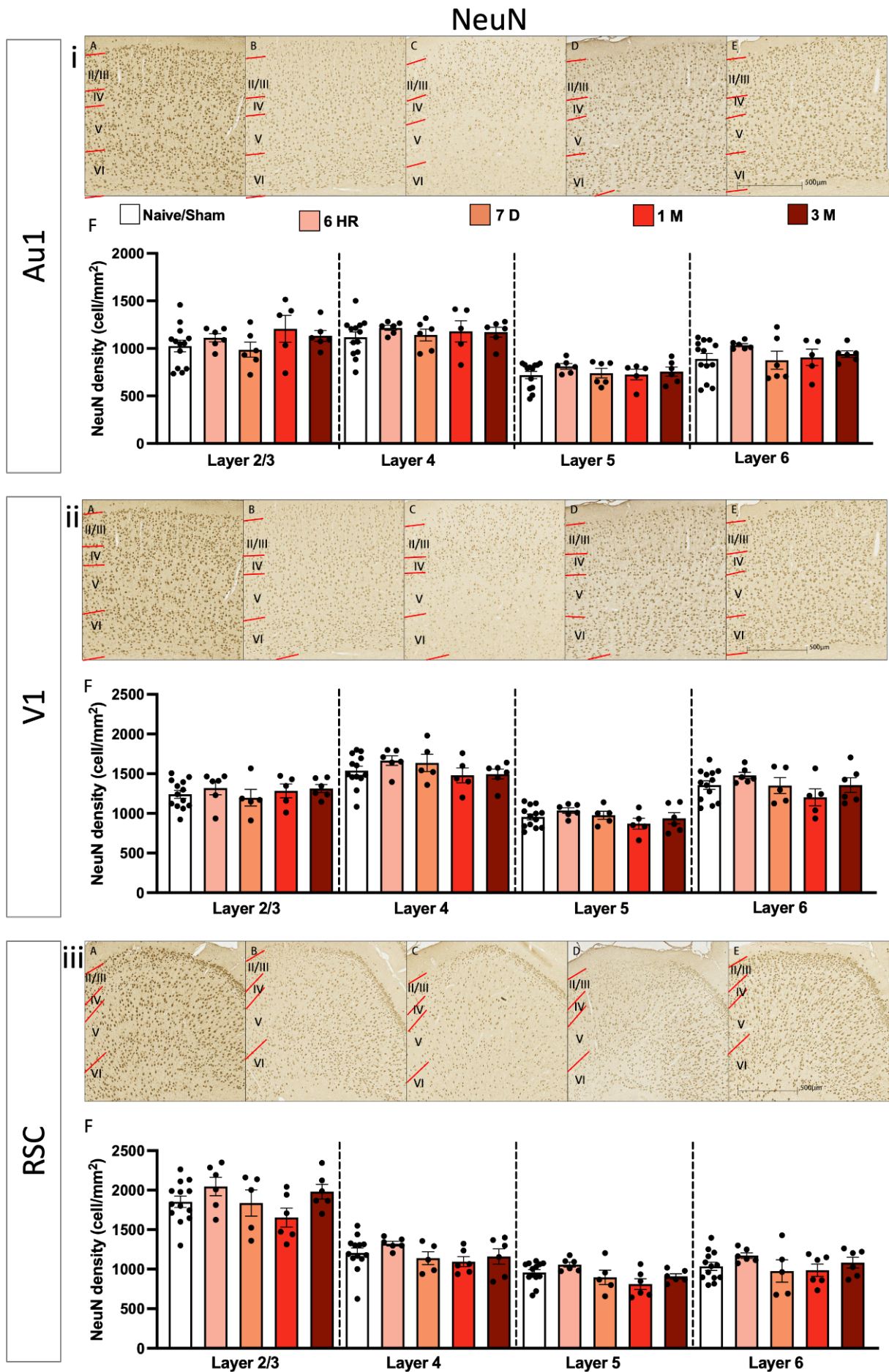


Figure 4.1 NeuN density changes in for the layers of cortical regions of interest.

No changes to NeuN density analysing the primary auditory cortex (Au1), the primary visual cortex (V1) and the retrosplenial cortex (RSC) at different time points post-blast traumatic brain injury. NeuN positive intraneuronal density following single blast traumatic brain injury in the primary auditory cortex for at (iA) Naïve/Sham, (1B) 6hours post-injury, (iC) 7 DPI post -injury, (iD) 1 month post-injury, (iE) 3 months post-injury. (iF) quantification of interneuron cell density using one-way ANOVA, corrected using BKY FDR. (iiA) Naïve/Sham, (1B) 6hours post-injury, (iiC) 7 DPI post -injury, (iiD) 1 month post-injury, (iiE) 3 months post-injury. (iiF) quantification of interneuron cell density using one-way ANOVA, corrected using BKY FDR. (iiiA) Naïve/Sham, (1B) 6hours post-injury, (iiiC) 7 DPI post -injury, (iiiD) 1 month post-injury, (iiiE) 3 months post-injury. (iiiF) quantification of interneuron cell density using one-way ANOVA, corrected using after Benjamini, Krieger and Yekutieli False Discovery Rate multiple comparison correction. All data presented mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. All data presented mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. All data presented mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ (Au1: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=6$, 1 month: $n=5$, 3 months: $n=6$. V1: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=5$, 1 month: $n=5$, 3 months: $n=6$. RSC: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=5$, 1 month: $n=5$, 3 months: $n=6$).

4.3.2 PV Positive Cells are Reduced in the Auditory Cortex at Chronic Times Post-Blast Injury

PV interneurons are an integral part of the E/I balance, and density changes may cause alterations it and thus the brain's network. Therefore, staining for PV neurons was carried out in the same three cortical areas of interest (**Au1**, **V1** or **RSC**) of the different groups of animals analysed (Naïve/Sham and post-blast exposure) at different times post-injury, as for the NeuN staining.

The PV positive neuron densities were analysed in all groups of rats (Au1: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=6$, 1 month: $n=7$, 3 months: $n=6$. V1: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=6$, 1 month: $n=7$, 3 months: $n=6$. RSC: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=6$, 1 month: $n=6$, 3 months: $n=6$). Mixed effects ANOVA were conducted with the main effects hemisphere and time post-injury to investigate whether changes were lateralised. The ANOVAs conducted showed that there were no significant differences for the main effect hemisphere for layers of the **Au1** (Supplementary Table 4), **V1** (Supplementary Table 5) or **RSC** (Supplementary Table 6). However, it was found that for the 6th layer of the **Au1**, significant differences for the main effect; time post-injury (Supplementary Table 4).

In light of these results, the PV densities for each hemisphere were pooled per layer for each region of interest and the data were then analysed using a one-way ANOVA, as reported in Table 4.2.

		PV		
		F (DFn, DFd)	p-val	adjusted p-val (q-val)
Au1	layer 2/3	F (4, 33) = 1.496	p=0.2258	q=0.9032
	layer 4	F (4, 33) = 1.594	p=0.1991	q=0.7964
	layer 5	F (4, 33) = 5.291	p=0.0021	q=0.0084
	layer 6	F (4, 33) = 4.670	p=0.0043	q=0.0172
V1	layer 2/3	F (4, 32) = 1.296	p=0.2923	q=0.8200
	layer 4	F (4, 32) = 0.085	p=0.9865	q=1.0000
	layer 5	F (4, 32) = 0.718	p=0.5857	q=0.8200
	layer 6	F (4, 32) = 1.034	p=0.4047	q=0.8200
RSC	layer 2/3	F (4, 33) = 1.749	p=0.1628	q=0.3665
	layer 4	F (4, 33) = 1.411	p=0.2521	q=0.3665
	layer 5	F (4, 33) = 1.154	p=0.3490	q=0.3665
	layer 6	F (4, 33) = 0.823	p=0.5197	q=0.3665

Table 4.2 Parvalbumin density One-way ANOVA results for the layers of cortical regions of interest.

Results shown are for primary auditory cortex (Au1), primary visual cortex (V1) and the Retrosplenial cortex (RSC) displaying two significant differences in layer 5 and 6 in the Au1. Significant differences were found after Benjamini, Krieger and Yekutieli False Discovery Rate multiple comparison correction in the primary auditory cortex after multiple comparison correction in layer 5 and 6 and are depicted with bold text ($q < 0.05$). (Au1: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=6$, 1 month: $n=7$, 3 months: $n=6$. V1: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=6$, 1 month: $n=7$, 3 months: $n=6$. RSC: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=6$, 1 month: $n=6$, 3 months: $n=6$).

Analysis of PV positive interneurons in the **Au1** cortex revealed significant reductions in both **layer 5** and **6** at chronic times post injury. In **layer 5**, the results showed a reduction of 29.53% at 7 DPI ($p=0.0084$), 45.73% at 1 month ($p=0.0006$) and 22.86% at 3 months ($p=0.0371$) post-injury relative to Naïve/Sham (Figure 4.2). In **layer 6**, the reduction of PV was of 33.88% at 7 days post-injury ($p=0.0041$) and 47.63% at 1 month post-injury ($p=0.0005$ (Figure 4.2i)). All other comparisons were not statistically significant ($p > 0.05$) (Naïve/sham: $n=13$, 6 hr: $n=6$, 7DPI: $n=7$, 1 month: $n=7$, 3 month: $n=6$).

There were no significant differences in PV positive interneurons in the **V1** (Figure 4.2ii) nor in the different layers of the **RSC** (Figure 4.2ii). One-way ANOVA results are shown in Table 4.2.

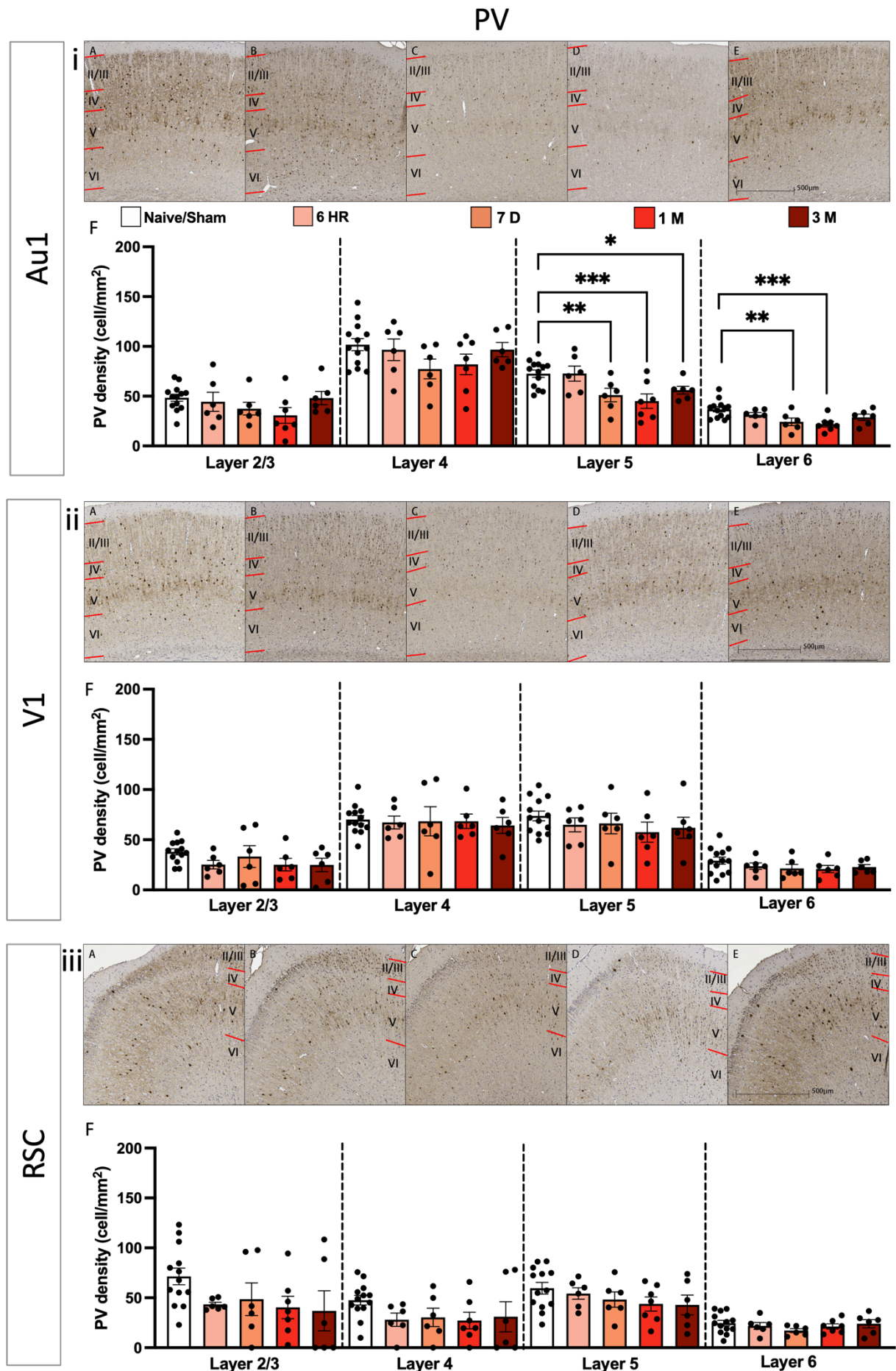


Figure 4.2 Parvalbumin (PV) density changes in for the layers of cortical regions of interest.

Parvalbumin density changes in the primary auditory cortex (Au1, primary visual cortex (V1) and the Retrosplenial cortex (RSC) following blast TBI. Parvalbumin positive intraneuronal density following single blast traumatic brain injury in the primary auditory cortex at (iA) Naïve/Sham, (1B) 6 hours post-injury, (iC) 7 DPI post-injury, (iD) 1 month post-injury, (iE) 3 months post-injury. (iF) quantification of interneuron cell density using one-way ANOVA, corrected using BKY FDR. (iiA) Naïve/Sham, (1B) 6 hours post-injury, (iiC) 7 DPI post-injury, (iiD) 1 month post-injury, (iiE) 3 months post-injury. (iiF) quantification of interneuron cell density using one-way ANOVA, corrected using BKY FDR. (iiiA) Naïve/Sham, (1B) 6 hours post-injury, (iiiC) 7 DPI post-injury, (iiiD) 1 month post-injury, (iiiE) 3 months post-injury. (iiiF) quantification of interneuron cell density using one-way ANOVA, corrected using BKY FDR. Results showed the Au1 in the 5th cortical layer. All data presented mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. All data presented mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ (Au1: Naïve/Sham: $n = 13$, 6 Hr: $n = 6$, 7 Day: $n = 6$, 1 month: $n = 7$, 3 months: $n = 6$. V1: Naïve/Sham: $n = 13$, 6 Hr: $n = 6$, 7 Day: $n = 6$, 1 month: $n = 7$, 3 months: $n = 6$. RSC: Naïve/Sham: $n = 13$, 6 Hr: $n = 6$, 7 Day: $n = 6$, 1 month: $n = 6$, 3 months: $n = 6$).

4.3.3 SST Positive Neurons are Reduced in all 3 Brain Regions Investigated, at Sub-Acute and Chronic Time Points

The second interneuron population of interest in the study was the SST IN. Their densities were calculated in the same regions of interest and time points post-injury as for NeuN and PV analysis (Naïve/sham: $n = 13$, 6 hr: $n = 5$, 7DPI: $n = 6$, 1 month: $n = 8$, 3 month: $n = 6$).

Initially, the densities were measured in each hemisphere per cortical layer for each region, as carried out before for the PV and the NeuN analysis.

The comparisons of the numbers of SST IN between hemispheres in **layers 2/3, 4 and 6** were not significantly different in the **Au1** for all time points shown by mixed effects ANOVA results, as reported in Supplementary Table 7.

However, significant decreases in the number of SST positive neurons were found in the ipsilateral side of the injury in the **5th Layer** of the **Au1** (Supplementary Table 7) across all time points post-injury compared with Naïve/Sham animals 6 hr: -60.86%, $p < 0.0001$, 7 DPI: -41.89%, $p = 0.0019$, 1 month: -26.68%, $p = 0.0337$ and 3 month: -41.58%, $p = 0.0020$ (Figure 4.3i). The number of SST neurons in the contralateral hemisphere also showed a decreased trend, although there were no significant differences among all time points compared with

Naïve/Sham animals (6 hr: $p=0.5668$, 7 DPI: $p=0.0832$, 1 month: $p=0.1350$ and 3 month: $p=0.2787$). This was most likely due to the fact that the Naïve/Sham group displayed a lower number of SST positive cells in the contralateral side (mean $\pm$ SEM = 30.42 ± 1.817) compared to the ipsilateral hemispheres (mean $\pm$ SEM = 37.23 ± 2.254) (Figure 4.3).

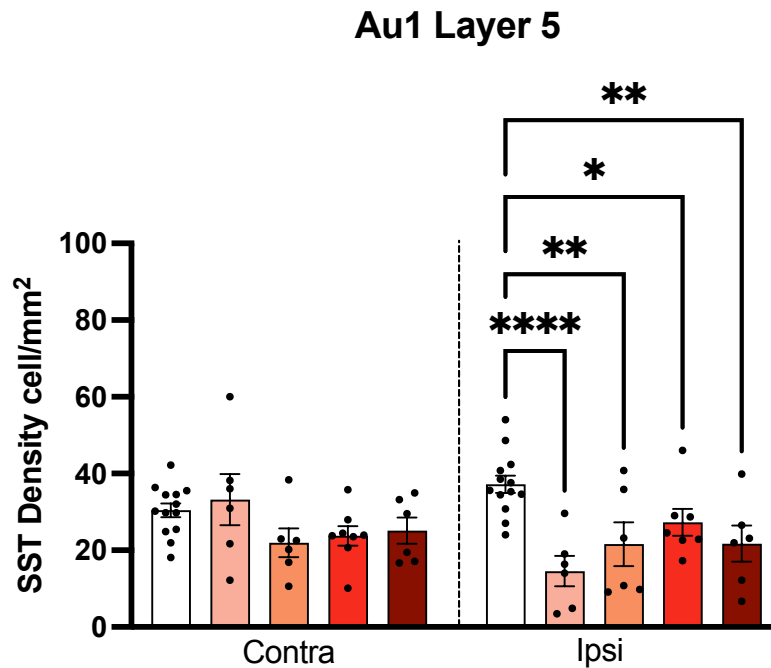


Figure 4.3 Somatostatin (SST) density decreases in the 5th layer of the primary auditory cortex (Au1).

Significant density decreases were observed in the ipsilateral hemisphere. Quantification of interneuron cell density using mixed effects ANOVA, corrected using Benjamini, Krieger and Yekutieli False Discovery Rate multiple comparison correction. All data presented mean $\pm$ SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$ Naïve/sham: $n=13$, 6 hr: $n=6$, 7DPI, $n=6$, 1 month: $n=8$, 3 month: $n=6$.

The analysis of the mixed effects ANOVA for the **SST** density in the **V1** revealed no changes between hemispheres for **layer 2/3, 4, 5 and 6** for all groups of animals, as shown in Supplementary Table 8. When the density for the immunopositive SST neurons was analysed for the **RSC** no significant changes between hemispheres were found in **layers 2/3, 4, 5 and 6** in all groups of rats, as reported in Supplementary Table 9.

Therefore, the hemispheric counts of SST positive IN were pooled per layer for each region and results are shown in Table 4.3. It was found that there were significant reductions in the **5th layer** of the **Au1**, the **5th layer** of the **V1** and the **2/3rd** and **5th layer** of the **RSC**.

SST				
		F (DFn, DFd)	p-val	adjusted p-val (q-val)
Au1	layer 2/3	F (4, 34) = 1.490	p=0.2270	q=0.2384
	layer 4	F (4, 34) = 1.567	p=0.2053	q=0.2384
	layer 5	F (4, 34) = 4.140	p=0.0077	q=0.0243
	layer 6	F (4, 34) = 0.9959	p=0.4232	q=0.3333
V1	layer 2/3	F (4, 34) = 1.049	p=0.3966	q=0.3123
	layer 4	F (4, 34) = 2.836	p=0.0393	q=0.0619
	layer 5	F (4, 34) = 4.241	p=0.0068	q=0.0214
	layer 6	F (4, 34) = 1.206	p=0.3263	q=0.3123
RSC	layer 2/3	F (4, 34) = 5.010	p=0.0028	q=0.0088
	layer 4	F (4, 33) = 1.608	p=0.1956	q=0.2054
	layer 5	F (4, 34) = 3.069	p=0.0292	q=0.046
	layer 6	F (4, 34) = 0.9797	p=0.4316	q=0.3399

Table 4.3 Somatostatin (SST) One-way ANOVA results for layers of cortical regions of interest.

Results shown for primary auditory cortex (Au1), primary visual cortex (V1) and the Retrosplenial cortex (RSC) displaying significant differences in the 5th layer of the primary visual cortex and the 2/3rd and 5th layer of the Retrosplenial cortex ($p < 0.05$). Significant differences are depicted with bold text that show the Au1, V1 and RSC have 5th layer changes. RSC is also significantly different in the 2/3rd layer after Benjamini, Krieger and Yekutieli False Discovery Rate multiple comparison correction. (Au1: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=6$, 1 month: $n=8$, 3 months: $n=6$. V1: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=6$, 1 month: $n=8$, 3 months: $n=6$. RSC: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=6$, 1 month: $n=8$, 3 months: $n=6$).

In the **AU1 cortex**, there were decreases in SST densities for all time points except 6 hours (**Au1**: 7 DPI: -35.52%, $p=0.0029$, 1 month: -25.89% $p=0.0147$, 3 month: -30.91% $p=0.0085$) (Figure 4.4i).

Similar findings were found in the **5th layer** of the **V1**, where decreases in SST densities were observed in the blast group when compared to Naïve/Sham group at 7 DPI (40% reduction, $p=0.0025$), 1 month (36% reduction, $p=0.0029$) and 3 months post-injury (32% reduction,

$p=0.0124$) (Figure 4.4ii). In addition, the number of SST positive cells in the **4th layer** of the **V1** was also reduced by 45.53% at 7 DPI ($p=0.0119$).

When the densities of SST were investigated in the **RSC**, reductions were also found at 3 months post-injury in the **5th layer** ($p=0.0201$, a 29.47% reduction relative to Naïve/Sham). However, decreased SST numbers were also detected in the **2/3rd** layers at 1 and 3 months post-injury (1 month 43% reduction, $p=0.0022$); 3 months (33.6% reduction $p=0.0252$), relative to Naïve/Sham (Figure 4.4iii). No significant differences were found in other layers of the other cortical ROIs (p and $q > 0.05$).

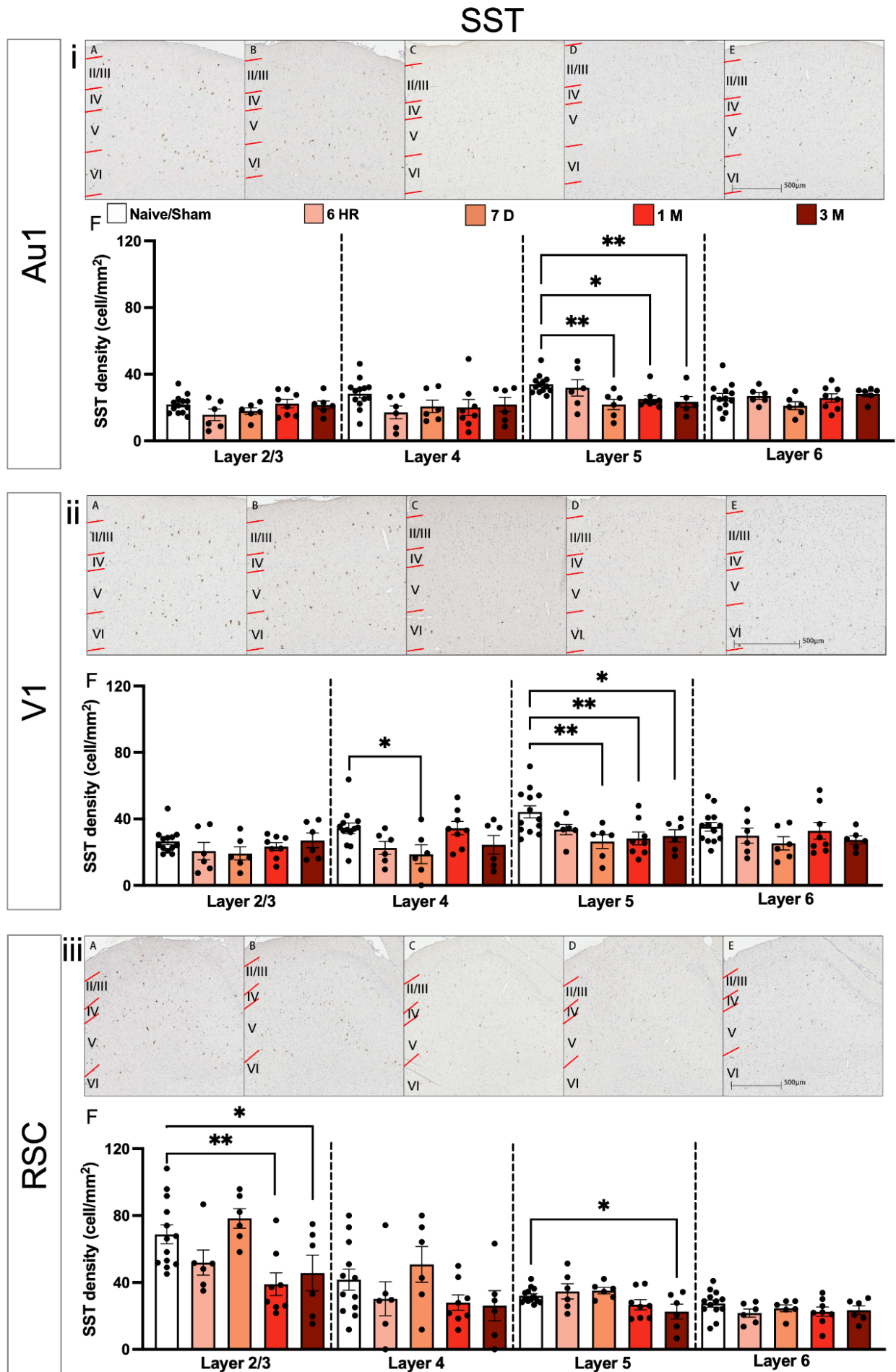


Figure 4.4: Somatostatin (SST) density changes in for the layers of cortical regions of interest.

Somatostatin density changes in the primary auditory cortex, the primary visual cortex and the retrosplenial cortex post-blast traumatic brain injury. Somatostatin positive intraneuronal density following single blast traumatic brain injury in the primary auditory cortex at (iA) Naïve/Sham, (1B) 6hours post-injury, (iC) 7 DPI post -injury, (iD) 1 month post-injury, (iE) 3 months post-injury. (iF) quantification of interneuron cell density using one-way ANOVA, corrected using BKY FDR. (iiA) Naïve/Sham, (1B) 6hours post-injury, (iiC) 7 DPI post -injury, (iiD) 1 month post-injury, (iiE) 3 months post-injury. (iiF) quantification of interneuron cell density using one-way ANOVA, corrected using BKY FDR. (iiiA) Naïve/Sham, (1B) 6hours post-injury, (iiiC) 7 DPI post -injury, (iiiD) 1 month post-injury, (iiiE) 3 months post-injury. (iiiF) quantification of interneuron cell density using one-way ANOVA, corrected using Benjamini, Krieger and Yekutieli False Discovery Rate multiple comparison correction. Results showed the Au1, V1 and RSC have significant increases mainly in the 5th cortical layer, but also in the 2/3rd layer of the RSC. All data presented mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. (Au1: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=6$, 1 month: $n=8$, 3 months: $n=6$. V1: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=6$, 1 month: $n=8$, 3 months: $n=6$. RSC: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=6$, 1 month: $n=8$, 3 months: $n=6$).

4.3.4 Increased Microglial Density at Acute and Chronic Time-Points Post- Injury in the Mild bTBI Model

A component of the inflammatory response post-injury is the activation of microglial and astrocyte cells within the brain. Following the observations of reductions in certain populations of interneurons in the brain post-blast injury, I investigated whether changes in the density of glial cells were associated with the reductions in INS. Therefore, the number and density of microglia and astrocytes were assessed by immunohistochemistry using IBA1 and GFAP DAB staining respectively, in the same regions of interest (Au1, V1 and RSC), as well as the corpus callosum (CC), at the same time points post-injury investigated above .

As it was previously analysed for the neuronal densities, each hemisphere was investigated separately to assess whether one of the hemispheres was affected by the blast more than the other. The density of microglial cells was analysed by counting the number of IBA1+ cells and normalising that result by the area. Morphology of the microglial cells was not calculated due to issues with the storage of the brains affecting the staining. Therefore, comparing morphology between brains was not possible, and only metrics that were relative to the area of the region of interest were included in this study.

Two-way ANOVA revealed that time post-injury was significant in the **Au1 and the RSC** for IBA1 density changes, but no differences were found for hemisphere or interaction between the different groups of animals (Naïve/sham: n=12, 6 hr: n=6, 7DPI, n=6, 1 month: n=5, 3 month: n=6). No main effect changes were detected also in the **V1** for differences of IBA1 between hemispheres and over time (Supplementary Table 10).

Therefore, the densities of IBA1 staining were pooled for both hemispheres and analysed using one-way ANOVAs for each region (Table 4.4). Significant differences were found in the **Au1 and the RSC**.

	IBA1	
	F (DFn, DFd)	p-val
Au1	F (4, 27) = 4.392	p=0.0073
V1	F (4, 27) = 2.293	p=0.0854
RSC	F (4, 27) = 5.504	p=0.0023
CC	F (4, 27) = 0.9094	p=0.4725

Table 4.4 One-way ANOVA for IBA1 density in all regions.

Results of One-way ANOVA for primary auditory cortex (Au1), primary visual cortex (V1), Retrosplenial cortex (RSC) and the corpus callosum (CC). Significant ANOVAs are depicted in bold ($p < 0.05$). Au1 and RSC were found to be significant. (Au1: Naïve/Sham: n=13, 6 Hr: n=6, 7 Day: n=4, 1 month: n=6, 3 months: n=6. V1: Naïve/Sham: n=13, 6 Hr: n=6, 7 Day: n=4, 1 month: n=6, 3 months: n=6. RSC: Naïve/Sham: n=13, 6 Hr: n=6, 7 Day: n=4, 1 month: n=6, 3 months: n=6. CC: Naïve/Sham: n=13, 6 Hr: n=6, 7 Day: n=4, 1 month: n=6, 3 months: n=6).

The quantification of number of IBA1 positive cells per area revealed significant elevations at 6h post injury (34% increase; $p = 0.0004$) and 1 month post-injury (18% increase; $p = 0.0414$) compared with sham animals in the **Au1 cortex** (Figure 4.5). All time-points investigated showed significant increases in IBA1 positive microglia in the **RSC**, except in the 3 months post injury group (6hour: 40% increase, $p=0.0001$, 7 DPI: 25% increase, $p=0.0207$ and 1 month post-injury: 20% increase, $p=0.0288$) when compared with Naïve/Sham animals (Figure 4.5).

There were no significant differences in IBA1 staining for the other brain regions analysed, although there was a trend of increased IBA1 density at 6 hours post-injury in the **CC** and **V1** (Figure 4.5).

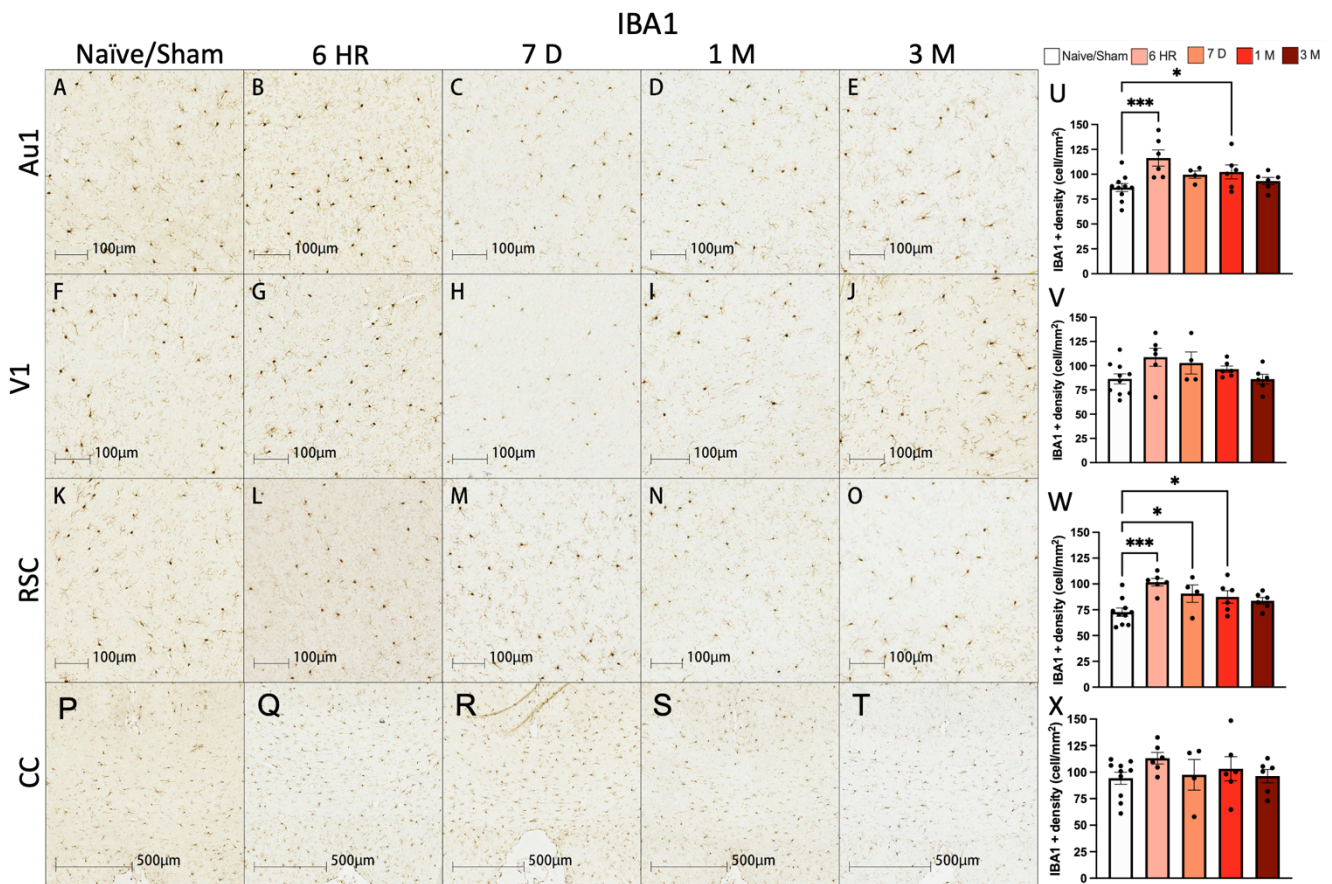


Figure 4.5 IBA1 density in all regions of interest.

(A-E) IBA1 staining of the auditory cortex (Au1). (F-J) IBA1 staining in the primary visual cortex (V1). (K-O) IBA1 staining in the retrosplenial cortex (RSC). (P-T) IBA1 staining in the corpus callosum. (A/F/K/P) Naïve/Sham, (B/G/L/M) 6 hr post-injury, (C/H/M/R) 7 DPI post-injury, (D/I/N/S) 1 month post-injury, (E/J/O/T) 3 months post-injury. U-X quantification of IBA1 for each region of interest using one-way ANOVA. Significant increases found in the Au1 and RSC at 6hr, and 1 month. Significance also found at 7 DPI in the RSC. No other comparisons were significant. Data presented mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. (Au1: Naïve/Sham: $n = 13$, 6 Hr: $n = 6$, 7 Day: $n = 4$, 1 month: $n = 6$, 3 months: $n = 6$. V1: Naïve/Sham: $n = 13$, 6 Hr: $n = 6$, 7 Day: $n = 4$, 1 month: $n = 6$, 3 months: $n = 6$. RSC: Naïve/Sham: $n = 13$, 6 Hr: $n = 6$, 7 Day: $n = 4$, 1 month: $n = 6$, 3 months: $n = 6$. CC: Naïve/Sham: $n = 13$, 6 Hr: $n = 6$, 7 Day: $n = 4$, 1 month: $n = 6$, 3 months: $n = 6$).

4.3.5 Microglia Increase in Coverage at 3 Months Post-Injury in All Regions of Interest

Based on the observed density increases of IBA1 cells, I assessed whether this increase was evident in the proportional area stained. If morphological alterations occurred, the cells would occupy a larger portion of the designated region of interest. This investigation

encompassed the regions **Au1**, **V1**, **RSC**, and **CC**, without considering hemisphere division, as prior analysis demonstrated that there were no differences in the staining between hemispheres on microglial densities.

One-way ANOVA analysis showed significant IBA1 increased in the **Au1**, **V1** and **CC** (Table 4.5).

IBA1 % Area		
	F (DFn, DFd)	p-val
Au1	F (4, 26) = 2.893	p=0.0417
V1	F (4, 26) = 3.266	p=0.0269
RSC	F (4, 26) = 2.374	p=0.0782
CC	F (4, 26) = 5.324	p=0.0029

Table 4.5 One-way ANOVA for IBA1 % area in all regions.

Results shown for primary auditory cortex (Au1), primary visual cortex (V1), Retrosplenial cortex (RSC) and the corpus callosum (CC). Significant ANOVAs are depicted in bold ($p < 0.05$). Au1, V1 and CC were found to have significance. Naïve/sham: n=12, 6 hr: n=6, 7DPI, n=6, 1 month: n=5, 3 month: n=6

The analysis revealed that there was an increase in the IBA1 percentage area stained at chronic time points post-injury, in all brain areas investigated, as shown in Figure 4.6 (**Au1**: area increase of 96.82%, $p=0.0326$, **V1**: 106.43% increase, $p=0.0250$, **CC**: 266.38% increase, $p=0.0006$) and the **RSC** followed the same increasing trend at this chronic timepoint (Figure 4.6).

IBA1

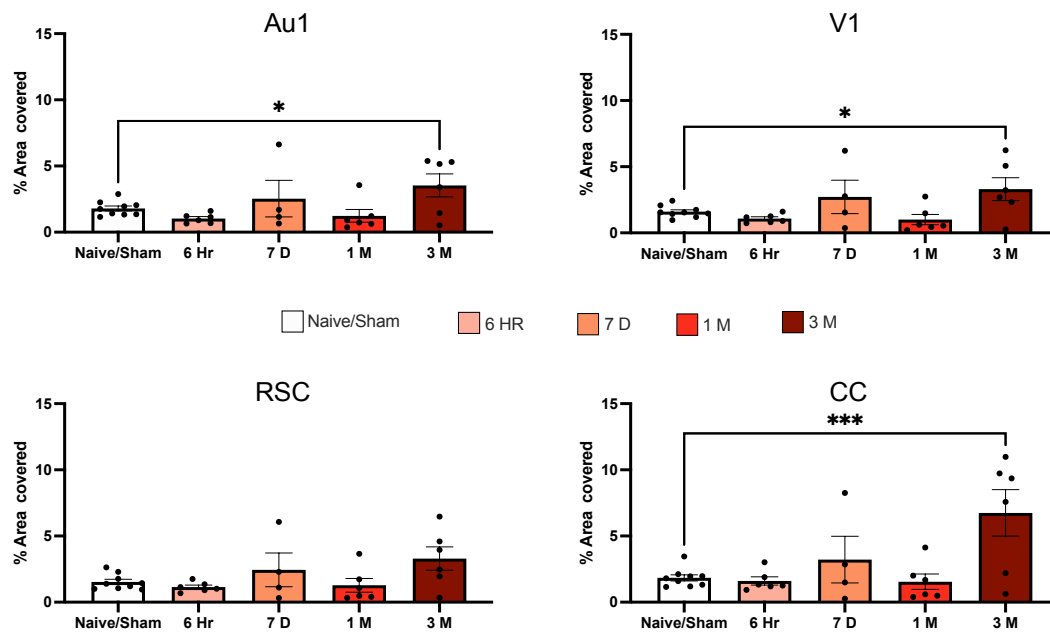


Figure 4.6 IBA1 % area covered analysis over the acute – chronic timepoints

The time points investigated spanned 6hr, 7 days post-injury, 1 month and 3 months post-injury, in the primary auditory cortex (Au1), Retrosplenial cortex (RSC), Primary visual cortex (V1) and the corpus callosum (CC). One-way ANOVA revealed significant increase in % area stained at 3 months. Quantification of IBA1 for each region of interest using one-way ANOVA data presented mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. (Au1: Naïve/Sham: $n=9$, 6 Hr: $n=6$, 7 Day: $n=4$, 1 month: $n=6$, 3 months: $n=6$. V1: Naïve/Sham: $n=9$, 6 Hr: $n=6$, 7 Day: $n=4$, 1 month: $n=6$, 3 months: $n=6$. RSC: Naïve/Sham: $n=9$, 6 Hr: $n=6$, 7 Day: $n=4$, 1 month: $n=6$, 3 months: $n=6$. CC: Naïve/Sham: $n=9$, 6 Hr: $n=6$, 7 Day: $n=4$, 1 month: $n=6$, 3 months: $n=6$).

4.3.6 No Astrocytic Changes after Single Mild Blast Exposure

Astrocytes are known to be an important part of the immune response to TBI. Therefore, I assessed the percentage area stained of GFAP+ astrocytes in the **Au1**, **V1**, **RSC** and **CC** for each hemisphere per region of interest, using a mixed effects ANOVA. Two-way ANOVAs were conducted and no differences were seen in any of the main factors in each of the groups of animals analysed (Supplementary Table 11) (Au1: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=6$, 1 month: $n=8$, 3 months: $n=6$. V1: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=4$, 1 month: $n=5$, 3 months: $n=6$. RSC: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=4$, 1 month: $n=5$, 3 months: $n=6$. CC: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=5$, 1 month: $n=8$, 3 months: $n=6$).

In light of the previous analysis, the values of GFAP % area stained for each hemisphere were pooled per region and analysed using a one-way ANOVA. GFAP % area covered as measurement of reactive astrogliosis showed no significant increases in the **Au1**, **V1**, **RSC** or the **CC** (Table 4.6) (Figure 4.7) among groups.

GFAP % Area		
	F (DFn, DFd)	p-val
Au1	F (4, 33) = 1.557	p=0.2088
V1	F (4, 29) = 0.5245	p=0.7185
RSC	F (4, 33) = 0.7405	p=0.5712
CC	F (4, 34) = 1.137	p=0.3558

Table 4.6 One-way ANOVA for GFAP % area in all regions.

The table shows one-way ANOVA for GFAP% in the primary auditory cortex (Au1), primary visual cortex (V1), Retrosplenial cortex (RSC) and the corpus callosum (CC). Significant ANOVAs are depicted in bold. No significance was found ($p>0.05$). (Au1: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=6$, 1 month: $n=8$, 3 months: $n=6$. V1: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=4$, 1 month: $n=5$, 3 months: $n=6$. RSC: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=4$, 1 month: $n=5$, 3 months: $n=6$. CC: Naïve/Sham: $n=13$, 6 Hr: $n=6$, 7 Day: $n=5$, 1 month: $n=8$, 3 months: $n=6$).

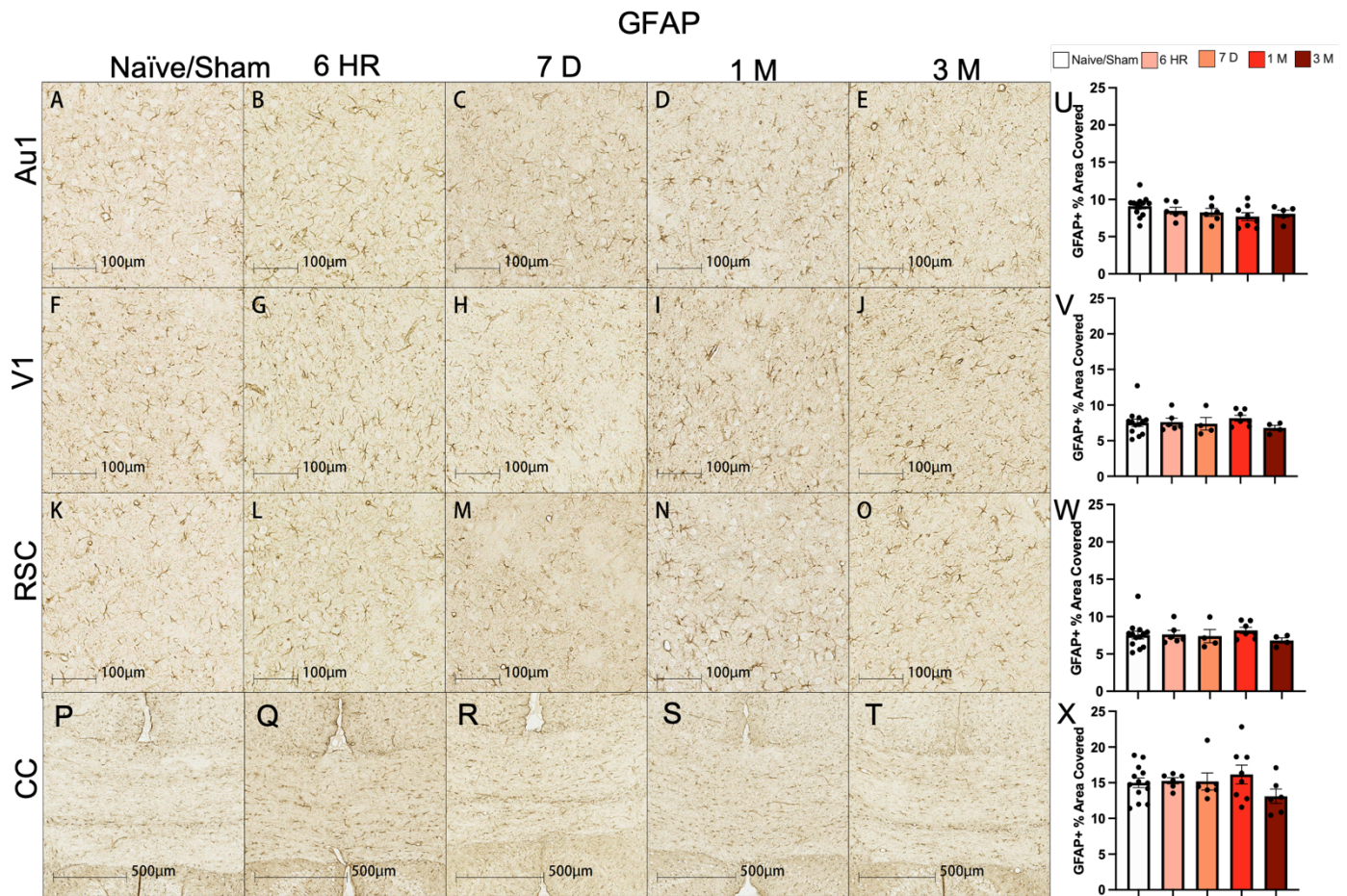


Figure 4.7 GFAP % area covered in all regions of interest.

GFAP analysis was conducted in the Au1, RSC, V1 and CC. GFAP % stained in all regions of interest (A-E) GFAP staining of the auditory cortex (Au1). (F-IJ) GFAP staining in the primary visual cortex (V1). (iK-iO) GFAP staining in the retrosplenial cortex (RSC). (P-T) GFAP staining in the corpus callosum. (A/F/K/P) Naïve/Sham, (B/G/L/M) 6Hr post-injury, (C/H/M/R) 7 DPI post-injury, (D/I/N/S) 1 month post-injury, (E/I/O/T) 3 months post-injury U-X. quantification of GFAP+ for each region of interest using one-way ANOVA found no significant differences data presented mean $\pm$ SEM. (Au1: Naïve/Sham: n=13, 6 Hr: n=6, 7 Day: n=6, 1 month: n=8, 3 months: n=6. V1: Naïve/Sham: n=13, 6 Hr: n=6, 7 Day: n=4, 1 month: n=5, 3 months: n=6. RSC: Naïve/Sham: n=13, 6 Hr: n=6, 7 Day: n=4, 1 month: n=5, 3 months: n=6. CC: Naïve/Sham: n=13, 6 Hr: n=6, 7 Day: n=5, 1 month: n=8, 3 months: n=6).

4.4 Discussion

The aim of this chapter was to investigate the neuronal and glial alterations in the neocortex in the same block of brain associated with electrophysiological changes observed following a single mild blast traumatic brain injury. The findings of this chapter indicate that this is a global effect and there are no differences between the two hemispheres (ipsilateral and contralateral) on neuronal or glial densities, implying that if the brain is affected, both hemispheres are equally impacted. The only exception was the number of SST interneurons in the auditory cortex, which seems to be reduced significantly in the ipsilateral hemisphere. This chapter also provides novel evidence that both PV and SST interneurons are affected by a single mild blast, and these alterations may be connected to the electrophysiological changes, analysed by Konstantinos Tsikonofilos (Supplementary Material 2). Additionally, the study reveals that the decrease in density of SST and PV interneurons occurs during sub-acute and chronic time-points, mirroring the findings from resting-state EEG results. Notably, these interneuronal changes predominantly occur mostly in the 5th layer of the neocortices, where the electrophysiological alterations were observed, in the absence of overall neuronal loss. Furthermore, this study demonstrates a mild inflammatory response at similar time points to the EEG changes, providing additional insights of the role of microglia in neuronal function.

4.4.1 Novel Interneuronal Density Reductions Post-Blast Exposure May be Associated with the Electrophysiological Changes

In this chapter, I investigated potential alterations in the PV and SST INs population density in the blast model. The EEG results showed global increases in power with a relative increase in the lower frequencies (Supplementary Material 2). This suggests changes in E/I balances in the blast model, as shown from more severe studies of TBI and post-traumatic epilepsy studies (Lowenstein, Thomas et al. 1992, Cantu, Walker et al. 2015). PV decreases were found in only one region, the Au1, similar to what was reported in the one of the two other blast TBI IN studies (Masri, 2020 #163). However, the novelty of our study was that these densities

were investigated at acute and chronic time points, whereas as previously stated, Masri 2020 (Masri 2020) only investigated up to 5 DPI. Similar to what had been found in other TBI studies, most of the changes were observed in the infragranular layers (Cantu, 2015 #167; Vascak, 2018 #176). The 5th layer is important for cortico-cortico interactions and signalling as well as sub-cortical interactions (Kim, Juavinett et al. 2015, Mease and Gonzalez 2021, Witcher, Bray et al. 2021), as the neurons are integrating inputs and distributing many outputs. The 6th layer is the location of long-ranged INs that can feed in to the CC (Tremblay, Lee et al. 2016), thus making them vulnerable to white matter damage and DAI. The reduction in PV interneurons in these layers could be contributing to the hyperexcitability seen in the EEG recordings, as the PV INs may not be providing their feed-forward inhibition (Ferguson and Gao 2018), that may result in increased signal to noise ratio within the brain (a potential lack of informational processing). Interestingly, increases in lower frequencies were reported in Gloor 1977 (cat brain lesion model) (Gloor, Ball et al. 1977), where lesions to the white matter caused delta waves to be generated from the above-lesion pyramidal cells, whereas a lesion solely to the grey matter did not produce delta wave activity.

With regards to the lack of changes in positively NeuN stained neurons in this study, it is important to note that just because a neuron is immunohistochemically positive, does not guarantee its pre-injury functionality is the same (Harris, Jin et al. 2022). There could be micro-DAI in the grey matter in the axons of the PV INs, or damage to their perinuclear net, which was not investigated in this thesis, but has been shown to burden PV INs post-TBI, and affect the functionality of PV INs and thus the functionality of principle neurons (excitatory pyramidal) (Vascak, Jin et al. 2018). Furthermore, despite the PV INs having no significant decrease in the other region of interest besides the Au1, these changes occurred in the absence of any general neuronal loss, as shown by the analysis of NeuN staining. This is a common finding in milder blast and non-blast TBI studies that have investigated IN populations (Cantu, Walker et al. 2015, Vascak, Jin et al. 2018).

In addition to changes in PV, we observed significant decreases in SST INs in all regions of interest (Au1, V1 and RSC), which was an unexpected result for a blast model. This is the first mild TBI study that has found SST INs to be affected, in contrast to nearly all mild blunt and blast studies of INs in TBI. This may be due to the longitudinal time course (6 hours – 3 months

post-injury) investigated in our study compared with other previous publications, such as , Masri, 2020 (1-5 DPI) (Masri, 2020 #163} and Harris, 2022 (3 Hour) (Harris, Jin et al. 2022). For example, had Masri, 2020 (Masri 2020) investigated more sub-acute and chronic timepoints post-injury, they may have seen SST alterations.

The fact that the SST reduction was more prevalent in the cortex than the PV cells is also surprising, as studies have found SST INs to be resistant to axotomy (Harris, Jin et al. 2022), unlike PV INs (Vascak, Jin et al. 2018). This suggests that there may be a different mechanism causing the reduction in SST cell density, as SST reductions have only been observed in lesions in penetrative CCI studies (Cantu, Walker et al. 2015). Nevertheless, this chapter's SST results shows that an IN population is preferentially affected by blast, since no general neuronal loss was observed, and that these changes happened in the 5th layer of the cortex, similar to other blunt TBI rodent studies (Cantu, Walker et al. 2015, Vascak, Jin et al. 2018). The implications of this density change could be contributing to the hyperexcitability observed by EEG recordings (Supplementary Material 2), as SST INs inhibit neighbouring PV and pyramidal neurons and usually have an effect at counteracting increasing levels of cortical excitability (Cossart, Dinocourt et al. 2001, Harris, Jin et al. 2022). In addition, this does not mean that the number of SST positive neurons is reduced by blast injury, but perhaps their SST expression. The reduced SST expression indicates that these neurons are no longer performing their inhibitive function and not able to compensate for the lack of PV inhibition, as they have been shown to do when PV INs are not able to function (Harris, Jin et al. 2022). Furthermore, these reductions in PV and SST are associated with a significant increase in microglial density particularly at acute times post-injury. Therefore, microglia may play a role in the functionality and networkings of the post-blast injury brain and may even contribute to the pruning of synapses and connections. Thus, there is a possibility of unbridled excitatory firing within the brain linked to an E/I imbalance and broadband excitability, as shown in the EEG results (Supplementary Material 2).

Here, this chapter has discussed these IN populations with respect to the power increases but has not discussed them with respect to functional connectivity. PV INs role in gamma-oscillation generation, and the specific gamma FC alterations seen in this model (Supplementary Material 2 Figure 6), suggest that these INs could be part of the mechanisms

as to why the EEG results observed an FC increase in the gamma band only. Cortical pyramidal cells in the 5th layer provide excitatory input to vasoactive intestinal polypeptide (VIP) cells. VIP cells are another IN cell that can inhibit a target region's SST INs (Modolo, Hassan et al. 2020). SST cells inhibit PV cells also. Thus, if a cortical pyramidal cell is hyperexcited and firing to a VIP cell, that VIP cell is inhibiting an SST cell, thereby disinhibiting a PV neuron. As a result, there are PV cells disinhibited and firing a flow of gamma-oscillations, unchecked, in that target region (Cottam, Smith et al. 2013, Xu, Jeong et al. 2013, Karnani, Jackson et al. 2016, Urban-Ciecko and Barth 2016, Muñoz, Tremblay et al. 2017). Furthermore, since there is a reduction in SST cells, seen in this study in more areas than the PVs, this may cause a gamma-oscillation also to flow, thus resulting in FC increase in the gamma band on a circuit level. Moreover, the intensified plasticity resulting from injury-induced hyperexcitation can potentially amplify this occurrence. It does so by fostering the formation of stronger synapses, leading to more dependable transmission of oscillations through pyramidal neurons (Witkowski, Gao et al. 2019). FC increase in the gamma band is seen in non-TBI studies of hyperexcitability pathologies where there is an E/I imbalance (Andreou, Nolte et al. 2015, Di Lorenzo, Daverio et al. 2015, Wang, Ethridge et al. 2017, Wang, Feng et al. 2022). Therefore, in this chapter, it could be suggested that there are two mechanisms at play, white matter alterations from axonal damage, to be discussed later, and an imbalance of excitation and inhibition, but more work would be required to confirm.

4.4.2 Blast Exposure is a Diffuse Type of Injury and the Histological Analyses of Markers for Inhibitory Neurons and Glial Cells do Not Show Lateralisation

For human EEG, it is possible to locate and regionalise some of the power or FC changes to locations in the brain. The EEG results show that the absolute power effect is mainly in the contralateral posterior temporal regions of the brains in the delta, theta, alpha and beta bands, whereas the gamma band was seen more globally spread across the brain (Supplementary Material 2 Figure 2). For relative changes, the results were similar except for alpha, it was found frontally. HFO for the relative power was also found widespread similar to Gamma (Supplementary Material 2 Figure 4). This asymmetry seen in most of the

frequency bands denotes the spatial sensitivity achieved using the array in this model, and could be explained by biomechanical understanding of the model's movement during blast. It would generate greater insight into the strain and strain rate undertaken by the axons in the brain if there was a 3D understanding similar to previously published works (Donat, Yanez Lopez et al. 2021). Information like this could aid preventative or therapeutic interventions.

These results served as the initial impetus for investigating whether there was a differential change in the density of the neurons and glial cells in a hemispheric manner. Previously, other studies explored the distribution of similar cell types in blast-related research (Readnower, Chavko et al. 2010, Masri 2020, Bishop, Won et al. 2022). Masri (2020) (Masri 2020) reported a reduction in PV density on the ipsilateral side of the blast in their rodent blast model. However, this change was only observed at 5 days post-injury (DPI). This does not rule out that this asymmetrical reduction in PV neurons at 5 DPI could progress to become equal in both hemispheres at 7 DPI or later, as occurs in our study. Nevertheless, Masri's findings suggested a lateralised effect of the impact, even from the blast noise itself in the Au1 region (Masri, 2020 #163). In contrast, the present chapter also investigated the effect of the blast in each of the hemispheres for the Au1 region and other brain areas, showing an equal effect in specific layers. This difference in results could be attributed to variations in blast parameters and the time points investigated. Furthermore, it is important to note that the theta band showed significant increases in both hemispheres (Figure 4.2), and the topoplots (Figure 4.2B) demonstrate that, although the statistical increases appear more lateralised, it is evident that both hemispheres are affected, which supports the histological analysis. Additionally, this equal effect in the auditory cortex could be influenced by the absence of hearing protection in the animals of our study at 1 and 3 months, whereas Masri's rodents had such protection. However, functional tests would be required to investigate whether hearing loss or alterations occur at chronic time points.

Interestingly, this study also revealed no hemispheric impacts on the reduction of somatostatin neurons or the increase in microglial cells. Together, the lack of asymmetry to these changes is possibly contributing to the global electrophysiological effects and demonstrating the diffusivity of the blast wave. The microglial findings align with those of Bishop et al., (Bishop, Won et al. 2022), who conducted a lateralised rodent blast study

involving both head mobile and head restrained conditions. Bishop et al., found significant increases in IBA1 positive stained cells from 1 to 30 DPI but did not observe significant differences between hemispheres. Thus, further supporting and displaying the diffuse nature of the blast injury.

4.4.3 Interneuronal Density Reductions do not Necessarily Mean Neuronal Loss/Death

The findings of reductions in the interneuronal population density following blast may suggest specific neuronal death of those INs. However, it should be noted the decreased density of these cells does not necessarily mean that they are dead, it may be that the neurons have downregulated expression of the proteins PV or SST, and therefore, are not staining positively for that cell type in the histological analysis (Lodge, Behrens et al. 2009, Filice, Vörckel et al. 2016, Urban-Ciecko and Barth 2016). This could potentially mean that the density changes are reversible through therapeutic intervention. Downregulation of expression of PV mRNA was shown by Masri, 2020 (Masri 2020) post-injury. Nevertheless, whether the cells have died due to injury or their expression has been downregulated, the functionality of the cells will have changed and result in electrophysiological changes (Filice, Vörckel et al. 2016), like those seen in this study.

Interestingly, it was found in Masri, et al. that the loss of PV was due to hearing loss from a ruptured tympanic membrane, rather than cell death (Masri 2020). In this chapter a reduction was observed in SST changes in the ipsilateral side of the injury, in layer 5 of the Au1. Nevertheless, it should be noted that tympanic membranes are able to recover and heal, and therefore they could be downregulated to enable plasticity or due to hearing damage, or both. Additionally, we did not observe differences between 1 and 3 months post injury, it seems that this loss is not recovered over time, suggesting that there is IN cell loss rather than downregulation of PV and SST markers. Therefore, further work doing genomic and proteomic assays and testing for plasticity markers (e.g synaptophysin and GAP-43) and cell death markers should be conducted in the future, to confirm if one or all theories are contributing to the functional changes within the brain post-blast injury.

4.4.4 Microglial Activation Coincides with Neuronal Functionality Post-Blast Exposure

It is important to note that while neuronal and axonal loss due to the biomechanical component of the injury can be contributing to the negative functional outcomes associated with blunt TBI and blast TBI, it is not the only factor impacting these changes. A combination of the other mechanisms mentioned, such as neuroinflammation and neurotransmitter imbalances, subunit changes, also play a role in the neuronal loss and development of the functional impairments seen in TBI and blast TBI.

There is a debate on the role of microglial activation in TBI and whether this is protective or detrimental. A study that investigated chronic microglial activation in a fluid percussion model of injury in mice found that microglia were causing detrimental neuropathology and functional impairments post-TBI, because animals that had the microglia removed did not present TBI-induced changes in their neurons 30 days post-injury (Witcher, Bray et al. 2021). They stated that, particularly during the subacute and chronic stages, microglia play a crucial role in disrupting the balance of neurons following TBI. However, contrasting investigations have shown that eliminating microglia may increase the detrimental effects in TBI models, unless microglia was allowed to repopulate (Willis, MacDonald et al. 2020). Findings from these studies indicate that the cognitive deficits resulting from TBI did not improve by microglia depletion alone, unless followed by repopulation during the acute phase of TBI. This repopulation process alters the phenotype of microglia, rendering them neuroprotective (Willis, MacDonald et al. 2020). However, an alternative report suggested that microglia depletion and subsequent repopulation can enhance both microglia phenotype, reduce oxidative stress and improve cognitive performance, but only when performed during the chronic phase of experimental TBI in mice, and not during the acute phase (Henry, Ritzel et al. 2020).

Microglia can therefore have neuroprotective functions, such as clearing up damaged cells, and can modulate neuronal circuit functions (Wake, Moorhouse et al. 2013). They are capable

of synapse elimination, neuronal surveillance, and neurogenesis, which will ultimately contribute to the maturation or new plastic neural circuits that shape cognition and behaviour (Wake, 2013 #1164)

In this chapter, the most significant changes in microglial numbers were observed at early times post-injury, particularly at 6 hours (increased 34-40%) compared with 1 month post-injury (18-20% increase) in the grey matter regions of the Au1 and RSC, although an increasing trend was seen in the other regions as well. There is varied preclinical blast literature on microglial activation as a sign of inflammatory response to injury. Our results align with other studies showing changes at acute time points (Huber, Meabon et al. 2016, Masri 2020), and mild TBI studies that reported microglial and astrocytic inflammatory responses at later time points (De Lanerolle, Bandak et al. 2011, Goldstein, Fisher et al. 2012, Ou, Clifton et al. 2022). Our findings, however, are in contrast with other blast studies. In the blast model of Hernandez et al. (Hernandez, Tan et al. 2018), they reported elevations in microglia/macrophages (IBA1) density at 1 DPI in the cerebral cortex lasting until 7 DPI, but results were normalised by their latest time point (21 DPI). On the other hand, some blast studies reported no microglial changes, which may be due to different blast parameters (orientation, pressure, head restrained/mobile and chest protected or not) and variation of methodologies for analysis.

An inflammatory response to injury can contribute to the degradation and compromise of axons in the brain (Huang, You et al. 2021). Microglia can contribute to the breakdown of the structural integrity of white matter that is seen in post-TBI patients (Sharp, Beckmann et al. 2011, Sharp, Scott et al. 2014, Huang, You et al. 2021, Mallas, Gorgoraptis et al. 2022) and preclinical studies (Donat, Yanez Lopez et al. 2021). However, their role to clear debris in the brain as well as maintaining a level of homeostasis can be neuroprotective. Additionally, microglia also secrete pro/anti inflammatory cytokines, chemokines and growth factors, contributing to the regeneration of the injured tissue. Transcriptomic studies have revealed that there are many different microglial phenotypes depending on the brain's state (wake, 2013).

In the present study, we did not observe any statistically significant increase in the number of microglia in the white matter. There was only a modest increase at the acute 6 hour time point until 1 month post-injury, with densities normalising to control levels at the 3 month time point in other regions of interest (Au1 and RSC). However, cell density is not the most sensitive way to assess microglial response to TBI. Therefore, the percentage area stained revealed that at 3 months post-injury there was a significant increase in the coverage of the microglia present in the white matter, Au1 and V1. This indicates that microglia seemed to have changed morphology, becoming bigger (as the number of cells were not different), such that they were covering more of the area. Change in morphology, suggests hypertrophy, and a different phenotype of microglia (Paolicelli, Sierra et al. 2022, Yu, Wang et al. 2022), one that may be contributing to the chronic EEG changes, and perpetuating the changes that resulted from the white matter alterations at the 1 month time point. This may be a consequence of the release of molecules from the damaged axons (modestly indicated in chapter 5), and cells to the microglia at this later time point, such as damaged cells releasing danger-associated molecular patterns (DAMPs) (Witcher, Bray et al. 2021), although there are many others (Shao, Wang et al. 2022). As a result, microglia can release noxious substances such as reactive oxygen species (ROS) and cause oxidative stress further affecting neurons (Donat, Scott et al. 2017).

Furthermore, white matter associated microglia have been shown to degrade myelin, another hallmark of white matter alteration in TBI (Roseborough, Jaremek et al. 2022). Further analysis on the morphology and measurements of cytokines released from the microglia throughout the time course post-injury would further help elucidating the phenotypes of microglia and the potential contribution to the electrophysiological and cellular changes in the blasted brain. Nevertheless, the indications of axonal damage as shown in chapter 5 and the microglial changes combined, support the fact that structural alterations can be a mechanistic substrate for the underlying electrophysiological changes.

This chapter's results show that astrocyte densities do not seem to change in blast models, when quantified as percentage area covered by GFAP, which would suggest a lack of response from the astrocytes, as they can become hypertrophic when activated due to injury. So far, in the blast literature there are contrasting findings regarding reactive astrogliosis in blast

models. Some studies have found elevation of astrocytes 72 hours post single-blast (VandeVord, Bolander et al. 2012), at 2 weeks (De Lanerolle, Bandak et al. 2011, Goldstein, Fisher et al. 2012, Svetlov, Prima et al. 2012) and others at 1 and 3 months post-injury (Kallakuri, Pace et al. 2018). However, similar to our work, other studies have reported no changes in astrocytes (Sajja, Galloway et al. 2012, Sosa, De Gasperi et al. 2013, Gama Sosa, De Gasperi et al. 2017, Murphy, Iacono et al. 2020, Rubio, Unnikrishnan et al. 2021). Interestingly, reports that compared the effect of different pressures in the blast model, found that the models performed with lower pressure (100kPa) showed no astrocytic changes beyond 4DPI, while models at higher pressure (450kPa) displayed elevated astrocyte density at 30DPI (Budde, Shah et al. 2013). This possibly highlights the importance of blast parameters and setup in terms of determining the nature of injury outcomes.

4.5 Conclusion

This chapter presents data from a preclinical model of single mild blast-induced traumatic brain injury to examine the pathological brain changes behind electrophysiological characteristics associated with this specific type of injury. Most of the hypotheses put forward in this work were successfully validated, with the exception of the lateralisation of the injury, the SST density reduction and the anticipated inflammatory response to the blast, which was not observed for astrocytes. By combining functional and histological approaches, this research achieved a clinically relevant aspect by utilising a preclinical model to investigate potential cellular-level mechanisms.

Furthermore, the study shed light on the long-term cellular factors contributing to hyperconnectivity and hyperexcitability following blast-induced injury. It is essential for future research to explore whether these cellular changes are also observed in human TBI cases. Additionally, combining the functional changes identified explored by my collaborators and understanding how these factors predispose individuals to neurodegenerative diseases should be prioritised.

5

Assessing Alterations in Axonal Damage in The White Matter as a result of blast TBI

5.1 Introduction

5.1.1. Imaging techniques for diagnosis and assessment of disease progression in TBI

TBI symptoms can persist for many years after the initial injury and have frequently been misunderstood as solely psychological in nature (Rose 2022). Given the higher prevalence of TBI in military environments, there is a pressing need to gain a better understanding of this condition in order to diagnose and monitor it. Consequently, ongoing efforts involve recruiting military personnel for studies aimed at enhancing our knowledge of TBI in the context of warzones (Graham, Blissitt et al. 2023).

TBI can produce a range of pathology, some of which is obvious and some not. It can often go unnoticed, or unreported, leading to challenges in diagnosis, treatment, and accurate reporting of injury statistics at later stages (Hoge, McGurk et al. 2008, Frost, Farrer et al. 2013, Robinson, Lindemer et al. 2015, Phipps, Mondello et al. 2020, Tenovuo, Diaz-Arrastia et al. 2021). Failure to diagnose TBI can result in further injuries and subsequent cognitive impairments and may even increase the risk of developing neurodegenerative diseases (Leiva-Salinas, Singh et al. , Sharp, Beckmann et al. 2011, Goldstein, Fisher et al. 2012, Mckee and Robinson 2014, Sharp, Scott et al. 2014, De Simoni, Grover et al. 2016, Tagge, Fisher et al. 2018, Mallas, De Simoni et al. 2021, Zimmerman, Laverse et al. 2021, Mallas, Gorgoraptis et al. 2022).

White matter tracts are enriched with a high concentration of axons, which are more prone to injury due to their inflexibility to shearing and tearing forces (Braun, Vaibhav et al. 2017). White matter damage, as demonstrated by diffusion tensor imaging (DTI) imaging, has proven to be a valuable diagnostic hallmark for TBI (Sharp and Ham 2011). It is often observed that most TBIs involve some degree of diffuse axonal injury (DAI) (Siedler, Chuah et al. 2014),

which can be detected through post-mortem assessment in humans (Siedler, Chuah et al. 2014).

Axons in the white matter can undergo demyelination, which can be seen as white matter integrity changes. The initial injury, whether focal or diffuse, can result in DAI and subsequent abnormalities in the white matter (Braun, Vaibhav et al. 2017). However, it is important to note that most of the axonal damage and white matter abnormalities can occur from secondary pathologies; demyelination and neuroinflammation, as the brain attempts to restore its equilibrium in response to the external assault (Mckee and Robinson 2014, Siedler, Chuah et al. 2014, Mckee and Daneshvar 2015, Braun, Vaibhav et al. 2017). Such patterns have been observed in both human and preclinical models of blunt and blast-related TBIs (Gentleman, Roberts et al. 1995, Povlishock and Christman 1995, Garman, Jenkins et al. 2011, Park, Eisen et al. 2013, Kim, Goodrich et al. 2020, Donat, Yanez Lopez et al. 2021, Graham, Zimmerman et al. 2021, O'Brien, Pham et al. 2021). Furthermore, it is important to note that brain injury is a dynamic process, and that time since injury can play an important factor in accurate diagnosis of TBI. The longer it takes to diagnose, the more likely hallmark symptoms can be confounded by co-morbidities (Hoge, McGurk et al. 2008, Sharp and Ham 2011, Frost, Farrer et al. 2013, Robinson, Lindemer et al. 2015, Phipps, Mondello et al. 2020, Tenovuo, Diaz-Arrastia et al. 2021).

Despite numerous studies confirming the usefulness of MRIs, and their utility in a clinical setting, they still are unlikely to identify all TBI, as not all injuries are in the white matter. This therefore makes it hard to diagnose and monitor a milder TBI using MRI. Therefore, there is a need for an alternative and complementary approach to diagnose and monitor TBI in the long term. Such an approach would greatly benefit individuals who lack access to advanced neuroimaging techniques, including those serving in active duty. One potential solution to enhance the diagnostic process is the incorporation of a fluid biomarker, which could be used in conjunction or independently of MRI.

5.1.2 Fluid biomarkers

A fluid biomarker that is able to sensitively detect DAI would be a useful for diagnosing TBI. Therefore, a useful biomarker should be able to detect alterations in DAI (from white matter or grey) early in mTBI cases, and aid in preventing the accumulation of injury. It must also be a way to confirm recovery, such as its concentration returning to “normal” levels, population depending. This would assist in diagnosis and prognosis and monitoring of injury development.

Fluid biomarkers can be sampled from different fluid samples, be that from cerebral microdialysis to measure cerebral proteins, a lumbar puncture to extract CSF or a blood sample to obtain serum or plasma. To fulfil the need to have a complementary method of TBI diagnosis and for it to be sensitive to DAI at acute time points, the fluid biomarker with a reliable correlation between cerebral spinal fluid (CSF) and blood concentrations is necessary. Although CSF is the most informative and accurate indicator of what has occurred in the brain, obtaining it is an invasive procedure. Therefore, the blood assessment should reflect what is happening in the brain, albeit at lower levels found in the CSF. Among different biomarkers used for axonal injury (Graham, Zimmerman et al. 2021, Holper, Watson et al. 2022, Youssef, Hughes et al. 2023) Neurofilament light (NFL) has been shown to do the aforementioned list of requirements (Shahim, Politis et al. 2020, Graham, Zimmerman et al. 2021, Halbgebauer, Steinacker et al. 2022).

The potential of neuronally specific neurofilaments (NF)s as fluid biomarkers in the field of neuroscience has generated promising results, as it has shown to indicate axonal injury as well as neurodegeneration (Chen, Meaney et al. 1999, Park, Liu et al. 2007, Chen, Johnson et al. 2009, Siedler, Chuah et al. 2014, Bergman, Dring et al. 2016, Shahim, Zetterberg et al. 2017, Khalil, Teunissen et al. 2018, Mages, Aleithe et al. 2018, Shahim, Tegner et al. 2018, Gaetani, Blennow et al. 2019, Kuhle, Kropshofer et al. 2019, Poesen and Van Damme 2019, Alirezaei, Pourhanifeh et al. 2020, Barro, Chitnis et al. 2020, Gao, Zhang et al. 2020, McCrea, Broglio et al. 2020, Nielsen, Soares et al. 2020, Shahim, Politis et al. 2020, Arun, Rossetti et al. 2021, Bittner, Oh et al. 2021, Graham, Zimmerman et al. 2021, McDonald, O'Brien et al. 2021,

McDonald, Shultz et al. 2021, O'Brien, Pham et al. 2021, Pekny, Wilhelmsson et al. 2021, Verde, Otto et al. 2021, Arsić and Nikić-Spiegel 2022, Nguyen, Malmstrom et al. 2022, O'Brien, Wright et al. 2022). The smallest subunit of the NFs, NFL, has demonstrated a long half-life of three weeks in humans, unlike other biomarkers, enabling information about the brain for longer (Barry, Millecamps et al. 2007). This is a useful characteristic for detecting TBI years post injury, which can be convenient for military personnel.

NFL is believed to be released from damaged axons into the CSF and blood, therefore it is easy to obtain and measure (Barry, Millecamps et al. 2007, Yue, Upadhyayula et al. 2020). Furthermore, NFL proteins are located abundantly in the cytoskeleton of the central nervous system (CNS), but in the peripheral nervous system (PNS) their prevalence decreases more than two-fold (Schlaepfer and Bruce 1990). This makes NFL concentrations less likely to be confounded by polytrauma.

Promisingly, NFL in rodents has the same polypeptide as the human NFL, it is highly conserved, thus making it very translationally useful for preclinical modelling of TBI. Moreover, NFL is the most abundant intermediate filament in the brain and it has the lowest molecular weight of the three NF subunits. Furthermore it is the most soluble relative to other subunits (Alirezai, Pourhanifeh et al. 2020). Consequently, NFL can be a valuable diagnostic tool in cases of TBI with delayed or sub-acute injury presentation seen in some cases, potentially due to their long half-life (Graham, Zimmerman et al. 2021). Furthermore, assessment of NFL by the ultrasensitive Quanterix single molecule assay (SIMOA) (Gaetani, Blennow et al. 2019), which is much more sensitive than traditional enzyme-linked immunoassay (ELISA), has allowed detection of ultra-low protein levels.

Studies investigating changes in NFs in TBI have shown that NF levels are elevated in milder forms of blunt TBI injury, in both the CSF and the blood (Zetterberg, Hietala et al. 2006, Shahim, Zetterberg et al. 2017, Ashton, Janelidze et al. 2021). This shows NFL as a potential fluid biomarker candidate for diagnosis in less severe cases of TBI. However, as previously stated, lumbar puncture for extracting CSF is painful and not practical for repeated sampling. Thus, the positive attribute of NFL is that plasma concentration levels reflect what occurs in the CSF (Graham, Zimmerman et al. 2021, Halbgebauer, 2022 #1057) and therefore it is a

suitable biomarker for identifying axonal injury in military and athletic populations using blood samples.

So far, studies investigating NFL as a fluid biomarker in blast injury have not reported clear changes at chronic time points. As an example, one report found a non-significant trend for an increase in the NFL of a rodent chronic blast model (Dickstein, De Gasperi et al. 2021). However, both the rats and human cohort of the same study had evidence of chronic CTE pathology (tau accumulations) after blast. It was also found that the human veterans of this study showed NFL levels that were not significantly different from the control comparison group. It was found, however, that patients with excess tau-ligand had higher NFL levels, showing that tau pathology was associated with increased NFL levels in the chronic phase. This was despite NFL levels not being significantly different from controls (Dickstein, De Gasperi et al. 2021).

Human blast studies of NFL are confusing in the acute phase, showing both an increase (Boutté, Thangavelu et al. 2019) and decrease in NFL levels (Edwards, Leete et al. 2020). Together, the use of NFL as a fluid biomarker should allow for an early diagnosis in blast and also to understand the progression of the TBI, by looking at the temporal profile in preclinical models and the location from within the brain.

5.2 Aims, Objectives and Hypotheses

5.2.1 Hypotheses

- Fluid biomarker neurofilament light will increase in both the plasma and the cerebral spinal fluid post-blast and peak at sub-acute timepoints and return to normal at 3 months (Chapter 5).
- Neurofilament light immunofluorescence intensity measured in the corpus callosum will decrease as a result of diffuse axonal injury and white matter damage (Chapter 5).

5.2.2 Aims and Objectives

1. To analyse the extent and temporal profile of axonal injury in the corpus callosum in the blast model.

Objective: Axonal damage in the white matter damage assessment will be conducted using immunostaining of neurofilament light and luxol fast blue in the corpus callosum.

2. To assess a potential traumatic brain injury biomarker that could be useful for the detection, diagnosis and progression of the brain damage.

Objective: I will investigate the axonal/neuronal damage using fluid biomarker NFL quantified by the Quanterix SIMOA® Assay at acute to chronic time points in the blast model, compare the results to histological results.

5.3 Methods

Immunofluorescent staining was conducted in the same manner as described in Chapter 2, section 2.1.6.1.

5.3.1 NFL antibody troubleshooting

For the purpose of identifying the source of the fluid biomarker NFL in the brain and measuring the expected loss of NFL in the brain tissue post-injury, antibodies were used to immunohistochemically stain for the location of NFL. Three antibodies for NFL that stain the same epitope regions and flanking regions as the Quanterix Simoa assay were used for IHC (MCA-1D33, MCA-1B11 and MCA-6h63) as the NFL fluid biomarker in the Quanterix SIMOA assay that are only available for antibody binding due to degenerating NFL or damaged

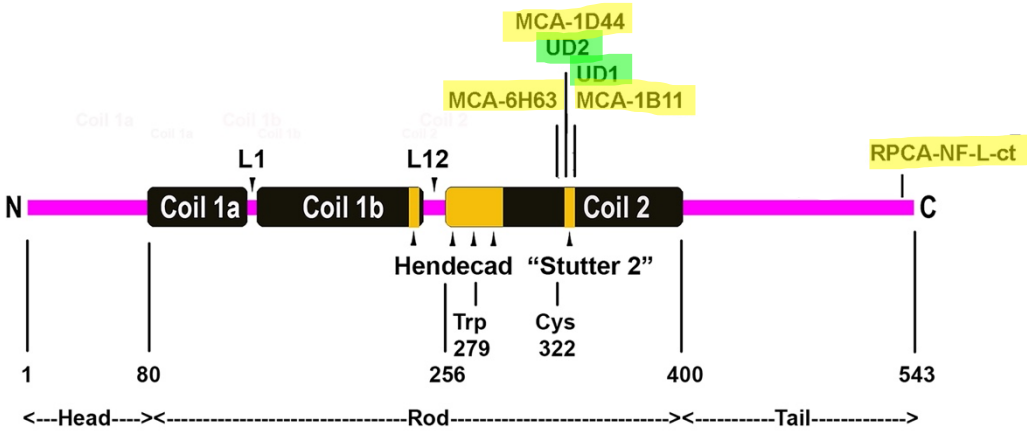


Figure 5.1 Diagrams of NF-L structure, landmarks and epitopes.

(A) Domain organization and epitope map of human NFL protein. The yellow blocks in Coil 1b and Coil 2 indicate the location of 11 amino acid 'hendecad' sequences, one of which is referred to here as 'Stutter 2' which is where the conserved coil changes/breaks down from the repeating heptad (7 amino acids) to 11 amino acid. And. Location of landmarks and relevant antibody epitopes are based on the human NFL protein sequence, those highlighted in yellow are the antibodies tested and those highlighted in green are the sequences that are found in the Quanterix Simoa Assay. The MCA-1D44, MCA-1B11 and MCA-6h63 bind to the same epitopes as the UD1 and UD2 antibody epitopes. This figure was adapted from (Shaw, Madorsky et al. 2022).

induced unmasked epitopes shown in coil 2 (Shaw, Madorsky et al. 2023) (Figure 5.1). One additional antibody obtained from Encor Biotechnology inc (RPCA-NFL-CT), stained the tail region (as described in chapter 1, section 1.1.10.1 -NFL structure of NFL) (Figure 5.1). The fluid biomarker Quanterix assay recognises two epitopes of NFL, U1 and U2, while the antibody MCA-1D44 binds to a site of U2 epitopes and the MCA-1B11 and MCA-6h63 antibodies bind to U1 epitopes (Shaw, Madorsky et al. 2023) (Figure 5.1 yellow highlight) (Figure 5.1).

They were tested first in controlled cortical injury (CCI) tissue (a focal injury model) as it had already shown to have a reduction in neurofilament heavy (Donat, Yanez Lopez et al. 2021). It was stained with the aim of identifying the damaged regions of NFL and the source of the NFL fluid biomarker measured in the SIMOA assay. The CCI tissue was generated by Dr Cornelius Donat as described in (Donat, Yanez Lopez et al. 2021) but the troubleshooting of these antibodies were conducted by me. The CCI 1mm depth injury was conducted at 14 DPI and referred to as mild. The CCI 2mm depth injury was conducted at 3DPI and 14 DPI and referred to as moderate.

5.3.1.1 Characterisation of the Staining with RPCA-NFL-CT Antibody

The first antibody, RPCA-NFL-CT, was tested in CCI sham tissue. This antibody should bind to the NFL C-terminal (the “tail”) of neuronal axons and the neuron body wherever NFL is expressed. In the white matter, the expectation from this staining was to detect individual axons and well as obtaining strong signal in the corpus callosum, although I was unable to detect individual axons within it as the NFL c-terminal was so highly expressed. In a TBI brain, it was expected that there would be a reduction of this C-terminal and thus the staining of this antibody would be reduced similar to previously published neurofilament heavy results (Donat, Yanez Lopez et al. 2021). However, it is possible that in a milder injury the C-terminal would stay intact in combination with partially degraded area in the rod which could be stained for with the other antibodies.

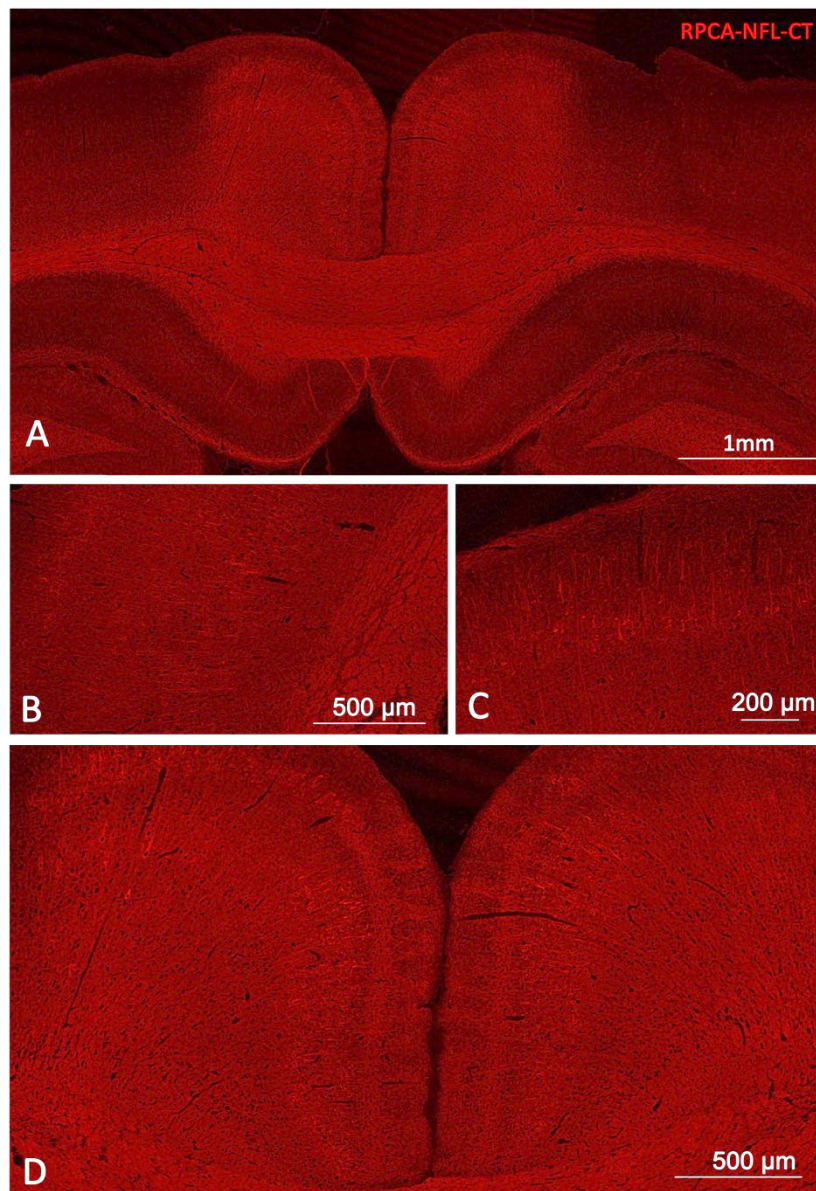


Figure 5.2 RPCA-NFL-CT staining in Sham CCI tissue.

A) corpus callosum and Retrosplenial cortex and the hippocampus. B) Auditory cortex, individual axons can be seen. C) Supragranular layers of the parietal cortex. D) Retrosplenial cortex.

Figure 5.2 illustrates the outcome of the positive RPCA-NFL-CT staining in a blast sham rat brain. As expected, NFL positive staining was located in neurons, both in cell bodies and axons. Higher intensities were found in the corpus callosum, although no individual axons were identified, as observed in the neocortex (Figure 5.2). Certain bright neuronal bodies and axons were detected in the cortical areas, as shown in Figure 5.2B-D. The staining appeared

bright in the neuronal bodies in specific layers, such as layer 4 of the auditory cortex (Figure 5.2B) and granular, c region (regions identified using Paxinos and Watson 2007 (Paxinos and Watson 2007)). Interestingly, there was not much positive staining in the cortical areas next to the Retrosplenial cortex, the medial parietal association cortex or the visual cortex layer 2/3 in the retrosplenial dysgranular layer and layer 5 of the Retrosplenial cortex.

5.3.1.2 Characterisation of the Staining of MCA-1D44 Antibody

MCA-1D44 staining has been shown in Dr Shaw's recent publication. This antibody can stain a similar region as the UD2 epitope that is found in the Quanterix Simoa assay. In this publication it was shown that it stained NFL with no overlap with antibodies that stain for intact neuronal processes but does stain linear sets of aggregates of damaged axons as well (Shaw, Madorsky et al. 2023). Therefore, I stained with the MCA-1D44 in CCI sections, as positive control tissue.

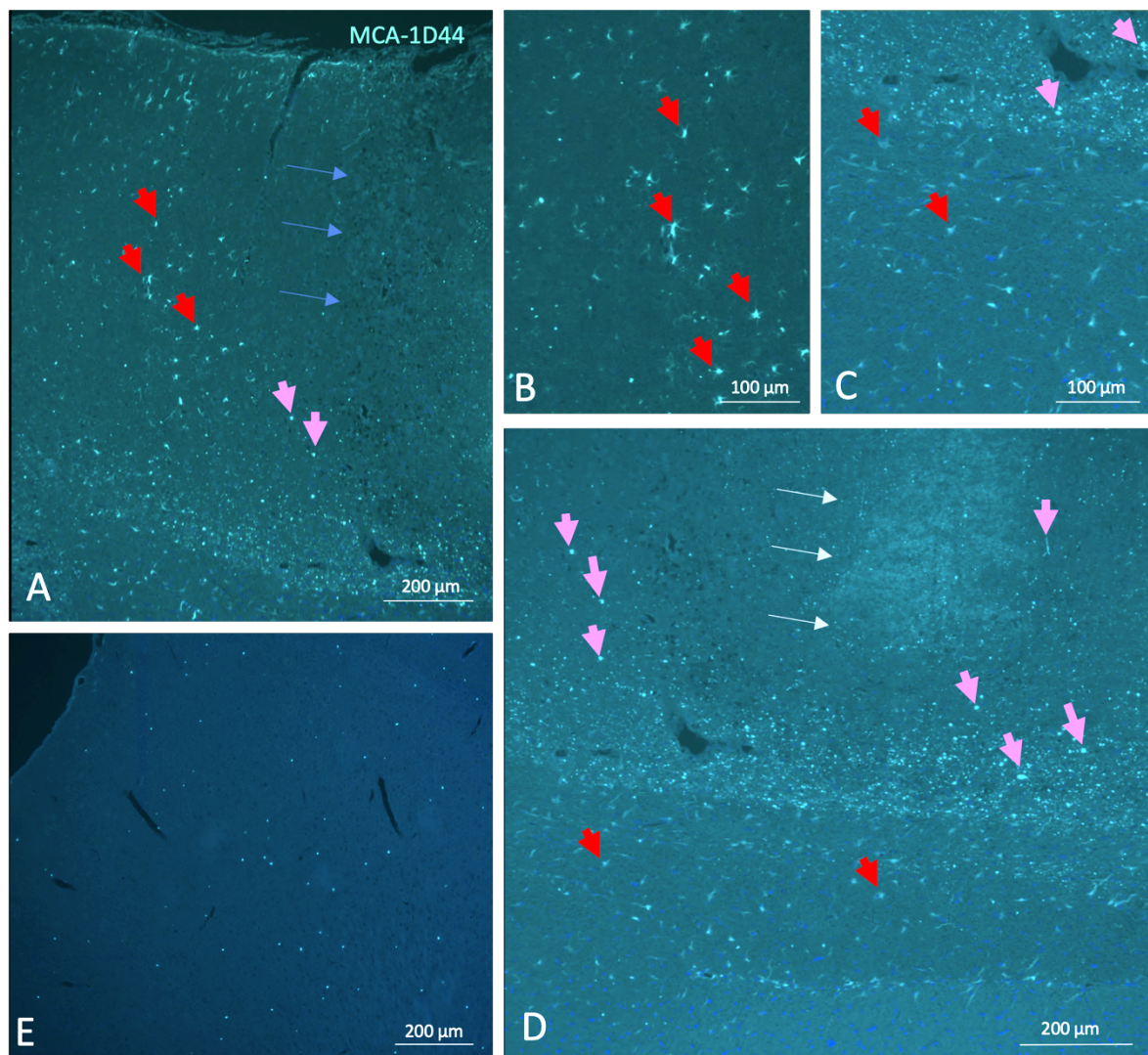


Figure 5.3 MCA-1D44 staining in CCI focal injury tissue at 3 DPI moderate.

A) Contusion site identified by blue arrows which shows per-contusion and corpus callosum. Red arrows showing suspected glial staining, pink arrows showing expected positive stain of MCA-1D44 punctate. B) Suspected glial stain pericontusionally, shown with red arrows. Is a high magnification of up of A C) Higher magnified image of the corpus callosum from D showing punctate, pink arrows, and potential glial staining, red arrows. D) Same image but more comprehensive of the contusion site, and positive signal, white arrows show highlighting of the contusion site. E) opposite hemisphere from the contusion site, seemingly random positive flecks of signal.

MCA-1D44 positive staining appeared in the contusion and pericontusion sites, as well as the subcortical white matter beneath the injury and near the site of the injury in CCI tissue. There were NFL positive aggregates that appear like dotty punctate around the contusion site, in the white matter and some highlighting of the contusion site, indicating damaged NFL in the contusion site (Figure 5.3A, C and D, pink arrows for punctate, D white arrows for highlight).

This antibody showed promising positive staining, however, it looked like it did not only identify the damaged NFL, but also positive signal appeared in glial cells (Figure 5.3, red arrows). When investigating the contralateral hemisphere, there was positive signal appearing as random dots. It is unclear whether it was flecks of artefacts or was part of genuine positive signal (Figure 5.3E). Therefore, in the interest of time, MCA-1D44 was not selected to identify damaged NFL within the blast brain.

5.3.1.3 Characterisation of the staining of MCA-6h63 antibody

As shown in the recent publication and on the Encor antibody website, MCA-6h63 also binds to distinct hidden NFL epitopes, similar to epitope UD1 recognised in the SIMOA Quanterix Assay (Shaw, Madorsky et al. 2023). Due to this, it should bind to damaged-induced NFL epitopes only (as damage can reveal the epitopes that are unable to be bound to when intact). It is also stated that the C-terminal antibody, such is the case of RPCA-NFL-CT, is not able to recognise the same degenerating profiles as the MCA-6h63. Therefore, I was expecting to see similar positive staining for MCA-6h63 as with the MCA-1D44, but with only punctate or lines of positive staining.

MCA-6h63 staining showed a more reliable signal than MCA-1D44, as no glia staining was identified, as shown in Figure 5.4. MCA-6h63 positive staining appeared as punctate signal in the contusion site. There was also signal adjacent to the injury site in the subcortical white matter. This suggested that damaged NFL was present in the white matter and cortex. The contusion site, where the tissue has degraded and there is great damage, appeared also more intensely stained, but not with the punctate signal, but more a brighter “wash” indicating high concentration of damaged NFL in that location (Figure 5.4A white arrows).

RPCA-NFL-CT and MCA-6h63 double staining in the CCI tissue showed that RPCA-NFL-CT stains the C-terminal NFL in neurons and their processes, whereas MCA-6h63 showed positive signal in expected areas of damage, the contusion and pericontusion site, as illustrated in Figure 5.5. It is clear from the images that in the contusion site there is very low to no signal from the RPCA-NFL-CT antibody, which is able to bind to the C-terminal, while in the area of low signal there is a positive signal for the MCA-6h63, (Figure 5.5A,D and G). It can also be observed that

there was an overall reduction on positive RPCA-NFL-CT signal in the white matter tract, but not a similarly proportioned increase in the MCA-6h63 positive staining.

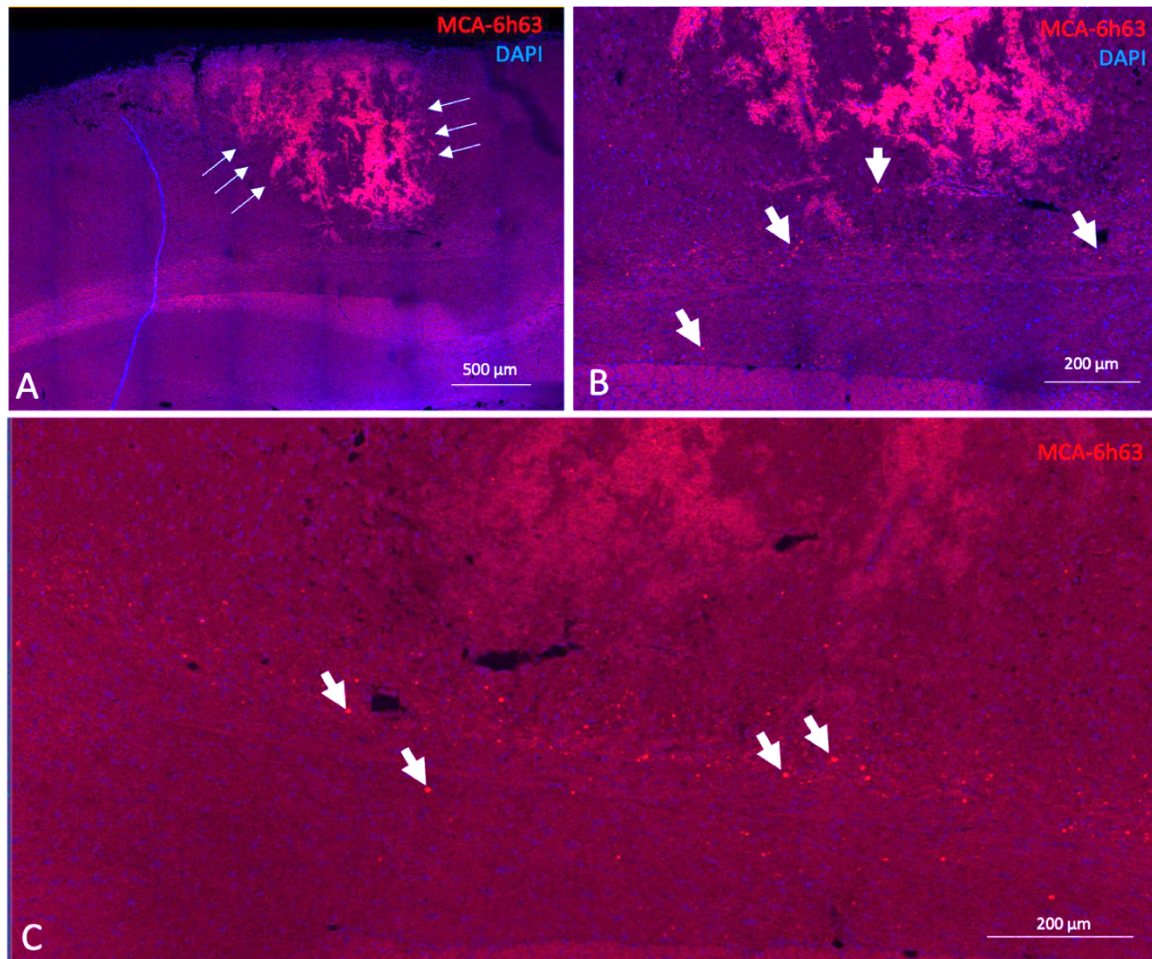


Figure 5.4 MCA-6h63 positive staining in the focal CCI model at 14 DPI moderate.

A) CCI contusion site shown by the long white arrows showing NFL highlighting the damaged cortex, and positive intact staining of the subcortical white matter away from contusion site, but less staining in the corpus callosum under the contusion site. B) High magnification image of the pericontusion area underneath showing positive signals of punctate as identified by the white arrows. This is counterstained with DAPI to show the structures of the brain. C) Higher magnified image of B, showing the positive signal in the contusion site as a mass higher intensity stain and underneath are white arrows indicating punctate of the MCA-6h63.

In addition, it can be observed in the area of colocalisation near the contusion site a positive signal of MCA-6h63 but no loss of positive signal from the RPCA-NFL-CT, (Figure 5.5B and C). Here it is visible that where there is RPCA-NFL-CT staining, there is also some MCA-6h63 in the same processes, but not all. When the positive signals are shown separately, as illustrated in (Figure 5.5E, F, H, and I), it appears that RPCA-NFL-CT stains the majority of the axons and

neuronal presence of intact NFL in that area. There were some processes which were also positive for MCA-6h63, indicating that those few axons were partially damaged from the injury and have areas of unmasked NFL epitope. Thus, it showed damaged epitopes available for binding as well as and an intact C-terminus (Figure 5.5, yellow arrows). This combination of antibodies is very promising to identify preserved, partially damaged, and damaged axons in TBI.

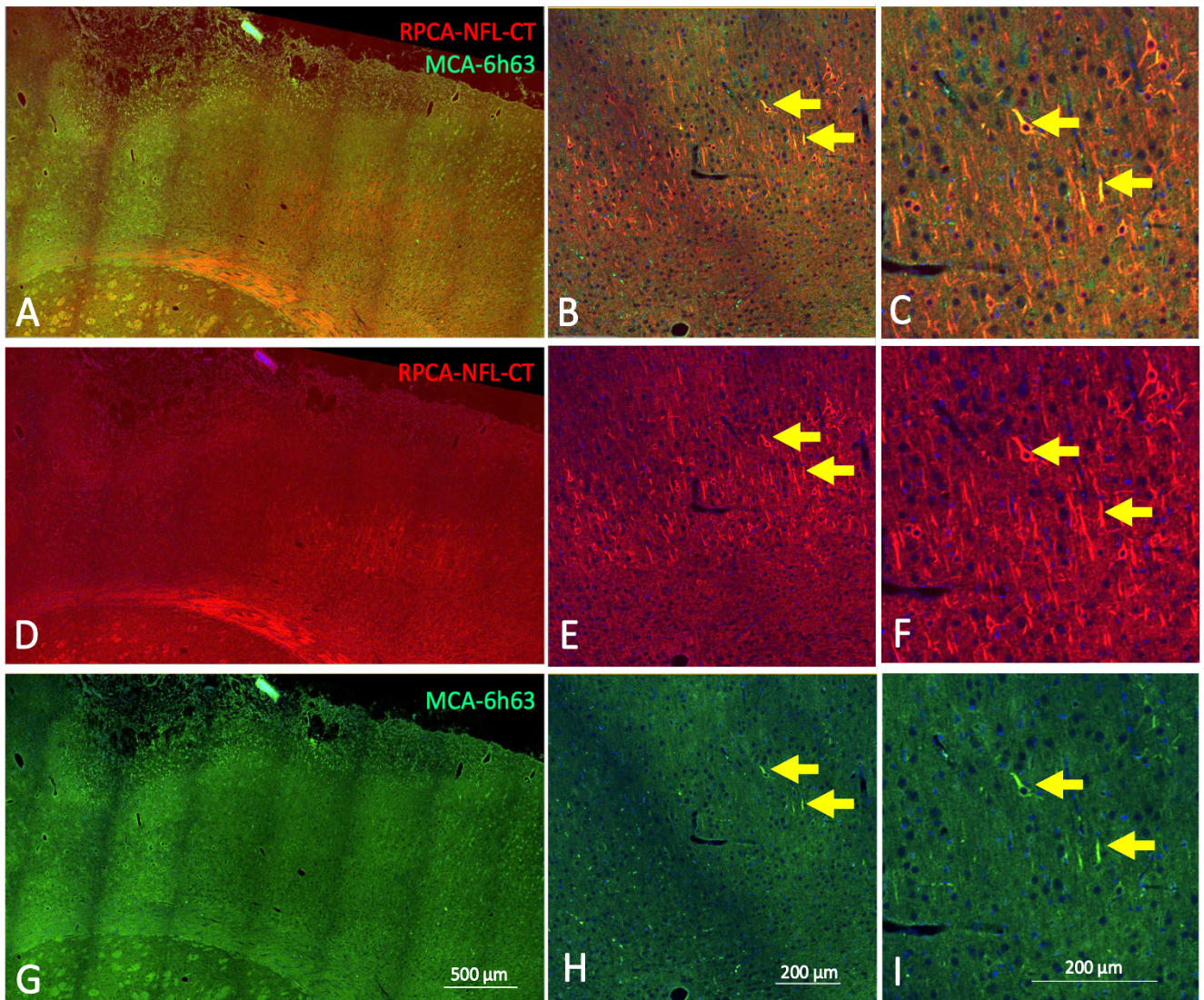


Figure 5.5 MCA-6h63 double stained with RPCA-NFL-CT in the 14 DPI moderate tissue.

A) Double stain of MCA-6h64 and RPCA-NFL-CT with some colocalization seen above the corpus callosum and beneath the contusion site. B) Higher magnification of the colocalisation site mentioned in A, yellow arrow indicates example colocalisation between the two positive signals showing as yellow stain when red and green overlap. C) Higher magnification of B, yellow arrows indicating the same example colocalisation. D) RPCA-NFL-CT stain of the contusion site, cortex and the white matter, clearly showing a lack of staining in the contusion site but higher positive signal in the white matter and the cortex where the colocalisation resides. E) The cortex is where the colocalisation resides at higher magnification showing only the intact NFL and area where the MCA-6h63 appears to be when the green channel is added, as shown by yellow arrows. G) shows the MCA-6h63 staining, at this magnification it is not bright and cannot be seen in the white matter, however it is present in the contusion site and in the cortex. H) MCA-6h63 in the same cortex site as B and E and only a few punctate and lines are positively stained. I) higher magnified image showing the more intensely stained areas are the process of the cell that appears as neuron shape. There is not as much positive staining as the RPCA-NFL-CT indicating that MCA-6h63 does indeed, not stain healthy NFL and appears to stain to the damaged.

5.3.1.4 Characterisation of the staining with MCA-1B11

MCA-1B11 is the last of the three antibodies from Encor Biotech that can detect the unmasked epitopes of the NFL polypeptide as a result from axonal damage. Therefore, the staining was expected to be similar to the MCA-1D44 and MCA-6h63 antibodies. MCA-1b11 binds to regions similar or the same to the UD1 epitope (Figure 5.1). According to previously published work by Dr Shaw, the MCA-6h63 and 1B11 bind very similarly in a contused rat spinal cord. Therefore, I expected to see very similar staining with the MCA-1B11 as to what was stained with the MCA-6h63.

MCA-1B11 staining was carried out in the same rats as the other antibodies, but on the next consecutive slice. It can be seen in Figure 5.6 that MCA-1B11 showed positive signal in the contusional and pericontusional areas (Figure 5.6A). Positive signal appeared as aggregates and punctate staining in the white matter, pericontusionally and in the contusion. Thus, this was a very similar staining as with the MCA-6h63. Double staining would be required between MCA-1B11 with the RPCA-NFL-CT, in order to decide which is the “best” combination to use in the blast tissue.

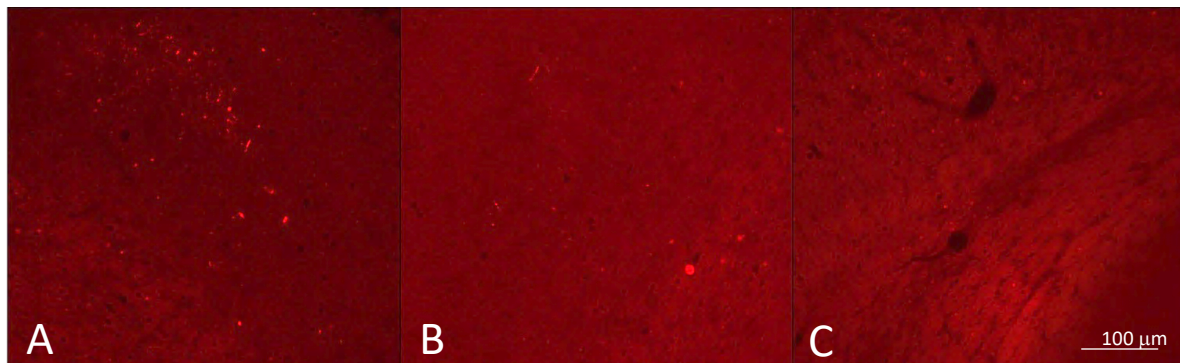


Figure 5.6 MCA-1B11 stained in 14 DPI moderate CCI focal injury tissue.

A) Pericontusion area is highly populated with positive stained punctate, and the contusion is highlighted in the lower left corner. B) Another area near the contusion where punctate is seen as lines and dots, indicating processes and NFL aggregates. C) Bright area of the corpus callosum, and punctate seen above it, indicating positive staining of the damaged NFL and unmasked epitope.

Double staining was conducted in 14 DPI moderate CCI focal injury tissue using RPCA-NFL-CT and MCA-1B11, as shown in Figure 5.7 and Figure 5.8. The staining demonstrated that in areas

of contusion and necrosed tissue, RPCA-NFL-CT showed weak positive staining, with only a few cells staining positively (Figure 5.7G and H, white arrows). Of those RPCA-NFL-CT positive cells, the majority co-localised with MCA-1B11 (Figure 5.7G-I, yellow arrows). In those areas, the main parenchyma that is necrosed was not stained positively for MCA-1B11 either, only neuronal shaped cells appeared to show positive MCA-1B11 signal (Figure 5.7I). This is also demonstrated in Figure 5.7A-E, which shows that in areas where there is *no* RPCA-NFL-CT there is neither MCA-1B11 staining, but instead there were only a few cells positively stained and some punctate signal (Figure 5.7D-F, white arrow is RPCA-NFL-CT loss, pink arrows show MCA-1B11 positive stain). This suggests a complete loss of any intact and undamaged C-terminal NFL, and only some unmasked fragments remaining in areas like the contusion site, and the white matter tract in the damaged area (Figure 5.7A-F).

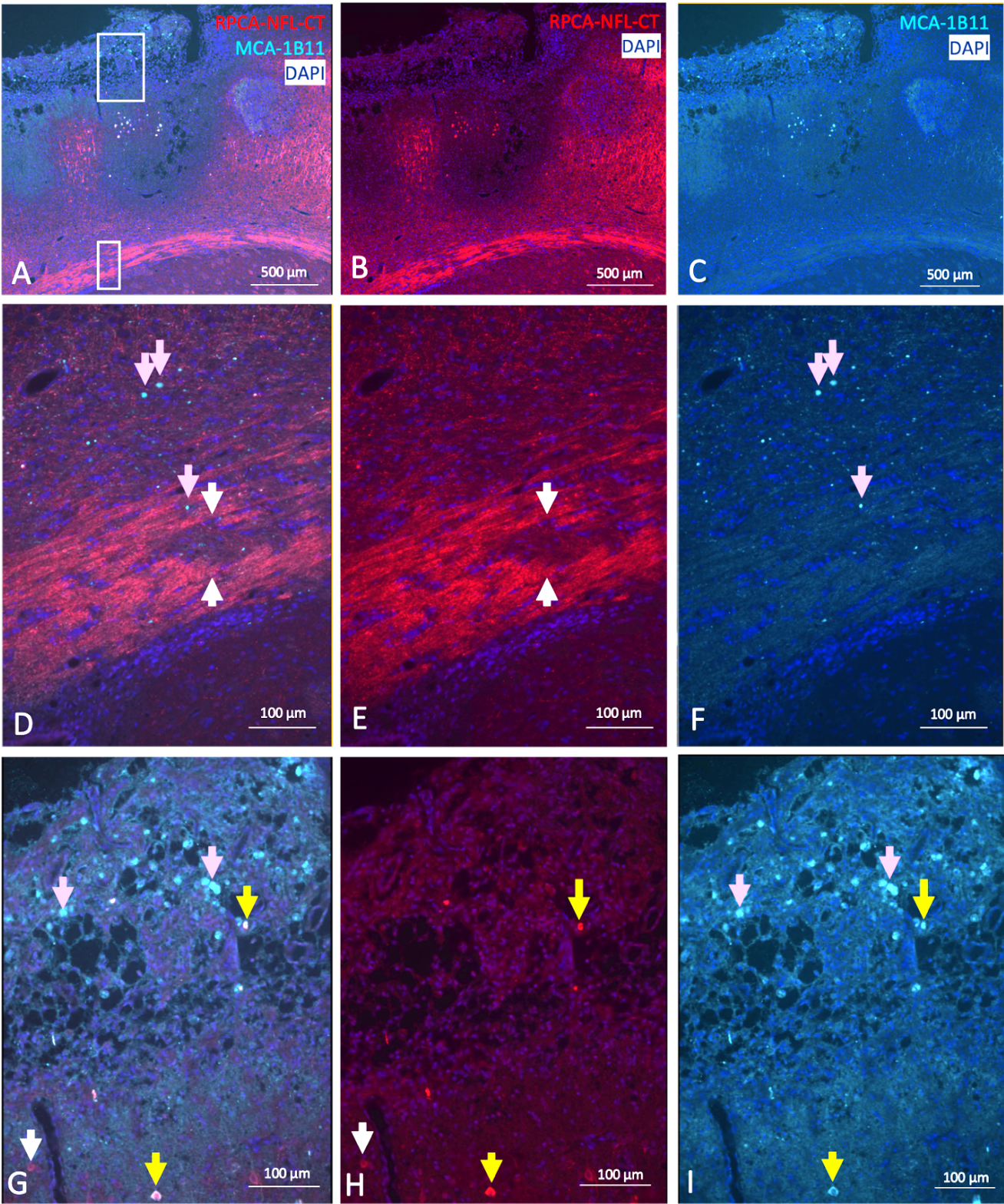


Figure 5.7 MCA-1B11 stained with RPCA-NFL-CT in 14 DPI moderate tissue.

A) Double stained contusion showing white matter damage in the corpus callosum with large gaps in the tissue. B) RPCA-NFL-CT showing reduction in damaged and necrosed tissue of the cortex and white matter tract. C) MCA-1B11 positive staining in contusion and per-contusion areas, white matter positive staining as punctate is hard to visualise but is present. Two boxes represent the area of higher magnification, D) Higher magnified area of the corpus callosum, pink arrows indicate MCA-1B11 positive punctate staining, white arrows indicate RPCA-NFL-CT loss. E) RPCA-NFL-CT loss shown by white arrows, and no sign of MCA-1B11 staining, but small flecks of red punctate seen in the image. F) MCA-1B11 positive staining shown as blue dots of punctate, corpus callosum not stained positively at all by the antibody. G) magnified image of the other box from A, double staining of the of the contusion and necrosed tissue, there are neuronal bodies positively stained with MCA-1b11 as shown with pink arrows, white arrows indicate RPCA-NFL-CT only stained neuronal bodies seen, yellow arrows indicate colocalisation. H) RPCA-NFL-CT only of G, showing a sample of which of the round cellular bodies are stained red, white arrow, yellow arrow indicates colocalized bodies. I) MCA-1B11 positive cell bodies in the contusion examples shown with pink arrows, the yellow arrows indicate colocalized cell bodies.

Figure 5.8 illustrates the co-staining of antibodies RPCA-NFL-CT and MCA-1B11 in an area next to the contusion where there is no tissue loss (Figure 5.8A). Most cells show RPCA-NFL-CT positively staining (Figure 5.8B and C), indicating there are still preserved axons and not fully damaged neurons. A few of the positive stains are RPCA-NFL-CT only, while a majority of the other the cells show co-localisation with MCA-1B11, suggesting they were damaged by the injury and have unmasked epitopes as well as preserved epitopes in the C-terminals. A whole neuronal body and axon is depicted in Figure 5.8 that shows progression of the damage. The image shows that the terminus of the axon appears to be almost entirely RPCA-NFL-CT positively stained (Figure 5.8C), while there is an area of co-localisation between both antibodies, followed by the neuronal body, which is stained almost entirely with MCA-1B11 (Figure 5.8B-D). There are other neurons showing co-localisation of both antibodies, indicating the same occurrence, although they are without long axons. The image also shows some unmasked NFL flecks, with MCA-1B11 positive punctate signal in the same image, without co-localisation with RPCA-NFL-CT (Figure 5.8B-D).

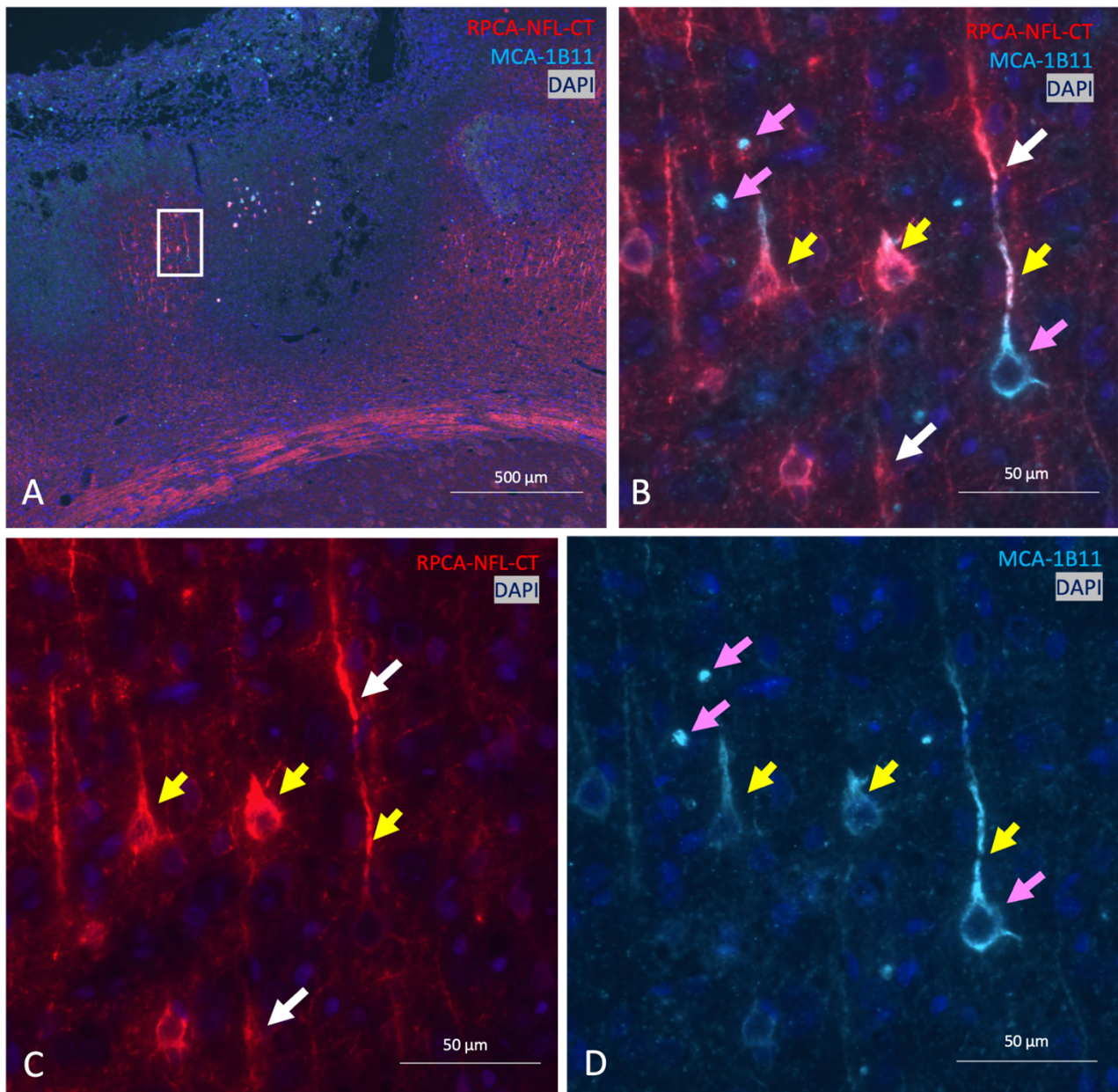


Figure 5.8 MCA-1B11 stained with RPCA-NFL-CT in 14 DPI moderate tissue.

A) white box indicates different focus are of colocalisation for the two antibodies. B) pericontusion site of where MCA-1B11 in the head of the neuron sand down the axon shown with pink and then yellow arrow showing where RPCA-NFL-CT and MCA-1B11 are both staining the axon followed by nearly totally red RPCA-NFL-CT axon terminus. Other colocalized neurons are shown with yellow arrows and white arrows shows an example where staining is exclusively RPCA-NFL-CT. C) RPCA-NFL-CT positive staining in the axon, reduced in the head of the neuron that is stained with MCA-1B11 in B. white arrows show RPCA-NFL-CT only staining and yellow arrows indicate locations of colocalisation. D) pink arrows show the MCA-1B11 only stain in the neuronal head and some punctate and yellow shows locations of colocalisation. This seems to indicate that these neurons are in a state of intact->damaged as indicated by single stain, colocalisation-> single stain.

Due to this promising staining, MCA-1B11 and MCA-6h63 were visually compared and MCA-1B11 was selected as the antibody to combine with RPCA-NFL-CT, as it seemed to capture the punctate signal with higher intensity in the contusion and pericontusion areas.

Therefore, staining with MCA-1B11 and RPCA-NFL-CT antibodies was carried out in the blast tissue, to assess whether it was sensitive enough to detect any axonal damage in the mild diffuse model and to identify the areas more prone to damage in this model. It was expected that there would not be as much punctate signal as with the tissue from CCI focal injury, due to the diffuse nature of the blast injury.

Based on the results obtained from fluid NFL biomarker peaking with highest CSF concentrations at 6 hours post-injury, we performed the staining with MCA-1B11 and RPCA-NFL-CT in brain tissue at this time point. Results of the staining showed strong positive signal for both antibodies at this time (Figure 5.9). The staining demonstrated numerous co-localisation sites in the brain, which was expected given the diffuse nature of the injury, where there would be no focal location of damaged NFL unmasked epitopes.

Figure 5.9 provides evidence that the white matter tract of the blast tissue exhibited positive signal for both antibodies, as indicated by yellow coloration. Figure 5.9B-J depicts magnified regions with high dual signal, indicating the presence of numerous processes stained positively. Within cortical areas, staining of MCA-1B11 was stronger in the neuronal bodies and axons of the infragranular layers (layer 5-6), compared to the supragranular layers (layer 1-3). Additionally, there was a notable double positive signal in the CA3 region of the hippocampus (as illustrated in Figure 5.9H-J), where co-localisation was so extensive that it was difficult to differentiate between the antibodies. These results suggest considerable NFL unmasking in the blast tissue.

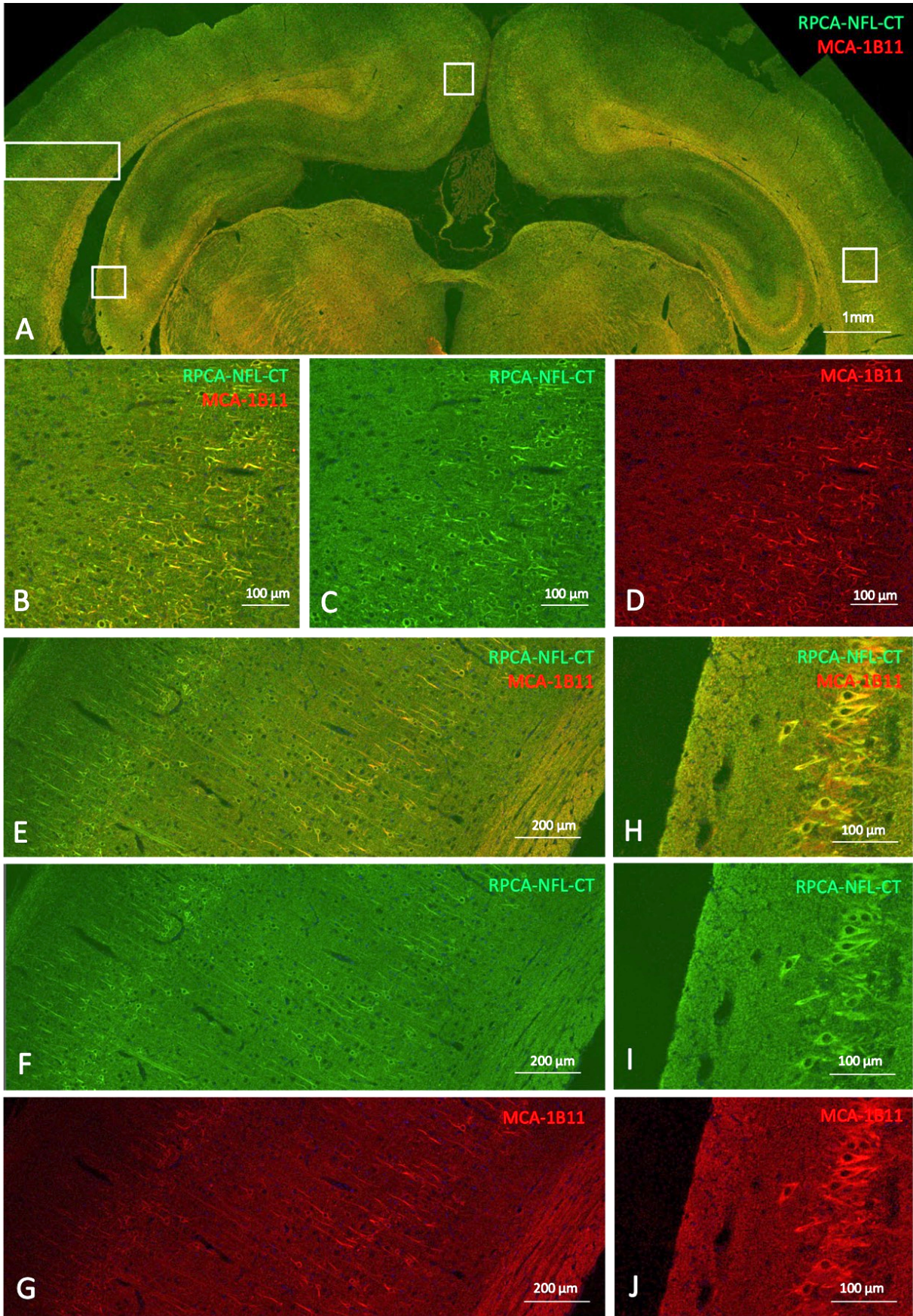


Figure 5.9 MCA-1B11 and RPCA-NFL-CT double stain in rat 6Hour mild blast TBI tissue, diffuse injury type .

A) whole tissue slice of the double stain, the tissue demonstrates a lot of colocalisation by presence of yellow in brain locations such as the retrosplenial cortex, auditory cortex and the corpus callosum as well as the mid brain. White boxes indicate areas of high magnification) Retrosplenial cortex at higher magnification, RPCA-NFL-CT showing good positive staining in layer 5, high proportion of axons and processes appear to be stained with both antibodies. B) double stain of the Retrosplenial cortex, many areas of yellow colocalisation, C) RPCA-NFL-CT in Retrosplenial cortex, high coverage of processes D) MCA-1B11 positive staining in the Retrosplenial cortex, in almost all the same processes as the RPCA-NFL-CT staining. E) Auditory cortex double stained with RPCA-NFL-CT and MCA-1B11, layers 5 and 6 are locations of yellow colocalisation from the joined positive staining of the two antibodies. F) RPCA-NFL-CT positive staining seen in all layers of the cortex around neuronal bodies and processes. It is also present in the corpus callosum. G) faint MCA-1B11 positive staining in 4th layer of the cortex but main positive stains are seen in the infragranular of the cortex. Positive staining seen in the corpus callosum. H) hippocampus staining in the CA3 with pyramidal cells staining positively for both antibodies. I)RPCA-NFL-CT positive signal in the pyramidal cells. J) MCA-1B11 positive signal in the same cells as RPCA-NFL-CT.

Due to the promising MCA-1B11 staining results in the blast tissue, I then investigated the naïve tissue used for the blast cohort. I was expecting no positive staining from the MCA-1B11, due to the fact there is no procedures conducted on the rat thus no injury inflicted on the tissue, nor any drugs administered. I predicted that the tissue would look similar to Figure 5.7F but with no punctate signal.

Results obtained in the naïve tissue indicated that the MCA-1B11 was not only capable of staining damaged NFL by binding to the unmasked epitope, but also recognised the intact C-terminal of NFL, as shown by Figure 5.10. It can be seen in various regions of the naïve tissue that there is co-localisation of the two antibodies, and in some areas were prominently stained with the MCA-1B11 antibody (Figure 5.10E). In magnified images of different cortical areas, it is evident that the high intensity staining of the RPCA-NFL-CT co-localised with high intensity MCA-1B11 in the same areas as in the 6Hour blast tissue (Figure 5.9). This signal was not observed in CCI tissue (Figure 5.7). This suggests that the antibody MCA-1B11 was not reliable to identify axonal damage associated with the changes detected by the fluid biomarker NFL in the brain.

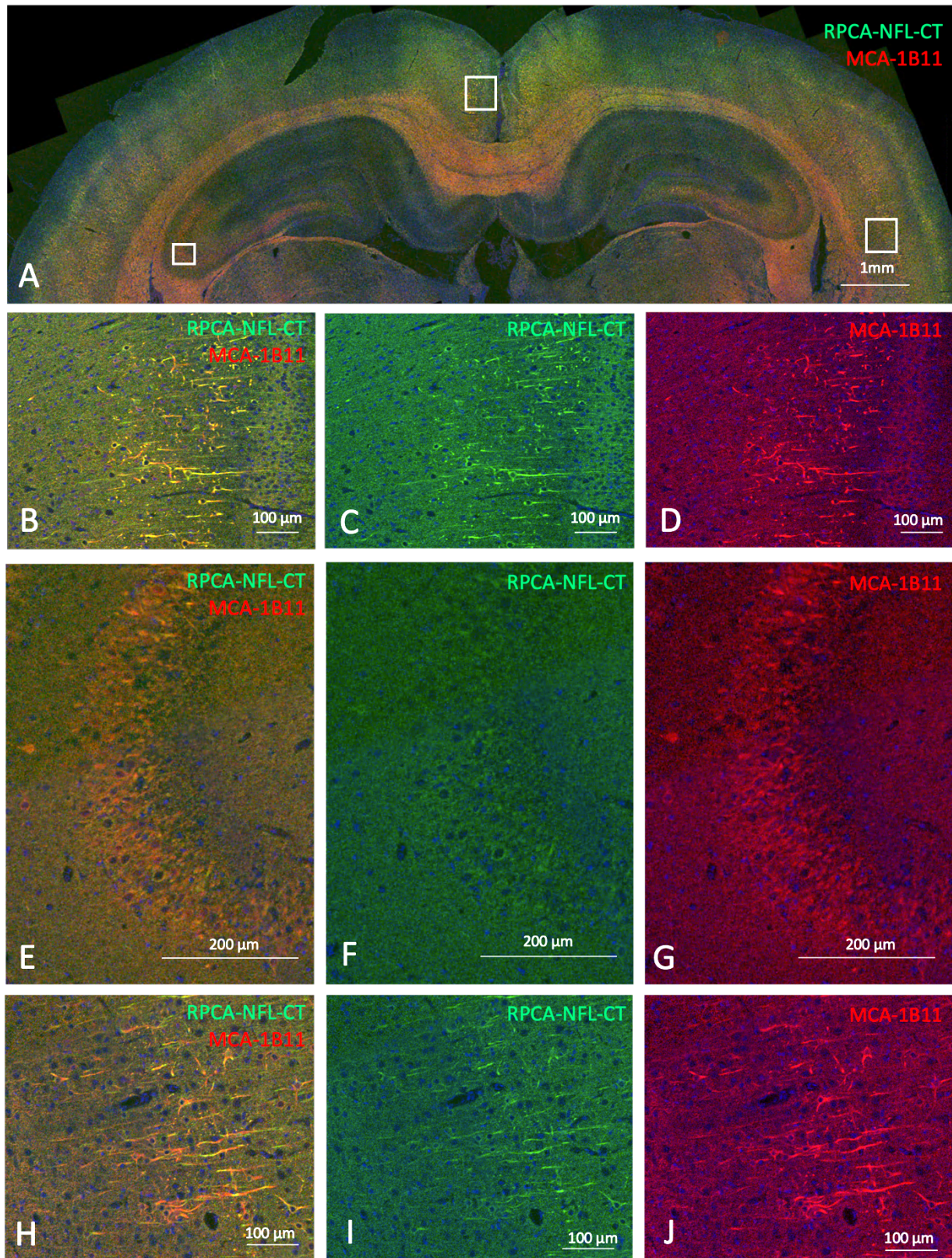


Figure 5.10 Double staining of MCA-1B11 and RPCA-NFL-CT in naïve rat brain.

A) Double stain of half brain, yellow colorations show colocalisation of the two antibodies. White boxes identify areas that are magnified in the figure. B) retrosplenial cortex showing lots of processes to have both antibodies' positive signal. C) RPCA-NFL-CT of Retrosplenial cortex. D) MCA-1B11 positive staining in retrosplenial cortex. E) hippocampus CA3. F)RPCA-NFL-CT in the CA3 not showing positive signal apart from a few outlines of neurons. G) MCA-1B11 positive staining in CA3. H) Auditory cortex showing processes of neurons strong staining for both antibodies. I) RPCA-NFL-CT many processes of neurons stained with high intensity in auditory cortex. J) MCA-1B11 in auditory cortex positive staining in the same processes as RPCA-NFL-CT.

In the interest of time for this chapter, only the RPCA-NFL-CT antibody was used of the three tested to measure the levels of NFL in the blast brains, with the assumption that if there was damage, there would be fewer C-terminals to bind to and there would be a reduction in the staining. This was shown in the moderate model of the contusion and white matter tract of the CCI focal injury model Figure 5.7, and the results of RPCA-NFL-CT staining in the CCI model as shown in Supplementary Figure 10 and Supplementary Figure 11

5.4 Results

5.4.1 Single mild blast exposure does not result in the thinning of the corpus callosum

I investigated whether blast head injury was also associated with similar corpus callosum thinning. Corpus Callosum thickness was unaffected by blast with no differences between segments or hemisphere ($p>0.05$). Qualitatively, there was no evident sign of demyelination in the corpus callosum in the blast model, except for slight pallor (less intense stain) at 7 DPI (Figure 5.11). To increase the sensitivity, I examined the thickness of the whole of the CC. Again, there were no differences found in thickness of the CC of the blast model at different timepoints, using one-way ANOVA ($F(4, 31) = 1.165, p=0.3454$) (Naïve/Sham: $n=13$, 6Hr: $n=5$, 7DPI: $n=6$, 1M: $n=7$, 3M: $n=5$.) (Figure 5.12).

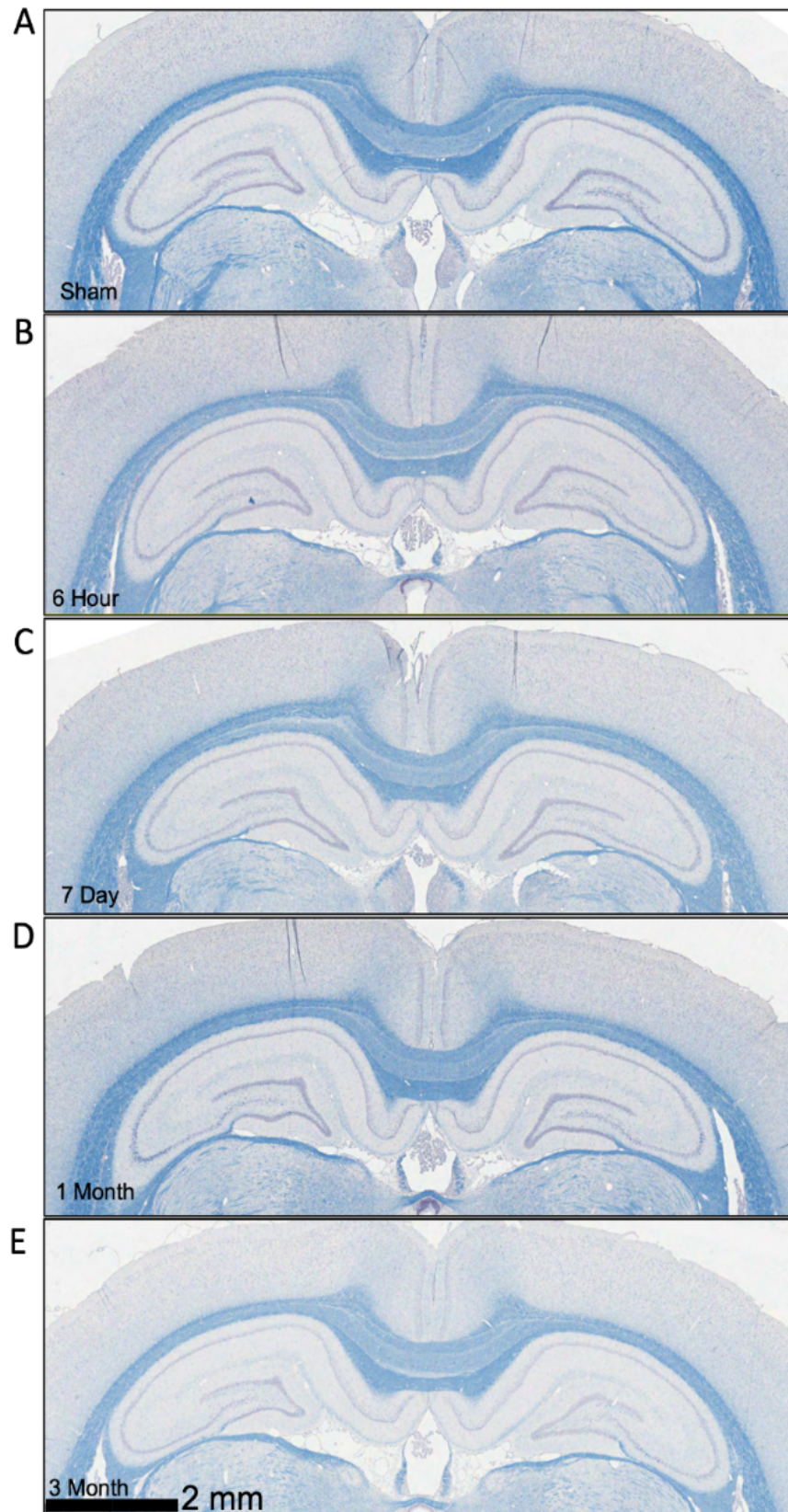


Figure 5.11 Luxol Fast Blue staining of the blast model of the corpus callosum.

A—E) Representative half micrographs of Luxol fast blue staining from Naïve/Sham to 3 months post-injury.

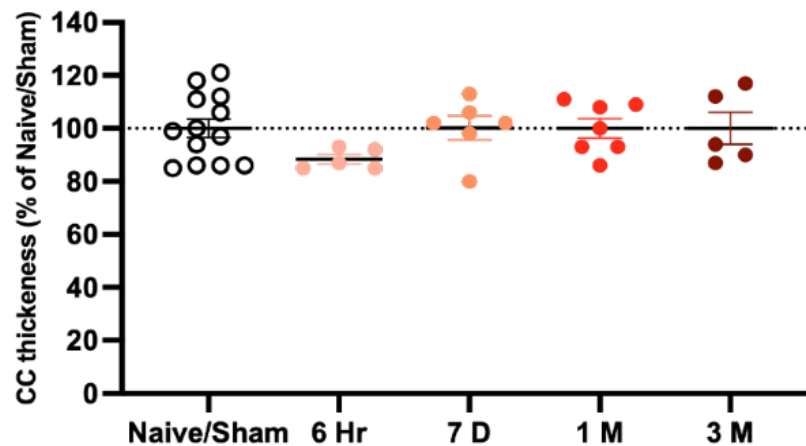


Figure 5.12 Quantification of corpus callosum thinning from Luxol Fast Blue staining in the blast model.

Data was normalised to the contralateral hemisphere and then compared against Naïve/Sham. Naïve/Sham: $n=13$, 6 Hr: $n=5$, 7 DPI: $n=6$, 1M: $n=7$, 3M: $n=5$. One-way ANOVA conducted. No differences were found. Data presented mean $\pm$ SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$

5.4.2 Blast Exposure Causes NFL Increase in the Corpus Callosum

I then performed the same staining with the RPCA-NFL-CT antibody in the brain sections of the blast injury model, in order to investigate the NFL damage caused by the blast exposure in the brain. Qualitatively, there was no obvious reduction in the RPCA-NFL-CT fluorescent intensity in the CC or elsewhere in the brain (Figure 5.13). Furthermore, unlike the CCI model's RPCA-NFL-CT staining, there was no obvious punctate signal observed in any of the time points. Instead, the CC of the 6hour, 7 day and 1 month post-injury blast injury groups looked slightly brighter than the control group or the 3 month post-injury group, particularly in the areas of increased intensity (nearest the midline and are the genu of the CC) (Naïve/Sham: $n=11$, 6Hr $n=6$, 7DPI $n=6$, 1M $n=7$, 3M: $n=6$).

Quantification of the average fluorescence intensity of the whole CC revealed a significant difference between the blast exposed groups (Naïve/Sham, 6hour, 7 DPI, 1 and 3 months post-injury) ($F(4, 31) = 2.882$, $p=0.0387$). Post-hoc analysis revealed a significant increase of

NFL at 7DPI relative to controls (23.6% increase, $p=0.0167$) (Figure 5.14). The comparison between the control group vs 6 hours and 1 month post-injury were borderline significant (17.60 % increase, $p=0.0672$ and 18% increase, $p=0.0613$, respectively). There was no significant difference found between controls and 3 months post-injury groups ($p=0.8230$). Furthermore, it should be noted that there is an absence of punctate staining in the corpus callosum.

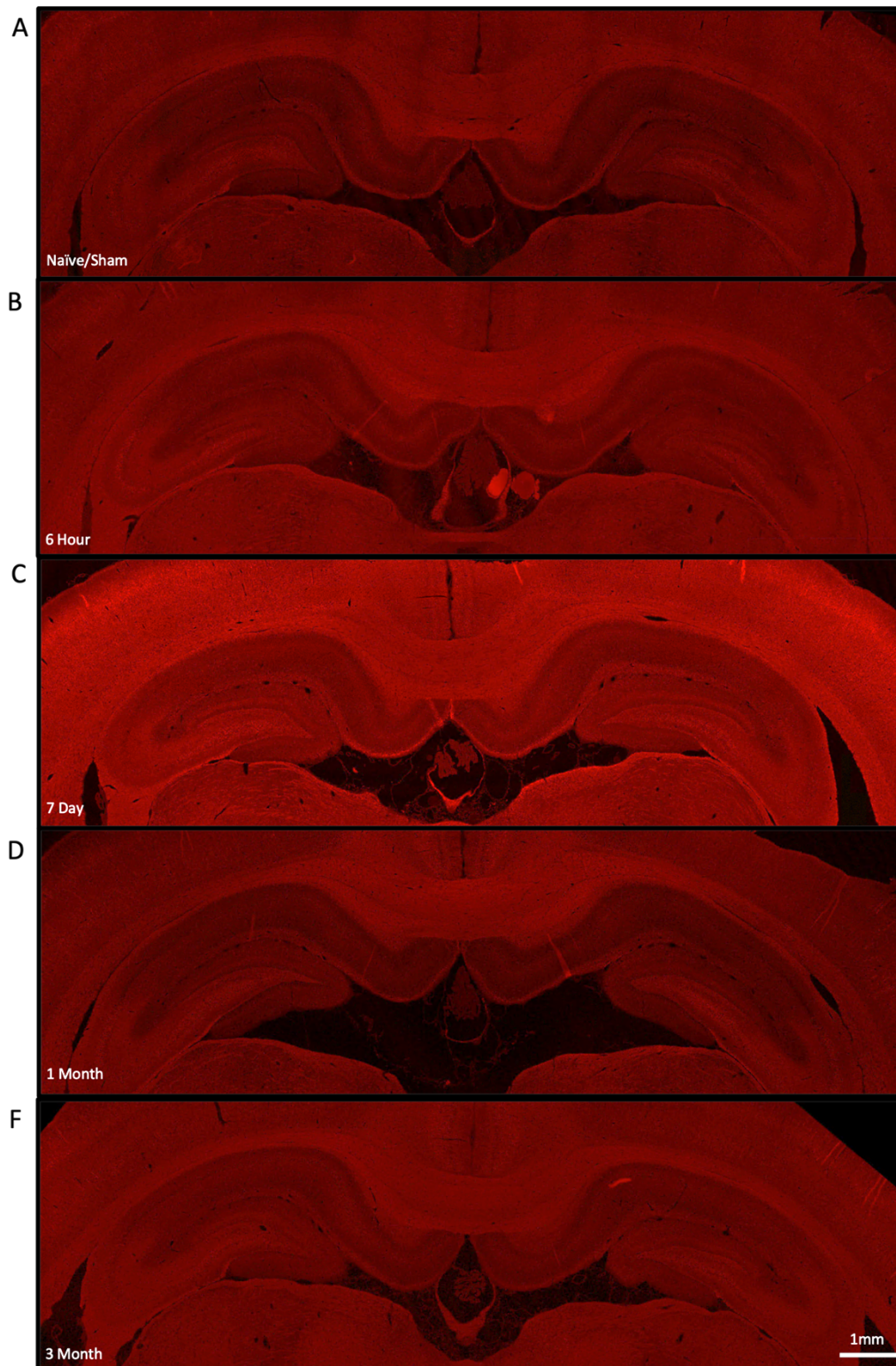


Figure 5.13 RPCA-NFL-CT neurofilament (NFL) antibody in blast exposed brains using AF546.

A-E) representative micrographs of NFL RPCA-NFL-CT from Naïve/Sham to 3 months post injury.

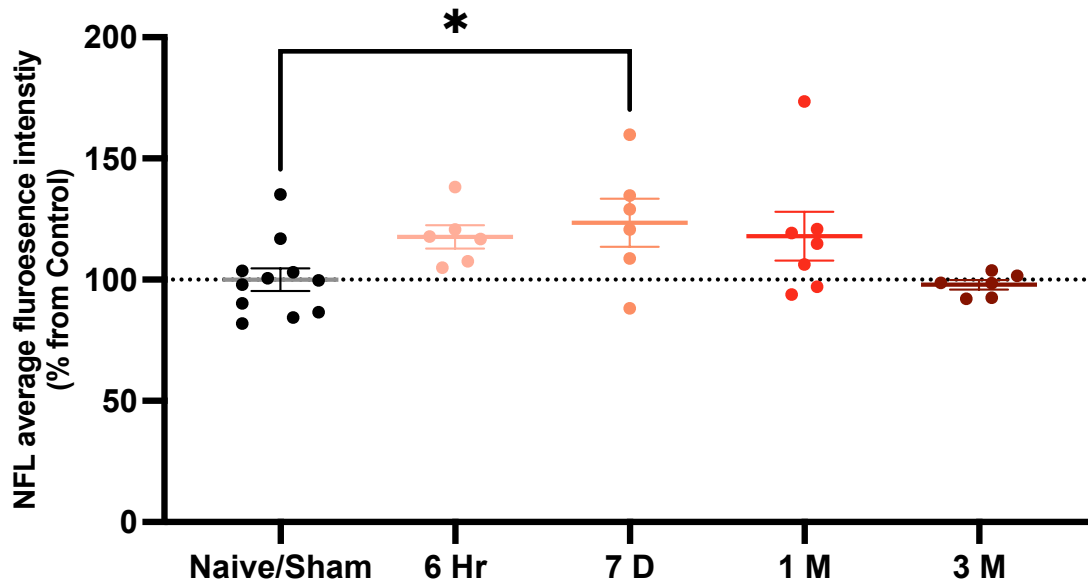


Figure 5.14 Quantification of Encor Biotech inc RPCA-NFL-CT neurofilament light (NFL) antibody of the blast model..

Neurofilament Light (NFL) average fluorescent intensity quantification of AF647 using one-way ANOVA. Significance found between Naïve/Sham and 7 DPI, no other difference found between other comparisons. Naïve/Sham: n=11, 6Hr n=6, 7DPI: n=6, 1M: n=7, 3M: n=6. Values show mean $\pm$ SEM, $* > 0.05$.

5.4.3 Blast Exposure Leads to NFL Increase in the White Matter Tract at Chronic Time Points

Neurofilament light from Synaptic Systems (Ssys) was also used to investigate white matter damage in the two models. This is an antibody that binds to the essential neuronal scaffolding protein in the conserved “AAA” epitopes of the “head” region of the NFL polypeptide. It was used to investigate if there were differences in the staining with the previous antibody, the RPCA-NFL-CT from Encor Biotechnology Inc, to assess whether the epitope influenced the sensitivity to any NFL loss. The staining with the Synaptic systems antibody showed a slight brightness increase at 7 DPI compared with controls, but most evident elevated fluorescence intensity changes were detected at 1 month post-injury (Figure 5.15) (Naïve/Sham: n=12, 6Hr n=5, 7 DPI n=6, 1M n=7, 3m: n=5). The analysis of the fluorescent intensity in the corpus callosum resulted in a significant one-way ANOVA ($F(4, 30) = 4.253$, $p = 0.0076$). This difference was due to a significant increase in the 1month post-injury group compared with sham controls (123.8%, $p = 0.0008$).

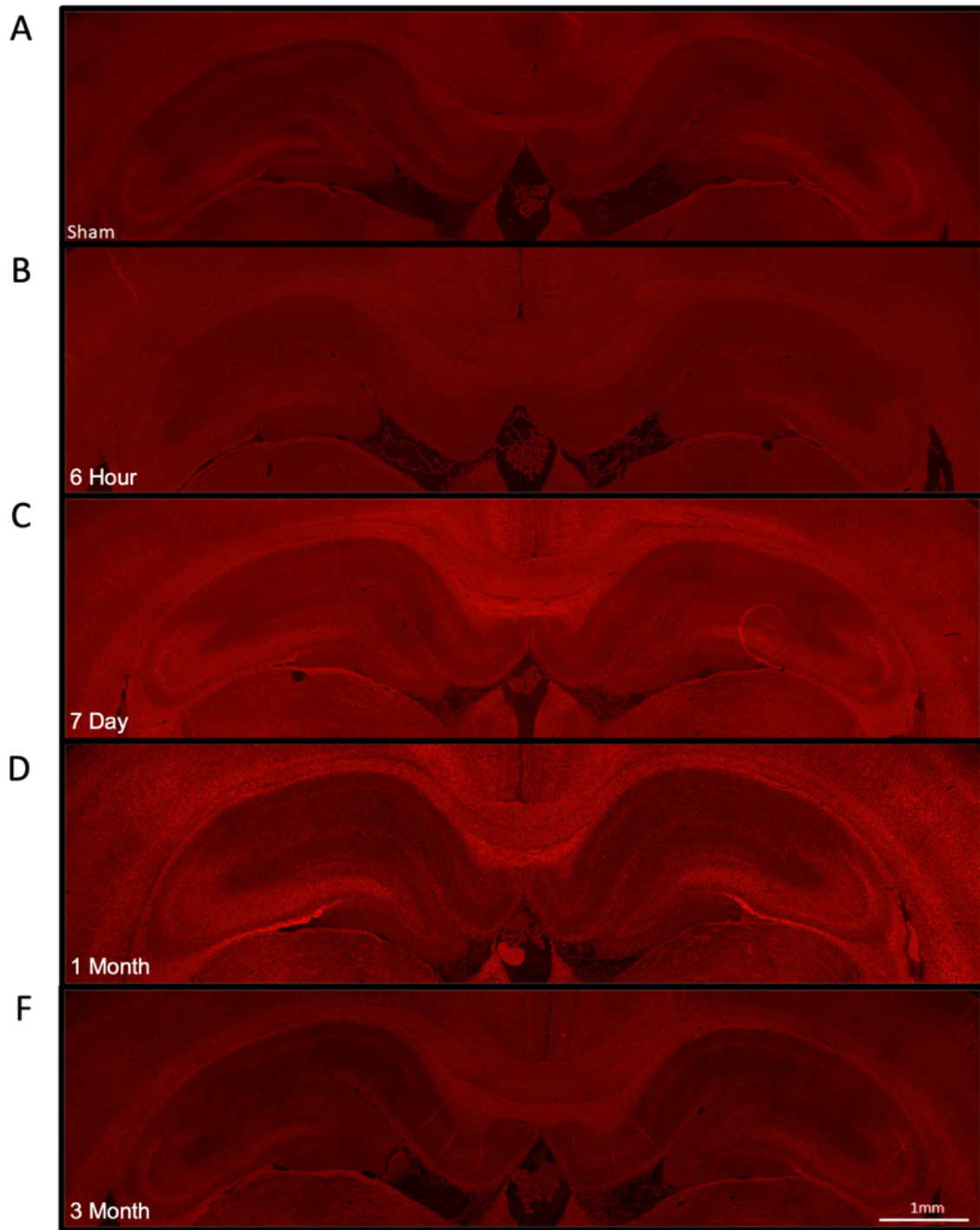


Figure 5.15 Synaptic Systems antibody for neurofilament light in the blast model.

Representative fluorescence intensity in the corpus callosum (A-E) the corpus callosum of AF647 of NFL of all time points post-injury.

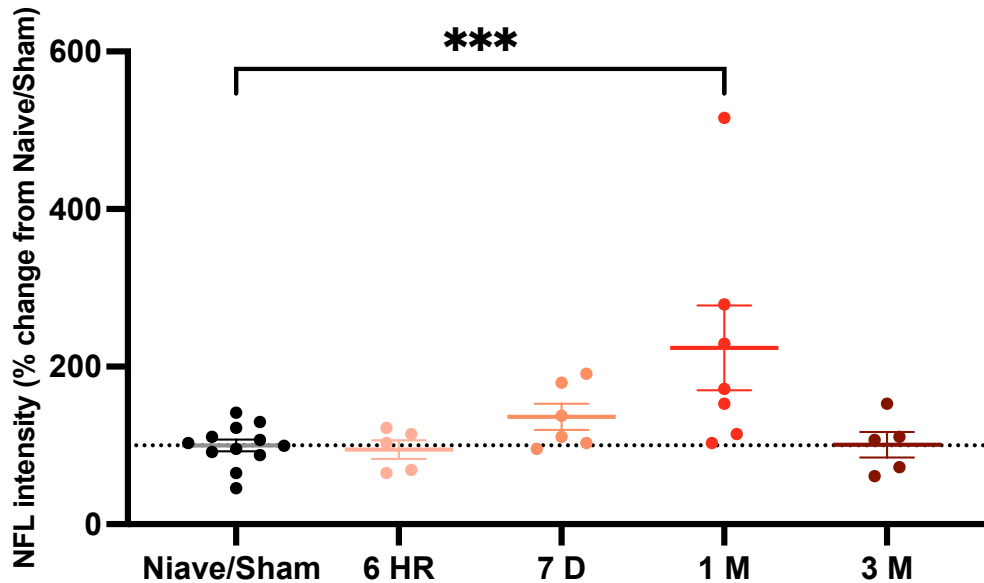


Figure 5.16 Quantification of Synaptic system neurofilament light (NFL) antibody of the blast model.

Quantification of Alexa 546 immunofluorescence intensity for NFL staining in the whole of the corpus callosum. Data was quantified using one-way ANOVA. Naive/Sham: $n=12$, 6Hr $n=5$, 7 DPI $n=6$, 1M $n=7$, 3M: $n=5$. Data presented mean $\pm$ SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$

5.4.4 Single Mild Blast Exposure Results in CSF NFL to Peak at Acute Time Point Post-injury that Remains Elevated at Chronic Timepoints

I extended the previous work on NFL in the CCI model to explore whether blast exposure also resulted in increased NFL levels. CSF NFL was sampled from the same blast-exposed rats as the brains used for histological analysis, at the same time points (Naïve/Sham: $n=16$, 6Hr: $n=10$, 7 DPI: $n=8$, 1M: $n=9$, 3m: $n=5$). Analysis using the Kruskal-Wallis test revealed a significant difference in CSF NFL concentrations ($H(4)=20.48$, $p=0.0004$). The following post-hoc analysis revealed levels of NFL in the CSF of blast-exposed animals increased and peaked at 6 hours post-injury compared with Naïve/Sham group (increase of 4262.63%, $p=0.0002$) (see Figure 5.17). At later time points (7 DPI, 1 month, and 3 months post-injury), the levels remained significantly increased from those in the Naïve/Sham group (7DPI: increased 2417.96%, $p=0.0006$, 1 month: 1617.56%, $p=0.0055$, 3 month: 967.23%, $p=0.0153$, respectively) but not as elevated (Figure 5.17). While the levels of NFL in the CSF gradually

decreased overtime, they remained significantly higher than those in the control group ($p < 0.05$) and not significantly different from each other ($p > 0.05$).

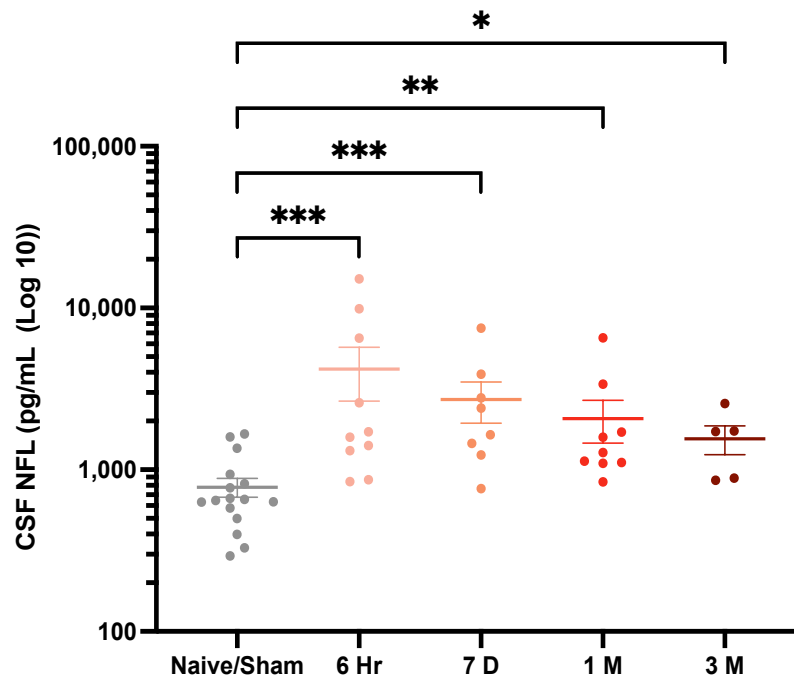


Figure 5.17 Blast exposed animal's CSF concentration of NFL.

CSF sampled and analysed at 6hour till 3months post-injury. Naïve/Sham: $n=16$, 6Hr: $n=10$, 7 DPI: $n=8$, 1M: $n=9$, 3m: $n=5$. Kruskal Wallis test found significance at 6Hr, 7 days post-injury and 1 month post-injury, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$. Values show mean $\pm$ SEM.

5.4.5 Single Mild Blast Exposure Leads to Increased NFL Plasma Concentrations Peaking at Acute Time Points but Returning Back to Baseline Levels in Later Time Points

We then investigated whether NFL measured in blood was altered at different times post-blast injury. Blood was sampled at more time intervals than the CSF, in order to get a greater understanding of the longitudinal temporal profile of NFL in the blast model.

Mixed effects analysis comparing blood plasma levels of the blasted animals was conducted using time, injury and their interaction as main factors (Sham 6hr: n=5, Sham 7DPI: n=6, Blast 6hr: n= 10, Blast 7DPI: n=8, Blast 3M: n=5, one animal was excluded from Sham 6hr, as it had been infected with murine pneumonia virus and was an outlier using ROUT outlier identifier $Q=0.5\%$). There was significance found in the main factors time ($F(4.000, 64.00) = 3.115$, $p=0.0209$) and interaction ($F(4, 64) = 2.754$, $p=0.0354$) but no significance found for injury ($F(1, 33) = 0.2319$, $p=0.6333$). A significant increase was found in NFL levels at 1DPI (144.28%, $p=0.0052$), a peak at 3DPI (309.21%, $p=0.0071$) and 7DPI (57.40%, $p=0.0378$) compared with the baseline. No significance was found between baseline and 6hour (104.35%, $p=0.0851$). Significant differences in plasma NFL were found between Naïve/sham and blast at 3DPI (141.05% increase, $p=0.0071$) whereby the 3 DPI was elevated relative to sham (Figure 5.18). Multiple comparison testing comparing baseline of the sham group and later sham timepoints showed no significant results ($p>0.05$).

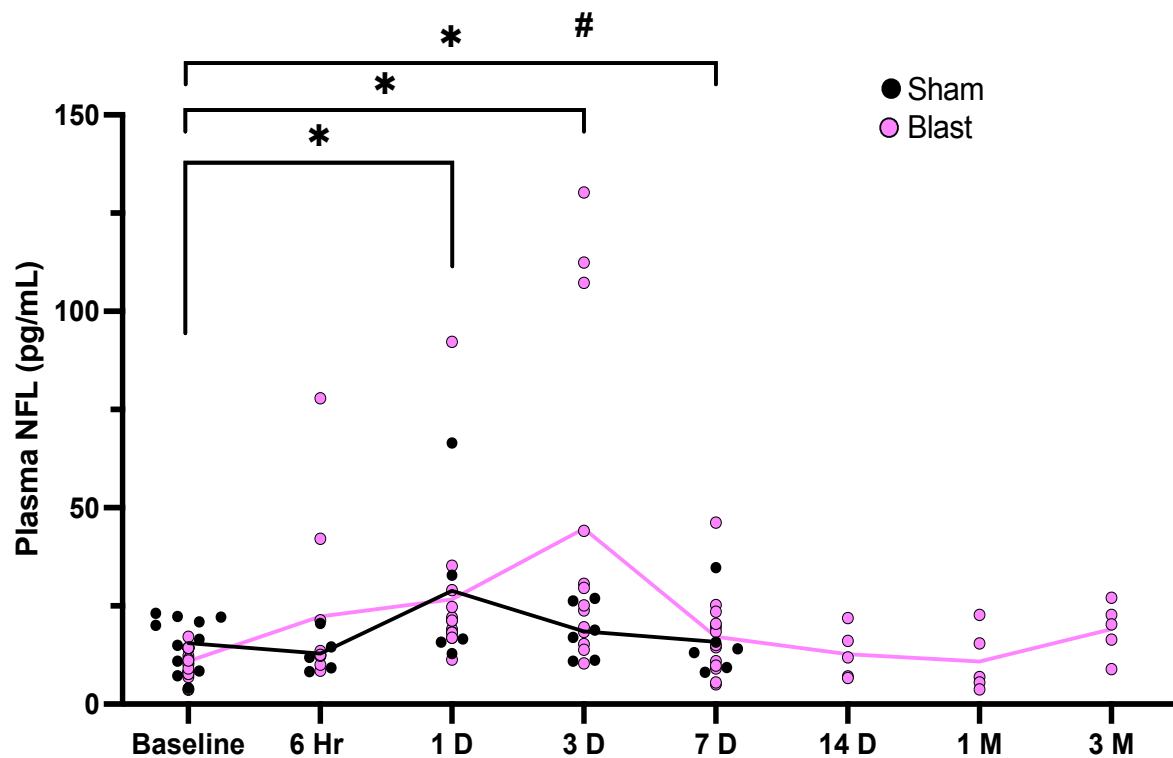


Figure 5.18 Blast NFL concentrations from blood plasma.

Significance found at 1,3, and 7DPI relative to control in the blast exposed blast samples using mixed effects ANOVA. Post-hoc test also revealed significant difference between sham and blast at 3DPI. Sham 6hr: n=6, Sham 7 days post injury (DPI): n=6, Blast 6hr: n= 10, Blast 7DPI: n=8, Blast 3M: n=5). *, Depicts comparison within treatment group relative to baseline. #=Depicts comparison between treatment groups. #,*<0.05. Values show mean $\pm$ SEM.

5.5 Discussion

In this chapter I present the results of how a single blast exposure affects the Corpus Callosum (CC) by analysing to what extent and over a 3 month time course the levels of NFL are altered and the thickness difference using luxol fast blue (LFB). Together with these results, this chapter assesses, for the first time, the use of fluid biomarker NFL in the CSF and blood over the same time course.

The results of the LFB staining show there was no evident white matter thinning observed in the blast model, by LFB staining and thickness analysis. In addition, the histological examination of NFL fluorescent intensity levels in the CC revealed a significant increase of around 20% at 7 DPI and 123% 1 month post-injury, depending on the antibody used. This suggests that the choice of antibody can influence the histological NFL results, highlighting the need for more sensitive antibodies in histological analysis. Interestingly, this study showed for the first time, results of the NFL fluid biomarker response to a single mild blast injury over a time course. The data revealed an acute peak of NFL in both CSF and blood plasma, with prolonged elevation in CSF NFL levels, while blood plasma returned to baseline levels after 7 DPI.

Thus, importantly, this study demonstrated that the fluid biomarker NFL is sensitive enough to detect diffuse DAI (or neurodegeneration) in a *mild* diffuse traumatic brain injury.

5.5.1 NFL Histology in the Blast Model

There was an increase in fluorescence intensity of NFL observed in the blast model which was an unexpected result. Two antibodies for NFL were used to assess changes in NFL levels. One of the antibodies used (RPCA-NFL-CT) was the same as that employed in the CCI model (Supplementary Material 3), and the second antibody referred to as SySy NFL from Synaptic Systems was used first. The use of two antibodies was necessary due to unexpected results observed with the SySy NFL antibody in the blast model. The results showed a 123% increase

in blast NFL fluorescence intensity compared with the sham control, instead of the expected absence of change or decrease, like those seen in focal injury stains of neurofilament heavy (Graham, Zimmerman et al. 2021). To ascertain whether similar results would be similar to the SySy more blasted brain tissue was subsequently stained with RPCA-NFL-CT. Contrary to expectations, both NFL antibodies used in the blast model demonstrated an increase. However, the increase with the RPCA-NFL-CT was only ~20% and occurred solely in one time point. This could be due to batch to batch variation of the immunofluorescent tissue (a common issue with this type of staining) and the fact that the microscope used for analysis was found to have two of its laser misaligned.

Interestingly, the peak fluorescence occurred at 7 DPI for the RPCA-NFL-CT antibody, whereas it was observed at 1 month post injury for the SySy NFL antibody. The potential reason for this observed increase in the mild blast model, more reliably demonstrated by the SySy antibody could be attributed to the mechanism of injury or the different binding abilities of the antibodies. The blast model represents a diffuse injury, potentially allowing for greater accessibility of the antibodies to the brain, a reason for increases using the SySy antibody put forward by a Stroke Study (Mages, Aleithe et al. 2018). Another possibility is that the smaller fragments of NFL present in the brain parenchyma could facilitate antibody attachment (Mages, Aleithe et al. 2018, Shaw, Madorsky et al. 2023). It is also possible that both explanations may have occurred together. Similar findings were observed in a stroke model investigating NFL, NFH and NFM (Mages, Aleithe et al. 2018). The researchers reported an increase in NFL fluorescence when using the SySy NFL antibody, which was found by conducting western blots that the SySy antibody was able to bind to 68kDa fragments and damaged aggregates of smaller molecular weights. In Mages study they chose a second antibody to confirm degradation of the NFL, as I have done in this chapter and found the expected NFL decreases in the stroke-damaged brain regions. Thus, in this study, another NFL antibody was used. This study used the RPCA-NFL-CT as discussed previously, and results suggest that both antibodies may be able to bind to smaller aggregates, however the RPCA-NFL-CT antibody had much smaller increases that could be attributed to methodological variation as western blots conducted by the manufacturing company show it only binds to 68kDa NFL. This suggests that the RPCA-NFL-CT would be necessary to combine it with an

antibody that binds only to unmasked epitopes (e.g MCA-1B11 or MCA-6h63) as a result from injury. Therefore, more work would be required to confirm this.

5.2.1 NFL Fluid Biomarker in the Blast Model

Despite the histological results in the blast model, this is the first study that has evaluated NFL concentration in the CSF and the blood in a blast model of TBI over acute-chronic time-points. It was found, despite the histological findings, that the blast model had increased NFL concentrations in the CSF that peaked at an acute time point. The observed NFL increases in the fluid biomarker, in combination with no overt decreases of NFL in the histology, indicate that there is a different source of axonal injury (grey matter) and/or a diffuse axonal damage such that there is not a statistically significant difference in the white matter in the blasted brains. This implies a more global effect of diffuse axonal injury (DAI) throughout the brain as a result of the blast wave and suggests future work should undertake broader investigation of axonal injury in the blasted brain.

The fluid biomarker increases observed in the blast model were a lot less pronounced relative to other models considered focal but mild (Graham, Zimmerman et al. 2021), thus indicating the milder nature of this diffuse blast model. It was observed that plasma NFL levels rose relative to baseline at 6 hours but had variation such that it was found not to be significant. Statistically significant elevations occurred in the blood plasma NFL at 1 DPI, peaked at 3 DPI and was significantly elevated up to 7 DPI. After 7 DPI, the NFL levels normalised to be non-significantly different from baseline.

The NFL elevation in the plasma followed a similar chronology to the CSF increases. However *statistically significant* elevation in the plasma does not occur until 1 DPI whereas there is a significant elevation in the CSF at 6 hours. This could be due to the speed at which the NFL is processed into the blood in a concentration that would be measured as significant.

5.2.1.1 NFL Fluid Biomarker Results in Relation to Other Studies

In this chapter I found elevated levels of NFL at 6 hours and 1 DPI, peaking at 3 DPI in the blood plasma of the blast model. This is consistent with non-blast mild TBI studies (Shahim, Zetterberg et al. 2017, Shahim, Tegner et al. 2018, Shahim, Politis et al. 2020). Shahim et al, 2018 investigated serum NFL in hockey players. Their findings revealed that increased NFL levels at 1 hour were able to discern between those athletes that were ready to return to play and those that could not, due to post-concussive symptoms (PCS). The study reported that those that returned to play had NFL levels that increased but normalised in 6 days post-sports related concussion (SRC), whereas those that did not had sustained elevated levels of NFL (Shahim, Tegner et al. 2018). This indicates that NFL is able to discern between injury severities. The study also reported that the NFL elevation peaked at 12 hours. The acute time point increase is similar to the work presented in this thesis. Therefore, with such an acute increase that indicated injury, NFL could be used to identify a window of opportunity for therapeutic input before the onset of other potentially debilitating secondary pathologies. It should be noted however, that NFL in CSF was not measured in these athletes, unlike it was in the animals used in this thesis.

The blast literature in human patients has lacked consistent results. The acute NFL plasma peak shown in our study aligns with some of the blast literature, but not all. Boutté, 2019, (Boutté, Thangavelu et al. 2019) investigated breachers, a subset of military personnel that use explosives to force entry. They can be exposed to multiple blasts in a given period. The study by Boutté found that serum collected within 1 hour showed elevated NFL levels and that the increase in NFL levels were associated with decreased simple reaction time, showing that blood taken at the most acute time can be informative of cognitive state. This statement is supported by others (Gao, Zhang et al. 2020). However, another human blast study was conducted by Vorn et al, (Vorn, Naunheim et al. 2022) that found no blood NFL concentration changes at 2 DPI, a time when other biomarkers they investigated had increased, but instead NFL increased at day 7, relative to baseline. It is not clear why there may be an instant NFL increase in one cohort and a delayed increase in the other, but the use of preclinical models can aid this investigation.

The lack of chronic NFL plasma elevation found in this chapter's single mild blast model is in alignment with one blast study that investigated NFL in a repetitive blast study. This report shows results from blast injury in a rat model as well as human veterans from the Operation Endurance Freedom and Operation Iraqi Freedom wars (Dickstein, 2021 #682}. The authors found increased NFL in humans in veterans. However, these NFL evaluations were in veterans that presented increases of tau pathology in areas of the brain, which are typical of CTE pathology, at chronic time points. This may suggest that NFL may be connected to increases in other biomarkers or offer predictive qualities for other biomarkers (not explored in this thesis) and pathologies. The NFL chronic plasma levels of this chapter align with the rat model's result from that same blast paper (Dickstein, De Gasperi et al. 2021). Dickstein did not investigate acute levels of NFL in serum, only chronic. They reported that they did not have significant findings in NFL serum at 4 or 10 months post-injury. Whilst the lack of chronic NFL elevation in this chapter aligns with this study, possibly indicating a recovery, the chronic CSF increases imply a longer lasting pathology of injury. This demonstrates that the chronic pathology of blast may require greater investigation using multiple modalities of NFL investigation.

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demonstrates that the chronic pathology of blast may require greater investigation using multiple modalities of NFL investigation.

However, the results from this chapter's NFL level are in contrast with a human blast study. Edwards et al., (Edwards, Leete et al. 2020) found significant decrease in NFL at acute time points after sub-concussive repeated blast. This could be due to an alteration in the BBB, where the NFL was unable to be "cleared" as part of the glymphatic system disruption, as they suggest. However, more preclinical work that tests a sub-concussive blast and BBB involvement would enlighten this blast scenario as no findings from this thesis could lend insight as to why this might be.

In view of the diversity of blast models possible to use (Chapter 3) and time points, it would be helpful to suggest that acute time points as well as chronic time points should always be investigated, in order to understand how blast TBI affects the brain biochemically. This could aid the prevention of repeated TBI and potential CTE pathology. However, it should be recognised that it can be very challenging to sample acute human blast blood samples, and therefore it is useful to use preclinical models which could inform these time points, and to take serial blood samplings over time, which this chapter did.

A possible reason why the blast model's fluid biomarker levels differ at later timepoints between the CSF and plasma, but are the same for the CCI model, is that there may be a role of the BBB in TBI. However, it is unknown how the BBB may play a role in the levels of NFL in the CSF and the blood. It has been seen in *in vitro* models of blast that, if the brain is not allowed to recover, the BBB may remain leaky, such as from repeat mTBI (Hue, Cao et al. 2013, Hue, Cao et al. 2014, Hue, Cho et al. 2015). This may explain the chronic levels of plasma NFL reported in human veteran/sports studies and the lack of chronic NFL plasma levels in this thesis, as shown by the blast model. Furthermore, the more severe the injury the more likely it is that the BBB remains leaky for longer or is intermittently leaky (Li 1995, Başkaya, Rao et al. 1997). This has been shown in humans, whereby the BBB can be intermittently leaky after repeated TBI (and seen chronically in mTBI) (Tomkins, Feintuch et al. 2011, O'Keeffe, Kelly et al. 2020, Wu, Wu et al. 2020). The problem with a chronically leaky BBB is that it can continuously let in leukocytes and other immunoreactive cells that can cause greater

secondary pathology (Sivandzade, Alqahtani et al. 2020) that may results in further axonal damage and neurodegeneration and thus increased NFL levels.

However, if the BBB did play a role in biomarker release, then it would be expected in human cases to see increased serum levels of NFL which peak acutely, rather than sub-acutely as shown by human TBI biomarker studies (Shahim, Zetterberg et al. 2017, Shahim, Tegner et al. 2018, Shahim, Politis et al. 2020, Graham, Zimmerman et al. 2021, Vorn, Naunheim et al. 2022). This is assuming the BBB was damaged at the time or acutely after injury, yet that may not be the case, as it could be sub-acute BBB damage that occurs. It is evident that more work would be needed to understand this relationship as to how and whether BBB is damaged in these different types of TBI and whether it affects the release of NFL into the blood. This could be done by investigating with electron microscopy in combination with dextrans and fluid biomarkers in animal models. There are already studies on human TBI using contrast dye MRI for BBB permeability identification and correlating it with white matter damage patterns (Veksler, Vazana et al. 2020).

Despite NFL's promising attributes and findings in the TBI literature, it could still be called into question as a viable biomarker for mTBI by some, as it has been utilised to identify neurodegenerative diseases and it cannot differentiate between them (Gaetani, Blennow et al. 2019, Ashton, Janelidze et al. 2021). This is due to confounding factors such as ageing, as older generations have naturally higher levels of NFL, and numerous neurodegenerative diseases release of NFL. However, for younger populations, such as military or young athletes, it is less likely that ageing or neurological disorder is the cause of elevated NFL levels (Ashton, Janelidze et al. 2021). It is more likely to be caused from a TBI. This was shown by Shahim et al., 2017 (Shahim, Zetterberg et al. 2017) where their serum NFL sampling detected higher NFL levels indicating mTBI in young boxers and professional hockey players relative to baseline, suggesting the SRC was the cause for the NFL increase, as there was no other reported head trauma or ailment. Therefore, NFL can be a valid diagnostic option for mTBI, despite its ability to be detected in other disorders in older patients and non-sporting populations.

5.6 Conclusion

This chapter also found preliminary NFL histological results that suggest smaller fragments that have been degraded by the blast injury are present and potentially the source of the NFL measured in the CSF and blood. However, further work with more sensitive antibodies is necessary to demonstrate this reliably. This chapter also presents for the first time a *single mild blast TBI* leads to elevated NFL levels over acute and chronic timepoints. I showed the acute-chronic temporal profile of the NFL fluid biomarker from a singular mild blast TBI. I show in this work that there are increased NFL levels acutely and chronically in the CSF, and acutely to sub-acutely in the plasma.

Furthermore, the NFL fluid biomarker results in spite of the inverse histological results suggest the sensitivity of NFL to detect a mild TBI at acute and chronic timepoints. This highlights the need to sample as acutely as possible, with repeated samples in order to assess injury development.

In conclusion, this chapter suggests that diffuse axonal injury (DAI) occurs as a result of blast in the CC. Furthermore, the NFL protein exhibits potential as a fluid biomarker for mild blast TBI, coinciding with some evidence from preclinical and human literature at acute and sub-acute time points. The fact that NFL is shown to increase despite unexpected histological analysis suggests that NFL can be employed in conjunction with MRI scans or as a standalone marker. Additionally, the study indicates that sampling NFL as early as possible and conducting frequent repeat samplings is crucial for comprehending injury progression. Overall, this investigation largely supports the hypotheses outlined in this chapter, although further histological examination is warranted.

6

General Discussion

6.1 Thesis overview

Mild traumatic brain injury (mTBI) caused by blast exposure is prominent in military personnel and can lead to a wide range of symptoms that may manifest immediately or months to years later. These symptoms may persist or dissipate, with uncertain reasons for their occurrence and resolution. Importantly, delaying the diagnosis of a brain injury can leave a brain vulnerable to further damage and contribute to the risk of developing neurological illness, such as epilepsy or neurodegenerative diseases (Wilson, Stewart et al. 2017). The time frame in which many studies conduct their investigations into the pathology of blast TBI is narrow. This observation is evident in the scientific literature, whereby human or animal blast studies focus solely on either acute or sub-acute time points (De Lanerolle, Bandak et al. 2011, Goldstein, Fisher et al. 2012, Cho, Sajja et al. 2013, Wang, Gao et al. 2014, Huber, Meabon et al. 2016, Clark, Merritt et al. 2018, Hernandez, Tan et al. 2018, Kallakuri, Pace et al. 2018, Edwards, Leete et al. 2020, Masri 2020, Vorn, Naunheim et al. 2022), while others focus exclusively on chronic times post-injury (Dickstein, De Gasperi et al. 2021, Graham, Blissitt et al. 2023) and they rarely cover the whole range (Säljö, Bao et al. 2000, Stemper, Shah et al. 2016).

In human blast studies, the lack of acute or sub-acute assessment and diagnosis can be a product of the inaccessibility to do so whilst in a war zone. Thus, a novelty of this thesis lies in the exploration of the temporal progression of blast effects using a preclinical model, covering a comprehensive temporal profile from the acute (6hours post-injury), sub-acute (7 days post-injury) and the chronic (1 and 3 months post-injury) stages post-injury.

To achieve this longitudinal exploration, this thesis set out to optimise a rodent mild blast TBI model using the Centre of Blast Injury Studies (CBIS) shock tube. The blast model was established taking into consideration the expectations that the model should have of a 0% mortality rate, a loss of righting reflex (LORR) that indicated injury, and a low post-blast welfare score. The scoring was to identify signs of pain or discomfort commonly seen in more severe TBIs.

There were no deaths due to the blast during the optimisation of the model or during the development of the experimental cohorts of the thesis. Furthermore, there was a significant increase in LORR time between sham and blast groups (two-fold increase, $p < 0.0001$). Moreover, there were no signs of pain, discomfort or weight loss in the animals used, either during optimisation or in the experimental cohort.

The functionality of the brain post-blast exposure was measured using resting-state EEG and was analysed by Konstantinos Tsikonofilios. The results showed hallmark power increases in the lower frequency bands and increased functional connectivity in the gamma band in the blast model at 1 and 3 months post-injury compared with sham animals (Supplementary Material 2). However, there were no changes in the acute phase, except for high frequency oscillations observed at 3-4 hours post-injury (Supplementary Material 2). These EEG results prompted histological investigations into the longitudinal pathology of neuronal populations post-blast. The blast model was used to induce the injury followed by first investigating two interneuronal (IN) subtypes, parvalbumin (PV) and somatostatin (SST), to assess whether their densities change and may contribute to these electrophysiological changes post-blast exposure. It was hypothesised that there would be reductions in the PV densities following the blast, while SST densities would remain unchanged. Surprisingly, the hypothesis was incorrect. My results revealed for the first-time reductions in the densities of both types of GABAergic INs as a result of blast injury. The greater decreases of these markers were detected in the SST IN populations in all cortical regions analysed, while PV reductions were found only in the primary auditory cortex (Au1) cortex. These changes in GABAergic INs occurred at all time-points (from 7 days post-injury (DPI) and 1 month post-injury, some lasting up to 3 months post-injury) except 6 hours post-injury in the absence of changes in general neuronal numbers, quantified by staining with the NeuN antibody. These results suggest that there is an E/I imbalance in the blast brain that may explain the electrophysiological patterns seen in this model.

Another aim of this thesis was to investigate the extent of microglial and astroglial pathology in the blast injury model over time. It was expected that there would be an inflammatory response, caused by microglial/macrophage and astroglial activation as a response of the blast lesion. Immunostaining with a microglia/macrophage marker (Ionized calcium-binding

adapter molecule 1, (IBA1)) revealed significant increases in the microglial densities, mostly at acute and sub-acute time points post-injury in the Au1 and the Retrosplenial cortex (RSC). Although no significant increases were seen in the primary visual cortex (V1) or the Corpus Callosum (CC). There were also changes in the percentage area covered with IBA1 microglial/macrophage staining at 3 months post-injury in all brain regions analysed (Au1, V1 and CC), except for the RSC, suggesting potential alterations in the microglial phenotype (cells becoming bigger). However, in contrast with some other blast TBI models, we did not observe changes in percentage area covered for GFAP positive astrocytes post-blast exposure.

In addition, I expected that the histopathological changes would be lateralised. I hypothesised that the changes to be more marked either in the ipsilateral hemisphere. This was due to the lateral exposure to the blast wave, as it hit the right side of the head, since the rat was placed perpendicular to the shock tube exit. Conversely the changes could be more marked in the contralateral side since that is where the electrophysiological changes were most presently presented (Supplementary Material 2). However, no differences between hemispheres were found for the parameters investigated, in most of the regions of interest. Thus, this is in line with the diffuse nature of the blast wave and its effects on the brain. The observation of the SST decline in the ipsilateral hemisphere at 6 hours post-injury, within the V1 cortex, may be attributed to the orientation of the rat in relation to the blast wave, as the rat's eye was not shielded from the oncoming blast exposure. This initial lateralisation observation in a rodent blast model is like that reported by Bishop et al. (Bishop, Won et al. 2022). However, in their paper, lateralised injury was due to the damage to the optic nerve and visual tract, while in our study we did not observe this pattern. Furthermore, no microglial density changes were observed in the V1 at any time point in this thesis.

Following interneuronal and glial density investigations, the thesis aimed to assess the effect of blast on the white matter, which is another pathological hallmark of TBI. Firstly, luxol fast blue (LFB) staining revealed no observable thinning of the corpus callosum (CC) at any times post-blast. However, when performing immunofluorescent (IF) staining of brain sections using an antibody for neurofilament light (NFL), as marker of neuroaxonal damage, an increase was observed in fluorescence intensity in the CC of the blast model at certain time points. This was contrary to the expected decreases in the NFL intensity. Nevertheless, the

concentration of NFL measured in cerebral spinal fluid (CSF) and blood plasma, as a biomarker for axonal damage, were found to be increased. Particularly, there was an acute peak of NFL at 6 hours post-injury in the CSF and the values remained significantly elevated up to 3 months post-injury compared with Naïve/Sham controls in the blast model. On the other hand, the plasma levels of NFL of the blast model showed less pronounced increases in concentration compared with the CSF values, peaking at 3 DPI until 7 DPI, after which the levels were no longer elevated above baseline. Nevertheless, the fluid biomarker seems sensitive enough to detect neuroaxonal damage in a diffuse mild model of TBI despite the unexpected histopathological results, and points towards its success to be used as a biomarker for milder forms of TBI.

	Measurement	ROI	3/4 hr or 6 hr	1 D	3 D	7 D	14 D	1 M	3 M
EEG*	Absolute power	EEG array	ns	-	-	-	-	↑All bands	↑All bands
	Relative power	EEG array	ns	-	-	-	-	↑Delta ↑Theta ↓Beta ↓Gamma ↓HFO	↑Delta ↑Theta ↓Alpha ↓Beta ↓Gamma ↓HFO
	Functional Connectivity	EEG array	↑HFO	-	-	-	-	↑Gamma	↑Gamma
Histology	NeuN density	Au1	ns	-	-	ns	-	ns	ns
		V1	ns	-	-	ns	-	ns	ns
		RSC	ns	-	-	ns	-	ns	ns
	PV density	Au1	ns	-	-	↓29.53% L5 ↓33.88% L6	-	↓45.73% L5 ↓47.63% L6	↓22.86% L5 ↓ L 6
		V1	ns	-	-	ns	-	ns	ns
		RSC	ns	-	-	ns	-	ns	ns
	SST density	Au1	ns	-	-	↓35.53% L5 ↓45.53% L4 ↓40.34% L5	-	↓25.89% L5 ↓36.10% L5	↓30.93% L5 ↓32.57% L5
		V1	↓34.43% L 4 ↓23.29% L 5	-	-		-		
		RSC	ns	-	-	ns	-	↓42.96% L2/3	↓33.61% L2/3 ↓29.47% L5
	IBA1 density	Au1	↑34%	-	-	ns	-	↑18%	ns
		V1	ns	-	-	ns	-	ns	ns
		RSC	↑40%	-	-	↑25%	-	↑20%	ns
		CC	ns	-	-	ns	-	ns	ns
	IBA1 % area	Au1	ns	-	-	ns	-	ns	↑96.82%
		V1	ns	-	-	ns	-	ns	↑106.43%
		RSC	ns	-	-	ns	-	ns	ns
		CC	ns	-	-	ns	-	ns	↑266.38%
	GFAP % area	Au1	ns	-	-	ns	-	ns	ns
		V1	ns	-	-	ns	-	ns	ns
		RSC	ns	-	-	ns	-	ns	ns
		CC	ns	-	-	ns	-	ns	ns
	SySy NFL	CC	ns	-	-	ns	-	↑123.82%	ns

	Encor Biotech NFL	CC	ns	-	-	↑ 23.61%	-	ns	ns
Fluid biomarker	NFL Conc.	CSF	↑ 4262.63%	-	-	↑ 2417.96%	-	↑ 1617.56%	↑ 967.23%
		Plasma	ns	↑ 144.28%	↑ 309.21%	↑ 57.40%	ns	ns	ns

Table 6.1 Summary of results

This table is displaying EEG (* = data presented in Supplementary Material 2 and conducted by Mr Tsikonofilos), histopathology and fluid biomarker results from the single mild blast exposure to the head of male Sprague-Dawley rats. -: no measurement taken in that modality or that timepoint, ns: no significance found $P > 0.05$, ↑: significant increase $p < 0.05$, ↓: significant decrease $p < 0.05$, ↘: tendency to significant decrease (p values between 0.05-0.06)

6.2 Effects of Blast Exposure at Early Time Points (Acute Phase) Post-Injury.

To dissect the potential effects in the brain that take place after the blast over time, I compared the different parameters (those by myself and by others) analysed in different phases post-injury. At the acute phase, (3-4 or 6 hours post-injury) it was observed that there were detectable changes in the EEG high frequency oscillation (HFO) (3-4h post-injury) (Supplementary Material 2 Figure 5), increases in the NFL biomarker in the CSF (6 hours post-injury, at 1 DPI and peak at 3 DPI), a decreasing trend of SST positive cells (in the V1), and an increase in microglia density in the Au1 and the RSC, without changes in the PV cell density or in CC thickness (Table 6.1).

6.2.1 Elevated Fluid Biomarker NFL in the Absence of Substantial Neuronal Loss

One of the key findings, evident not only in the acute phase but also in subsequent chronic phase, centres on a significant elevation of NFL in both CSF and plasma. Curiously, despite this surge in NFL levels, there is an absence of neuronal loss, as evidenced by the NeuN results in each region of the neocortex analysed. This apparent incongruity highlights the sensitivity of the fluid biomarker NFL in detecting even milder forms of TBI and indicates a neuroaxonal source of NFL rather than neurodegeneration.

Moreover, while it's conceivable that changes in the IN population could contribute to elevated NFL levels, no significant reductions in IN density were demonstrated during the acute and sub-acute (nor at chronic) timepoints of the observed trends. Given that INs constitute approximately 20% of all neurons, and considering that SST INs make up 20% of the IN population, which translates to 4% of total brain neurons, even a notable decrease of -60% and -40% in SST neuron count at 6 hours and 7 days post-injury in one hemisphere would only account for a reduction of 2.4% or 1.6% of overall neurons, respectively. Therefore, it's improbable that any potential IN neurodegeneration would significantly contribute to the measured NFL concentrations in plasma and CSF.

Within the context of this blast model, the findings strongly suggest that the heightened NFL levels primarily stem from axonal damage rather than neurodegeneration. Furthermore, if there was mass neuronal loss from the blast injury at the acute timepoints, it would be evidenced by the EEG recordings (refer to Supplementary Material 2), as the brain would have its networking profoundly affected (power changes).

However, it's important to acknowledge that this suggestion of DAI instead of neurodegeneration is not without its limitations. The histological observations of NFL, that would indicate axonal damage in the main white matter tract of the rodent brain, do not reflect the NFL fluid biomarker results. This could potentially be attributed to two factors: the antibody's sensitivity employed in the analysis and the extent of diffusivity associated with the blast injury and subsequent axonal injury.

Interestingly, the results of the fluid biomarker concentrations of NFL in combination with the absence of corroborative NFL histological evidence also manifested in a controlled cortical impact (CCI) model I worked on, using the same antibodies. In this instance, the milder CCI injury did not induce overt damage to the corpus callosum that was visually discernible. This parallels the outcomes from the blast model, where elevated NFL concentrations were observed in the blood plasma in absence of change in the NFL histology (refer to Supplementary Material 3). Yet, in the CCI model, MRI scans were conducted, and diffuse tensor imaging unveiled detectable impacts on the corpus callosum due to the milder trauma at both acute and subacute timeframes. Consequently, the immunohistochemical antibody

used to measure NFL changes in the brain tissue may not have high enough sensitivity for milder models. That fact that the antibody's sensitivity might underpin the lack of observation of expected NFL reduction suggesting diffuse axonal injury (DAI) in the CCI and the blast model. This prompts the need for further investigation employing more sensitive antibodies, capable of detecting and discerning the changes of NFL in the milder models. Further, it should detect the degraded fragments, should they be present, rather than binding indiscriminately to both intact and degraded counterparts, as expounded upon in Chapter 5.

6.2.2. High Frequency Oscillations and Microglial Density Change in the Absence of Measured White Matter and Interneuronal Alterations

Intriguingly, the observation of functional connectivity increases in high-frequency oscillations (HFOs) already evident in the acute phase, without accompanying power changes or evident neuronal alterations except with some trends observed in the visual cortex, raises interesting questions as to the function of HFOs (Table 6.1). These connectivity enhancements may stem from short, synchronised bursts originating from the thalamus and reaching the somatosensory cortex (Ozaki and Hashimoto 2011), suggesting their potential involvement in information processing. Some researchers have proposed HFOs as novel biomarkers of epileptogenic tissue. Although none of the animals in this study exhibited seizures, the observed electrophysiological patterns might indicate a propensity towards such activity, despite it not manifesting as post-blast epileptic activity (Zweiphenning, van 't Klooster et al. 2016). An alternative theory posits that these HFOs are linked to information processing mechanisms that are associated with memory (Engel and da Silva 2012). Despite these intriguing findings, the purpose of HFOs in both healthy and injured brain remains poorly understood in the scientific field. Notably, this time point lacks hallmark EEG pathology, such as increased power in the low frequencies (Delta: 1-4 Hz and Theta: 4-8 Hz, refer to Supplementary Material 2 Figure 4 and Table 6.1), which could be attributed to the absence of significant interneuronal changes and white matter alterations (Table 6.1), further supporting the previously mentioned suggestion that the hyperexcitability observed in this

blast model can be due to an excitatory/inhibitory imbalance due to the reduction on IN density (Chapter 4).

The aforementioned EEG detected HFO changes may also be due to the biomechanical strain from the blast exposure, which may cause micro damage to the neurons and axons (axonal damage suggested by increase plasma and CSF concentrations of NFL), and thus, results in a proportionally mild neuroinflammatory response (Donat, Yanez Lopez et al. 2021). Interestingly, the present study showed increases in microglia density in the RSC and the Au1 at the acute phase, suggesting microglial proliferation, typical of an inflammatory response but not in all regions analysed. A potential reason of the microglial increase in the Au1 could be due to the fact that it is the cortical area closest to the initial impact of the blast wave, as well as the region area that is directly opposite, which may be a product of the coup-contrecoup of the brain from the resulting movement (as shown in chapter 3). It may also be because the cortical area is affected by the loud noise from the air-driven simulated explosion (Säljö, Bao et al. 2000, Masri 2020), although hearing loss was not measured in this study. Another reason for this increase could be derived due to the particular orientation of the axons in the cortex, as axonal stress and strain can cause damage to axons and an inflammatory response (Donat, Yanez Lopez et al. 2021). However, we did not observe changes in microglia number at the CC during the acute phase or alterations in CC thickness by Luxol fast blue or overt axonal loss by NFL staining. However, it is possible that other areas of white matter could have been damaged at this point, as NFL concentrations in the CSF were found increased early on after injury. This is in agreement with a report investigating a swine model of diffuse TBI, whereby DAI and microglial activation occurred in those regions already at 6 h post-injury (Lafrenaye, Todani et al. 2015).

Based on these results, increased NFL concentrations and alterations in EEG can be used as potential biomarkers for bTBI. These results also suggest that they should be measured as acutely as possible, followed by serial tests; however, it is important to note that acute time points are not always feasible/available for early diagnose in the battlefield.

6.3 Effects of Blast Exposure at the Chronic Phase Post-Injury

Following the acute phase, single mild blast exposure resulted in further pathological alterations over time, as secondary injury developed and manifested as interneuronal density reduction, white matter alterations and electrophysiological changes.

In the blast model, the major changes in brain function occurred at 1 month and 3 months post-injury, manifested as EEG power alterations and functional connectivity (FC) increases in gamma band (see Supplementary Materials 2). These changes coincided with reductions in INs, including decreases of PV density in the auditory cortex (from 7 days to 3 months post-injury) and SST densities in all regions investigated in the chronic phase. Concomitant with IN reductions, the percentage area of IBA1 staining increased at 3 months post-injury (Table 6.1), without changes in the number of cells, suggesting a change in microglia phenotype. However, previous research by Masri (2020) (Masri 2020) demonstrated a dissociation between activated microglia and PV IN loss, implying that microglia may not directly contribute to the observed changes in the IN density. Conversely, due to the intricate complexity of microglia and the various phenotypes they can adopt, it is possible that microglia play a more significant role than can be conclusively determined in this thesis. For instance, a study by Wang et al. (2018) (Wang, Zinsmaier et al. 2018) found that pharmacologically blocking the pro-inflammatory cytokine tumour necrosis factor alpha (TNF- α) with dithiothalidomide (dTT), for 5 days after the blast reduced the loss of PV interneurons in the hippocampus after blast injury. It would be insightful to explore whether similar effects occur in the cortex and how they may impact the observed EEG power changes at chronic time points. However, microglia are complex in their phenotype and function and inhibiting certain phenotypes of microglia can cause detrimental effects, such as what was found by Scott et al, (Scott, Zetterberg et al. 2018). In that study, they blocked chronic microglial activation with Minocycline in patients with TBI which resulted in increased plasma NFL, a sign of neurodegeneration, thus suggesting that the chronic microglia had reparative effects on the brain that were lost when blocked.

As indicated in the summary Table (Table 6.1), no significant changes were observed in white matter at chronic time points, except for some increases in NFL staining 23% and 123% at 7 days and 1 month post-injury. However, NFL concentrations in the CSF and the plasma

remained elevated at 7DPI and 3 months (CSF) despite no indication of general neuronal loss (NeuN). This indicates that even for a *single* mild diffuse TBI, NFL is sensitive enough to detect axonal injury in the brain and that sub-acute time points are still suitable to assess NFL in CSF or Plasma as biomarkers and employed as a means of diagnosis.

As previously mentioned in chapter 5, the BBB and NFL release may be linked, but what should also be considered is the role of BBB and electrophysiological outcomes. Understanding and detecting BBB disruption post-blast could also enlighten the EEG post-injury hallmarks. Korn et al found BBB disruption in symptomatic TBI patients with negative MRI scans for TBI diagnosis but instead had increases in low frequency power (Korn, Golan et al. 2005). This suggests that the BBB could be a substrate to the electrophysiological outcomes in this model as well as E/I imbalance or DAI and highlights the need for future work on blast TBI and BBB disruption.

6.4 Translational Implications of this Study

Blast exposure is associated with TBI, neuropsychiatric symptoms, and long-term cognitive disability in patients. A model that reproduces this type of diffuse injury is the blast model, which aims to mimic the effects of explosions. The advantage of using an animal blast model is that the injury is very reproducible, allows the manipulation of the different parameters of the blast wave, as well as the ability to perform a longitudinal study of the functional and anatomical changes in the brain, which can be quite invasive. Rodents such as rats, used in this thesis, are useful despite the obvious limitations, because they have similar genetics and the same neuronal properties as the humans. One of the advantages of using rats over mice is that they have a larger brain (Ellenbroek and Youn 2016), which enables better resolution imaging (MRI) and the use of a more translationally relevant EEG array, which allowed for greater spatial as well as temporal sensitivity relative to other preclinical EEG studies. This array allowed the use of the FC metric debiased weighted phase lag index (DWPLI), which is much more translationally relevant due to the number of electrodes employed.

The observed power shift, characterised by changes in delta/theta frequency bands at 1 and 3 months post-blast injury in this study has been consistently identified in human studies.(Franke, Walker et al. 2016, Huang, Harrington et al. 2017, Kaltiainen, Helle et al. 2018, Lewine, Plis et al. 2019, Mallas, Gorgoraptis et al. 2022). These findings have been reported to be associated with alterations in structural connectivity (Ball, Gloor et al. 1977, Gloor, Ball et al. 1977). Huang et al., 2009 (Huang, Theilmann et al. 2009) conducted DTI and MEG analysis on patients with negative CT and MRI scans and found slow wave power increases that correlated with white matter alterations, shown as reduced fractional anisotropy (FA). However, for some patients their DTI results showed no abnormalities, but they did have increased MEG delta waves. These structural alterations (e.g white matter damage or DAI) can be hard to detect for some mild TBI cases, as structural damage may not appear on MRI scans (Yue, Upadhyayula et al. 2020) or not be present in the white matter. However, they can still occur, despite no mass lesioning or MRI detection, which is why electrophysiology may prove a good addition to the diagnosis protocol, as it can be more sensitive than other assessment metrics (Huang, Theilmann et al. 2009, Huang, Nichols et al. 2012). Furthermore, these structural changes involved in the low frequency power shift might also lead to cognitive impairment (Sharp, Beckmann et al. 2011, Sharp, Scott et al. 2014, Mallas, Gorgoraptis et al. 2022).

Nevertheless, the preclinical work of this thesis uniquely enabled the combination of acute histological tissue analysis with EEG analysis, a research approach that is unfeasible in human subjects. The study by Mallas, 2020 (Mallas, Gorgoraptis et al. 2022) investigating patients with TBI and patients with post-traumatic amnesia (PTA) revealed impaired association memory, accompanied with a shift toward slower-frequency activity on EEG, similar to what was shown in our study. However, due to the limitations of human studies, it was not possible to explore the events preceding the investigated timepoints or to investigate changes at the cellular level in the brains of these patients. Thus, our blast animal study offers a significant advantage in providing insights that are otherwise unattainable in humans.

The persistence of the observed changes of the CSF NFL acutely and up to 3 months post-injury, has not been extensively investigated in the human blast TBI field. Instead, only blood plasma has been assessed in human blast cohorts (Edwards, Leete et al. 2020, Dickstein, De

Gasperi et al. 2021, Vorn, Naunheim et al. 2022). Of the non-blast CSF NFL levels reported in the field, like Neselius's study of Olympic boxers, CSF-NFL remained elevated approximately 3 months after the initial measurement was taken (Neselius, Brisby et al. 2012). This is of interest, since the CSF-NFL pattern of this thesis's blast model seems to be similar to that of repetitive injury. However, the TBI literature is inconsistent, as an amateur boxing cohort revealed no CSF-NFL increases in those who had a boxing bout of <15 hits (milder injury), and normalised levels of those who had more than 15 hits (more severe injury) after a 3-month rest period (Zetterberg, Hietala et al. 2006). This may be due to the number of previous hits experienced by both studies, as it could be assumed that Olympians train and compete more often than amateurs and therefore are likely to receive more hits to the head during training as well as in competition. Nevertheless, comparing the NFL levels of blast model from this thesis to these studies, indicates the blast model caused overt axonal damage, (despite the lack of histological proofs) and was sensitive to injury/abnormality in the brain (as shown by the EEG) even at chronic timepoints.

However, this overt chronic axonal injury is not depicted in NFL plasma concentrations in the blast model of this thesis. The chronic NFL blood plasma levels of this blast study are more in keeping with the only study investigating human and rat blast NFL levels at chronic timepoints shown by Dickstein et al. (Dickstein, De Gasperi et al. 2021). The authors found no blood NFL increases in their rats at 4 and 10 months post injury furthermore no differences were found in the veterans in the study. unless the veterans increased tau-pathology ([¹⁸F]AV1451 ligand retention). However, acute and sub-acute increases in the plasma NFL, which was observed in this thesis, have also been reported in some blast and non-blast human studies (Shahim, Tegner et al. 2018, Boutté, Thangavelu et al. 2019, Vorn, Naunheim et al. 2022), whilst others have shown negative or no changes (Edwards, Leete et al. 2020). It should be noted that what is missing from these studies is a chronic timepoint to assess whether their NFL increases persisted chronically or not. Therefore, the advantage of this rodent blast model is that it allowed for longitudinal investigation to assess this effect over time and showed that plasma NFL elevations did not persist chronically.

Future investigations using EEG assessments at acute and sub-acute time points could potentially identify the timing of hallmark shifts in EEG power and elucidate the existence of

a therapeutic window before these changes occur. Discovering such a therapeutic window would have substantial implications for human therapeutics, particularly if combined with increased concentrations of NFL as a fluid biomarker for TBI. Furthermore, a portable way to diagnose TBI for those out on deployment or to be used by military doctors would be of great use and enable faster diagnosis, prognosis, monitoring and hopefully aid the long-term health of soldiers.

6.5 Limitations and Future Directions

6.5.1 Limitations of the Study Design

This study, as with any scientific work, has its limitations, which have influenced the results. These include factors, such as methodological design, tools used, time constraints, among others. The limitations are important to be recognised and considered, because they can help explain the outcomes of the study and they can be overcome by future work.

First of all, the use of rodents in a preclinical study implies some challenges, compared with other higher mammals or investigations in patients. Using rodents in biomedical research has been common practice, combined with the fact that they are cheaper to use, with less ethical implications and enable controlled experimental conditions (Marklund 2016, Shah, Gurdziel et al. 2019). The problem of using rodents is that their brains are smaller and less complex than those of humans (Marklund 2016), which may result in the limited applicability of some findings. For example, there is less white matter in the rodent brain relative to the human, less microglia in the white matter than humans and a rodent's micro- and astro-glial cells are less complex and may not have the same array of phenotypes and may have different transcriptional signatures (Verkhatsky, Bush et al. 2018, Jurga, Paleczna et al. 2020). This all may play a role in inhibiting full translatability of the results. Furthermore, the architecture of the rodent brain is very different to a human, with the cerebellum sitting at the back of the brain rather than underneath (Ellenbroek and Youn 2016). This may affect the biomechanics of the blast wave interacting with a rodent brain, as it is likely different relative to the human

brain (Needham, Ritzel et al. 2015). Additionally, the rodent brain is lissencephalic whereas a human brain is gyrencephalic. This may have an effect on the interpretation of the post-TBI pathology outcomes, as human studies can find inflammatory cells activated and tau pathology at the sulci the brains in CTE cases, which can occur after blast (Goldstein, Fisher et al. 2012, Shively, Horkayne-Szakaly et al. 2016). Furthermore, when investigating white matter tracts in the rat brain, the CC is the only prominent tract relative to a human brain and that was the only white matter tract used in this thesis. Nevertheless, rodents can be very useful in answering specific questions that cannot be achieved in human patients and it enables the validation of theories and hypotheses.

An aspect that should also be considered is the fact that the experimental groups for the histological cohort of the blast model did not have time-matched sham controls. There was a smaller sham subset of animals at the beginning of the study. However, I combined the results of the shams, as it was found that there were no observable differences between the sham groups. This was performed despite some of the animals having slightly larger brains due to older age (in the case of the 3 month post-injury shams). Although there were no differences between the sham groups, it is still a possible factor that may have influenced the results.

Another limitation of this study is that it was carried out only in male rats. Previous studies have found that TBI can affect males and females differently, and the outcomes can differ in humans and in animal models (Gupte, Brooks et al. 2019). However, some research has shown that men are more likely to have a TBI than women, in the general population (CDC 2022). In addition, there are sex differences in the incidence and mortality caused by TBI, with males showing 3 times higher death rate than females (CDC 2022). Yet, in the athletic environment, women are more likely to receive a concussion than men (Shannon, Mira et al. 2017, Gupte, Brooks et al. 2019, Koerte, Schultz et al. 2020). Female TBI incidences for TBI in the military are lower, but that is due to fewer women in general in the military. However, despite lower female deployment rates, there *are* females in the military, and therefore there will be increased instances of blast injury in that population demographic (Harding 2021). Therefore, in future studies, both sexes should be investigated, because the impact of blast TBI and its treatment can be different between males and females.

An additional potential limitation pertains to the selected post-injury timepoints investigated. The analysis of blast-induced TBI pathology excluded informative timepoints such as 3 DPI, which can aid in detecting the development of secondary pathology and blood-brain barrier (BBB) damage. Additionally, the absence of 14 DPI, a commonly studied timepoint post-injury, prevents insights into the persistence or resolution of secondary pathology. Moreover, the lack of a longer-term chronic timepoint, such as 6 months post-injury, impedes the evaluation of whether mild bTBI leads to the resolution of observed CSF NFL and EEG alterations detected at 3 months, or if these alterations persist. Additionally, the potential presence of delayed pathologies and/or neurodegenerative conditions remains unknown for this model. Future investigations should incorporate these timepoints to enhance our understanding of the temporal dynamics of blast injury pathology.

Concerning the functional element of this thesis, only resting EEG was conducted in the cohort of blast animals in the lab of Dr Andriy Kozlov. Interestingly, some auditory tests were carried out, investigating signs of tinnitus and hearing loss after blast, since the wave could affect the auditory sensory organ as well as the central auditory system.

Furthermore, behavioural tests could have been performed to understand the functional impact of an injury type on cognition, anxiety, memory and motor deficits (Hausser, Johnson et al. 2018), which are also affected by TBI and should be conducted in future studies.

Finally, the animals of this thesis were separated between EEG and histopathology cohorts. Since the model was repeatable and reliable, it can be assumed that the blasted animals would have the same pathology in each cohort, nevertheless, it has prevented correlations and predictive statistics for greater post-blast comprehension between the cohorts.

6.5.2 Technical Issues with the Pathological Assessment

Other technical limitations of the study include the fact that the pathological assessment was conducted only in one particular section (block) of the brain. The rat brain was divided in different sections (blocks) for neuropathological analysis, and we explored only in block 4,

because it was the region where most of the EEG differences were located on the topoplots. However, this means that alterations in other areas of the brain could have been missed. Since this study indicated that the blast wave caused a diffuse injury (chapter 4), there is reason to believe that maybe the frontal cortex (Eierud, Nathan et al. 2019) as well as the cerebellum (Bishop, Won et al. 2022) could have been affected by the wave and damaged. Therefore, given more time, and with hindsight other regions of the brain should be investigated potentially by slicing the brain sagittal rather than coronally.

Another point for improvement would be to carry out the morphological analysis of the microglia and astrocytes. Future work should investigate the morphological changes as well as the inflammatory profile of these cells by measuring cytokine and chemokine alterations, to understand the changes in phenotype of these cells over different times post-injury (Escartin, Galea et al. 2021, Amor, McNamara et al. 2022, Paolicelli, Sierra et al. 2022, Yu, Wang et al. 2022, Yuan and Wu 2022). Additionally different antibodies specific for activated microglia, rather than IBA1 that binds to macrophages and microglia. Furthermore, genomic and proteomic studies would help to characterise more specific alterations of the inflammatory response, which may enable further understanding of the temporal pathological profile post-blast injury and to select the right therapeutic window to target neuroinflammation.

Besides investigating white matter damage and neuronal death, future work could include assessment of synaptic density and connectivity. It is important to consider that this may have an influence on neuronal functionality and EEG measurements (Witcher, Bray et al. 2021). In order to ascertain the occurrence of synaptogenesis in the brain as a potential component of post-blast neuroplasticity, the evaluation of markers such as Synaptophysin (a presynaptic marker) and PSD-95 (a postsynaptic marker) or the use of genetically modified mice or rats that have fluorescent neurons (Thy-1-GFP animals) can provide insights into the generation of new synapses subsequent to the blast event.

Additionally, future work could involve the use genetically modified mice instead of rats to investigate IN loss, such as the Cre-mice as used in Vascak et al (Vascak, Jin et al. 2018) or

transgenic animals overexpressing tau (to investigate CTE in the blast model) and use markers for apoptosis and necrosis.

6.5.3 Limitations of Axonal Injury Markers

6.5.3.1 Axonal Damage in the White Matter using Immunostaining

One of the modalities that were used in this thesis to measure white matter damage, involved staining using a NFL antibody, which did not reveal reductions in immunofluorescence as expected. Instead, it was observed that there were increases in immunofluorescence intensity, which coincided with previous publications in diffuse injury models (Mages, Aleithe et al. 2018). However, staining of axonal damage could have been assessed in combination with other antibodies, such as the amyloid precursor protein (APP), to ascertain whether there was axonal injury or varicosities. This particular stain is known in the field as the gold standard of identifying axonal injury (Gentleman, Nash et al. 1993, Sherriff, Bridges et al. 1994, Bruggeman, Haitsma et al. 2021).

Additionally, double staining with MCA-6h63 could have helped to identify and differentiate the overall NFL polypeptide and the damaged NFL polypeptides, by binding to the sites on the polypeptides rendered available due to damage that would otherwise be masked. This was shown by Shaw et al., (Shaw, Madorsky et al. 2023), who demonstrated the co-localisation of intact and injury-induced unmasked NFL polypeptides in a rodent spinal cord injury model. The results of this thesis suggests that the NFL antibody used in the study may lack the required sensitivity to identify mild axonal injury, highlighting the need for a more sensitive antibody, to ensure alignment with the results obtained from fluid biomarker assays and to accurately identify the true source of the epitopes detected in these assays. A double stain for intact C-terminal and unmasked NFL would prove more insightful to better explain the source of the NFL fluid biomarker results for both models of TBI (the CCI and the blast) in this work (Jakimovski, Kuhle et al. 2019) and could also be the source of the NFL biomarker increases. As axonal damage it has been shown in other research that grey matter can have

axonal varicosities at 6 hours post-injury (Grady, McLaughlin et al. 1993), leading to functional alterations (Vascak, Jin et al. 2018).

Another potential limitation is the use of immunofluorescence as a way to quantify the histological NFL changes in a mild TBI model. There is inevitable variation between immunofluorescent intensities between different groups and sections, that perhaps could have been influenced by different staining days or variation in perfusion quality. These factors can influence the results of IF measurements even when the data is normalised to an internal control.

In Chapter 5 I demonstrated differences in the analysis of NFL staining between the Synaptic Systems (SySy) NFL antibody and the Encor Biotech (RPCA-NFL-CT) NFL antibody. At a molecular level, the disparity between the two NFL antibodies could lie in the epitopes they bind to. The SySy NFL antibody binds to the conserved AAA region of the NFL "head," (N-terminal) while the RPCA-NFL-CT antibody binds to the NFL C-terminal, the "tail". Despite not binding to the same epitope, both antibodies yielded an increased outcome in the diffuse model. However, the SySy antibody resulted in a greater difference from the RPCA-NFL-CT relative to Naïve/Sham control. Nevertheless, this emphasises again the lack of sensitivity in these two antibodies in detecting milder injury NFL changes, likely due to the subtle axon damage that can occur after blast exposure, which is not necessarily readily detected by the standard immunohistochemical markers (Murphy, Iacono et al. 2020). Furthermore, it underscores the importance of using antibodies recognising the damage induced unmasked regions of NFL, such as MCA-6h63 (troubleshoot in chapter 2) (Shaw, Madorsky et al. 2022), and confirming the findings with an APP stain (triple stain), in order to comprehensively understand the alterations in NFL polypeptides in milder and more diffuse models of TBI.

Additionally, quantitative measures for demyelination that may result in CC thinning were carried out using LFB staining, which have some issues of reproducibility when the intensity is quantified. Therefore, to investigate demyelination in the future, one should use more reliable markers of demyelination, such as myelin basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG), as these are commonly used markers for pathologies with demyelination, such as MS (Egg, Reindl et al. 2001). It is also possible to use other methods like electron microscopy.

6.5.3.2 NFL as Fluid Biomarker

In many other TBI studies, investigators have compared NFL with other fluid biomarkers (Shahim, Zetterberg et al. 2017, Shahim, Tegner et al. 2018, Hossain, Mohammadian et al. 2019, Shahim, Politis et al. 2020, Graham, Zimmerman et al. 2021, McDonald, O'Brien et al. 2021, McDonald, Shultz et al. 2021, O'Brien, Pham et al. 2021, O'Brien, Wright et al. 2022, Graham, Blissitt et al. 2023). It would be useful in future studies to measure NFL alterations in relation to other commonly tested fluid biomarkers, such as GFAP, tau, UCH-L1. Although they do not all mark for axonal injury, they can also be informative of injury severity and type of damage allowing for fuller comprehension of its position within the realm of blast traumatic brain injury.

It is crucial to acknowledge the limitations of biomarkers and to understand the factors that can impact their concentration levels in order to be useful in humans, as no biomarker exhibits absolute precision.

Age is a factor that could affect NFL levels. This is more of a concern for humans than controlled experimental studies, but it could affect a diagnosis due to higher concentration levels in older patients. NFL was found to be increased 2.2% every year of age in healthy controls in an MS study (Disanto, Barro et al. 2017). Another study reported that between a 50 and 80 year old person, there is a six-fold increase in NFL levels in the blood serum (Gaetani, Blennow et al. 2019). Therefore, the age of a patient or animal model should be considered, because it could lead to different diagnostic outcomes. The measurement of neurofilament light chain (NFL) has demonstrated promise as a biomarker of injury severity, particularly in young individuals (Barro, Chitnis et al. 2020). As mentioned previously, NFL has shown a dose-dependent response of NFL levels to injury (O'Brien, Pham et al. 2021), making it particularly informative in young military personnel returning from a war zone or young athlete from a playing field. Therefore, an elevated level of NFL in a young individual, who would typically have low baseline levels compared to individuals who were very old or had neurological conditions such as multiple sclerosis (MS) or Alzheimer's disease (AD), would indicate traumatic brain injury and its severity. However, in order to explore this potential

confounding effect on NFL concentration in an experimental setting, a study could be designed to assess the effect of blast on older blasted animals and measure their NFL concentrations.

NFL levels are also affected by a higher body mass index (BMI) (Barro, Chitnis et al. 2020, Manouchehrinia, Piehl et al. 2020). Those who have greater BMI, whether through muscle mass or fat, would have a greater blood volume. However, it has been found that despite an inverse relationship between NFL and BMI/blood volume, this variation did not affect the NFL levels in CSF (Manouchehrinia, Piehl et al. 2020). Although this is less of an issue for small rodent studies, it should be noted that blood volume and its cause should be considered a confounding effect on NFL measurements in humans (Barro, Chitnis et al. 2020).

6.5 Final Conclusions

In conclusion, this thesis has presented significant and novel findings, indicating that mild blast TBI elicits functional deficits and more pronounced effects on GABAergic interneurons compared to other blast models, concomitant with an inflammatory response. Additionally, this study has revealed that a single mild bTBI results in increased levels of NFL, in both the CSF and blood plasma at early times post injury, despite a lack of neuronal loss suggesting its detection of diffuse axonal injury and sensitivity in diagnosing and monitoring blast related mild TBI in human patients. Overall, this investigation holds promise and contributes to the advancing understanding of blast TBI pathology, presenting novel interneuronal changes and a portable, sensitive tool for the assessment of mild TBI.

7

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Appendix

Specific Immunostaining protocols

3,3'-diaminobenzidine (DAB) staining using the supersensitive kit

Paraffin sections were deparaffinised and rehydrated using xylene and decreasing concentration of ethanol. The tissue was then washed in PBS followed by permeabilisation and endogenous peroxidase quenching using 1% Hydrogen Peroxidase (H_2O_2) in 1x PBS + 1% Triton-100-X (PBS-Tx) (w/v) for 30 minutes. After this, the slides were washed in PBS and underwent antigen retrieval in 0.01 M Citrate Buffer (pH 6) for 20 minutes in a steamer. Slides were then washed and incubated overnight at 4°C with the primary antibody (Table 2.3).

On the following day, the primary antibody was washed off with PBS, followed by the super sensitive Kit (Table 2.5 and Table 2.6), which was used according to manufacturer's protocol. The slides were then washed and stained with DAB (Table 2.5 and Table 2.6), (GFAP: 2 mins, NeuN: 90 seconds) and then washed with tap water, dehydrated and coverslipped as previously described.

3,3'-diaminobenzidine (DAB) Staining using Avidin-biotin complex (ABC) Horse radish Peroxidase (HRP)-kit

Paraffin sections were deparaffinised and rehydrated using xylene and decreasing concentrations of ethanol. The tissue was then washed in PBS followed by permeabilisation and endogenous peroxidase quenching using 1% Hydrogen Peroxidase (H_2O_2) in 1x PBS + 1% Triton-100-X (PBS-Tx) (w/v) for 30 minutes. Following this, the slides were washed in PBS and underwent antigen retrieval in 0.01M Citrate Buffer (pH6) for 20 minutes in a steamer. The tissue was then blocked using 10% foetal bovine serum (FBS), 10% Normal Goat Serum (NGS) and 1% Bovine Serum Albumin (BSA) for 2 hours. Slides were then washed and incubated

overnight at 4 degrees with the primary antibody (Table 2.3 in 2%FBS/2%NGS/0.2%BSA PBS + 1% Triton-100-X (PBS-Tx) (w/v).p

On the following day, the primary antibody was washed off with PBS, followed by incubation with the biotinylated secondary antibody in 2%FBS/2%NGS/0.2%BSA PBS + 1% Triton-100-X (PBS-Tx) (w/v) (Table 2.3) for 90 min. The slides were then washed and incubated with avidin-biotin complex (ABC) solution in PBS for 40 minutes before being washed again. The slides were then stained with DAB (Table 2.5 and Table 2.6) (PV: 2 minutes) and then washed with tap water. For IBA1, the slides were dehydrated and coverslipped as previously described. For the PV they were counterstained with haematoxylin from Solmedia for 20 seconds and then washed in tap water before dehydration and coverslipping.

[3,3'-diaminobenzidine \(DAB\) Staining using ImmPRESS®HRP Anti-Rabbit IgG Polymer Detection Kit](#)

The impress kit was used particularly for somatostatin staining.

Paraffin sections were deparaffinised and rehydrated using xylene and decreasing concentration of ethanol. The tissue was then washed in PBS followed by permeabilisation and endogenous peroxidase quenching using 1% Hydrogen Peroxidase (H₂O₂) in 1x PBS + 1% Triton-100-X (PBS-Tx) (w/v) for 30 minutes. After this, the slides were washed in PBS and underwent antigen retrieval in 0.01M Citrate Buffer (pH6) for 20 minutes in a steamer. The tissue was then blocked using 10% foetal bovine serum (FBS), 10% Normal or Normal Horse Serum (NHS), depending on the Impress kit, and 1% Bovine Serum Albumin (BSA) in 1% Triton-100-X (PBS-Tx) (w/v) for 2 hours. Slides were then washed and incubated overnight at 4 degrees with the primary antibody (Table 2.3) in 2%FBS/2%NGS or 2% NHS/0.2%BSA + 1% Triton-100-X (PBS-Tx) (w/v).

On the following day, the primary antibody was washed off with PBS, followed by incubation with the Impress kit (1-2 drops/brain) (Table 2.5) for 30 mins. The slides were then washed and then stained with DAB for 2 minutes for IBA1 and 5 minutes for SST (Table 2.5 and Table 2.6),) and then washed with tap water. For IBA1, the slides were dehydrated and

coverslipped as previously described. For the SST they were counterstained with haematoxylin from Solmedia (Table 2.5 hydration and coverslipping).

Supplementary Material

Mixed ANOVA results for NeuN, PV and SST immunostaining for all regions of interest

Au1	NeuN			
	layer 2/3			
		F (DFn, DFd)	p-val	adjusted p-val (Q-val)
	Hemisphere	F (1, 28) = 0.1197	p=0.732	Q=0.8385
	Time post-injury	F (4, 31) = 1.020	p=0.4126	Q=0.8385
	Hemisphere x Time post-injury	F (4, 28) = 0.5788	p=0.6804	Q=0.8385
	layer 4			
		F (DFn, DFd)	p-val	adjusted p (Q-val)
	Hemisphere	F (1, 29) = 0.1631	p=0.6893	Q=0.8385
	Time post-injury	F (4, 31) = 0.2636	p=0.8990	Q=0.9440
	Hemisphere x Time post-injury	F (4, 29) = 1.227	p=0.3212	Q=0.8385
	layer 5			
		F (DFn, DFd)	p-val	adjusted p (Q-val)
	Hemisphere	F (1, 29) = 0.1749,	p=0.6788	Q=0.8385
	Time post-injury	F (4, 31) = 0.6529	p=0.6292	Q=0.8385
	Hemisphere x Time post-injury	F (4, 29) = 1.385	p=0.2638	Q=0.8385
	layer 6			
		F (DFn, DFd)	p-val	adjusted p (Q-val)
	Hemisphere	F (1, 28) = 0.4226	p=0.5209	Q=0.8385
	Time post-injury	F (4, 31) = 0.7959	p=0.5370	Q=0.8385
	Hemisphere x Time post-injury	F (4, 28) = 1.841	p=0.1490	Q=0.8385

Supplementary Table 1 Mixed effect ANOVA results for NeuN immunostaining in the primary auditory cortex (Au1).

Main factors are hemisphere (ipsilateral/contralateral) and time post-injury and their interaction. After Benjamini, Krieger and Yekutieli False Discovery Rate multiple comparison correction (p and $Q > 0.05$) (Naïve/sham: $n=13$, 6 hr: $n=6$, 7DPI, $n=7$, 1 month: $n=6$, 3 month: $n=6$).

V1	NeuN			
	layer 2/3			
		F (DFn, DFd)	p-val	adjusted p-val (Q-val)
	Hemisphere	F (1, 28) = 0.06500	p=0.8006	Q=0.9167
	Time post-injury	F (4, 30) = 0.4843	p=0.7471	Q=0.9167
	Hemisphere x Time post-injury	F (4, 28) = 0.6617	p=0.6238	Q=0.9167
	layer 4			
		F (DFn, DFd)	p-val	adjusted p (Q-val)
	Hemisphere	F (1, 29) = 2.559	p=0.1205	Q=0.8656
	Time post-injury	F (4, 30) = 0.8919	p=0.4809	q=0.8656
	Hemisphere x Time post-injury	F (4, 29) = 1.445	p=0.2446	q=0.8656
	layer 5			
		F (DFn, DFd)	p-val	adjusted p (q-val)
	Hemisphere	F (1, 29) = 0.02601	p=0.8730	q=0.9167
	Time post-injury	F (4, 30) = 1.111	p=0.3695	q=0.8656
	Hemisphere x Time post-injury	F (4, 29) = 0.9405	p=0.4546	q=0.8656
	layer 6			
		F (DFn, DFd)	p-val	adjusted p (q-val)
	Hemisphere	F (1, 29) = 0.03694	p=0.8489	q=0.9166
	Time post-injury	F (4, 30) = 1.205	p=0.3290	q=0.8656
	Hemisphere x Time post-injury	F (4, 29) = 1.217	p=0.3252	q=0.8656

Supplementary Table 2 Mixed effect ANOVA results for NeuN immunostaining in the primary Visual (V1).

Main factors are hemisphere (ipsilateral/contralateral) and time post-injury and their interaction. After Benjamini, Krieger and Yekutieli False Discovery Rate multiple comparison correction (p and $Q > 0.05$) (Naïve/sham: $n=13$, 6 hr: $n=6$, 7DPI, $n=7$, 1 month: $n=6$, 3 month: $n=6$).

RSC	NeuN			
	layer 2/3			
		F (DFn, DFd)	p-val	adjusted p-val (q-val)
	Hemisphere	F (1, 29) = 0.02224	p=0.8825	q=0.9974
	Time post-injury	F (4, 31) = 1.528	p=0.2184	q=0.4368
	Hemisphere x Time post-injury	F (4, 29) = 1.590	p=0.2034	q=0.4368
	layer 4			
		F (DFn, DFd)	p-val	adjusted p (q-val)
	Hemisphere	F (1, 29) = 0.006323	p=0.9372	q=0.9974
	Time post-injury	F (4, 31) = 1.739	p=0.1665	q=0.4368
	Hemisphere x Time post-injury	F (4, 29) = 0.03582	p=0.9974	q=0.9974
	layer 5			
		F (DFn, DFd)	p-val	adjusted p (q-val)
	Hemisphere	F (1, 30) = 0.8007	p=0.3780	q=0.6480
	Time post-injury	F (4, 31) = 2.122	p=0.1017	q=0.4068
	Hemisphere x Time post-injury	F (4, 30) = 4.079	p=0.0093	q=0.1116
	layer 6			
		F (DFn, DFd)	p-val	adjusted p (q-val)
	Hemisphere	F (1, 30) = 3.515	p=0.0706	q=0.6480
	Time post-injury	F (4, 31) = 0.9532	p=0.4468	q=0.4068
	Hemisphere x Time post-injury	F (4, 30) = 0.6647	p=0.6214	q=0.1116

Supplementary Table 3 Mixed effect ANOVA results for NeuN immunostaining in the Retrosplenial cortex (RSC).

Main factors are hemisphere (ipsilateral/contralateral) and time post-injury and their interaction after Benjamini, Krieger and Yekutieli False Discovery Rate multiple comparison correction (p and $q > 0.05$) (Naïve/sham: $n=13$, 6 hr: $n=6$, 7DPI, $n=7$, 1 month: $n=6$, 3 month: $n=6$).

Au1	PV			
	layer 2/3			
		F (DFn, DFd)	p-val	adjusted p-val (q-val)
	Hemisphere	F (1, 30) = 3.515	p=0.3257	q=0.3800
	Time post-injury	F (4, 33) = 1.372	p=0.3478	q=0.3478
	Hemisphere x Time post-injury	F (4, 32) = 0.5084	p=0.7299	q=0.6699
	layer 4			
		F (DFn, DFd)	p-val	adjusted p (q-val)
	Hemisphere	F (1, 32) = 5.847	p=0.0215	q=0.0753
	Time post-injury	F (4, 32) = 2.268	p=0.0836	q=0.2195
	Hemisphere x Time post-injury	F (4, 32) = 1.028	p=0.4080	q=0.4284
	layer 5			
		F (DFn, DFd)	p-val	adjusted p (q-val)
	Hemisphere	F (1, 32) = 2.739	p=0.1077	q=0.2262
	Time post-injury	F (1, 32) = 0.09043	p=0.7656	q=0.6699
	Hemisphere x Time post-injury	F (4, 32) = 1.487	p=0.2292	q=0.3438
	layer 6			
		F (DFn, DFd)	p-val	adjusted p (q-val)
	Hemisphere	F (1, 32) = 0.09043	p=0.7656	q=0.6699
	Time post-injury	F (4, 33) = 4.252	p=0.0069	q=0.0362
	Hemisphere x Time post-injury	F (4, 32) = 1.675	p=0.1801	q=0.3152

Supplementary Table 4 Mixed effect ANOVA results for parvalbumin immunostaining in the Primary Auditory Cortex (Au1).

Main factors are hemisphere (ipsilateral/contralateral) and time post-injury and their interaction. No significant findings were found ($p>0.05$) other than time post-injury in layer 6 after Benjamini, Krieger and Yekutieli False Discovery Rate multiple comparison correction highlighted in bold text. (Naïve/sham: $n=13$, 6 hr: $n=6$, 7DPI, $n=6$, 1 month: $n=7$, 3 month: $n=6$)

V1	PV			
	layer 2/3			
		F (DFn, DFd)	p-val	adjusted p-val (q-val)
	Hemisphere	F (1, 30) = 0.5906	p=0.4482	q=0.7059
	Time post-injury	F (4, 32) = 1.202	p=0.329	q=0.6909
	Hemisphere x Time post-injury	F (4, 30) = 1.525	p=0.2202	q=0.5549
	layer 4			
		F (DFn, DFd)	p-val	adjusted p (q-val)
	Hemisphere	F (1, 30) = 0.1176	p=0.7341	q=0.925
	Time post-injury	F (4, 32) = 0.04696	p=0.9956	q=1.0000
	Hemisphere x Time post-injury	F (4, 30) = 1.029	p=0.4084	q=0.7059
	layer 5			
		F (DFn, DFd)	p-val	adjusted p (q-val)
	Hemisphere	F (1, 31) = 3.441	p=0.0731	q=0.5377
	Time post-injury	F (4, 32) = 0.3560	p=0.8379	q=0.9598
	Hemisphere x Time post-injury	F (4, 31) = 1.767	p=0.1607	q=0.5377
	layer 6			
		F (DFn, DFd)	p-val	adjusted p (q-val)
	Hemisphere	F (1, 31) = 1.967	p=0.1707	q=0.5377
	Time post-injury	F (4, 32) = 0.6171	p=0.6535	q=0.9149
	Hemisphere x Time post-injury	F (4, 31) = 1.819	p=0.1502	q=0.5377

Supplementary Table 5 Mixed effect ANOVA results for parvalbumin immunostaining in the primary visual cortex (V1).

Main factors are hemisphere (ipsilateral/contralateral) and time post-injury and their interaction. No differences were found after Benjamini, Krieger and Yekutieli False Discovery Rate multiple comparison correction (p and q>0.05) (Naïve/sham: n=13, 6 hr: n=6, 7DPI, n=6, 1 month: n=7, 3 month: n=6).

RSC	PV			
	layer 2/3			
		F (DFn, DFd)	p-val	adjusted p-val (q-val)
	Hemisphere	F (1, 33) = 0.1012	p=0.7523	q=0.8617
	Time post-injury	F (4, 34) = 2.445	p=0.0653	q=0.2637
	Hemisphere x Time post-injury	F (4, 33) = 1.929	p=0.1288	q=0.2637
	layer 4			
		F (DFn, DFd)	p-val	adjusted p (q-val)
	Hemisphere	F (1, 32) = 0.03013	p=0.8633	q=0.9065
	Time post-injury	F (4, 34) = 1.875	p=0.1374	q=0.2637
	Hemisphere x Time post-injury	F (4, 32) = 1.431	p=0.2465	q=0.3882
	layer 5			
		F (DFn, DFd)	p-val	adjusted p (q-val)
	Hemisphere	F (1, 32) = 2.791	p=0.1045	q=0.2637
	Time post-injury	F (4, 34) = 2.152	p=0.0957	q=0.2637
	Hemisphere x Time post-injury	F (4, 32) = 2.242	p=0.0865	q=0.2637
	layer 6			
		F (DFn, DFd)	p-val	adjusted p (q-val)
	Hemisphere	F (1, 32) = 0.3344	p=0.5672	q=0.7941
	Time post-injury	F (4, 34) = 1.827	p=0.1465	q=0.2637
	Hemisphere x Time post-injury	F (4, 32) = 0.4786	p=0.7512	q=0.8617

Supplementary Table 6 Mixed effect ANOVA results for parvalbumin immunostaining in the retrosplenial cortex (RSC)

. Main factors are hemisphere (ipsilateral/contralateral) and time post-injury and their interaction. After Benjamini, Krieger and Yekutieli False Discovery Rate multiple comparison correction no differences were found (p and $q > 0.05$) (Naïve/sham: $n=13$, 6 hr: $n=6$, 7DPI, $n=6$, 1 month: $n=7$, 3 month: $n=6$).

SST

Au1

layer 2/3			
	F (DFn, DFd)	p-val	adjusted p-val (q-val)
Hemisphere	F (1, 30) = 3.515	p=0.3257	q=0.3800
Time post-injury	F (4, 33) = 1.372	p=0.3478	q=0.3478
Hemisphere x Time post-injury	F (4, 32) = 0.5084	p=0.7299	q=0.6699
layer 4			
	F (DFn, DFd)	p-val	adjusted p (q-val)
Hemisphere	F (1, 67) = 0.1953	p=0.6599	q=0.5774
Time post-injury	F (4, 67) = 1.931	p=0.1154	q=0.4039
Hemisphere x Time post-injury	F (4, 67) = 0.7004	p=0.5944	q=0.5774
layer 5			
	F (DFn, DFd)	p-val	adjusted p (q-val)
Hemisphere	F (1, 33) = 1.204	p=0.2804	q=0.5627
Time post-injury	F (4, 34) = 4.317	p=0.0062	q=0.0394
Hemisphere x Time post-injury	F (4, 33) = 4.184	p=0.0075	q=0.0394
layer 6			
	F (DFn, DFd)	p-val	adjusted p (q-val)
Hemisphere	F (1, 33) = 0.5050	p=0.4823	q=0.5627
Time post-injury	F (4, 34) = 1.016	p=0.4132	q=0.5627
Hemisphere x Time post-injury	F (4, 33) = 0.9855	p=0.4289	q=0.5627

Supplementary Table 7 Somatostatin mixed effects analysis in the primary auditory cortex (Au1).

SST

findings were found ($p>0.05$) other than time post-injury in layer 5 after Benjamini, Krieger and Yekutieli False Discovery Rate multiple comparison correction highlighted in bold text. (Naïve/sham: $n=13$, 6 hr: $n=5$, 7DPI, $n=6$, 1 month: $n=8$, 3 month: $n=6$).

V1

layer 2/3			
	F (DFn, DFd)	p-val	adjusted p-val (q-val)
Hemisphere	F (1, 30) = 1.436	p=0.2401	q=0.9067
Time post-injury	F (4, 34) = 0.8487	p=0.5044	q=0.9067
Hemisphere x Time post-injury	F (4, 30) = 0.9531	p=0.4473	q=0.9067
layer 4			
	F (DFn, DFd)	p-val	adjusted p (q-val)
Hemisphere	F (1, 32) = 0.4506	p=0.5069	q=0.9067
Time post-injury	F (4, 34) = 2.934	p=0.0347	q=0.2186
Hemisphere x Time post-injury	F (4, 32) = 0.4219	p=0.7916	q=0.9067
layer 5			
	F (DFn, DFd)	p-val	adjusted p (q-val)
Hemisphere	F (1, 32) = 0.08857	p=0.7679	q=0.9067
Time post-injury	F (4, 34) = 3.839	p=0.0111	q=0.1399
Hemisphere x Time post-injury	F (4, 32) = 0.6107	p=0.6579	q=0.9067
layer 6			
	F (DFn, DFd)	p-val	adjusted p (q-val)
Hemisphere	F (1, 32) = 0.02151	p=0.8843	q=0.9285
Time post-injury	F (4, 34) = 1.166	p=0.3429	q=0.9067
Hemisphere x Time post-injury	F (4, 32) = 0.6984	p=0.5987	q=0.9067

Supplementary Table 8 Somatostatin (SST) mixed effects analysis in the primary visual cortex (V1).

Main factors are hemisphere (ipsilateral/contralateral) and time post-injury and their interaction. No significant findings were found after using Benjamini, Krieger and Yekutieli False Discovery Rate multiple comparison correction (p and $q > 0.05$) Naïve/sham: $n=13$, 6 hr: $n=5$, 7DPI, $n=6$, 1 month: $n=8$, 3 month: $n=6$.

RSC	SST			
	layer 2/3			
		F (DFn, DFd)	p-val	adjusted p-val (q-val)
	Hemisphere	F (1, 32) = 0.2282	p=0.6361	q=0.9819
	Time post-injury	F (4, 34) = 3.026	p=0.0309	q=0.3074
	Hemisphere x Time post-injury	F (4, 32) = 0.3920	p=0.8128	q=0.9819
	layer 4			
		F (DFn, DFd)	p-val	adjusted p (q-val)
	Hemisphere	F (1, 31) = 0.03291	p=0.8572	q=0.9819
	Time post-injury	F (4, 34) = 0.4576	p=0.7662	q=0.9819
	Hemisphere x Time post-injury	F (4, 31) = 0.05064	p=0.9949	q=1.0000
	layer 5			
		F (DFn, DFd)	p-val	adjusted p (q-val)
	Hemisphere	F (1, 32) = 2.036	p=0.1633	q=0.5478
	Time post-injury	F (4, 34) = 2.669	p=0.0488	q=0.3074
	Hemisphere x Time post-injury	F (4, 32) = 1.529	p=0.2174	q=0.5478
	layer 6			
		F (DFn, DFd)	p-val	adjusted p (q-val)
	Hemisphere	F (1, 33) = 1.617	p=0.2124	q=0.5478
	Time post-injury	F (4, 34) = 0.5832	p=0.6769	q=0.9819
	Hemisphere x Time post-injury	F (4, 33) = 0.8453	p=0.5066	q=0.9819

Supplementary Table 9 Somatostatin (SST) mixed effects analysis in the retrosplenial cortex (RSC).

Main factors are hemisphere (ipsilateral/contralateral) and time post-injury and their interaction. No significant findings were found ($p > 0.05$) (Naïve/sham: $n=13$, 6 hr: $n=5$, 7DPI, $n=6$, 1 month: $n=8$, 3 month: $n=6$).

IBA1		
	F (DFn, DFd)	p-val
Au1	Hemisphere	F (1, 59) = 0.01627
	Time post-injury	F (4, 59) = 5.299
	Hemisphere x Time post-injury	F (4, 59) = 0.2335
	F (DFn, DFd)	p-val
V1	Hemisphere	F (4, 53) = 0.5167
	Time post-injury	F (1, 53) = 1.986
	Hemisphere x Time post-injury	F (4, 53) = 2.157
	F (DFn, DFd)	p-val
RSC	Hemisphere	F (4, 54) = 0.9067
	Time post-injury	F (1, 54) = 0.7657
	Hemisphere x Time post-injury	F (4, 54) = 5.534

Supplementary Table 10 IBA1 two-way ANOVA results for primary auditory cortex (Au1), primary visual cortex (V1) and the Retrosplenial cortex (RSC).

Main factors are hemisphere (ipsilateral/contralateral) and time post-injury and their interaction. Significant findings for time post-injury or interaction ($p < 0.05$) and shown in bold. No other significant findings were found ($p > 0.05$). (Naïve/sham: $n=12$, 6 hr: $n=6$, 7DPI, $n=6$, 1 month: $n=5$, 3 month: $n=6$)

GFAP		
	F (DFn, DFd)	p-val
Au1	Hemisphere	F (1, 34) = 0.4523
	Time post-injury	F (4, 34) = 0.7771
	Hemisphere x Time post-injury	F (4, 34) = 0.3437
	F (DFn, DFd)	p-val
V1	Hemisphere	F (1, 28) = 0.6666
	Time post-injury	F (4, 31) = 1.553
	Hemisphere x Time post-injury	F (4, 28) = 0.4820
	F (DFn, DFd)	p-val
RSC	Hemisphere	F (1, 33) = 0.1681
	Time post-injury	F (4, 34) = 1.113
	Hemisphere x Time post-injury	F (4, 33) = 1.060

Supplementary Table 11 GFAP two-way ANOVA results for primary auditory cortex (Au1), primary visual cortex (V1) and the Retrosplenial cortex (RSC).

Main factors are hemisphere (ipsilateral/contralateral) and time post-injury and their interaction. Significant findings for time post-injury or interaction ($p < 0.05$) and shown in bold. No other significant findings were found ($p > 0.05$). (Naïve/sham: $n=12$, 6 hr: $n=6$, 7DPI, $n=6$, 1 month: $n=8$, 3 month: $n=6$)

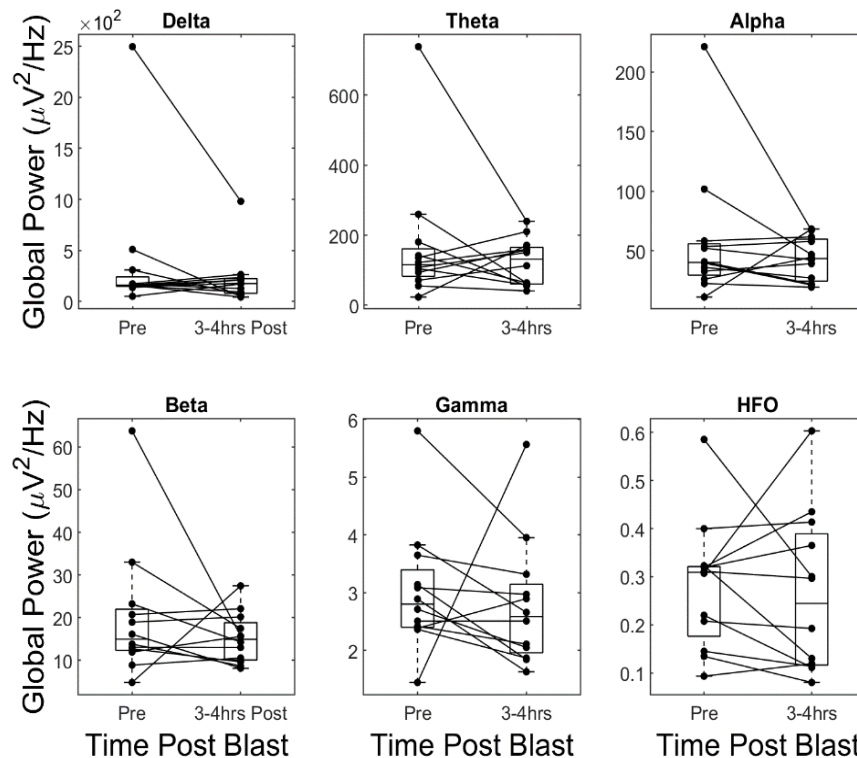
Supplementary Material 2

Collaborative project blast TBI electroencephalogram results

Electrophysiological Investigations were conducted by collaborating lab using the blast model. The animals were blast by me in combination with Dr Bruyns-Haylett and Konstantinos Tsikonofilos but the electrophysiological experiments were conducted by them. Investigations into absolute and relative power were conducted in male Sprague Dawley rats. Analysis was conducted by Konstantinos Tsikonofilos.

Animals were split into two separate EEG cohorts, one for the acute time points, where animals had a pre-blast EEG recording followed by single blast exposure. After this blast exposure, the rats had a second EEG recording at 3-4 hours post-injury. This was to understand what acute changes occurred soon after a blast (n=12 animals). The other cohort had no pre-blast EEG recording. Instead, these animals were exposed to a single blast exposure and allowed to recover. At 1 (n=13) or 3 (n=20) months post-injury, these animals were then subject to EEG recordings. Shams for the 1-3 months (n sham 1 month = 8, n sham month = 9) were conducted in the same manner, except they had no blast wave exposure, but the same anaesthesia and analgesia protocol was used.

Absolute power measures the total power of each frequency band. It represents the overall magnitude of brain activity in a specific frequency band in the EEG signal. Higher values would indicate that a greater amount of activity was occurring in that frequency band. The results from the acute time (3-4 hours post-injury), referred to as “0day”, showed that there were no absolute power changes in any of the frequency bands between pre and 3-4hours post-blast which was unexpected ($n=12$, $p>0.05$) (Supplementary Material 2 Figure 1

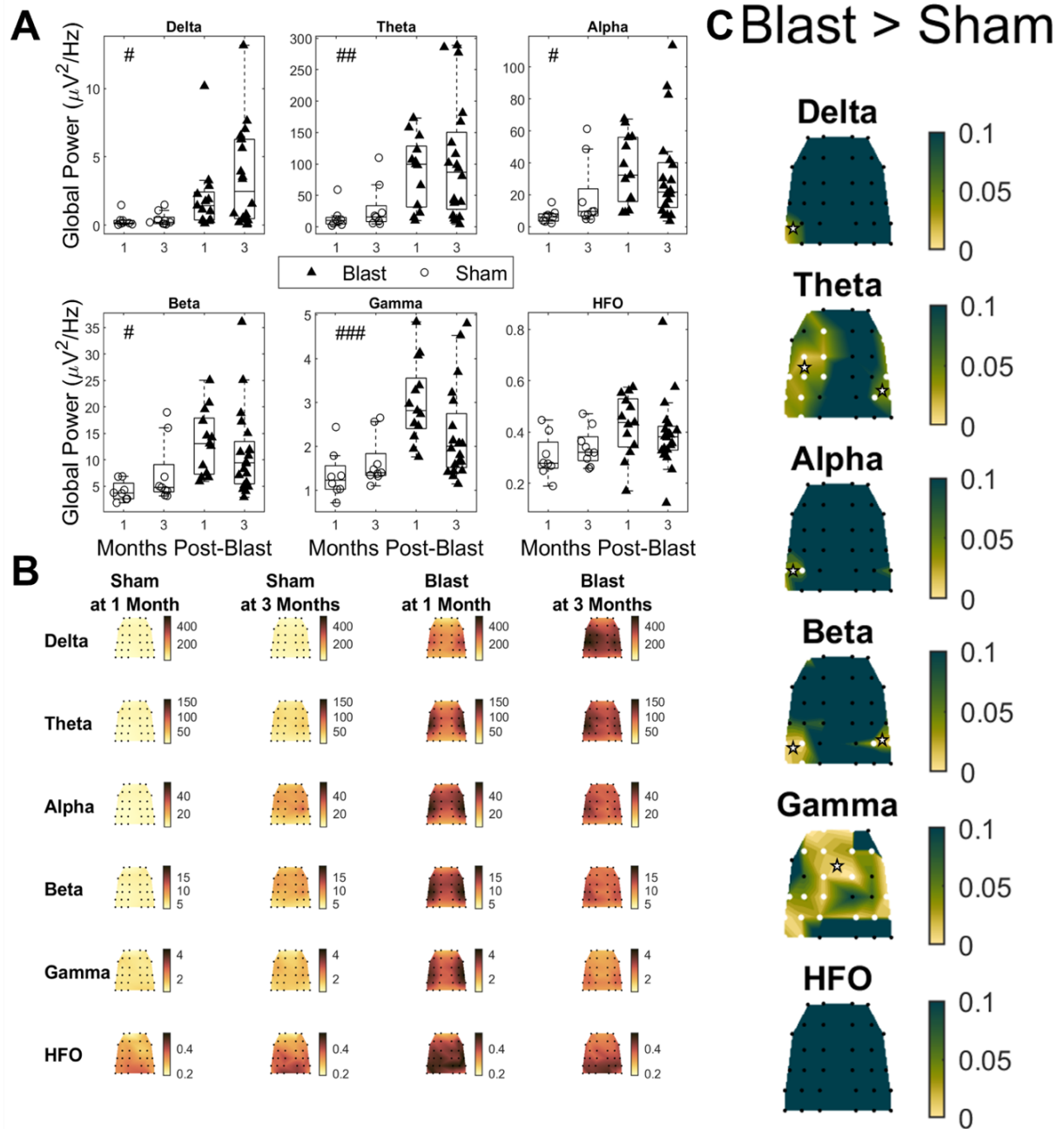


Supplementary Material 2 Figure 1 Absolute power analysis in single mild blast cohort at pre-blast and 3-4 hours post-blast.

Analysis found no differences seen in any of the frequency bands. Paired t-tests with Bonferroni correction for multiple comparisons were conducted on pre blasted EEG absolute power measurements and 3-4 hours post-blast measurements on each of the frequency bands ; Delta: 1-4 Hz, Theta: 4-8 Hz, Alpha: 8-12 Hz, Beta: 12-25 Hz, Gamma: 25-80 Hz, HFO: 80-200Hz. $P\text{-adj}>0.05$. ($n= 12$) Analysis and figure by Konstantinos Tsikonofilios.

For the chronic cohort of animals, without pre-blast EEG recording, the comparison group were time-matched shams. It was observed that in all frequency bands, there was an increase in absolute power for both 1 and 3 months post-injury irrespective of time post-injury in all frequency bands (Supplementary Material 2 Figure 2A). The mean power is depicted in

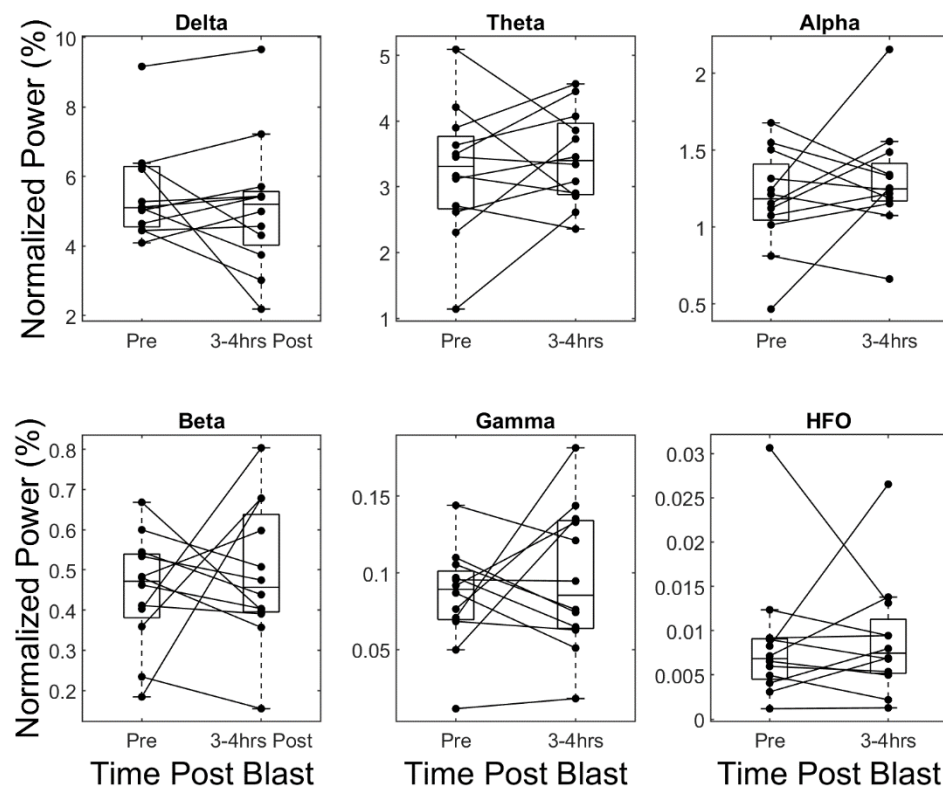
topoplots (Supplementary Material 2 Figure 2B), which showed on the electrode array where these changes were occurring. Interestingly, the blasted groups had their most significant power increases (Supplementary Material 2 Figure 2C, white stars) on the contralateral side of the brain to the blast wave for most frequency bands, except for gamma, which appeared more widely spread. This absolute power increase suggests post-TBI hyperexcitability within the brain at the chronic time points of 1 and 3 months post-injury.



Supplementary Material 2 Figure 2 Absolute power in Blast and Sham groups at 1- and 3-months post-injury.

(A) Global absolute power for all groups across all frequency bands. Delta: 1-4 Hz, Theta: 4-8 Hz, Alpha: 8-12 Hz, Beta: 12-25 Hz, Gamma: 25-80 Hz, HFO: 80-200Hz. Permutation-based 2-way ANOVA (Group x Time Post-Blast with interaction, 10000 iterations) with Bonferroni correction for multiple comparisons ($n = 6$ bands). Significant main effects of group were found for all frequency bands except for the HFO band. Significance values for all tests #, $P\text{-adj} < 0.05$ main effect of group, ##: $P\text{-adj} < 0.01$ main effect of group, ###: $P\text{-adj} < 0.001$ main effect of group, (B) Topoplots depicting mean group absolute power for all groups and frequency bands, across the multielectrode array. C) Topoplots depicting p -values for the main effect of group (permutation-based 2-way ANOVA, 10000 iterations), Bonferroni corrected for multiple comparisons ($n = 32$ channels) for all bands. Channels for which the adjusted p -value is below a threshold of 0.05 are marked with white electrode dots. Blast electrode dots denote > 0.05 . Regions that are significant is denoted by white stars.. (blast 1 month $n = 13$, blast month 3 $n = 20$, sham 1 month $n = 8$, sham 3 month $n = 9$) Analysis and figure made by Konstantinos Tsikonofilios.

Relative power was also analysed for the acute and chronic time points. Relative power (also known as normalised power) measures what proportion or percentage of power within a specific frequency band is, relative to all the rest of the frequency bands. For the 0 day cohort there was no difference in the normalised power (Supplementary Material 2 Figure 3).

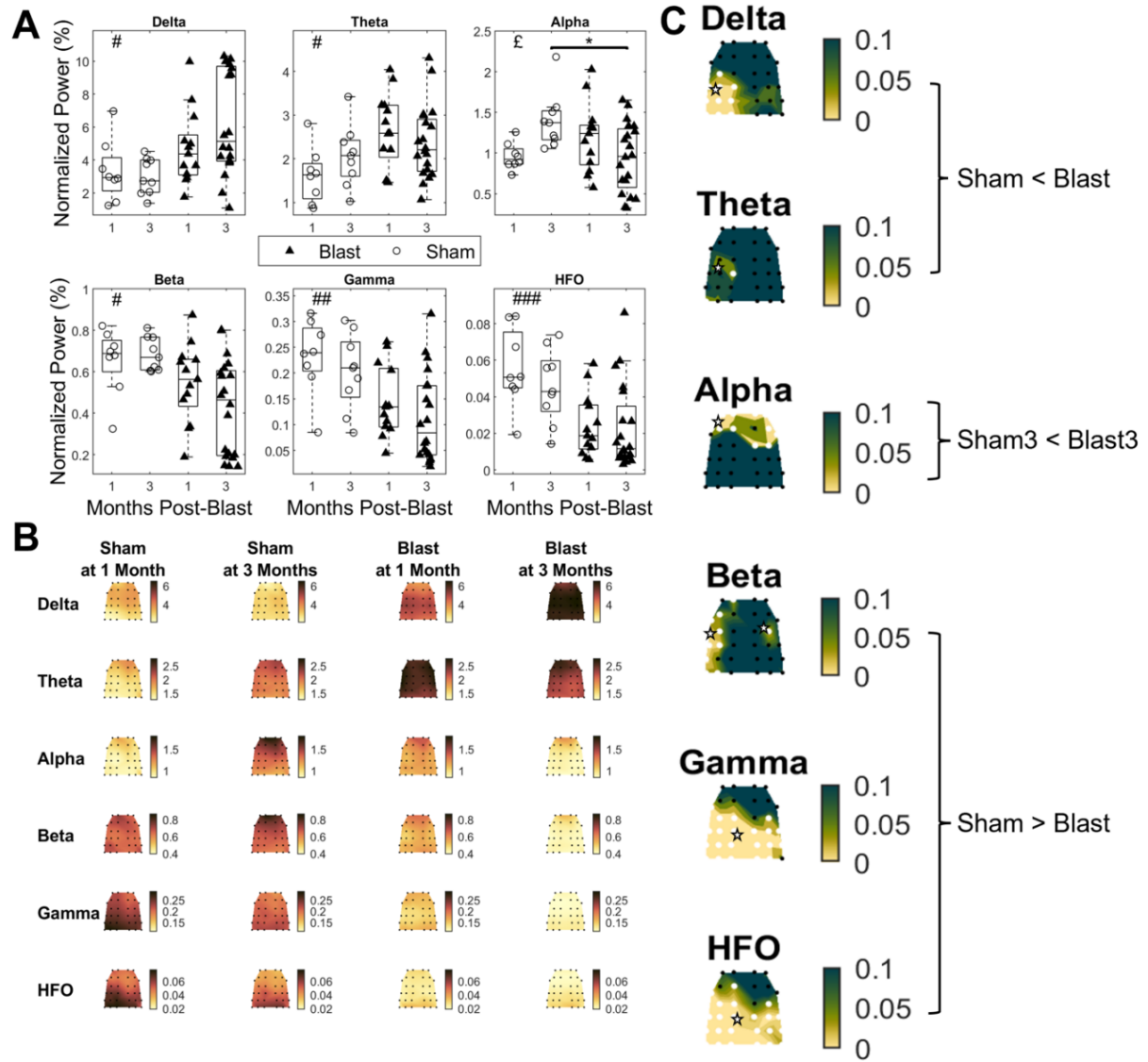


Supplementary Material 2 Figure 3 Normalised power in blast exposed 0day animals between pre-blast and 3-4 hours post-blast.

No differences were found after analysis. Paired t-tests with Bonferroni correction for multiple comparisons. $P_{adj} > 0.05$. (n=12) Analysis and figure made by Konstantinos Tsikonofilios.

There were differences in the chronic time points of relative power between 1 month blast and 3 months post-injury relative to their control groups (Supplementary Material 2 Figure 4A). There was a significant increase in the low frequencies (delta and theta) and no statistical significance between 1 and 3 months post-injury. In addition, the higher frequencies such as beta, gamma and high frequency oscillations were significantly decreased. These findings are similar to the hallmark pathologies reported in post-TBI patients (Mallas, Gorgoraptis et al. 2022).

The topoplots also revealed the electrode locations of this shift towards the low frequency powers in animals that were subject to single mild blast, and depicted that the contralateral side to the injury was the location of the greater mean change apart from gamma, which was located more widely and caudally (Supplementary Material 2 Figure 4B and C).

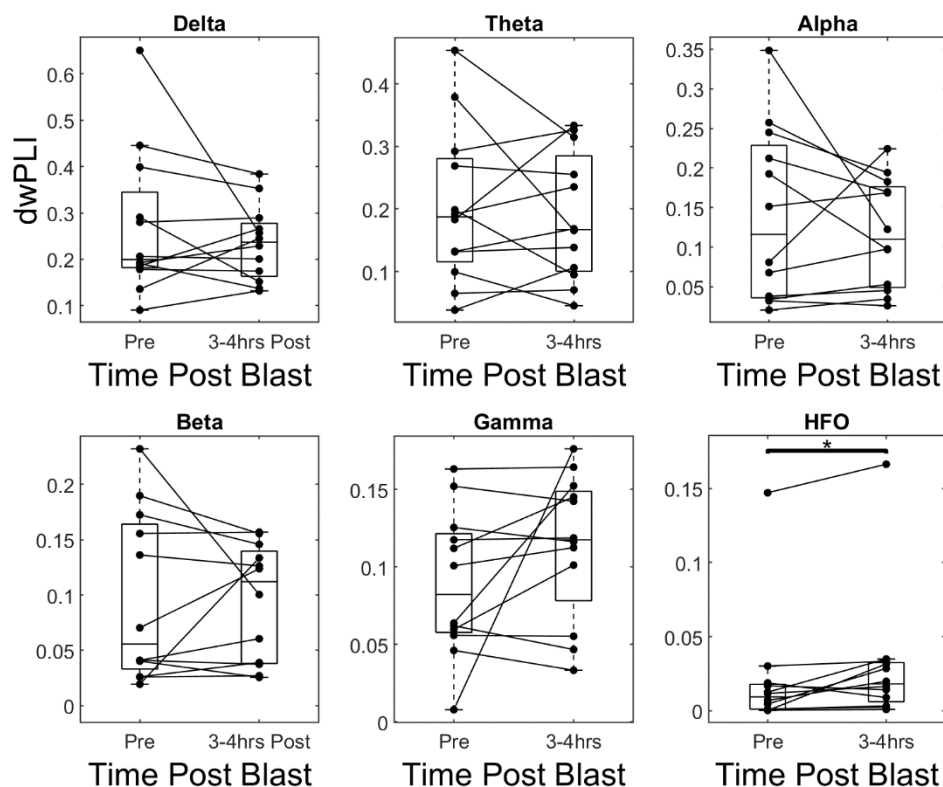


Supplementary Material 2 Figure 4 Absolute power in Blast and Sham groups at 1- and 3-months post-injury.

(A) Global absolute power for all groups across all frequency bands. Delta: 1-4 Hz, Theta: 4-8 Hz, Alpha: 8-12 Hz, Beta: 12-25 Hz, Gamma: 25-80 Hz, HFO: 80-200Hz. Permutation-based 2-way ANOVA (Group $\times$ Time Post-Blast with interaction, 10000 iterations) with Bonferroni correction for multiple comparisons (n = 6 bands). Significant main effects of group were found for all frequency bands except for the HFO band. Significance values for all tests #, $P\text{-adj} < 0.05$ main effect of group, ##: $P\text{-adj} < 0.01$ main effect of group, ###: $P\text{-adj} < 0.001$ main effect of group, (B) Topoplots depicting mean group absolute power for all groups and frequency bands, across the multielectrode array. (C) Topoplots depicting p-values for the main effect of group (permutation-based 2-way ANOVA, 10000 iterations), Bonferroni corrected for multiple comparisons (n = 32 channels) for all bands. Channels for which the adjusted p-value is below a threshold of 0.05 are marked with white electrode dots. Blast electrode dots denote > 0.05 . Regions that are significant is denoted by white stars.. (blast 1 month n = 13, blast month 3 n = 20, sham 1 month n = 8, sham 3 month n = 9) Analysis and figure made by Konstantinos Tsikonofilios.

Functional connectivity analysis was conducted using phase-based functional connectivity metric, called debiased weighted phase lag index (DWPLI). The debiased weighted Phase Lag Index (DWPLI) is a measure used to quantify phase synchrony between two brain regions, whilst taking into account partial volume. Phase synchrony refers to the temporal coordination or alignment of the phase of neural oscillations between different brain regions. It indicates that these regions are more likely to be functionally connected and engaged in coordinated information processing.

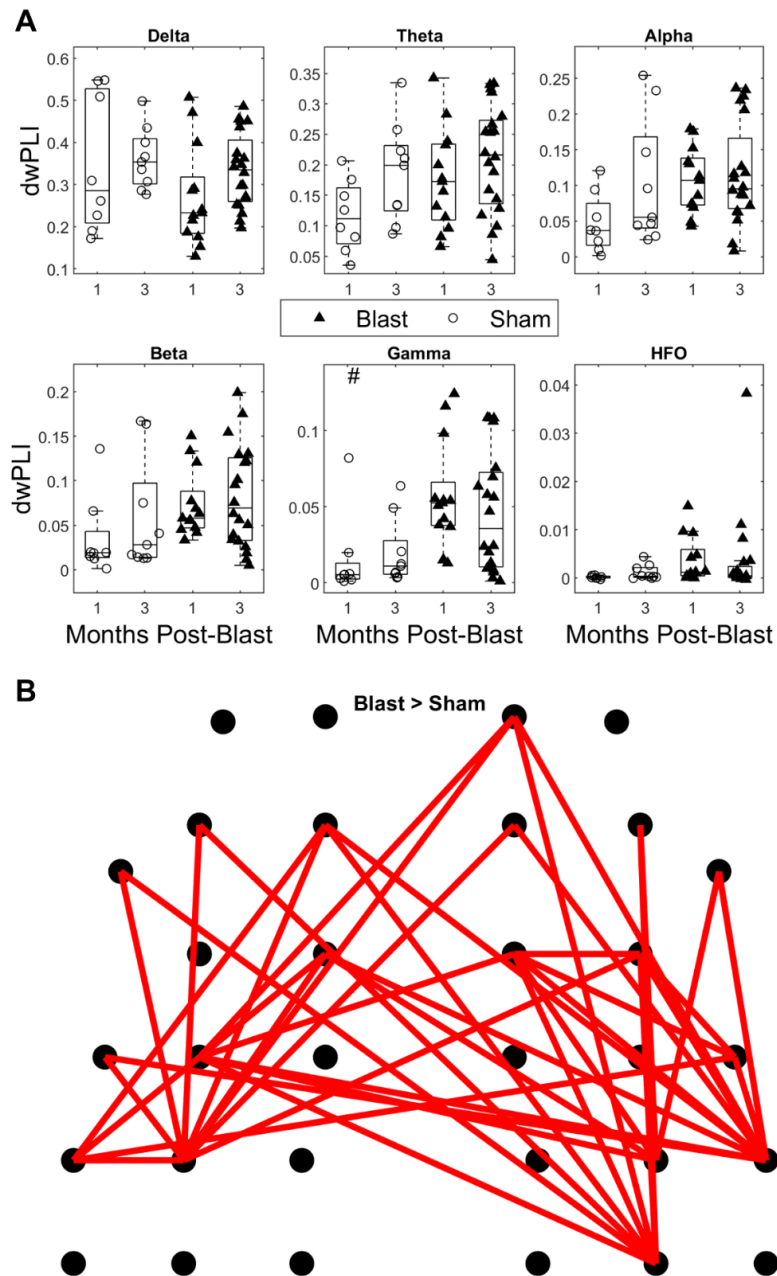
The analysis showed that there were no changes in the 0 day animals except for a significant increase in the high frequency oscillations (HFO) (Supplementary Material 2 Figure 5).



Supplementary Material 2 Figure 5 Phase-based functional connectivity (DWPLI) in pre-blast and 3-4hours post-blast.

Permutation-based paired *t* test, 10000 permutations, Bonferroni corrected for $n=6$ bands. $P\text{-adj}>0.05$ ($n=12$). Increased functional connectivity at 3-4 hours in the high frequency oscillation band only. Analysis and figure made by Konstantinos Tsikonofilios.

FC in the chronic time points was conducted also using DWPLI and the results showed no significant changes in the frequency bands, other than gamma. The gamma frequency band was found to be significantly increased at both 1 and 3 months post-injury (Supplementary Material 2 Figure 6). The results of the 1 and 3 month post-blast data were then pooled, as they showed no differences between the time points. An edge analysis was conducted between the electrodes to investigate between which electrodes had the connectivity increase. The electrodes that had hyperconnected pairs were located in the middle of the brain, not too frontal nor caudal. These results show the heightened functional connectivity in the gamma band following blast injury.



Supplementary Material 2 Figure 6 Phase-based functional connectivity (dwPLI) in Blast and Sham groups at 1- and 3-months post-injury.

(A) Global connectivity for all groups. Permutation-based 2-way ANOVA (Group x Time Post-Blast with interaction, 10000 iterations) with Bonferroni correction for multiple comparisons ($n = 6$ bands). A significant main effect of group was found only for the gamma band. Significance values for all tests *: $P\text{-adj} < 0.05$. (B) Topoplot depicting a significantly hyperconnected network for the blast group. Independent-samples t-test (blast vs sham) based on 50000 permutations with an FDR-adjusted threshold of $p = 0.05$, using the Network-based statistic toolbox. (blast 1 month $n = 13$, blast month 3 $n = 20$, sham 1 month $n = 8$, sham 3 month $n = 9$). Analysis and figure made by Konstantinos Tsikonofilios.

Overall findings of the EEG results in the blast-exposed rats

The collaborative EEG analysis conducted by Konstantinos Tsikonofilos revealed that animals exposed to blasts exhibited an overall increase in power across all frequency bands in the brain at chronic time points. Furthermore, the analysis demonstrated a shift towards lower frequency bands when examining relative power at chronic time points. This indicates that, although the entire brain displayed increased activity across all bands, the majority of this increase was observed in the lower frequency bands.

Additionally, the EEG analysis revealed that the gamma band was the only frequency band showing increased functional connectivity at chronic time points. This suggests enhanced transfer of information and synchronisation in the neural signals within the gamma band. No significant differences were found in most of the analysed measures when considering acute time points, except for a change in functional connectivity in the high-frequency oscillation band.

Supplementary Material 3

White matter analysis using MRI-DTI and Neurofilament Light in a blunt injury model

Dr Cornelius Donat conducted the CCI experiments on Male Sprague Dawley rats (methods 2.4) and Dr Maria Yanez-Lopez and Dr Nicoleta Baxan conducted the MRI scans in the CCI model, to determine white matter damage at different time points and severity levels. MRI scans were conducted on the day of termination, at 3 and 14 days post-injury (DPI) for the moderate injury severity (2mm deep CCI) and at 14 DPI for the mild severity (1mm deep CCI) (as described in (Donat, Yanez Lopez et al. 2021)).

The main white matter tract in the rodent brain, the CC was divided into 5 segments per hemisphere (as described in methods and in (Graham, Zimmerman et al. 2021)). Analysis was carried out in each CC segment of the CCI injury model. I analysed the 3 day post injury (DPI) data (Naïve/sham 3 DPI n=8, naïve/Sham 14 DPI: n=10, Moderate 3 DPI: n=8) and compared it to already previously published 14 DPI data (mild 14 DPI: n=10, moderate 14 DPI: n=11) (Donat, Yanez Lopez et al. 2021). I conducted repeated two-way ANOVAs on all the injury groups, (factors: segments, hemisphere and interaction) on all MRI measures.

MRI DTI and NODDI indicates loss of white matter integrity at acute and sub-acute time points and in both mild and moderate CCI

Fractional anisotropy (FA) is a scalar value that quantifies the degree of anisotropy or directionality of water diffusion within a voxel. It reflects the coherence and organisation of white matter fibres. Higher FA values indicate more restricted and directionally aligned diffusion, suggesting greater white matter integrity and fibre density. Conversely, lower FA values suggest less organised or disrupted white matter tracts.

FA changes were compared between ipsilateral and contralateral sites of the injury, having divided the CC in different segments, 5 segments per hemisphere. Segment 1 for each hemisphere represents the segment closest to the midline.

FA at 3 DPI in the moderate injury model, revealed significant main effects of segment (1-5) and hemisphere (ipsilateral/contralateral) but no interaction (Supplementary Material 3 Table 1). Post-hoc testing revealed the ipsilateral (side of the impact) hemisphere was significantly reduced (-12.62%, $p < 0.0001$) (Supplementary Material 3 Figure 1A). Results for mild CCI at 14 DPI were similar to the moderate model at 3 DPI. The mild 14 DPI data (Supplementary Material 3 Table 1 and Supplementary Material 3 Figure 1B) had significant main effects of interaction, segment but not hemisphere (Supplementary Material 3 Table 1) driven by FA reductions in the ipsilateral segments 3 and 4 (segment 3: -10.40%, $p = 0.0102$ segment 4: -13.62%, $p = 0.0002$) these segments are closest to the injury site.

The moderate impact at 14 DPI showed a greater reduction in FA (Supplementary Figure 7C and Supplementary Table 1). There was a significant interaction between segment and hemisphere (Supplementary Material 3 Table 1 and Supplementary Material 3 Figure 1). This resulted from significant reductions of FA in the ipsilateral segments 3 and 4 compared to the contralateral hemispheres (segment 3: -31.26%, $p = 0.0001$ and segment 4: -30.86%, $P < 0.0001$).

FA			
3 DPI moderate	F (DFn, DFd)		P value
	Segment x Hemisphere	F (4, 35) = 1.680	p=0.1768
	Segment Hemisphere	F (4, 35) = 50.35 F (1, 35) = 39.92	P<0.0001 P<0.0001
14 DPI moderate	F (DFn, DFd)		P value
	Segment x Hemisphere	F (4, 50) = 3.786	p=0.0091
	Segment Hemisphere	F (4, 50) = 22.10 F (1, 50) = 30.72	P<0.0001 P<0.0001
14 DPI mild	F (DFn, DFd)		P value
	Segment x Hemisphere	F (4, 45) = 7.960	P<0.0001
	Segment Hemisphere	F (4, 45) = 14.74 F (1, 45) = 1.947	P<0.0001 p=0.1697

Supplementary Material 3 Table 1 Effect of CCI on fractional anisotropy (FA) values in the segments of the CC.

FA two-way ANOVA results comparing hemisphere (ipsilateral/contralateral) and segment (1-5) in 3 day post injury (DPI) 2mm (moderate), 14 day post injury (mild) 1mm and 14 day post injury 2mm (moderate). Significant differences are indicated in bold ($P<0.001$ and $P<0.0001$). (3 day post injury (DPI) data (Naïve/sham 3 DPI n=8, naïve/Sham 14 DPI: n=10, Moderate 3 DPI: n=8, mild 14 DPI: n=10, moderate 14 DPI: n=11)

Mean diffusivity (MD), represents the average displacement of water molecules within a voxel. It quantifies the magnitude of diffusion in all directions, irrespective of directionality. MD is influenced by various factors, including cell density, cell membrane integrity, and the presence of barriers to diffusion. Increased MD values often indicate cellular damage, reduced tissue complexity, or increased extracellular space.

MD for the moderate injury at 3 DPI (Supplementary Material 3 Figure 1D-F), did not reveal differences between hemispheres or interaction (Supplementary Material 3 Table 2) although there were differences between segments.

MD in the mild CCI at 14 DPI demonstrated a significant interaction (Supplementary Material 3 Table 2 This interaction was revealed at segment 2, as there was a significant decrease in the ipsilateral side relative to the contralateral side (-4.76%, $p=0.0021$). No other segments showed significant differences in this model at 14 DPI ($p>0.05$).

MD			
3 DPI moderate	F (DFn, DFd)		P value
	Segment x Hemisphere	F (4, 35) = 0.7804	p=0.5456
	Segment	F (4, 35) = 24.12	p<0.0001
14 DPI moderate	Hemisphere	F (1, 35) = 1.395	p=0.2455
	F (DFn, DFd)		P value
	Segment x Hemisphere	F (4, 50) = 1.191	p=0.3261
14 DPI mild	Segment	F (4, 50) = 4.144	p=0.0056
	Hemisphere	F (1, 50) = 4.829	p=0.0326
	F (DFn, DFd)		P value
14 DPI mild	Segment x Hemisphere	F (4, 45) = 2.620	p=0.0473
	Segment	F (4, 45) = 5.558	p=0.0010
	Hemisphere	F (1, 45) = 3.000	p=0.0901

Supplementary Material 3 Table 2 Effect of CCI on mean diffusivity (MD) values in the segments of the CC.

MD two-way ANOVA results comparing hemisphere (ipsilateral/contralateral) and segment (1-5) in 3 day post injury (DPI) 2mm (moderate), 14 day post injury (mild) 1mm and 14 day post injury 2mm (moderate). Significant differences are indicated in bold ($P<0.05$, $P<0.01$, $P<0.001$ and $P<0.0001$). (Naïve/sham 3 DPI $n=8$, naïve/Sham 14 DPI: $n=10$, Moderate 3 DPI: $n=8$, mild 14 DPI: $n=10$, moderate 14 DPI: $n=11$)

14 DPI in the moderate injury showed significance in the hemisphere and segment, but no interaction (Supplementary Material 3 Figure 1F). The ipsilateral hemisphere was significantly increased relative to the contralateral irrespective of segment (increase of 4.4% over all segments for ipsilateral hemisphere, $p=0.0167$).

Neurite Density Index (NDI) is a metric used to estimate the density or packing density of neurites, such as axons and dendrites, within a voxel. It provides an indirect measure of neuronal density and can reflect changes in axonal integrity or density in white matter regions. A higher NDI value typically indicates a higher density or more intact neurites within the voxel.

In the CCI moderate model at 3 DPI, the only main effect that was found to be significant was segment (Supplementary Material 3 Figure 1G). but no other significance was found.

NDI in the mild 14 DPI model there was a significant main effect of hemisphere and segment (Supplementary Material 3 Table 3) post-hoc tests revealed a modest ipsilateral increase of 3.71% ($p=0.0041$). At 14 DPI in the moderate CCI model (Supplementary Material 3 Figure 1) only a significant main effect of segment was found (Supplementary Material 3 Table 3).

NDI			
3 DPI moderate	F (DFn, DFd)		P value
	Segment x Hemisphere	F (4, 35) = 1.467	p=0.2331
	Segment	F (4, 35) = 30.68	P<0.0001
14 DPI moderate	Hemisphere	F (1, 35) = 1.023	p=0.3188
	F (DFn, DFd)		P value
	Segment x Hemisphere	F (4, 50) = 1.414	p=0.2431
14 DPI mild	Segment	F (4, 50) = 5.695	p=0.0007
	Hemisphere	F (1, 50) = 0.008	p=0.9290
	F (DFn, DFd)		P value
14 DPI mild	Segment x Hemisphere	F (4, 45) = 2.305	p=0.0728
	Segment	F (4, 45) = 6.901	p=0.0002
	Hemisphere	F (1, 45) = 10.07	p=0.0027

Supplementary Material 3 Table 3 Effect of CCI on neurite dispersion index (NDI) values in the segments of the CC.

NDI two-way ANOVA results comparing hemisphere (ipsilateral/contralateral) and segment (1-5) in 3 day post injury (DPI) 2mm (moderate), 14 day post injury (mild) 1mm and 14 day post injury 2mm (moderate). Significant differences are indicated in bold ($P<0.05$, $P<0.01$, $P<0.001$ and $P<0.0001$). (Naïve/sham 3 DPI $n=8$, naïve/Sham 14 DPI: $n=10$, Moderate 3 DPI: $n=8$, mild 14 DPI: $n=10$, moderate 14 DPI: $n=11$).

The Orientation Dispersion Index (ODI), is a quantitative measure used to evaluate the dispersion or distribution of fibre orientations within a voxel. It provides information about the coherence or alignment of white matter fibres in a particular region. A lower ODI value indicates greater fibre alignment and directionality, suggesting more organised and intact white matter tracts.

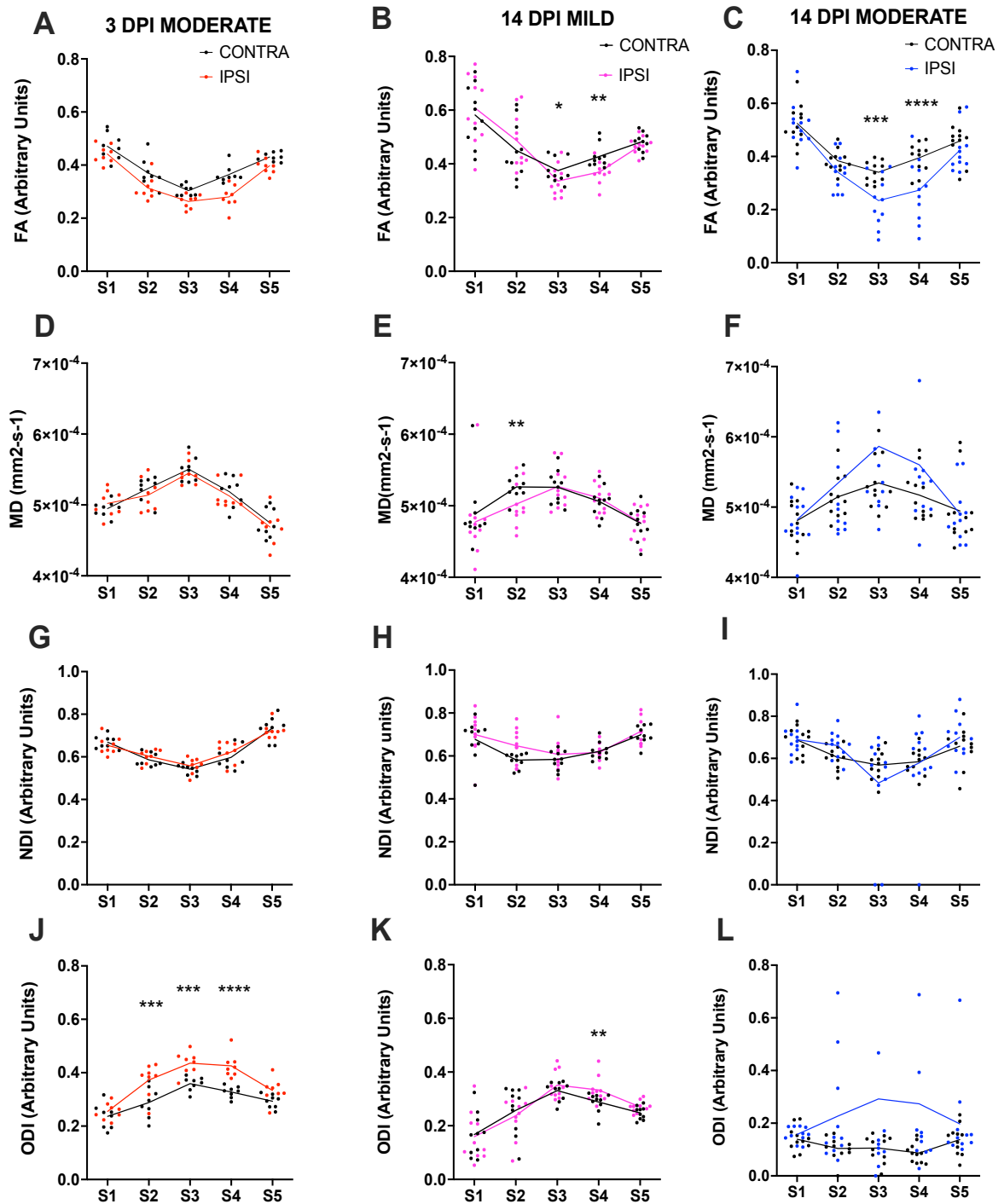
The moderate CCI at 3 DPI revealed a significant interaction (Supplementary Material 3 Table 4 and Supplementary Material 3 Figure 1J) and post-hoc tests showed significantly increased ODI values for segments 2-4 in the ipsilateral hemispheres compared to contralateral hemispheres (29.39%, $p=0.0002$, 21.37%, $p=0.0006$ and 29.88%, $p<0.0001$ respectively).

Mild CCI 14 DPI revealed significant interaction as shown in Supplementary Material 3 Table 4. Post-hoc test revealed segment 4 was significantly increased (15.59% increase, $p=0.0002$) (Supplementary Material 3 Figure 1). ODI in the moderate CCI model at 14 DPI revealed a significant main effect of hemisphere (Supplementary Material 3 Table 4). The ipsilateral hemisphere was significantly increased relative to the contralateral (100.09% increase, $p=0.0002$).

ODI			
3 DPI moderate	F (DFn, DFd)		P value
	Segment x Hemisphere	F (4, 35) = 2.681	p=0.0475
	Segment	F (4, 35) = 29.66	P<0.0001
14 DPI moderate	Hemisphere	F (1, 35) = 49.44	P<0.0001
	F (DFn, DFd)		P value
	Segment x Hemisphere	F (4, 50) = 1.221	p=0.3136
14 DPI mild	Segment	F (4, 50) = 0.2145	p=0.9292
	Hemisphere	F (1, 50) = 14.13	p=0.0004
	F (DFn, DFd)		P value
14 DPI mild	Segment x Hemisphere	F (4, 45) = 3.558	p=0.0132
	Segment	F (4, 45) = 14.00	P<0.0001
	Hemisphere	F (1, 45) = 3.855	p=0.0558

Supplementary Material 3 Table 4 Effect of CCI on orientation dispersion index (ODI) values in the segments of the CC.

ODI two-way ANOVA results comparing hemisphere (ipsilateral/contralateral) and segment (1-5) in 3 day post injury (DPI) 2mm (moderate), 14 day post injury (mild) 1mm and 14 day post injury 2mm (moderate). Significant differences are indicated in bold ($P<0.05$, $P<0.01$, $P<0.001$ and $P<0.0001$). (Naïve/sham 3 DPI $n=8$, naïve/Sham 14 DPI: $n=10$, Moderate 3 DPI: $n=8$, mild 14 DPI: $n=10$, moderate 14 DPI: $n=11$).



Supplementary Material 3 Figure 1 Diffusion tensor imaging (DTI) results of the focal injury model

DTI measures show corpus callosum white matter integrity changes over time and display differences in severity. Diffusion tensor imaging measures displayed per segment for each hemisphere of the CC. ((A-C) is FA for each injury group, (D-F) is MD for each injury group. (G-I), is NDI for each injury group and (J-L), is ODI for each injury group. (A/D/G/J) is moderate injury severity at 3 DPI. (B/E/H/K) is mild injury at 14 DPI, (C/F/I/L) is moderate injury at 14 DPI. All data is present as mean $\pm$ SEM. Naïve/sham 3 DPI: n=8, naïve/Sham 14 DPI: n=10, Moderate 3 DPI: n=8, mild impact 14 DPI: n=10, moderate 14 DPI: n=11. * P <0.05, ** P <0.01, *** P <0.001 and **** P <0.0001 as compared to the contralateral side. Results adapted from (Donat, Yanez Lopez et al. 2021).

LFB staining reveals White matter thinning at 14 DPI in moderate CCI

MRI analysis suggested white matter damage in the segments from the ipsilateral side of the injury in the moderate CCI model relative to the contralateral hemisphere at 3 DPI, similar to the changes at 14 DPI previously published (Donat, Yanez Lopez et al. 2021). Following this, I investigated whether this axonal damage was associated with demyelination and CC thinning, measured using Luxol fast blue staining (Naïve/Sham n=11 , 3 DPI moderate, n=8 , 14 DPI mild, n=6, 14 DPI moderate, n=6).

Luxol fast blue staining was used to measure the Corpus Callosum (CC) thickness in the different injury groups (3 DPI and 14 DPI moderate and 14 DPI mild) for each segment. Two-way ANOVA (main effects injury group (injury) and segment and interaction) showed a significant interaction in Supplementary Material 3 Table 5. Post-hoc analysis revealed only the 14 DPI moderate injury group had significant LFB thinning in the ipsilateral side of the corpus callosum to the injury site. The other injury groups did not show significant differences ($p>0.05$)

	Factors	F (DFn, DFd)	P value
CCI LFB thickness	Injury	F (3, 130) = 16.37	P<0.0001
	Segment	F (4, 130) = 3.941	p=0.0047
	Interaction	F (12, 130) = 3.411	p=0.0002

Supplementary Material 3 Table 5 Effect of CCI on orientation dispersion index (ODI) values in the segments of the CC.

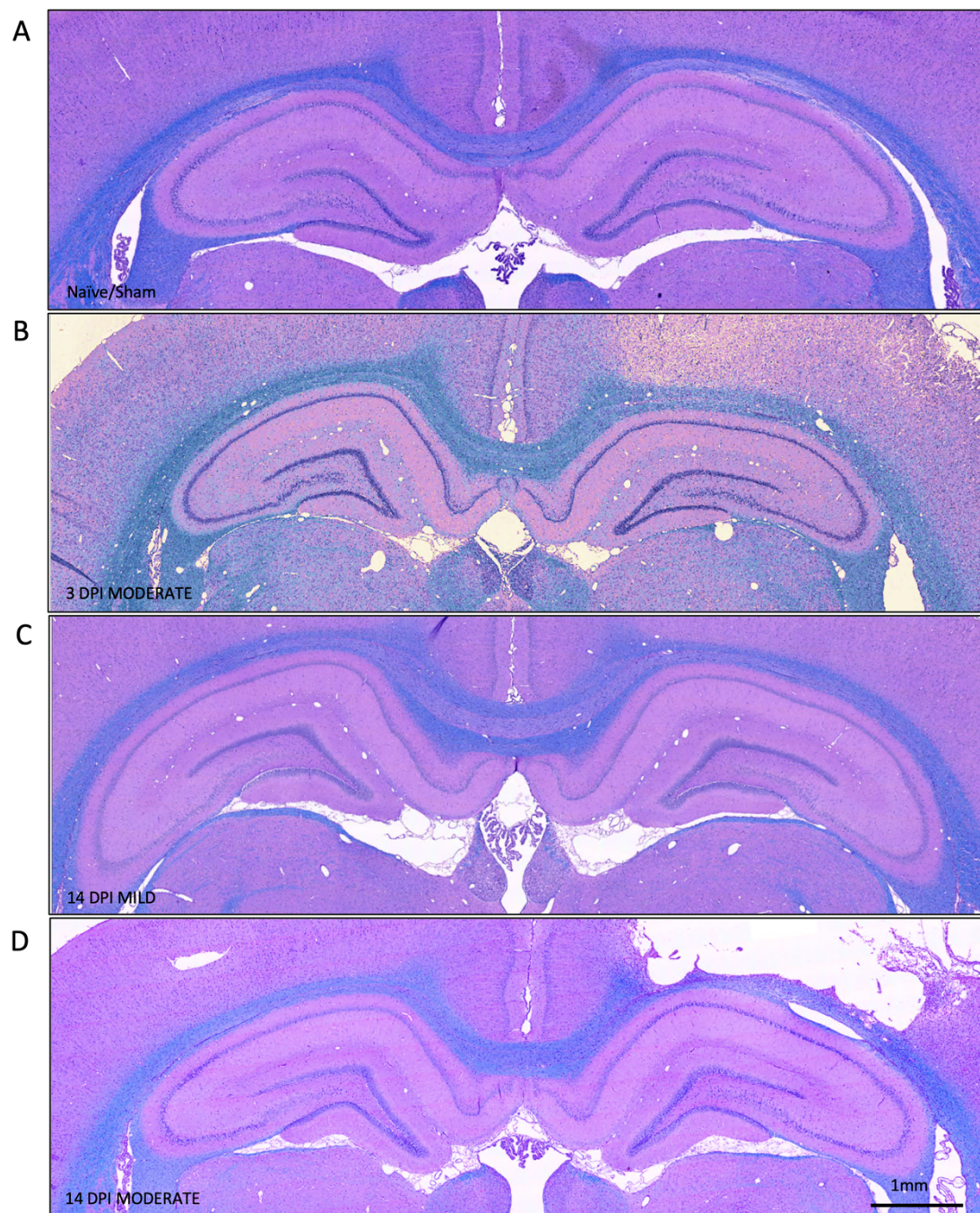
ODI two-way ANOVA results comparing hemisphere (ipsilateral/contralateral) and segment (1-5) in 3 day post injury(DPI) 2mm (moderate), 14 day post injury (mild) 1mm and 14 day post injury 2mm (moderate). Significant differences are indicated in bold ($P<0.05$, $P<0.01$, $P<0.001$ and $P<0.0001$). (Naïve/sham 3 DPI n=8, naïve/Sham 14 DPI: n=10, Moderate 3 DPI: n=8, mild 14 DPI: n=10, moderate 14 DPI: n=11.

The interaction resulted from reduced thickness of the CC between the 14 DPI moderate and Naïve/Sham in segments 2-4 (segment 2: -29.89% $p<0.0001$ and segment 3: -43.94, $p<0.0001$, segment 4: -20.89%, $p=0.0030$) (Supplementary Material 3 Figure 3). Similar reductions of

thickness were seen between 14 DPI moderate relative to the 14 DPI mild CCI in the same segments (segments 2: -32.29% $p<0.0001$, segment 3: -41.87% $p<0.0001$, segment 4: -23.31% $p=0.0023$).

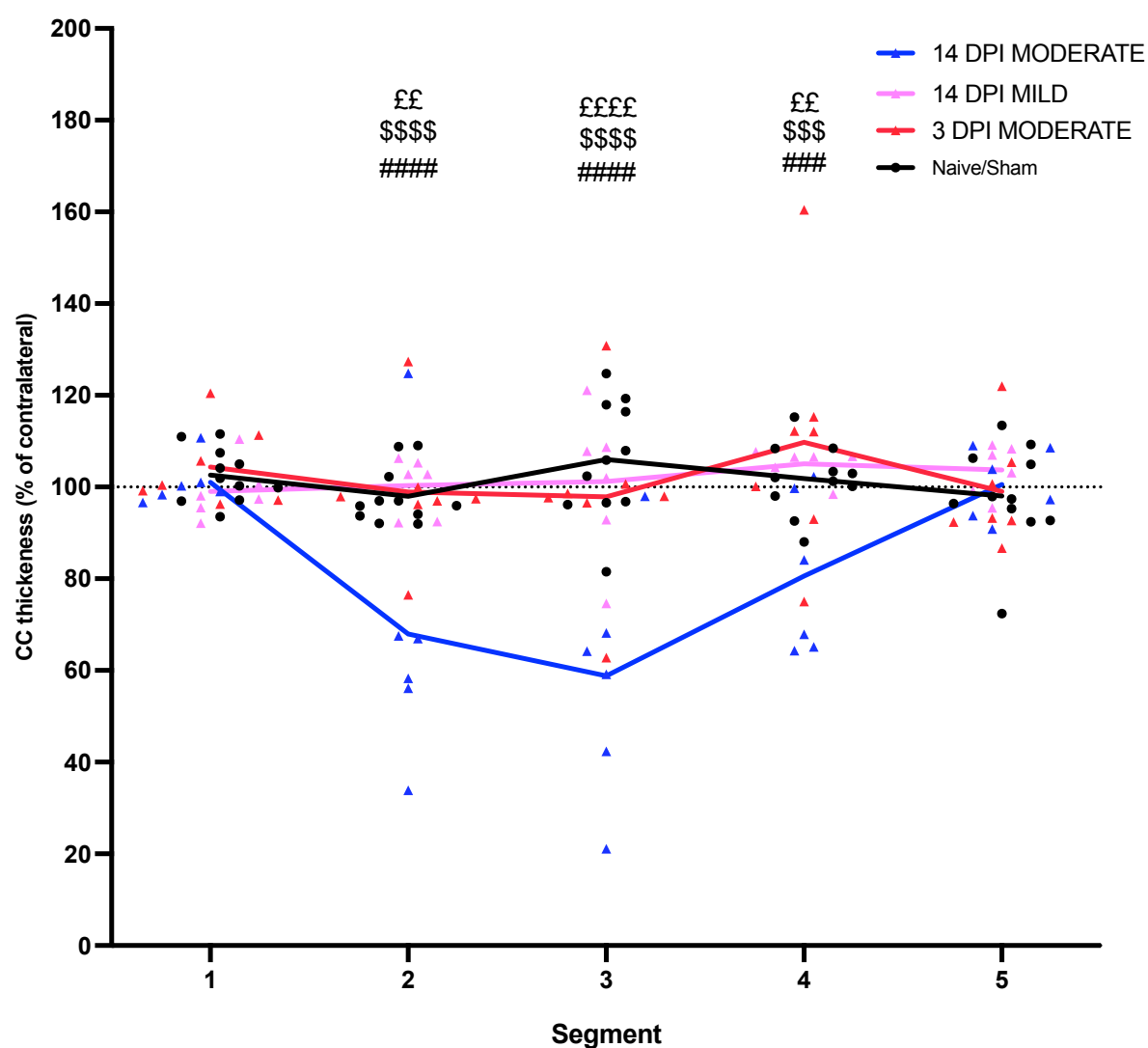
Then, I compared the 14 DPI moderate CCI with the 3D DPI, showing differences between them in segments 2-4 (segments 2: -32.18% $p=0.0001$, segment 3: -41.29%, $p<0.0001$ and segment 4: -25.94%, $p=0.0002$). For the 3DPI there was no significant difference in thickness when compared the Naïve/Sham group (Supplementary Material 3 Figure 2 and Supplementary Material 3 Figure 3).

To summarise, there were no reductions in LFB staining in the CC for any TBI group, except for the moderate CCI at 14 days post –injury.



Supplementary Material 3 Figure 2 Luxol Fast Blue staining of the CCI focal injury model of the corpus callosum.

A-E) Representative micrographs from Naïve/Sham, 3 DPI moderate, 14 DPI mild, 14 DPI moderate. Thinning is clearly seen on the injury side of the hemisphere in D where the cortex has lost tissue.



Supplementary Material 3 Figure 3 Quantification of corpus callosum thinning from Luxol Fast Blue staining in the CCI focal injury model

Naïve/Sham $n=11$, 3 DPI moderate, $n=8$, 14 DPI mild, $n=6$, 14 DPI moderate, $n=6$. # = Naïve/Sham vs 14 DPI moderate and #, ##, ###, ##### indicate significant difference ($P<0.05$, 0.01, 0.001 and 0.0001), \$ = 14 DPI mild vs 14 DPI moderate, \$,\$,\$,\$,\$,\$,\$,\$,\$,\$ indicate significant difference ($P<0.05$, 0.01, 0.001 and 0.0001), £ = 3 DPI moderate vs 14 DPI moderate, £,££,£££,££££, indicates significant difference ($P<0.05$, 0.01, 0.001 and 0.0001).

Focal CCI injury leads to a reduction in the neurofilament stained with RPCA-NFL-CT antibody in the corpus callosum

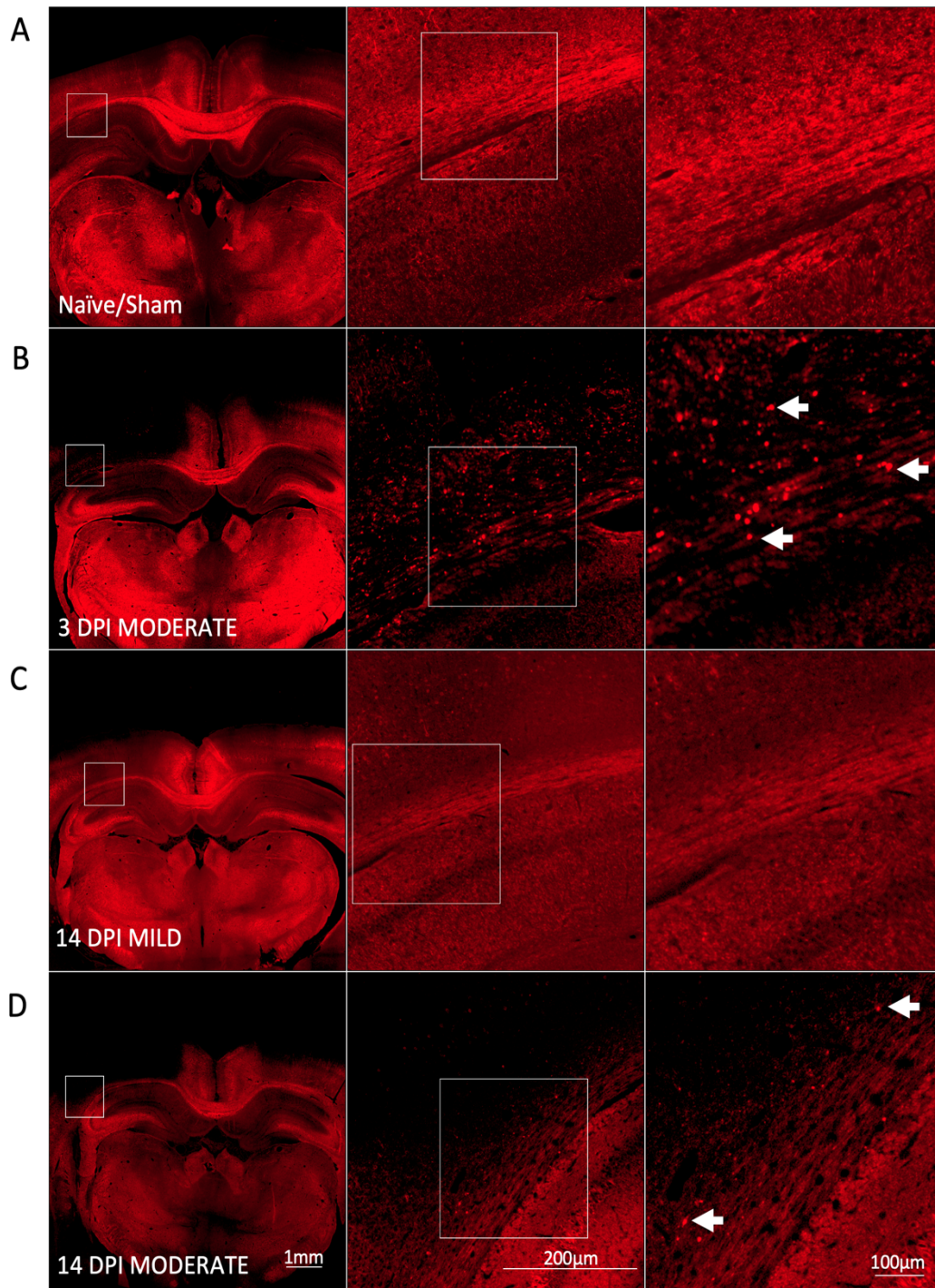
I next investigated whether axonal damage was associated with a loss of C-terminal (the “tail”) NFL in the white matter. For this purpose, the CCI brain tissue was stained with the RPCA-NFL-CT antibody, which binds to the C-terminal of the NFL polypeptide. It is expected that if the axons were damaged, then the NFL polypeptide would be damaged, and/ or released from the damaged area, resulting in a reduction of C-terminals present in the CC to bind to. In addition to the staining with this antibody, other antibodies (MCA-1B11, 6H63 and 1D44) were also used. These antibodies were specific for an unmasked epitope of NFL that appears as a result of injury, and would recognise the same epitope of NFL as the fluid biomarker assay (Shaw, Madorsky et al. 2023), however two of these antibodies were found to be unreliable (as shown in methods of Chapter 5 - NFL antibody troubleshooting).

The middle panel of Supplementary Material 3 Figure 4B and D illustrates a reduction in the intensity of the RPCA-NFL-CT positive stain in the injury site of the CCI model, and the presence of punctate staining in the CC segments, showing significant reductions of fluorescence intensity. In the moderate CCI model, the punctate staining was denser at 3DPI compared with 14DPI (Supplementary Material 3 Figure 4). However, no differences were observed between the mild CCI model with the control group, even at higher magnification.

As in the MRI analysis, (described in (Donat, Yanez Lopez et al. 2021) whereby Dr Maria Yanez Lopez conducted the analysis on the 14 DPI, I conducted similar analysis on the 3 DPI tissue) the CC was divided into 5 segments per hemisphere and RPCA-NFL-CT staining value was quantified as average positive fluorescent intensity. This value was then divided by its contralateral segment and represented as % of contralateral, as shown in Supplementary Material 3 Figure 5. Some CCI animals could not be used due to damage to the tissue, and therefore the n numbers were smaller than those used for LFB (Naïve/Sham n=5, 3 DPI moderate CCI, n=6, 14 DPI mild CCI, n=8, 14 DPI moderate CCI, n=5).

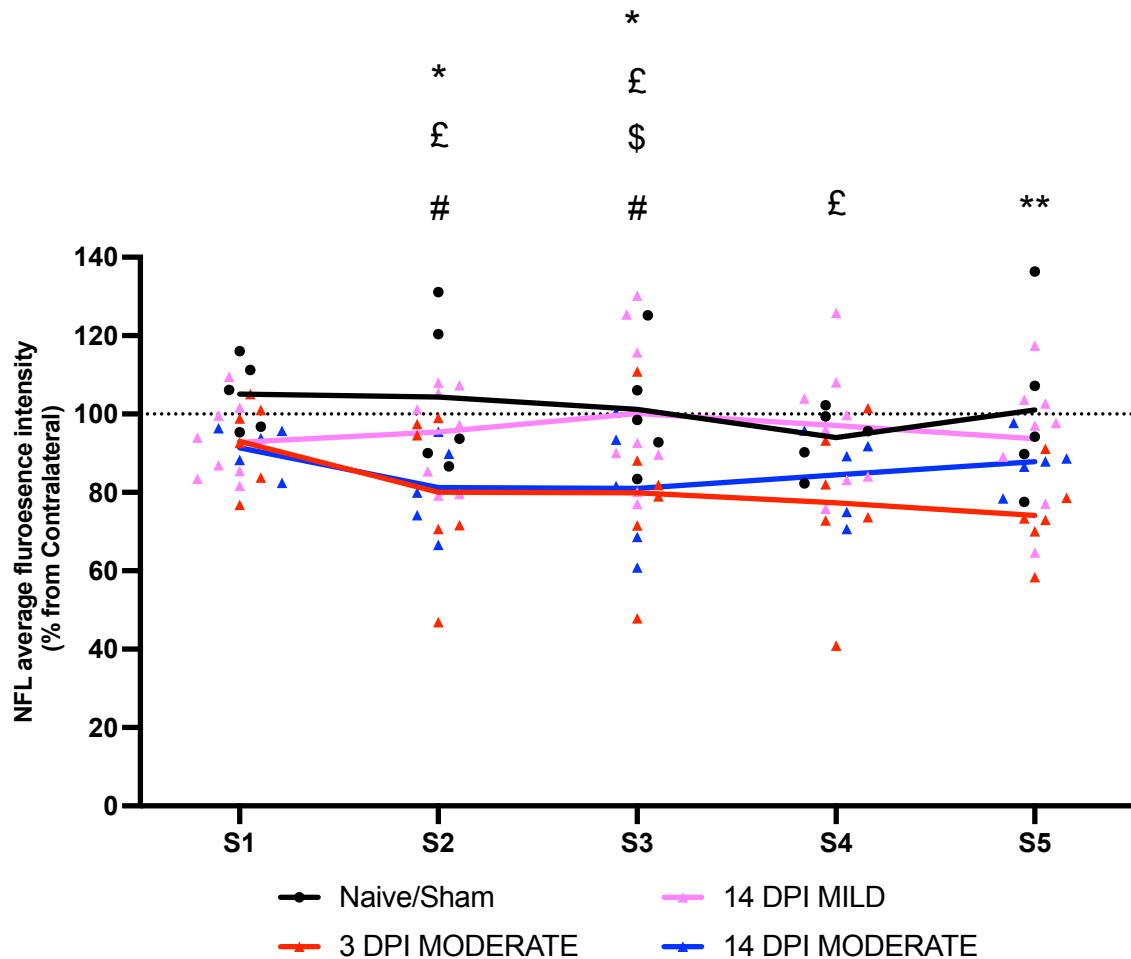
Two-way ANOVA revealed a main effect of injury ($F(3, 100) = 10.40, p < 0.0001$) but no significance was found for segment or their interaction ($F(4, 100) = 0.7900, p = 0.5344$ and $F(12, 100) = 0.5930, p = 0.8433$, respectively). Follow-up analysis for each injury group found a significant decrease in NFL in the moderate CCI at 3DPI in segments 2,3 and 5 relative to the Naïve/Sham control group (segments 2: -23.29%, $p = 0.0016$, segments 3: -21.05%, $p = 0.00246$ and segments 5: -26.65%, $p = 0.0048$) and a trend in segment 4: -17.67% $p = 0.0783$ (Supplementary Material 3 Figure 5).

This effect was also observed in the moderate CCI at 14DPI compared to Naïve/Sham, showing a significant decrease in fluorescence intensity in segments 2 (-28.48%, $p = 0.0195$) and 3 (-4.42%, $p = 0.0409$). In addition, significant differences in NFL were also found between 14DPI mild and the 14DPI moderate group in segments 3 (-19.09%, $p = 0.0319$), whereby the moderate injury showed much less NFL intensity than the mild. There were also differences in intensity between the 3DPI moderate CCI and 14DPI mild CCI in segments 3-5 (segment 3: -20.20% $p = 0.0168$, segment 4: -20.32% $p = 0.0197$ and segment 5: -20.90%, $p = 0.0206$), as the 3DPI moderate CCI group showed reduced NFL intensity compared with the 14 DPI mild CCI group.



Supplementary Material 3 Figure 4 Neurofilament light (NFL) RPCA-NFL-CT immunofluorescent staining in focal injury (CCI) tissue for all injury groups.

Staining is of Encor Biotech NFL antibody the using AF647 Left panel showing most of the brain stained then the middle and right panel indicate higher magnifications of the white matter tract in segments 3-4 on the ipsilateral site. A) Naïve/Sham showing no obvious loss of white matter or reduction of RPCA-NFL-CT, B) 3 DPI moderate injury showing considerable reduction of staining in the cortex and white matter, middle panel showing punctate in higher magnification, right panel shows examples of the punctate with white arrows. C) 14DPI mild tissue demonstrating no obvious changes in the white matter or staining. D) 14 DPI moderate tissue showing considerable tissue loss in the ipsilateral cortex and the subcortical white matter and punctate.



Supplementary Material 3 Figure 5 Focal injury quantification of neurofilament light antibody from Encor Biotech RPCA-NFL-CT ANOVA.

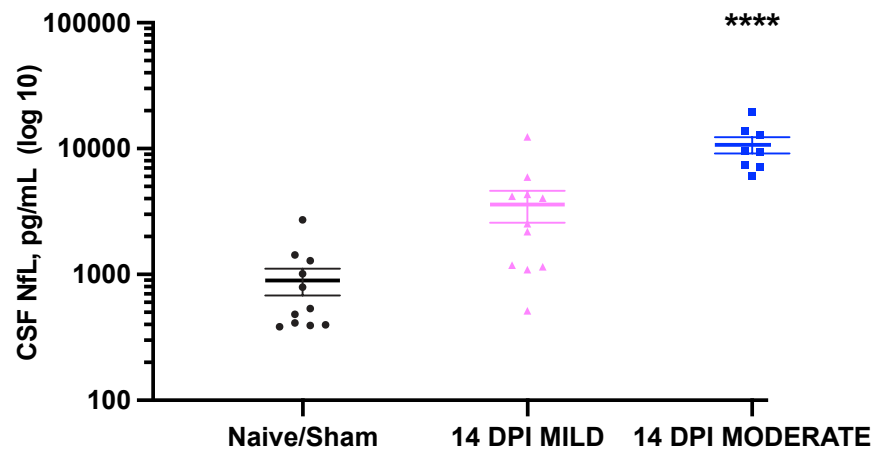
The results show a significant reduction of NFL in the corpus callosum in the moderate injury at both timepoints but not the mild injury. Graph is depicted without individual datapoints to aid clarity therefore Naïve/Sham $n=5$, 3 DPI moderate, $n=6$, 14 DPI mild, $n=8$, 14 DPI moderate, $n=5$. # = Naïve/Sham vs 14 DPI moderate and. #, ##, ###, #### indicate significant difference ($P<0.05$, 0.01, 0.001 and 0.0001), * = Naïve/Sham vs 3 DPI moderate, and *, **, ***, **** indicate significant difference ($P<0.05$, 0.01, 0.001 and 0.0001), \$ = 14 DPI mild vs 14 DPI moderate, \$, \$\$, \$\$\$, \$\$\$\$, indicate significant difference ($P<0.05$, 0.01, 0.001 and 0.0001), £ = 3 DPI moderate vs 14 DPI moderate, £, ££, £££, ££££, indicates significant difference ($P<0.05$, 0.01, 0.001 and 0.0001).

Focal injury leads to increased levels of NFL in the CSF and blood plasma

After investigating the loss of NFL in the histopathology of the CCI and blast animals, I then explored whether changes in concentration of NFL in fluids would be detected in the CSF and blood plasma for both models. This was conducted in collaboration with Prof Henrick Zetterberg's lab at University College London. Samples were analysed using the ultrasensitive Quanterix single molecule assay (SIMOA) in both CCI and blast animals.

CSF was sampled from the moderate CCI cohort at 14 DPI (time of termination), while blood samples were obtained at different time points (baseline, 3, 7, 10 and 14 DPI) by Dr Donat as published in Graham, 2020 (Graham, Jolly et al. 2020).

The analysis of NFL concentration in the CSF revealed a significant one-way ANOVA ($F(2, 27) = 24.22, p < 0.0001$). Investigation into what caused this significance between the injury severities revealed an increase at 14 DPI in the moderate CCI group compared with Naïve/sham animals (increase of 1098.80%, $p < 0.0001$) and borderline significance in the 14 DPI mild CCI (302.08% increase from Naïve/Sham, $p = 0.05$), (Supplementary Material 3 Figure 6) (Naïve/Sham: $n=11$, 14 DPI mild: $n=11$, 14 DPI moderate: $n=8$), as published in Graham et al, 2021 (Graham, Zimmerman et al. 2021)



Supplementary Material 3 Figure 6 Cerebral Spinal Fluid (CSF) Neurofilament (NFL) 14 days after CCI focal injury.

Naïve/Sham: n=11, 14 DPI mild: n=11, 14 DPI moderate n=8. Values shown as mean $\pm$ SEM.

**** indicates a significance of $P < 0.0001$ compared to and Naïve/Sham. Figure adapted from (Graham, Zimmerman et al. 2021), differences from the figure that is published consist of combining the Naïve/Sham group and the post-hoc tests. Data presented mean $\pm$ SEM

Plasma NFL concentration levels were increased over 2 weeks post-mild and post-moderate injury. Mixed-effects analysis with time and injury as fixed effects showed significance for time ($F(1.186, 26.68) = 64.07$, $p < 0.0001$), injury ($F(1, 28) = 21.06$, $p < 0.0001$) and interaction between time and injury severity ($F(4, 90) = 14.86$, $p < 0.0001$), (Naïve/Sham baseline: n=11, Naïve/Sham 3-14DPI: n=4, 14 DPI mild: n=12, 14 DPI moderate: n=14). Post-hoc analysis revealed all timepoints to be significantly increase from baseline for both mild and moderate animals (for mild cohort relative to baseline, 3DPI: increase of 2616.60%, $p = 0.0006$, 7DPI: 893.70% $p = 0.0008$, 10DPI: 684.61% $p = 0.0137$, 14DPI: 251.59%, $p = 0.0004$. For moderate injury; 3-10DPI: $p < 0.0001$, (3 DPI: 6353.76%, 7 DPI: 1915.28%, 10 DPI: 1096.90%) and 14 DPI: 600.26%, $p = 0.0002$) (Supplementary Material 3 Figure 7). No difference was found in the control group over time ($p > 0.05$) (Graham, Zimmerman et al. 2021).

