Blast Injury Outcome Study in Armed Forces Personnel (BIOSAP)

and

Blast Injury In Pigs Study (BIIPS)

PhD Thesis

Mr. David LJ Baxter, February 2016

List of publications

Jamall O, Feeney C, Zaw-Linn J, Malik A, Niemi ME, Tenorio-Jimenez C, Ham TE, Jilka SR, Jenkins PO, Scott G, Li LM, Gorgoraptis N, Baxter D, Sharp DJ, Goldstone AP.
Clin Endocrinol (Oxf). 2016 Feb 27, Prevalence and Correlates of Vitamin D Deficiency in Adults after Traumatic Brain Injury.

Kwok H-T, Baxter D, DeFelice J, Hellyer P, Kirkman E, Watts S, Midwinter M, Gentleman S, Sharp Det al., 2014, The neuropathology of blast traumatic brain injury in a porcine polytrauma model, *BRAIN INJURY*, Vol: 28, Pages: 779-780, ISSN: 0269-9052

Baxter D, Sharp DJ, Feeney C, et al., 2013, Pituitary Dysfunction after Blast Traumatic Brain Injury:
The UK BIOSAP Study, *Annals of Neurology*, Vol:74, ISSN:0364-5134, Pages:527-536

Synopsis

Operation Herrick is the codename under which all British operations in the war in Afghanistan have been conducted since 2002. It consists of the British contribution to the NATO-led International Security Assistance Force (ISAF) and support to the American-led Operation Enduring Freedom (OEF). Operation Herrick superseded Operation VERITAS and Operation FINGAL.

The UK ceased all combat operations in Afghanistan and withdrew the last combat troops on the 27 October 2014. Between 2001 and 12 December 2014, a total of 453 British military personnel died during operations in Afghanistan and over 2116 were injured.

The **Blast Injury Outcome Study in Armed forces Personnel (BIOSAP)** began in 2010. At that time, I was a neurosurgical trainee at the Royal London Hospital in North London and had recently returned to hospital-based clinical work after being deployed to Sangin forward operating base (FOB), Helmand province, Afghanistan, as a medical officer for Charlie company (C coy) of the 2nd Battalion the Royal Regiment of Fusiliers (2RRF).

In 2007 through 2008, I was the Regimental Medical Officer (RMO) for 2RRF. We were based in Cyprus and, during my time with the Battalion, they deployed to both Iraq and Afghanistan. The British army posts soldiers, with their families, to many of its bases throughout the world. During my time in Cyprus with 2RRF, I was responsible for the day-to-day healthcare of the soldiers and their families. I saw first hand how a soldier's ability to perform his role impacted on others around him. Infantry soldering requires both extraordinary physical fitness as well as mental robustness both in terms of cognition and emotion. Injuries, whether they are immediately visible, such as a traumatic limb amputation or invisible, such as a traumatic brain injury, have implications for the whole battalion and the family, as well as the individual who has been injured.

In 2008, I deployed to Sangin, Afghanistan. There, I lived and worked with the soldiers from C-coy. I developed an understanding of the character required to fight in front line combat. I witnessed, first hand, the manner in which contacts (a sterile word used to describe what happens when we engage with the enemy) are fought and how injuries are sustained. I treated, at the point of injury, soldiers who had been

exposed to blast weapons and saw how the Mobile Emergency Response Team (MERT) recovered and treated them.

Following my return to the UK, I became a research fellow in the Academic Departments of Military Surgery and Trauma (ADMST) and Emergency Medicine (ADMEM). At ADMEM, I catalogued the number of head injuries sustained by UK personnel as well as their causes and outcomes in order to understand the impact of head injury from an epidemiological perspective.

In the civilian literature, head injuries in the context of polytrauma (a term used to describe when more than one system in the body is injured e.g. a soldier who has sustained both a head injury and a fracture of the femur) are associated with higher mortality and worse functional outcomes (Gawande 2004). This is true for military injuries as well. As the conflict in Afghanistan matured, the weapons systems used by the enemy changed from mainly small arms to explosive devices. At the same time our equipment, specifically personal protective equipment (PPE), and tactics, evolved. PPE evolved to protect against lethal injuries such as catastrophic haemorrhage from femoral artery injuries or penetrating head injuries. This led to an increasing number of soldiers surviving previously non-survivable injuries; many of these had been exposed to blast (Penn-Barwell 2015).

The pathology and outcome from penetrating traumatic brain injury (TBI) have been well understood since the First and Second World Wars and this is true, albeit to a lesser extent, of blunt TBI since the 1980s. At the time BIOSAP began, there was very little information about the mechanism by which blast caused injury and no data about the mid to long-term outcome of these injuries. There was an increasing body of civilian literature that demonstrated endocrine dysfunction was frequently associated with TBI and that modern magnetic resonance (MR) imaging techniques could offer new insight into both structural and functional injuries. I began work at the Computational, Cognitive and Clinical Neuroimaging laboratory at Imperial College London with Dr. David Sharp (soon to become Professor Sharp) and Dr. Anthony Goldstone. Here we identified soldiers who had been injured in Afghanistan by explosive devices and, using these modern techniques, began to understand blast traumatic brain injury (bTBI).

In 2011, an opportunity to work with the Defence Science and Technology Laboratory (DSTL), at Porton Down, arose. In conjunction with Dr. Emrys Kirkman and Dr. Sarah Watts at DSTL and Professor Steve Gentleman in the Department of Medicine at Imperial College London, we developed a method to examine porcine brains that had been injured by blast. This study, as far as I am aware, is unique in combining both MR imaging results with neuro-pathological correlates of injury and early immune activation, and has come to be referred to as the Blast Injury In Pigs (BIIPs) study.

I returned to Afghanistan in 2012 to work at the Role 3 field hospital in Camp Bastion as a trauma surgeon. By this time, media coverage had brought the subject of injured personnel into the spotlight. Mainstream media began to draw links between post-traumatic stress disorder (PTSD) and traumatic brain injury (TBI), whilst the academic community, drawing on animal studies and observational work in American National Football League (NFL) players, focused its attention on the effect of repetitive minor TBIs. Journalists and academics drew parallels between NFL players and soldiers who had suffered repetitive TBIs and the resulting debate led to a class action by retired NFL players and a settlement of 800 million dollars by the NFL (NFL 2016). Whilst the disease process is not yet fully understood, repetitive TBI is now thought to cause a chronic activation of the brain's immune system that leads to a persistent encephalopathy. The ongoing debate has raised awareness of what we now call chronic traumatic encephalopathy (CTE). Although the last decade of research has led to advances in our understanding of TBI, outcomes from severe TBI remain poor and from what we generally consider mild TBI remain variable and difficult to predict. Many of the questions raised remain unanswered.

This thesis is a summation of both the BIOSAP and BIIPs studies.

Abbreviations

11-DOC	11-deoxycortisol
ACTH	Adrenocorticotropic hormone
ADMEM	Academic Department of Military Emergency Medicine
ADMST	Academic Department of Military Surgery and Trauma
AIS	Abbreviated Injury Scale
APP	Amyloid Precursor Protein
BBB	blood brain barrier
BET	brain extraction tool
BIIPs	Blast Injury In Pigs
BIOSAP	Blast Injury Outcome Study in Armed forces Personnel
BMI	body mass index
BOP	blast overpressure
BSA	Bovine serum albumin
bTBI	Blast traumatic brain injury
CC	Corpus callosum
CSF	cerebro-spinal fluid
CT	Computerised tomography
CTE	chronic traumatic encephalopathy
DAB	3-3'-diaminobenzidine-tetrahydrochloride
DAI	diffuse axonal injury
DKEFS	Delis-Kaplan Executive Function System
DSTL	Defence Science and Technology Laboratory
DTI	diffusion tensor imaging
FA	fractional anisotropy
FAI	free androgen index (100x testosterone / SHBG)

FBC	Full blood count
FDG-PET	fluorodeoxyglucose positron emission tomography
FFP	Fresh frozen plasma
FOB	forward operating base
FSL	FMRIB Software Library
GCS	Glasgow Coma Scale
GE	Gradient echo
GH	Growth hormone
Gn	Gonadotrophin
GRE	gradient recalled echo
GST	glucagon stimulation test
H&E	Haematoxylin and Eosin
HARDI	High Angular Resolution Diffusion Imaging
HS	haemorrhagic shock
IEDs	Improvised explosive devices
IGF-1	Insulin-like growth factor-1
IMS	industrial methylated spirits
ISAF	International Security Assistance Force
ISS	Injury Severity Score
ITT	insulin tolerance tests
MERT	Mobile Emergency Response Team
MR	magnetic resonance
MRI	magnetic resonance imaging
ms	milliseconds
MST	morphine sulphate
MTBI	Mild traumatic brain injury

NATO	North Atlantic Treaty Organization
nbTBI	Non-blast traumatic brain injury
NMR	Nuclear Magnetic Resonance
ND	not done
NR	normal range
OEF	Operation Enduring Freedom
OIF	Operation Iraqi Freedom
PCL-M	PTSD Checklist–Military
PCS	post-concussion syndrome
Psi	Pounds per square inch
PPE	personal protective equipment
PRBC	packed red blood cell
PTA	Post traumatic amnesia
PTSD	post-traumatic stress disorder
RF	Radio frequency
RMO	Regimental Medical Officer
ROI	region of interest
RTA	Road traffic accident
SIS	second impact syndrome
SWI	susceptibility weighted imaging
TSH	Thyroid-stimulating hormone
TBI	Traumatic Brain Injury
TBSS	Tract based spatial statistics
TE	Echo time
TR	Repetition time

WASI	Wechsler Abbreviated Scale of Intelligence
WTAR	Wechsler Test of Adult Reading
WM	White matter
WWI	World War I

Table of contents

Table of figures

Table of tables

1 Blast Injury Outcome Study in Armed Forces Personnel (BIOSAP) and Blast Injury in Pigs Study (BIIPs)

1.1 Introduction

The brain has myriad functions, including motor and sensory information processing, cognition and regulation of endocrine function. By necessity the tools used to investigate brain injury include clinical assessment, neuropsychological and endocrine tests as well as computerised tomography (CT) and magnetic resonance (MR) radiological studies. In the following introduction I outline the prerequisite information in order to understand the investigative rationale we employed for this work. I start the chapter by discussing blast because this area may be most unfamiliar to some readers, before moving on to traumatic brain injury (TBI), MR imaging, endocrine functions of the brain and the current use of animal models in TBI.

In order to appreciate the value of this research, one must first recognise why blast poses a significant problem for military populations and, crucially, that the blast itself may generate a different mechanism of injury to those seen in non-blast traumatic blast injury (nbTBI).

The Blast Injury Outcome Study in Armed forces Personnel (BIOSAP) examined the imaging, neuropsychological and endocrine effects of moderate and severe blast traumatic blast injury (bTBI) in a group of soldiers injured by blast in Afghanistan and compared these to civilians with a similar severity of non-blast injury, as well as uninjured civilian controls.

The Blast Injury In Pigs (BIIPs) study used 4.7 Tesla magnetic resonance imaging (MRI) scanning and immunohistopathological assessment to compare the presence, pattern and extent of brain injury between a group of pigs that had been exposed to a blast wave and one that had not. In the final section of this chapter I explain the utility of animal models of injury and summarise the relevant literature to date.

This work was a collaboration between UCL, where I am a registered doctoral student, the Computational, Cognitive and Clinical Neuroimaging Laboratory (C3NL) at Imperial College London, Imperial Healthcare Trust (Charing Cross and

Hammersmith Hospitals), the Academic Department of Military Surgery and Trauma (ADMST) and the Defence Science and Technology Laboratory (DSTL) Porton Down.

Injured soldiers were recruited using the injured personnel database at ADMST. They underwent neuropsychological testing and MR imaging in the Robert Steiner Unit of Hammersmith Hospital and clinical endocrine assessment at Charing Cross Hospital. The data was analysed at C3NL. The porcine blast injury experiment took place at DSTL, Porton Down. The brains were processed at DSTL and stored at Imperial College London. They were analysed in the Department of Pathology at Imperial Hospital Trust.

Given the multi-institutional collaboration that has been involved with these studies, there are many individuals who have been invaluable in their assistance. In particular, I would like to thank Surgeon Captain Mark Midwinter, Mr. Neil Kitchen and Professor David Sharp for overall supervision of the projects; Robert Leech and Peter Hellyer for their help processing the imaging data and statistical analysis; Dr. Anthony Goldstone and Dr. Claire Feeney for their assistance in performing and interpreting the endocrine assessments; Dr. Maneesh Patel for his help interpreting the human and animal structural MR imaging; Dr Marina Arridge for her assistance designing the porcine scanning protocol; Dr Emrys Kirkaman and Dr Sarah Watts, who ran the porcine blast model and supported retrieval of the pig brains; Ms Ting Kwok who assisted with analyzing the histology specimens; and Professor Steven Gentlemen for his supervision of the immunohistological component of the porcine work.

For the purposes of submitting this thesis it is important to clarify my role in the research. I identified, recruited and performed neuropsychological testing, MR imaging as well as some of the clinical assessment of the soldiers in the study. I analysed the neuropsychological and imaging data and helped to analyse the endocrine function results. I developed a methodology for retrieving and processing the porcine brains and helped to develop the scanning protocol that we used. I supervised an MRes student (Ting Kwok) and together we performed the immunohistological preparation and analysis of the specimens.

1.2 Blast

Explosive devices have been used extensively in Afghanistan. They have caused over 66% of coalition casualties that occurred there since 2001 (Kirkman 2011).

Improvised explosive devices (IEDs) are comprised of an activator, an initiator, a container, explosive and a power source (see Figures 1-1 and 1-2). IEDs designed to destroy vehicles contain a shaped charge that will penetrate the vehicle's armour. Antipersonnel IEDs contain objects (metal casing, nails, rocks and other materials) that act as ballistic fragments, causing injury at greater distance than the blast alone would. The IEDs used in Afghanistan were primarily made from ammonium nitrate (which was obtained from ammonium based fertilisers) or potassium chlorate (which is used in match making). IEDs can be initiated by mechanical, igniferous, improvised, electronic or chemical methods, and triggered by either the victim (trip wires, pressure sensitive plates or magnetic triggers) or by an attacker (command wire, remote control and infrared).

Figure 1-1. Different types of IED initiators (Military Annual Training Test MATT 9 C-IED Training J)

This figure demonstrates the variety of initiators that are used to trigger an explosion. The initiator produces heat energy that starts the explosive reaction. This is achieved by chemical reaction, electrical spark or heat energy from a burning fuse.

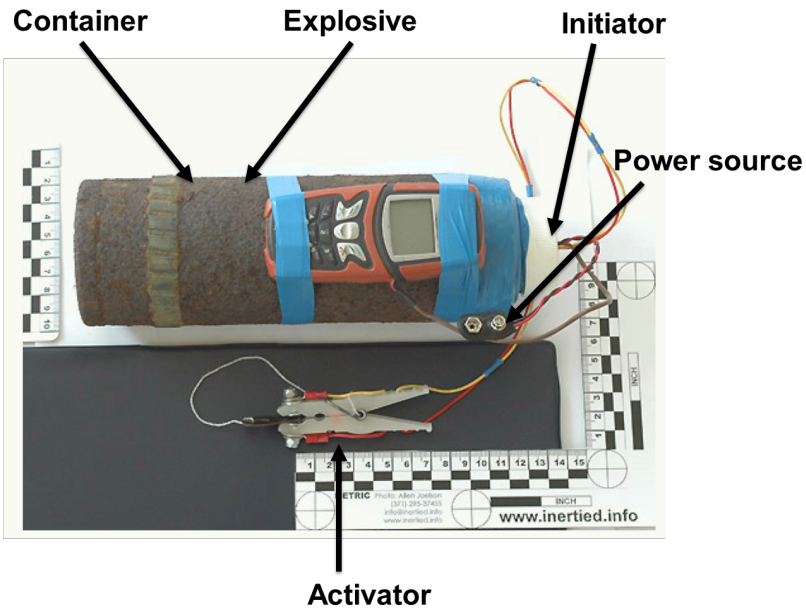


Figure 1-2. Components of an IED (image from http://www.inertproducts.com)

This figure shows what the activator, initiator, container, explosive and power source can look like. This is a training device that is activated by a mobile phone call and triggered by the victim dislodging a spacer that allows the two parts of the clothes peg to make contact. An electronic current that has been generated by a battery (not shown) powers the initiator causing the explosive to detonate. In this example the explosive is an old munitions shell and its casing fragments will cause additional injury.

Explosives are reactive materials that release energy in the form of light, heat, sound and pressure. They are classified by the speed at which they detonate. If the detonation front of the material moves faster than the speed of sound it is said to be a high explosive, and it will produce a shock wave. If the detonation front moves slower than the speed of sound it is called a low explosive. Both generate high temperatures and rapid release of gases, low explosives do not generate a shock wave. Low explosives produce subsonic explosions that spread by rapid combustion of materials (deflagration). In contrast, high explosives generate a supersonic explosion where the blast overpressure (BOP) wave expands outwards rapidly from the centre of detonation. The explosion generates a pulse of increased air pressure (the BOP peak), lasting milliseconds, it expands rapidly from the point of detonation and is followed by an area of negative pressure. The BOP peak is followed by an area of negative pressure and they are collectively known as the shock wave. The

magnitude of the shock wave dissipates rapidly as it expands away from the point of detonation by an inverse cube relation (Kirkman 2011). The rapidly expanding gases released by the explosion displace an equal volume of air that expands outwards at high velocities. This is referred to as the blast wind.

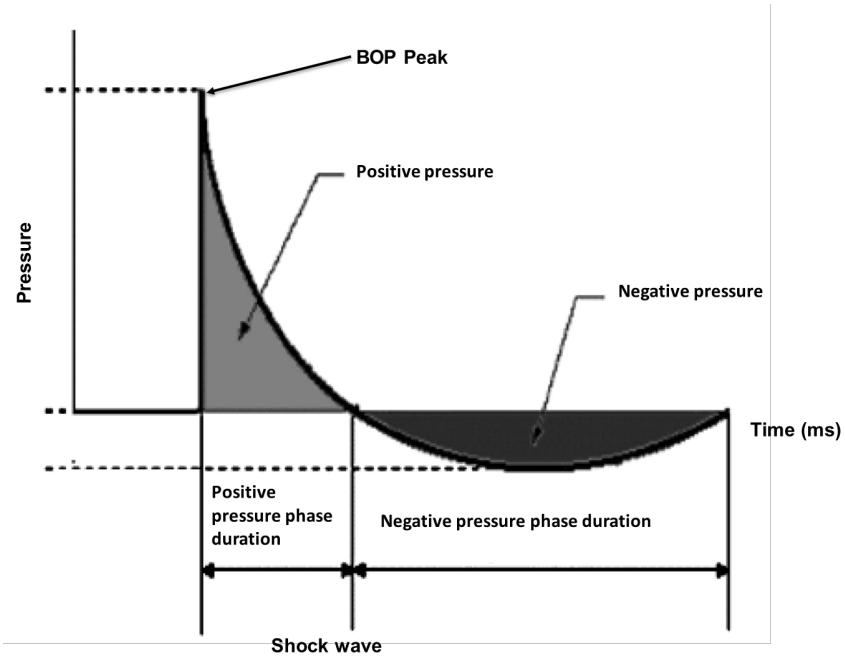


Figure 1-3. Friedlander waveform modified from Bauman 2009

A drawing of an ideal Friedlander wave showing the peak pressure, positive pressure wave and negative pressure wave.

When the expanding shock wave makes contact with hard solid surfaces, such as the ground and walls, it is reflected and this causes amplification of the peak pressure and greater injury. Blast victims in buildings have a greater degree of injury compared to those in the open (Bauman 2009) (CDC - Explosions and Blast Injuries 2015).

The IEDs used in Afghanistan were primarily high explosives. The extent of the damage they cause was dependent on the explosive used (as this determined the size of the high pressure peak and its duration), the container (as this fragments and acts as a fragmentation weapon), the distance of the victim from the point of detonation, and whether the explosion was in a confined space (as this determines the degree of focusing and amplification of the shock wave). BOP waves are

normally described in kPa or psi and the range of energy that soldiers are exposed to is wide depending on the size of the explosive, their distance from it and their PPE. Exposures of 690kPa or 100psi are considered potentially lethal (Champion 2009).

1.2.1 Classification of blast injury

Injuries sustained from blast are classified as primary, secondary, tertiary and quaternary. Primary blast injuries result from the initial BOP wave. A blast wind follows this overpressure wave and can propel objects such as shrapnel from the IED and other debris into the body, causing secondary effects. Tertiary injuries may result from rapid acceleration and deceleration as the victim is thrown and impact surrounding objects. Quaternary effects encompass a range of miscellaneous injuries, sustained from heat, toxins, chemicals and radiation given off by the blast (Elder 2010b, DeWitt 2009). The primary, secondary and tertiary effects of blast are shown in Figure 1-4.

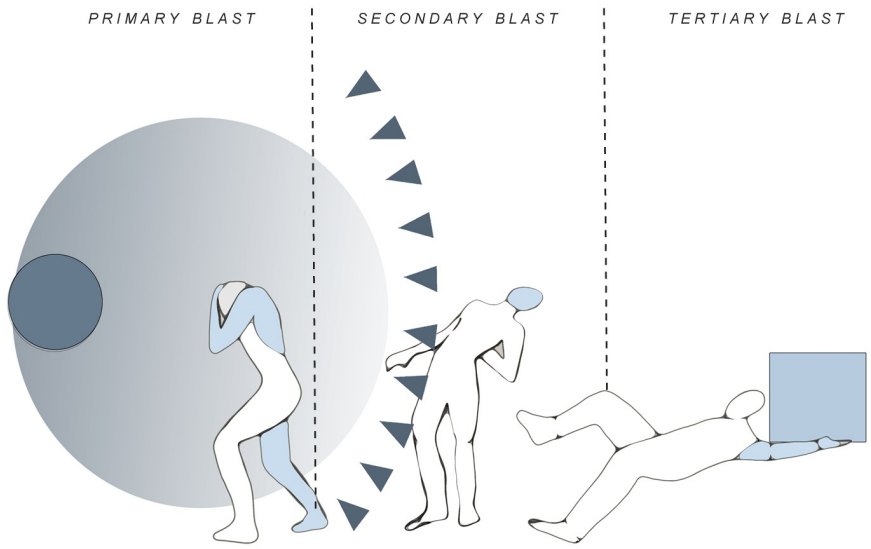


Figure 1-4. The primary, secondary and tertiary effects of blast

Primary blast injury is caused by the effects of the BOP wave itself. Secondary blast injuries are caused by shrapnel and debris being propelled by the blast force. Tertiary blast injuries are caused by impact of the body with other objects.

It is difficult to tease apart the direct effects of the BOP wave from the subordinate injuries in human clinical data. For this reason, animals have been used to model blast, controlling for the other effects. The question of whether the primary BOP wave can indeed cause brain injury is still in contention and, if so, the mechanisms through which injury occurs are unknown (Champion 2009). We hope that the BIIPs project will offer unique insights into the effects of primary blast in a porcine model.

1.2.2 Impact on the body

When a shockwave impacts the body, most of the energy is absorbed and propagated while the rest is reflected (Howe 2009). This generates high-frequency stress waves and lower-frequency shear waves, which in turn injure the tissues by mechanisms including spallation, implosion, and inertial effects (Nakagawa 2011, Leung 2008). Spallation is the disruption that occurs between materials of differing densities, and the compression is reflected at the material interface, leading to fragmentation of the denser material.

Implosion occurs as gas bubbles in the tissue are compressed by the shockwave. This inflicts damage as the tissue itself collapses and, as the gas re-expands following the wave passage, it impacts surrounding tissue. As the BOP propagates, lighter density tissues will accelerate more than the denser tissues, resulting in large stress forces at the interface. This is known as the inertial effect. As such, the most vulnerable organs affected in blast are those with air/liquid interfaces, such as the auditory canals, lungs and abdomen (Elder 2010b, Champion 2009). It is therefore thought that injuries resulting from purely primary blast effects (the BOP wave) are characterised by parenchymal damage to the air filled organs such as the lungs, inner ear and intestines. As these tend to have few external signs of injury, this may have led to an underestimation of the true presence and extent of injury (Cernak 2010).

1.2.3 Impact on the brain

The mechanics by which the BOP wave affects the brain are not well understood. Theories about how the BOP wave can cause an immediate primary brain injury include: direct stress wave coupling through the skull or the foramina (the orbits, auditory canals and sinuses) (Nakagawa 2011); mechanical acceleration or rotation of head impacting the brain within the cranium; the standard TBI coup-contrecoup injury (Svetlov 2009); and shearing of white matter (WM) regions causing diffuse axonal injury (DAI) as well as skull flexures that can cause direct trauma to the underlying brain. The skull may also reflect and amplify the shockwave increasing the magnitude of the BOP wave and the damage it causes (Moss 2009).

As well as the direct impact of the shockwave passing through the head, energy may be transferred from elsewhere in the body. The BOP wave can compress the abdomen and chest, which may pressurise systemic blood and cerebrospinal fluid (CSF) within the spinal canal to impact brain tissue near the cerebral vessels and ventricular system (Courtney 2009, Bauman 2009). The effect of the shockwave on other parts of the body may also influence the nervous response back to the brain via the vasovagal response, leading to cerebral hypoxemia (Cernak 2010). In reality these mechanisms may co-exist (see Figure 1-5).

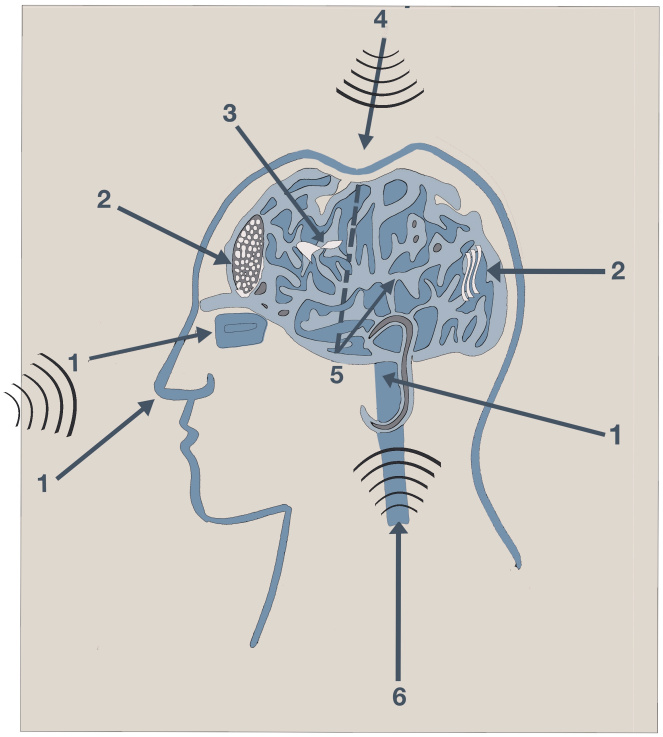


Figure 1-5. Theoretical mechanisms by which BOP wave causes primary brain injury

This figure shows the different mechanism by which the BOP is theorised to cause primary brain injury. 1. Direct stress wave coupling through the skull or the foramina. 2. Acceleration or rotation of head impacting the brain within the cranium, the coup-contrecoup injury. 3. Shearing of WM regions causing diffuse axonal injury. 4. Skull flexure causing direct trauma to the underlying brain. 5. Reflection and amplification of the shock wave by the skull. 6. Energy transfer from pressurisation of systemic blood and CSF.

Secondary brain injury can occur from the activation of the neuroendocrine-immune system since haemorrhage and mechanical tissue damage can reult in the release of pro-inflammatory cytokines into systemic circulation, which can induce apoptosis (Bhattacharjee 2008) (see Figure 1-6).

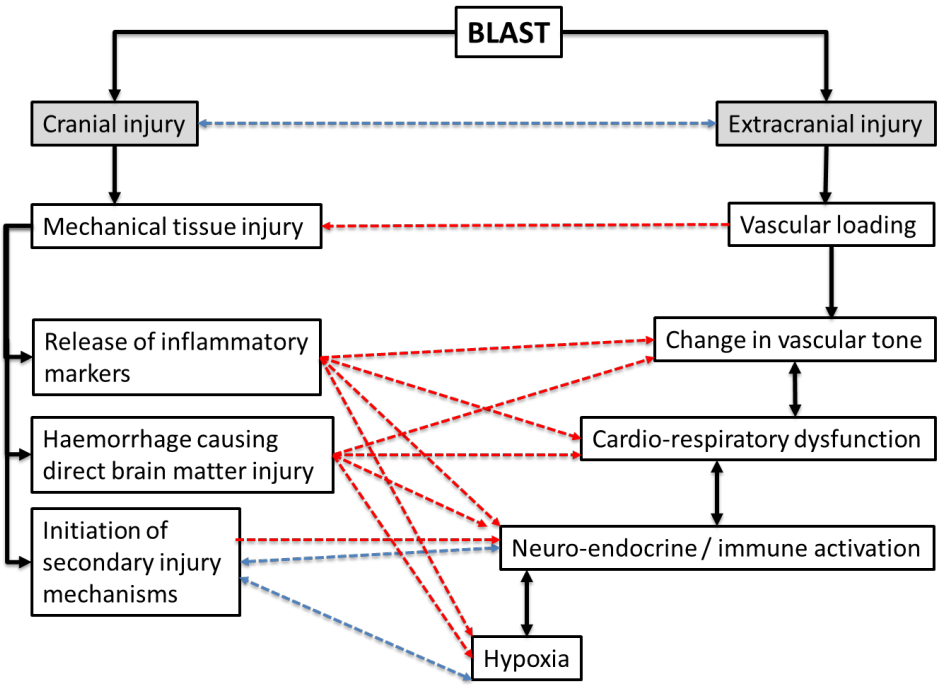


Figure 1-6. Interactions between intracranial and extracranial injuries

This figure demonstrates the complex interaction between intracranial and extracranial injuries (adapted from Cernak 2010).

1.3 Traumatic brain injury

1.3.1 Definition

Traumatic brain injury (TBI) can be defined as “an alteration in brain function, or other evidence of brain pathology, caused by an external force” (Menon 2010). Alterations in brain function include: loss of consciousness; altered level of consciousness; or memory loss at the time of the injury. Radiological investigations, including MR imaging and laboratory tests, such as lumbar puncture, can also be used to confirm brain damage.

1.3.2 Epidemiology

It is estimated that approximately 10 million people per year suffer a TBI worldwide (Hyder 2007). In Europe, about 235 per 100,000 individuals suffer a head injury each year leading to approximately one million hospital admissions (Tagliaferri 2006). In

the United Kingdom (UK) 700,000 people attend A&E departments every year with a TBI; 140,000 of those will be admitted for treatment (Brain injury – the facts 2015).

TBI is one of the major causes of death and disability among people under the age of 40 years. The incidence of TBI is bimodal with peaks in the young (<19 years old) and old (>75). Adults over 75 years have the highest incidence of hospitalisation only (Langlois 2006b). In the UK, 30% of the 115,000 estimated people suffering a TBI per year are younger than 15 years old (Thornhill 2000). There are approximately 1 million people in the UK currently living with the long-term effects of a brain injury (Brain injury – the facts 2015). Overall, males are about twice as likely as females to sustain a TBI, which is likely due to males engaging more often in risk-taking behaviour (CDC 2001).

1.3.3 Aetiology

In the UK, falls (22-43%) and assaults (20-50%) are the most common causes of TBI, followed by road traffic accidents (RTAs) (~ 25%) (NCCAC 2007). The rate of TBI secondary to falls is highest among children and adults over 75 years, and the rates of both RTA and assault are higher among adolescents (Langlois 2006a). RTAs account for a greater proportion of the more severe cases of TBI.

Alcohol consumption increases the risk of RTAs, while risk-taking behaviour, falls and violence are contributory factors in many adult TBIs (Kraus 1989, Tagliaferri 2006).

Exposure to blasts is a leading cause of TBI among active duty military personnel in war zones. Veterans' advocates in the United States (US) believe that 10 to 20% (150,000 to 300,000) of Iraq veterans have some level of TBI (Facts About TBI in the USA 2015).

1.3.4 Outcomes

TBI often results in focal neurological deficits, cognitive slowing, behavioural and emotional impairments, sleep disturbance, endocrine dysfunction and epilepsy (Langlois 2006b). Forty percent of TBI survivors in Western Europe continued to

have persistent disabilities (Maegele 2007). In 2010, the direct and indirect annual costs of TBI in Europe were estimated at €64.1 billion, representing an economic challenge for health care systems (Gustavsson 2011).

The increase in survival following TBI (Fuller 2011), and in particular that it is younger people surviving, results in long lasting disabilities that increase the requirement for rehabilitation (Patel 2005). TBI represents a significant medical and socioeconomic burden for modern society (Murray 1997).

Despite the knowledge of lesion location in TBI, outcomes in terms of functional deficit are difficult to predict. This may be because there is widespread disruption of WM leading to disruption of the cognitive networks they serve (Kinnunen 2011).

1.3.5 Classification of TBI

There are several ways of classifying TBI. Classification may be: by mechanism of injury; by severity; based on clinical examination at the time of presentation; by the extent of injury (focal vs diffuse); and by patho-anatomical type (extradural haematoma, contusion, diffuse axonal injury, subdural haematoma, subarcahnoid haemorrhage and diffuse swelling). Classification systems are used to help medical professionals communicate information consistently, as a guide to investigation and treatment in the acute setting, for predicting outcomes, and as a means of comparing the efficacy of treatments. The most commonly used TBI classification systems are:

- Primary vs secondary
- Blunt vs penetrating
- Focal vs Diffuse Axonal Injury (DAI)
- Glasgow Coma Score (GCS)
- Proposed patho-anatomical system
- The Mayo Classification System

Here, we have used the Mayo Classification System because it addresses the lack of reliability of using the patient's GCS alone and can still be calculated if some of the initial medical documentation is missing. This is particularly important in the military population, who were injured in battle and where the requirement to win the

firefight may prevent medics from immediately recording the GCS. I describe the different TBI classification systems in more detail below.

1.3.6 Classification of extracranial injuries

In polytrauma, extracranial injuries modulate and are modulated by the presence of TBI. We have used Abbreviated Injury Score (AIS) and the Injury Severity Score (ISS) classification systems, which are described below.

1.3.7 Primary vs secondary injury

Primary injury is traumatic damage to the brain, caused by an external mechanical force and occurs at the moment of injury. It is usually caused by an object striking the head or by acceleration-deceleration forces causing shearing of WM tracts (Silver 2005). Primary injuries are currently untreatable. The secondary injury occurs in the hours, days and months following trauma and results from processes initiated by the trauma. Secondary injuries are thought to be preventable and so is the focus of most medical treatments. The secondary injury comprises a multitude of processes including; ischaemia, hypoxia, hypotension, cerebral oedema, hypercapnia, acidosis, infection and excitotoxicity secondary to neurotransmitter release. More recently, spreading waves of depolarisation on the cortex of the brain (Hinzman 2014, Hartings 2014) and microglial activation, causing a prolonged state of inflammation predisposing patients to diseases like dementia, have been proposed as additional mechanisms of secondary injury (Hernandez-Ontiveros 2013).

1.3.8 Blunt vs penetrating

Blunt injuries occur when the head is struck by an object or experiences rapid acceleration-deceleration forces. Crucially, the skull and dura surrounding the brain are not penetrated. With regard to later classifications, blunt injuries can cause both focal and diffuse injury. Most injuries occurring in peacetime are blunt injuries (Prognosis in penetrating brain injury, 2001). In contrast penetrating injuries occur when the skull and dura are breached and the brain is directly injured. Penetrating

brain injuries have a higher mortality than blunt injuries (Prognosis in penetrating brain injury, 2001). A far greater proportion of brain injuries occurring in wartime are penetrating and it is this mechanism of injury that helmets are designed to protect against.

1.3.9 Focal v diffuse axonal injury (DAI)

Brain damage following a TBI can be described as focal or diffuse, based on clinical and radiological examination (Silver 2005). These mechanisms are usually found together.

1.3.10 Focal injury

Focal brain injuries mainly result from inertial forces, such as objects striking the head or the brain striking the inside of the skull, causing structural disruption of neural tissues (Ommaya 1974). They principally consist of micro-vascular injury resulting in contusions to the cortical grey matter (Gennarelli 1998). They most commonly occur when the brain collides with a dural ridge or bony protuberance and thus have characteristics locations, such as the orbitofrontal cortex and temporal poles, where the brain is in close proximity to the skull or a dural ridge (Gentry 1988).

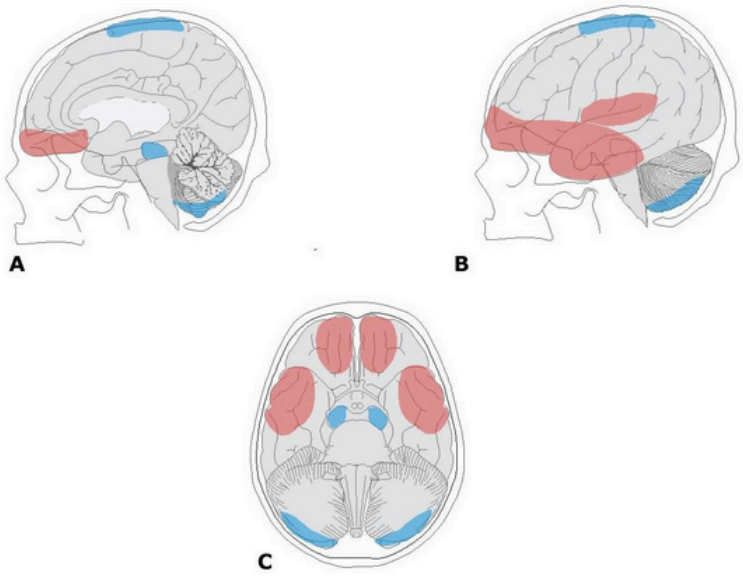


Figure 1-7. Common locations of intracerebral contusions

Schematic diagram showing the contusion location in the A, midline sagittal, B, lateral sagittal and C, axial planes. The areas in red are those most commonly affected. The areas that are most commonly affected are the orbito-frontal cortex, the temporal lobe and adjacent parietal opercular and superior temporal gyrus (Morales 2015).

1.3.11 Diffuse axonal injury

Diffuse axonal injury (DAI) refers to a range of WM injury with the most severe being tearing of axons at the time of injury to disruption of the axon's cytoskeleton and subsequent axonal death. It is the most common pathologic finding of TBI (Gentry 1988). It can occur in the absence of impact forces and is thought to be the result of the rapid acceleration- deceleration forces experienced in RTAs and some falls and assaults (Adams 1989). DAI occurs throughout the deep and subcortical white matter, and is common in midline structures, such as the corpus callosum and in the frontal lobes (Gentry 1988). Violent head movements lead to the tearing or stretching and deformation of axons (Povlishock 2005). Larger neurons and those that change direction experience greater shearing when exposed to acceleration-deceleration forces (Grady 1993, Povlishock 1993, Yaghmai 1992). In addition, axons are more easily injured in areas of differing tissue density, such as at the subcortical grey-WM interface (Grady 1993, Povlishock 1993). Axonal fibres are the most vulnerable to these shearing forces and so are injured first. If the forces are sufficiently strong, the perforating blood vessels within the WM will also be damaged leading to the small petechial haemorrhages, known as microbleeds, that are currently the diagnostic sign of underlying WM injury on radiological examination (Bigler 2001). Tearing of the axons at the time of injury, known as "primary axotomy", is relatively rare. In most cases disruption of the cytoskeleton causes a failure of axonal transport of ions leading to "secondary axotomy" and subsequent disconnection (Johnson 2013). Figure 1-8 illustrates DAI.

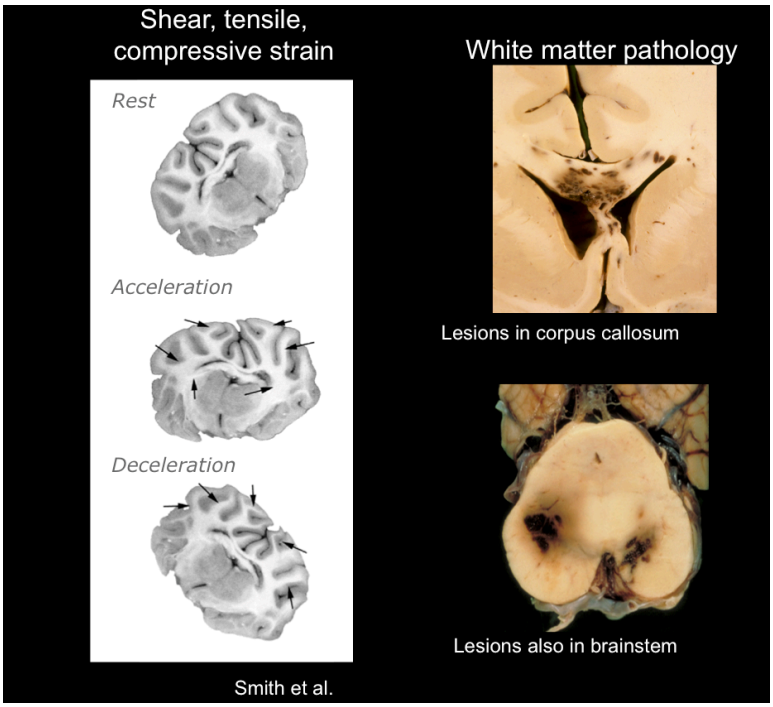


Figure 1-8. Diffuse axonal injury

The three images on the left show the coronal slice through a porcine brain. The top image shows the normal position of the white matter tracts and gyri in the brain. The middle image shows the distortion of the normal white matter anatomy when the brain is subjected to large acceleration forces and the bottom image shows the areas of compressive strain when the brain is exposed to large deceleration forces. The image on the right shows post mortem examination of a human brain with macroscopic evidence of haemorrhage in the corpus callosum and brainstem (Smith 2003).

1.3.12 Classification by injury severity

1.3.12.1 Glasgow Coma Score

The severity of brain injuries is commonly described using the modified Glasgow Coma Scale (GCS). As shown in Table 1-1, this 15 point scale assesses the patient's best eye, motor and verbal response and has been validated as a predictor of in-hospital mortality (Moore 2006). Whilst being validated and reproducible, the GCS has been criticised for being inaccurate in intubated patients, in intoxicated patients and those with orbital injuries. It is mathematically weighted towards the motor response and lacks reliability when monitoring the level of consciousness in

patients with moderate brain injury (Segatore 1992). Crucially, for the previously mentioned reasons, it can only be performed in 61% of patients in the pre-hospital setting (Glasgow Coma Scale 2015). In the context of brain injury a GCS of 13-15 is defined as mild, 9-12 as moderate and 3-8 as severe.

Table 1-1. GCS scoring system

Glasgow Coma Scale (GCS, Teasdale and Jennett 1974)

Glasgow Coma Scale		
Eye response	Open spontaneously	4
	Open to verbal command	3
	Open in response to pain	2
	No response	1
Verbal response	Talking / orientated	5
	Confused speech / disorientated	4
	Inappropriate words	3
	Incomprehensible sounds	2
	No response	1
Motor response	Obeys commands	6
	Localises to painful stimuli	5
	Withdraws from painful stimuli	4
	Abnormal flexion	3
	Extension	2
	No response	1

1.3.12.2 Patho-anatomical classification

Critics of the GCS have argued that it does not provide specific information about the pathophysiological mechanisms, which are responsible for neurological deficits and targeted by interventions. As a result the GCS alone cannot direct treatment and, by grouping injuries of different pathologies together, is a barrier to finding effective therapies (Saatman 2008). Many different types of TBI can produce the same GCS score (See Figure 1-9). The treatment and outcomes from these may be very

different and a more detailed classification system has been proposed in order to determine whether therapies are effective. The new patho-anatomical classification system is based on the premise that injuries with similar patho-anatomical features would share patho-physiological mechanisms (Saatman 2008). This system would be used in conjunction with measures of injury severity, like GCS, and look at specific outcome relevant to the patho-anatomic subtypes of TBI. It is currently a research tool whose main utility will be in the evaluation of therapies.

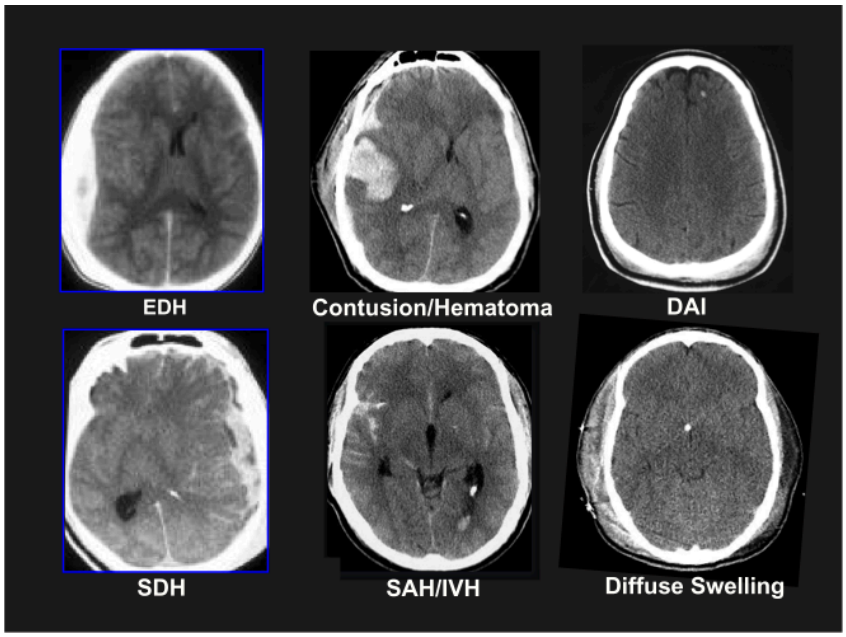


Figure 1-9. Heterogeneity of TBI

Each of these patients had a GCS of <8. However they have different pathologies, treatment and outcomes. CT scans of six different patients with severe TBI, highlighting the significant heterogeneity of pathological findings. CT scans represent patients with extradural haematomas (EDH), contusions and intraparenchymal haematomas (Contusions/Hematoma), diffuse axonal injury (DAI), subdural haematoma (SDH), subarachnoid haemorrhage and intraventricular haemorrhage (SAH/IVH) and diffuse brain swelling (Saatman 2008).

1.3.12.3 The Mayo Classification System

The Mayo Classification System for TBI severity (shown in Table 1-2) was developed to address the unreliability of the GCS in assessing awareness and the frequency of missing documentation in medical notes. It classifies patients into three groups; definite moderate to severe TBI, probable mild TBI (MTBI) and possible TBI. It takes into account episodes of loss of consciousness, the duration of posttraumatic amnesia as well as radiological evidence on CT scanning of injury (for example most commonly skull fractures and contusions) (Malec 2007).

Using this classification system a TBI is classified as definite moderate-severe if one or more of the following are present: death due to this TBI, loss of consciousness of 30 minutes or more, post traumatic amnesia (PTA) of 24 hours or more, and GCS <13 in the first 24 hours. In addition, if there were radiological evidence of neurological injury such as a contusion, then the TBI would be classified as definite moderate-severe. If there was a loss of consciousness for less than 30 minutes, PTA less than 24 hours, then the injury would be classified as probable mild. If there are one or more of the following symptoms: blurred vision, confusion, feeling dazed, dizziness, headache or nausea, the injury is classified as a probable TBI (Malec, 2007). The Mayo classification system has been shown to have both a high sensitivity and specificity for moderate-severe TBI (Malec 2007).

Table 1-2. Mayo Classification of TBI (Malec 2007)

MAYO TBI Severity Classification System	
A. Moderate-Severe (Definite) TBI if one of more of the following criteria apply:	Death due to this TBI Loss of consciousness of 30 minutes or more Post-traumatic anterograde amnesia of 24 hours or more Worst GCS in the first 24 hours <13 One or more of the following is present;

	• Intracerebral haematoma • Subdural haematoma • Extradural haematoma • Cerebral contusion • Penetrating TBI • Subarachnoid haemorrhage • Brain Stem Injury
B. If none of the Criteria A apply, classify as Mild (Probable TBI) if one or more of the following criteria apply:	Momentary loss of consciousness <30 minutes Post-traumatic anterograde amnesia less than 24 hours Depressed, basilar or linear skull fracture (dura intact)
C. If none of Criteria A or B apply, classify as Symptomatic (Possible) TBI if one or more of the following symptoms are present:	• Blurred vision • Mental state changes (confusion) • Dazed • Dizziness • Focal neurological symptoms • Headache • Nausea

1.3.12.4 Injury Severity Score (ISS) and Abbreviated Injury Scale (AIS)

It is important to consider extracranial injuries because outcomes for a given ISS have been shown to be worse if there is also a brain injury. In addition the outcomes from head injuries are worse if there is associated hypoxia or hypotension which can

be caused by extracranial injuries (Fuller 2011). In both civilian and military trauma, head injuries frequently occur in the context of polytrauma (where there is an injury to more than one body system). This is especially true in the context of soldiers injured by explosive devices, which indiscriminately injure the whole body. The Injury Severity Score (ISS) (shown in Table 1-3) is an anatomical scoring system originally developed by the automotor industry, which provides an overall score for patients with multiple injuries. Each injury is assigned an Abbreviated Injury Scale (AIS) score and is allocated to one of nine body regions (head, face, neck, chest, abdomen, pelvis, spine, upper and lower extremities and external, which encompasses the skin). Only the highest AIS score for each region is used. The AIS scores for the 3 most severely injured body regions are squared and added together to produce the ISS score. The ISS score ranks injuries from 0 to 75. If an injury is assigned an AIS of 6 (unsurvivable injury), the ISS score is automatically assigned to 75. The ISS score has been shown to correlate with mortality, morbidity, hospital stay and other measures of severity (Injury Severity Score, Trauma.org 2015).

Table 1-3. Example of the Injury Severity Score for a fictional polytrauma patient

This table shows how the ISS is calculated for a fictional patient who has suffered a head, chest, liver and spleen injury as well as a femoral fracture. Using the AIS classification system these injuries were scored and the top three were then squared and added together, giving an ISS of 50 out of a total possible score of 75.

Region	Injury description	AIS	Square of the Top Three
Head & Neck	Cerebral contusion	3	9
Face	No injury	0	
Chest	Flail chest	4	16
Abdomen	Minor contusion of liver	2	
	Complex rupture spleen	5	25
Extremity	Fractured femur	3	
External	No injury	0	
Injury Severity Score			**50**

The AIS requires knowledge of the precise anatomical damage that has occurred. This information is often not known at the time of initial assessment. An error in AIS scoring increases the ISS error and, as a consequence, many different injury patterns can yield the same ISS score. For these reasons, the ISS is not useful as a triage tool. However, it has been shown to be a good method post-triage for describing patients with multiple injuries and evaluating their care (Baker 1974).

The Mayo Classification System and the Injury Severity Score were both used to assess the subjects in this study. We chose to use the Mayo system because it addressed missing or inaccurate clinical information at the time of the soldiers' injury

and has been shown to be more sensitive and specific in classifying severity of TBI when compared to single indicators such as PTA, GCS and loss of consciousness alone (Friedland, http://www.acnr.co.uk/2013/07/classification-of-traumatic-brain-injury/). The ISS was used in order to make the comparisons across subjects and groups more accurate by taking into account extra-cranial injury.

1.4 Radiological assessment

1.4.1 Computerised Tomography (CT)

At the initial time of injury, patients may be rapidly deteriorating from their brain injury or may be physiologically unstable from injuries to other body systems. These clinical conditions require urgent treatment and the most rapid investigations possible including radiological imaging. In addition, because the equipment (for example a ventilator) used to assess and treat unconscious patients is usually ferromagnetic, CT scanning is the first line imaging modality employed in TBI.

CT scanning uses X-rays to create a two dimensional picture of the patient's anatomy. X-rays are fired from an X-ray tube that rotates around the patient. The X-rays pass through the patient's body and are absorbed to differing degrees depending on the density of the tissues they passing through. The denser the tissue, the more X-ray radiation is absorbed and the region appears brighter on the picture produced. The globin molecule in haemaglobin is relatively dense and so an acute haemorrhage appears white on CT – thus CT provides a rapid assessment of structural brain injuries and an accurate means of diagnosing intracranial haematomas. It is available in most trauma centres or facilities where trauma patients are managed in the UK and is faster and cheaper than MRI. MRI is, however, more sensitive than CT in detecting traumatic lesions (Paterakis 2000). In addition, CT underestimates the severity of many forms of cerebral injury such as non-haemorrhagic cortical contusions, when there is little or no change in tissue density (Gentry 1988).

1.4.2 Magnetic resonance imaging (MRI)

1.4.2.1 MRI summary

MRI uses a magnetic field to produce images of biological tissue. It is based on the resonance of nuclei in a magnetic field (nuclear magnetic resonance). These nuclei are stimulated using oscillating radiofrequency (RF) pulses, known as pulse sequences, whilst in a magnetic field. When this energy is released by the nuclei, a signal containing spatial and structural information from the material under investigation can be detected. These signals are reconstructed into images. Different sequences detect different tissue properties and can differentiate tissues of differing densities such as the grey matter and WM of the brain. MR imaging has been shown to be more sensitive than CT for the detection of traumatic lesions (Paterakis 2000). MR imaging can be performed using several different sequences, which are sensitised to the differing properties of the tissues. Some sequences are more sensitive than others for investigating traumatic lesions. Susceptibility weighted imaging (SWI) is very sensitive to the properties of haem, making it a useful investigation of microbleeds. DTI is sensitive to the diffusion of water and can be used investigate WM integrity (Smith 2006). In the following section, I describe the basis of MR imaging and the main sequences (T1, T2, T2* and SWI) used. I then describe DTI, which is one of the main imaging techniques used in this PhD (Gentry 1988).

1.4.2.2 Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) describes the interaction of an externally applied magnetic field with the magnetic moment of the nucleus of an atom. All the particles of an atom spin on their own axis. The rotation of the nuclear particles creates a magnetic field, which is described as a magnetic moment. It is possible to consider these particles' behaviour as similar to miniature magnets. In the context of this work, the hydrogen nucleus absorbs RF energy and then subsequently emits it. The NMR signal is detected as a function of time and demonstrates the presence of hydrogen atoms but not their location in space.

In a hydrogen atom, there is a single proton (^{1}H), constituting a spinning positive charge. The interaction of the magnetic moments with an applied magnetic field generates signals that provide spatial information.

In medical imaging, signals are usually collected from the nuclei of hydrogen (^{1}H) atoms. However it is also possible to obtain signals from other elements including ^{13}C, ^{23}Na and ^{31}P, which is the basis of MR Spectroscopy.

1.4.2.3 Repetition time and echo time

There are two important factors that govern the time at which MR images are collected. One is the time interval between successive excitation pulses, known as repetition time (TR). The other is the time interval between excitation and relaxation, known as echo time (TE). Variations in these parameters will affect whether signal intensity is primarily due to T1, T2 or T2* relaxation (Figure 1-10).

1.4.2.4 T1-weighted images

T1-weighting imaging is commonly used for imaging anatomical structures. Images are called T1-weighted if the relative signal intensity of voxels depends upon the T1 value of the tissue. This type of imaging requires an intermediate TR, to generate a contrast between the different tissue types, and a short TE, to minimise T2 contrast. T1-weighted images depict the spatial distribution of T1 values, so that voxels with short T1 values are bright and those with long T1 values are dark. Fluid appears as black, grey matter appears as dark grey and white matter appears as light grey.

1.4.2.5 T2-weighted images

To have an exclusive T2 contrast, the TR must be long, so that the longitudinal recovery is almost complete in all tissues and T1 contrast is minimal, with an intermediate TE. On these images, fluid appears bright, grey matter appears as light grey and white matter is dark. This type of image is particularly useful for investigating pathological conditions such as tumours and inflammation in which there is an increase in the water content of the tissues making the areas appear brighter.

1.4.2.6 T2-weighted images*

T2* are generated by pulse sequences with long TR and medium TE. The pulse sequence uses magnetic field gradients to generate the signal echo. T2*-weighted images are sensitive to the amount of deoxygenated haemoglobin, iron and calcification present in tissues. It is the basis of gradient echo (GRE) and susceptibility weighted imaging (SWI) (Chavhan 2009).

1.4.2.7 Susceptibility weighted images

SWI are generated using a flow compensated, long echo and gradient recalled echo (GRE) pulse sequence. This technique takes advantage of the difference in susceptibility between tissues and uses the phase image to detect them. The magnitude and phase data are combined to produce an image, which is extremely sensitive to venous blood, haemorrhage and iron storage.

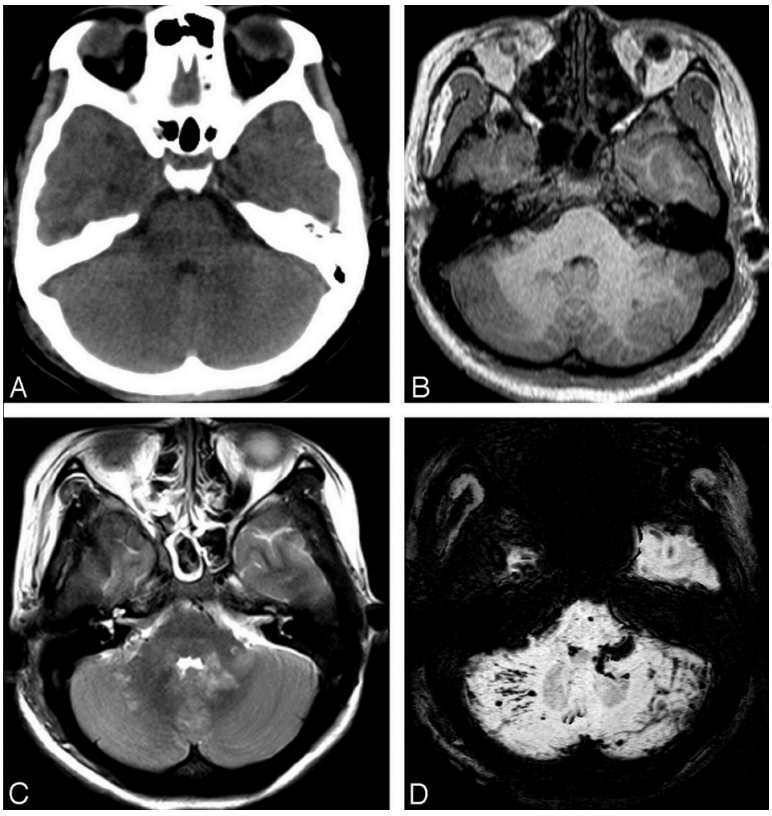


Figure 1-10. T1, T2, T2* SWI image

A, non-enhanced CT scan shows areas of low attenuation in the pons and bilateral brachium pontis. B, T1-weighted image shows similar hypointense

lesions in the pons and right hemisphere of the cerebellum. C, T2-weighted image shows multiple hyperintense lesions in the pons and right cerebellar hemisphere. *D*, SWI demonstrates multiple hypointense lesions in the pons and both hemispheres of the cerebellum. There are more lesions shown on SWI, illustrating its increased sensitivity for microbleeds.

1.4.3 Diffusion Tensor Imaging

Diffusion tensor imaging (DTI) is an MR technique used to reveal the microstructure and anatomy of the brain by characterising the diffusion of water molecules. It can be used to provide information about anatomical connectivity in the brain, by measuring the anisotropic diffusion of water in white matter tracts (Smith 2006).

In an unrestricted environment, water molecules can move in all directions (Brownian motion), and the diffusion is said to be isotropic. In brain tissue, water movement is limited by cell membranes and myelin and the diffusion is said to be restricted or anisotropic. The extent of restriction depends on brain tissue type. In CSF water can move in all directions, and diffusion is unrestricted or isotropic. White matter tracts have underlying directionality because of myelinated axons, which restricts the movement of water molecules in the direction of the tracts producing an anisotropic diffusion pattern. DTI can be used to estimate axonal orientation and myelin integrity.

1.4.4 Measuring water diffusion with MRI

In diffusion weighted MRI, the detection of water diffusion is made possible by the use of a pair of magnetic gradient pulses. The first gradient causes protons along the gradient axis to dephase. The amount of dephasing is dependent on the position of the proton along this magnetic gradient, which allows encoding the position of the protons along one axis by their relative phase. The second gradient is applied after approximately 20-50 milliseconds (ms) along the same axis but with opposite polarity. This has the effect of cancelling out the dephasing induced by the first gradient. The second pulse should completely rephase the proton to its initial status, but because the amount of rephasing is also dependent on the position of the proton along the gradient axis, any displacement of water molecules along this axis occurring between the two gradients results in incomplete rephasing, which causes a

detectable loss in signal. The more diffusion that occurs along the direction of the applied gradient, the more attenuated the signal is compared to the signal obtained with no diffusion gradients applied (b=0). A diffusion coefficient D can thus be calculated for each imaging voxel in the diffusion-weighted image based on the change in signal intensity, using the following equation:

$$S = S_0 e^{-bD}$$

Where S is the signal intensity in the diffusion-weighted image, S_0 is the signal intensity in the reference image (non-diffusion-weighted or b_0 image) and b is the diffusion-weighting gradient factor (Mori 2006).

Importantly, diffusion weighted MRI is only sensitive to water diffusion occurring along the axis of the applied magnetic gradient. To detect the diffusion of water in different directions, the gradients must be applied along multiple axes. DTI is typically carried out by applying many diffusion gradients along specific directions. The strength of these gradients is characterised by a known parameter b ('b-value'). The more directions, the better the direction of diffusion will be described.

1.4.5 DTI measures

By calculating the distance which water diffuses in a given voxel in a given amount of time for several (at least six) non-collinear directions, it is possible to reconstruct a 3D ellipsoidal shape that best describes the pattern of water diffusion occurring in a given voxel. This ellipsoid can be mathematically described as a 3D tensor that can be characterised by 3 eigenvalues λ_1 (major axis), λ_2 (median) and λ_3 (minimum).

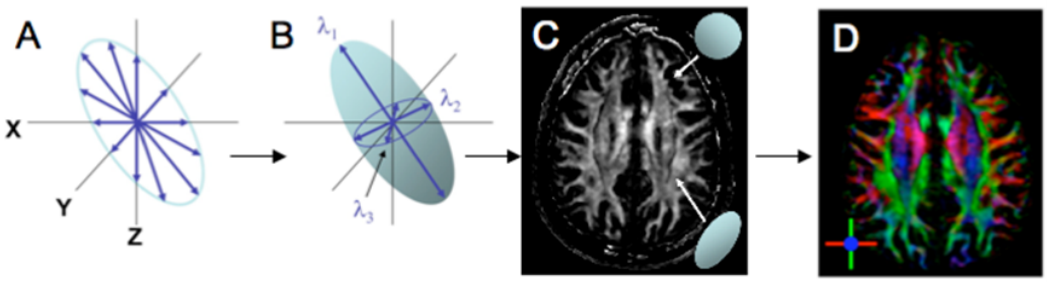


Figure 1-11. DTI image production

A) Diffusion is measured along multiple axes by applying various gradients. B)

This allows the estimation of the shape of the diffusion ellipsoid that can be described by 3 eigenvalues. C) An anisotropic map can be created, in which regions with higher anisotropy are brighter. D) The principal orientations of each pixel can also be colour coded to produce an orientation map. (Adapted from Mori 2006).

By employing this diffusion tensor model, several diffusion parameters can be derived. The most frequently used is fractional anisotropy (FA), which estimates the degree of diffusion directionality. FA is a function of the 3 eigenvalues characterising the diffusion tensor.

An FA of 0 indicates a complete isotropic diffusion, and values can increase from 0 to 1 with increasing diffusion anisotropy. FA provides important information about the composition of tissue within a voxel. In a WM bundle, reduced FA is generally assumed to reflect damage to the axon membrane, reduced axonal myelination, reduced axonal packing density, and/or reduced axonal coherence (Arfanakis 2002, Song 2002).

1.4.6 DTI data analysis

There are several accepted methods to compare diffusion data across subjects. Some researchers have summarised the diffusion characteristics (primarily FA) of the whole brain by performing a whole brain histogram analysis and comparing these values between subjects. This approach is limited because it does not take into account where in the brain the difference occurs (Smith 2006). Histogram analysis describes changes to the whole brain, but does not provide any regionally specific information.

Voxel based morphometry is another method by which FA can be compared across subjects. Using this method each subject's FA image is registered into a standard space and then statistics are carried out on each voxel to find areas that correlate. There is concern about the alignment of images and a lack of certainty that the voxels at any given standard space represent data from the same part of the same WM tract from each and every subject.

1.4.7 Region of interest (ROI) analysis

ROI analysis can be used to test any tract or specific region and is particularly useful in the investigation of TBI, where specific tracts/regions are more frequently damaged than others. This approach simply consists of extracting averaged measures of diffusion (e.g. FA) from specific brain regions or WM tracts.

1.4.8 Tract-based spatial statistics (TBSS)

Voxel-based analysis can be fully automated using software such as FSL (FSL is an analysis tool for FMRI, MRI and DTI brain imaging data created by FMRIB, Oxford, UK) and hence easily reproducible. It can be used to examine the entire brain in a single subject and to compare groups of subjects. TBSS is a voxel-based technique to analyse WM structure across the whole brain (Smith 2006). TBSS allows complex patterns of WM disruption to be identified and their relationships (such as with cognitive or endocrine function) studied. TBSS analysis creates a mean FA skeleton at the centre of the WM tracts by thinning the mean FA image. This reduces partial-volume confounds and reduces inter-individual variability.

1.4.9 Magnetic Resonance Imaging in TBI and bTBI

The brain has localised functions with each cerebral hemisphere having a contralateral relationship with the side of the body that it controls. The brain is divided into the Cerebrum, Cerebellum, Limbic system and Brainstem. These are further sub-divided.

The cerebrum is made up of the frontal, parietal, temporal and occipital lobes. The frontal lobe is responsible for creative thought, problem solving, intellect, attention and executive function. The parietal lobe is responsible for the initiation of movement, orientation, recognition and perception of stimuli. The occipital lobe is responsible for visual processing and the temporal lobes associated with the perception and recognition of auditory stimuli, memory and speech.

The cerebellum is associated with regulation and coordination of movement, posture and balance. More recently it has been shown to have a role in cognition, in particular episodic memory (Andreasen 1999).

The limbic system, is comprised of the amygdala, hippocampus, thalamus, hypothalamus, basal ganglia and cingulate gyrus. It is the emotional centre of the brain and is responsible for memory formation.

The brainstem is made up of the midbrain, pons and medulla. It plays an important role in the regulation of cardiac and respiratory function, consciousness and the sleep cycle. In addition it contains nuclei that relay signals from the cerebum to the cerebellum and in this way acts as a vehicle for sensory information.

Given this localisation of function, it should be relatively simple to predict the neurological deficits that would occur following TBI. However, the anatomical location of lesions in TBI does not fully explain the neurological deficits experienced by patients (Bigler 2001). Despite the heterogeneity in the cause, severity, and distribution of pathology in TBI, common neuropsychological and cognitive deficits are frequently observed and these are likely to result from the widespread disruption of WM networks that occurs in DAI (Kinnunen 2011).

Whilst large focal haemorrhagic lesions are seen on CT and conventional T1-weighted MR, the microbleeds associated with DAI are not (Ommaya 1971). The best available imaging technique to identify microbleeds is susceptibility weighted imaging (SWI) (Akiyama 2009). SWI is a MR technique that allows the visualisation of small amounts of haem (from red blood cells) products by accentuating their magnetic properties (Haacke 2004). The extent of SWI-identified haemorrhages has been shown to correlate with the initial severity of injury, duration of coma, long-term outcome as well as specific neuropsychological deficits (Tong 2004, Babikian 2005). However, the presence of microbleeds alone underestimates the extent of DAI, as some DAI may be non-haemorrhagic (Gentry 1994, Paterakis 2000). Therefore, in this study, in addition to conventional MR, we have used diffusion tensor imaging (DTI), a relatively new MRI modality that provides *in vivo* indices of WM integrity in order to identify otherwise undetected DAI (Arfanakis 2002, Bazarian 2007, Sugiyama 2009, Sharp 2011).

CT and standard MRI structural images (T1, T2 and T2*) can demonstrate large focal contusions or bleeds (Van Boven 2009). Whilst CT is essential in the early assessment of brain injury, it has been shown to be less sensitive than MR in detecting intraparenchymal, shearing and haemorrhagic lesions as well as in injuries

of the posterior fossa (Chastain 2009). T2WI and FLAIR imaging are more sensitive than T1 for evaluating brain lesion (Chastain 2009) and they have also been shown to discriminate between good and poor outcomes by volume and number of lesions as well as by anatomical distribution (Chastain 2009). Standard structural MRI is not sensitive to DAI, which it can only detect indirectly once the injury is in the chronic phase and brain atrophy has occurred by measuring brain volume loss (measured using volumetric analysis) (Van Boven 2009).

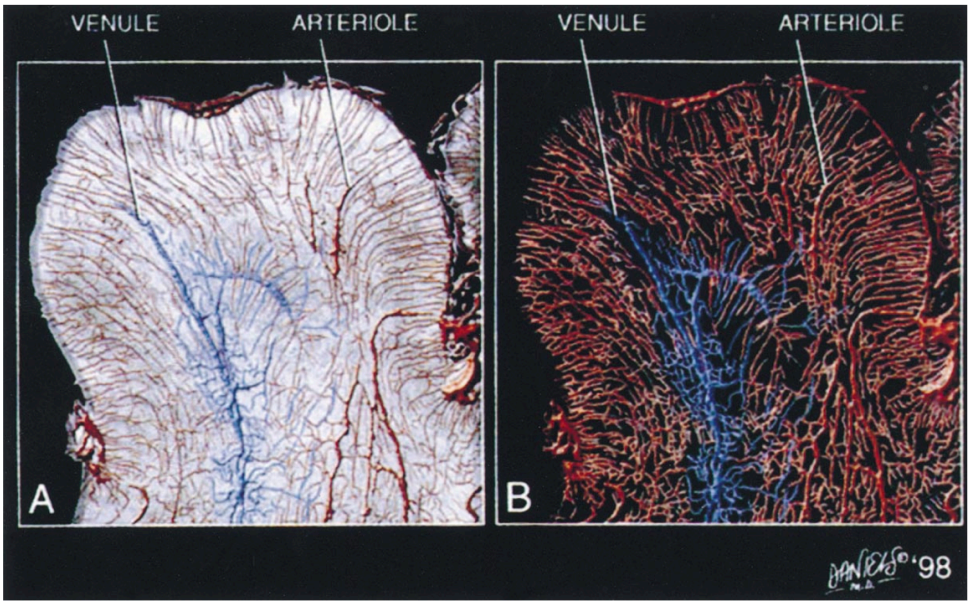


Figure 1-12. The vascular supply of a single gyrus

The vascular supply of a single gyrus (A and B). Arterioles are depicted in red with venules in blue. Neural cells are considerably smaller. In (B), brain tissue has been removed to aid in visualising of the vasculature (from Haughton 1998).

Figure 1-12 shows the vascular supply to a single gyrus. The arterioles form a complex matrix of delicate vessels. Each of these vessels supplies even more delicate neurons and glial cells that cannot be seen at this magnification. Both nerves and blood vessels have limited elasticity, are of a fixed length and are held in position by other cells. Shearing forces will first damage the axonal fibres that are the most fragile and hence susceptible to injury. As the whole brain experiences these forces, the damage will be widespread and more pronounced in those areas, such as the corpus callosum, where the fibres are most vulnerable. This widespread axonal injury is referred to as diffuse axonal injury. If the shearing forces are stronger, then the micro-vascular supply will also be ruptured causing the vessels to

bleed. These small bleeds are known as microbleeds and hence are a surrogate marker of DAI (Bigler 2001).

Both Gradient Echo (GE) MR and Susceptibility Weighted Imaging (SWI) can detect microbleeds. The Microbleed Anatomical Rating Scale has been shown to be a reliable tool across different MRI sequences and levels of observer experience (Gregoire 2009).

SWI has a significantly higher sensitivity to haemorrhagic lesions than GE. Using SWI the number and volume of hemorrhagic lesions has been shown to correlate with the Glasgow Coma Scale score (Tong 2006) as well as with other clinical measures of TBI severity and with outcome at 6 to 12 months post-injury (Tong 2004). Using GE and SWI sequences underestimates the extent of brain injury because they are visualising the associated haemorrhage but not the underlying axonal injury.

Pathology studies examining brains with DAI have shown multifocal WM lesions that are occasionally associated with petechial haemorrhage (Adams 1984). DAI most frequently affects the WM, corpus callosum and the upper brain stem. Studies have linked DAI to tearing of the axons, known as “primary axotomy” and more frequently to focal misalignments of the cytoskeletal network and to changes of the axonal permeability (Povlishock 1995, Christman 1994, Grady 1993, Pettus 1994, Gennarelli 1997), depending on the severity of the injury, leading to disconnection, or “secondary axotomy” (Johnson 2013) (see Figure 1-13).

.

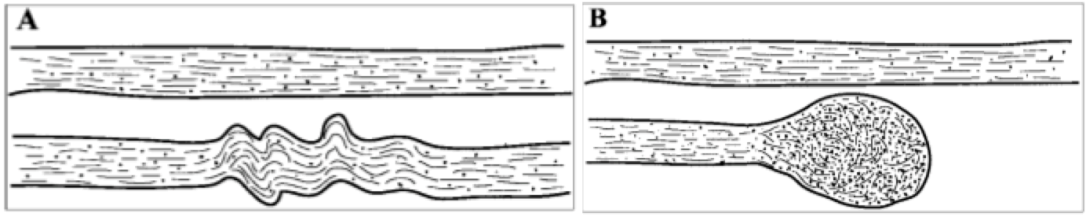


Figure 1-13. Secondary axonal injury

Illustration of axonal changes secondary to cytoskeletal perturbation from mild TBI. A. The top neuron is healthy, in the bottom neuron neurofilamentous and cytoskeletal misalignment is visible a short time after injury, this impairs axonal transport. B. Organelles accumulate in the injured region, causing the

axon to swell locally and subsequently disconnect from the rest (Adapted from Arfanakis 2002).

The first evidence of DAI is focal neurofilament misalignment, which becomes prominent within the first 6 hours post injury (Povlishock 1995, Christman 1994, Grady 1993, Pettus 1994, Gennarelli 1997). This misalignment causes impairment of axoplasmic transport and the accumulation of organelles. This continues for several hours after injury, causing swelling and expansion of the axon. The axon becomes lobulated by further swelling and becomes disconnected at 30–60 hours after injury. After disconnection, the segments of the axon are sealed and enveloped by a myelin sheath. In severe TBI the axons may be directly torn in addition to the effects described (Povlishock 1995, Christman 1994, Arfanakis 2002).

These changes initially decrease the diffusion of water along axons and increase the diffusion in directions perpendicular to them. Over time, as the axons are sealed by myelin, the water is no longer able to diffuse perpendicular to the axon. These changes in diffusion can be detected using DTI and are the basis behind which it has been shown to indicate the approximate time since injury (Mac Donald 2007).

DTI measures water diffusion in multiple directions. In tissues with underlying directionality such as the axonal alignment seen in white matter tracts, diffusion is limited to the direction of the axons. This directionality is described as being highly anisotropic. Anisotropy is usually expressed relative to the magnitude of the diffusion tensor as the fractional anisotropy. This is an index ranging from 0 to 1 (Sidaros 2008). Intact WM fibres would have a high FA value. When axons are injured, water diffusion changes for the reasons described above. Diffusion can now occur in more directions and is said to be isotropic and the FA value would be reduced (Arfanakis 2002).

DTI studies have shown reductions in FA at sites of DAI and have been shown to correlate with both outcome (Sidaros 2008) and cognition (Kraus 2007). DTI provides an objective means (the FA value) for determining the relationship of cognitive deficits to TBI (Kraus 2007).

1.5 Hypothalamic-Pituitary-Adrenal axis

The endocrine system's role is the maintenance of a stable internal environment

despite changes in the external environment (homeostasis). The hypothalamus acts as a master gland to this system, controlling the anterior pituitary gland and certain peripheral organs. It consolidates signals derived from the cortex with environmental cues, such as light and temperature and peripheral endocrine feedback and signals the pituitary gland to release hormones. Within the endocrine system, the hypothalamic pituitary adrenal (HPA) axis (shown in Figure 1-14) is of particular importance because it controls the body's response to stress, regulates digestion, the immune system, metabolism and, through its connections to the limbic system, affects emotions. Cortisol, a hormonal end product made in the adrenal cortex, acts to inhibit the hypothalamus and pituitary gland in a negative feedback loop (Bowen 2001).

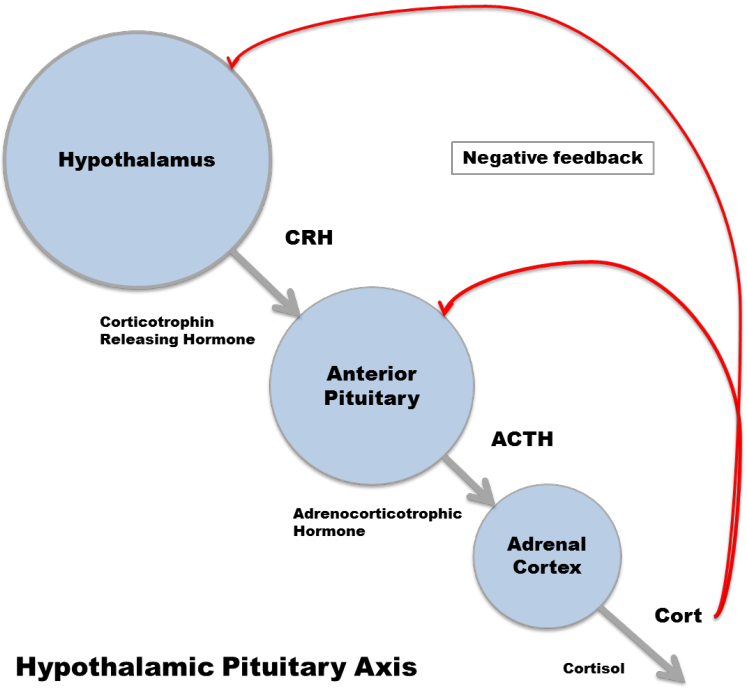


Figure 1-14. Hypothalamic-Pituitary-Adrenal Axis

The hypothalamus controls anterior pituitary gland function which in turn controls cortisol by the adrenal cortex, both are inhibited by cortisol.

1.5.1 Hypothalamic-Pituitary Axis dysfunction in TBI

The pituitary gland (as shown in Figure 1-15) and infundibulum are contained with the sella turcica, a hard boney and ligamentous structure of the skull. They are surrounded by a friable hypophyseal portal vascular system. The hypothalamus is in

close proximity to the sella. It is connected to the pituitary gland by the infundibulum. Trauma can directly cause damage to the cell bodies within the pituitary gland or hypothalamus, the WM fibres of the infundibulum or the hypophyseal vessels supplying the gland and infundibulum. Blood in the subarachnoid space around the gland may cause superficial siderosis and inflammation that may impair pituitary function. In addition, in the context of haemorrhage associated hypovolaemia, the gland may become ischaemic as seen in Sheehan's syndrome.

TBI is a recognised cause of pituitary dysfunction, in particular growth hormone (GH) deficiency (Schneider 2007). Reported prevalence rates of pituitary dysfunction following TBI vary between 2 and 68% (Schneider 2007, Kokshoorn 2011). This variability is due, in part, to differences in the heterogeneity, severity and time since injury, as well as the normal range of the tests used (Schneider 2007, Kokshoorn 2011, Kokshoorn 2010). In addition to adverse metabolic consequences, hypopituitarism causes multiple symptoms impacting on physical and psychological well-being that will impair recovery after TBI and thus hormone replacement represents an important therapeutic opportunity (Salvatori 2005, Cherrier 2009, Molitch 2011, Bondanelli 2007). It is unknown how often bTBI leads to pituitary dysfunction (Guerrero 2010).

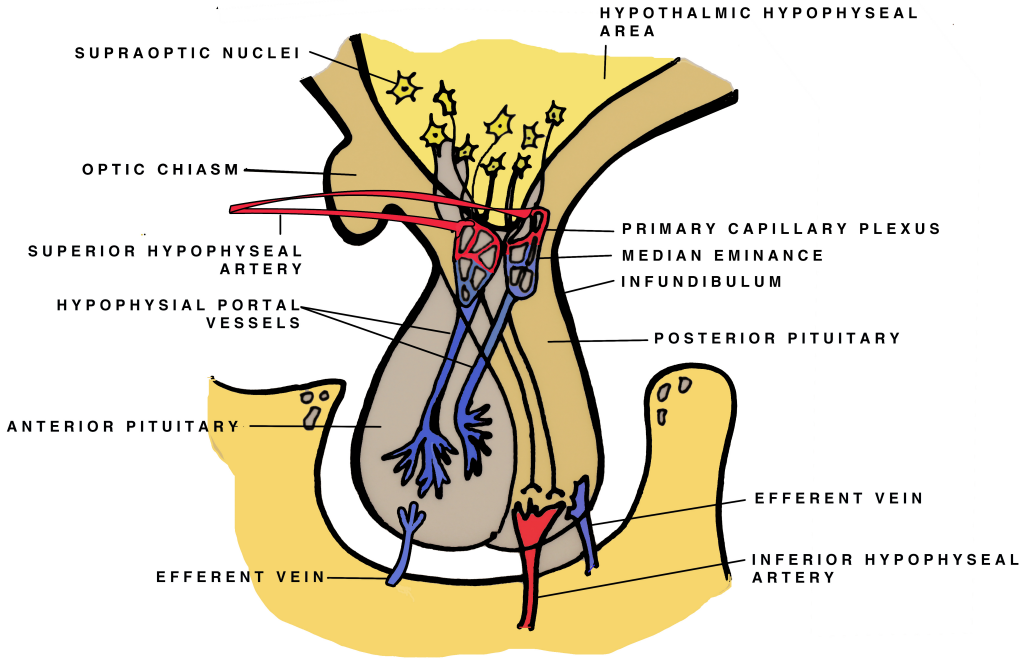


Figure 1-15 Pituitary gland

Anatomy showing the proximity to the sella turcica and the friable portal

system.

1.6 Animal models of traumatic brain injury

1.6.1 Why do we need animal models of TBI?

There is a vast body of literature on animal models of non-blast TBI. Many of these are post mortem pathological studies, as the imaging techniques to examine the brain *in vivo* have only been readily available in the last two decades. In part because of a lack of knowledge about the underlying processes that were involved in TBI, many studies treated TBI as a homogenous entity. We now know that this is not the case and there are multiple forces as well as biochemical mechanisms that contribute to the overall injury. Protective equipment (such as a helmet) are optimised to resist specific forces and therapeutic agents used to disrupt or enhance specific biochemical mechanisms. Therefore, the fact that therapeutic trials in TBI (such as the use of steroids in TBI as studied in the CRASH randomized control trial) (Edwards 2005) have failed to show any benefits may be, in part, because they have treated TBI as a homogenous disease (Saatman 2008). Since MR imaging has become more widely available, we now recognise that TBI is a heterogeneous group of pathologies. As a consequence, animal models are being developed that can reliably reproduce specific aspects of the injury.

1.6.2 Focal injury

In TBI, the primary injury involves mechanical tissue deformation and causes diffuse neuronal depolarisation and the release of excitatory neurotransmitters including glutamate and aspartate (Andriessen 2010, Muir 2006). These neurotransmitters bind to glutamate receptors and induce a massive influx of calcium ions (Lee 2004). Calcium activates calcium-dependent phospholipases, proteases and endonucleases that degrade lipids, proteins and nucleic acids. Calcium is sequestrated in mitochondria leading to calcium disturbance, energy deficits, free radical formation and initiation of apoptosis (Xiong 1997, McCall 1987). There is increased formation of oxygen and nitrogen reactive species that oxidise lipids, proteins and nucleic acids (Bains 2012). The pro-inflammatory cytokines released as a consequence of TBI up-regulate several transcription factors, inflammatory

mediators and neuroprotective genes as well as down-regulate neurotransmitter receptors and their release mechanisms (Raghavendra 2003). Increased expression of cytokines and chemokines induces brain oedema, blood–brain barrier damage and cell death (Ziebell 2010). The result of these complex cascades after TBI eventually leads to cell damage and death, causing a range of functional deficits.

Recent work has indicated that glial cells, the immune cells of the CNS, can become chronically activated leading to a progressive aggregation of tau protein in the brain, and that this tauopathy has been implicated as the cause of chronic traumatic encephalopathy (CTE) (Goldstein 2012).

1.6.3 Diffuse axonal injury (DAI)

In a mouse model of DAI, Cernak *et al.* demonstrated an increase in the permeability of the blood brain barrier with associated oedema as well as apoptosis in the cerebral hemispheres and brain stem (Cernak 2004).

The cerebral blood supply has the ability to adapt its arterial tone and thus maintain cerebral perfusion across a wide range systemic blood pressure. This mechanism is known as auto-regulation and has been shown to be damaged in animal models of TBI (DeWitt 2003, Bauman 2009), as well as being seen in soldiers with moderate to severe bTBI (Armonda 2006).

1.6.4 Why are animal models of blast necessary?

Under battlefield conditions, it is not possible to study human subjects in the very early stages of brain injury, also it is not possible to use sophisticated MR imaging techniques. Therefore, knowledge acquired from animal models may be important for understanding the basic science behind brain injury.

Animal models can also be used to correlate pathological and physiological processes with imaging findings and validate other assessment tools, such as chemical biomarkers from the central nervous system or peripheral circulation.

Ultimately, animal models could be used to test the efficacy of protective equipment (such as helmets and body armour) and novel therapies (such as substance p antagonists and cyclosporine A) (Vink 2002).

The brain may be exposed to several different forces during an explosion and so care must be taken to ensure that the model only exposes the animal to the mechanism being investigated. Animals have different sized and shaped skeletons and connective tissues with different mechanical properties to humans. The effect of blast on the thorax, and in generating whiplash injuries in humans is of particular concern. An identical BOP wave applied to the thorax will have a different effect on a person compared to a pig because the two skeletons vary greatly in their mechanical properties. Similarly a whiplash injury will be very different between a human and a pig because the human head is supported by a thin and flexible upright neck, whereas a pig's head has very thick muscle support.

It is likely that different animal models will be needed to demonstrate the different mechanisms occurring in blast injury.

1.6.5 The current literature for animal models in bTBI

Currently there is less research on the pathophysiology of bTBI compared to TBI. Recent animal studies include rodent and swine models of bTBI, most use blast/shock tubes (see Figure 1-16) which have varied in size depending on the size of the animal. In rodent models, BOP wave exposures have been tested between 30 and 170 kPa, whilst porcine investigations have modeled BOP exposures of 1MPa (Ritzel 2008, Personal Communication). At the lower end of the spectrum, this range of BOP wave results in a mild injury with no visible injury on the animal, whereas at the high end, exposures can result in an immediate fatality rate of around 80%. Animals are evaluated after exposure for cognitive performance, pathology, changes in gene expression and other biochemical signatures. The animals were found to have blast-induced neuronal dysfunction, as well as morphological, cellular and behavioural changes (Cernak 2005).

Crucially, the scientific community remains divided as to whether BOP wave can cause neuronal injury at the pressures experienced by soldiers. Since virtually all human injuries involve multiple mechanisms, animal models have been used to try to isolate the effect of the BOP wave.

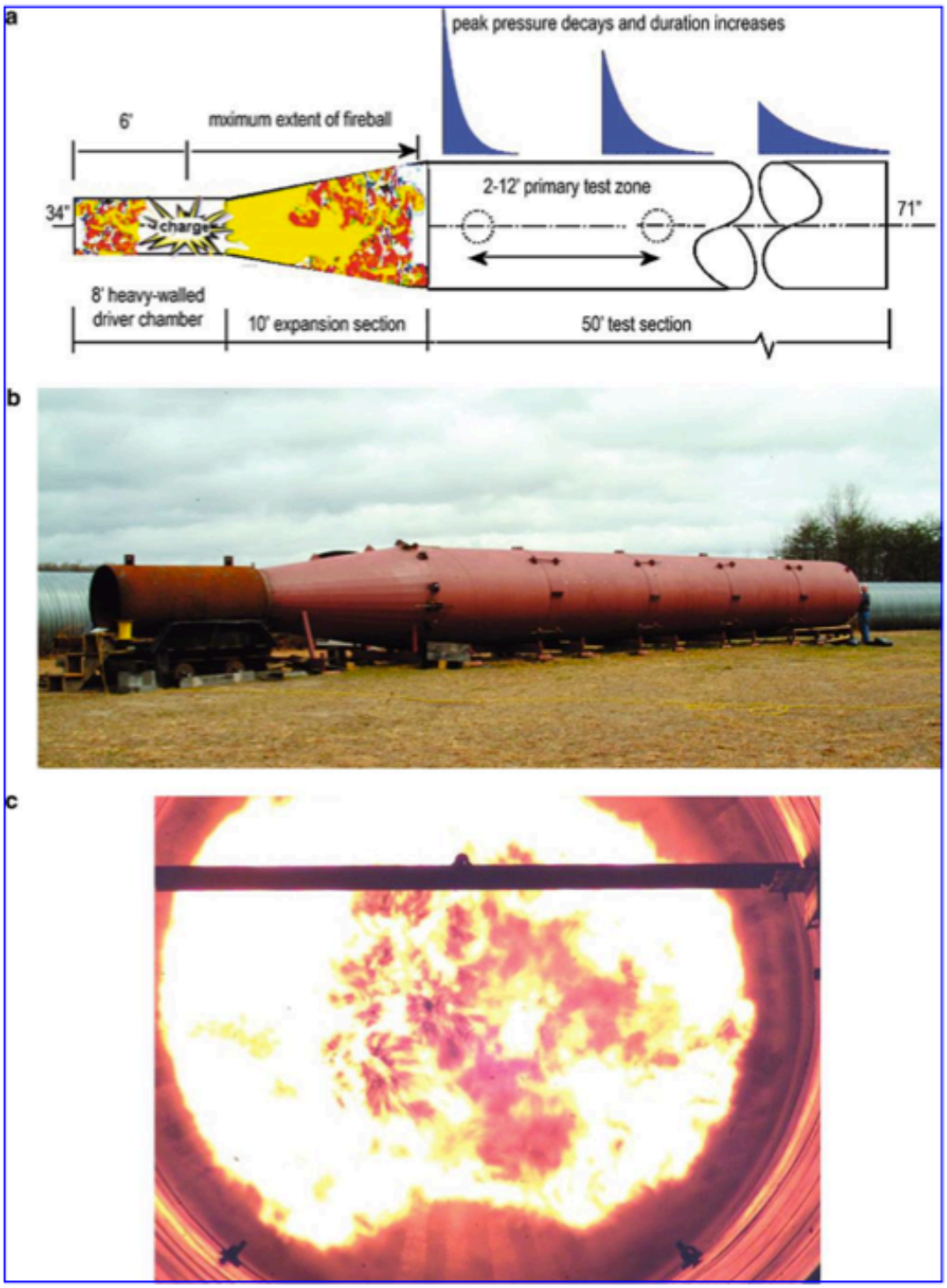


Figure 1-16. An example of a blast tube

(a) Two-dimensional diagram of the blast tube, including the driver, expansion cone and conduction segments of the blast tube. The swine is positioned 20 feet from the centre of the blast, just outside the fireball, and 44 feet from the end of the tube. (b) Photograph of the blast tube. (c) Front photograph of the blast tube during a calibration test (Bauman 2009).

1.6.6 What happens in a bTBI

Acutely, blast exposure in animals causes prominent vasospasm and decreased cerebral blood flow along with blood-brain barrier breakdown and increased vascular permeability. Chronically after blast exposure there are alterations of the vascular extracellular matrix as well as sustained microglial and astroglial activation (Elder 2015). Goldstein *et al. (2012)* demonstrated that blast-exposed mice show phosphorylated tauopathy, myelinated axonopathy, microvasculopathy, chronic neuroinflammation and neurodegeneration in the absence of macroscopic tissue damage or haemorrhage. Head immobilisation during blast exposure prevented blast-induced learning and memory deficits, indicating that head rotation may play an important role in generating these deficits (see Diffuse axonal injury Figure 1-8, earlier in this chapter). It has become increasingly clear that brain pathology, the underlying mechanisms and potential biomarkers associated with primary blast exposures may be different from those imposed by focal mechanical head trauma (Bhattacharjee 2008). Animal placement locations along the length of the shock tube (that is, inside, outside or near the exit) has an important role in the biomechanical loading on the animal and thereby alters the injury type, its severity and the probability of lethality (Sundaramurthy 2012). Considering the variations in the current blast injury models, comparison of the results between different laboratories is virtually impossible. Characterisation and implementation of relevant standard experimental blast models is of particular importance for understanding the mechanisms of blast injury, the identification of biomarkers and, eventually, the development of strategies for mitigating blast-induced brain injury.

1.7 Chapter summary

In this chapter I have shown that bTBI is a significant problem for the military population and described how it may produce WM injury through a separate mechanism not present in nbTBI. In reality, because of the primary, secondary, tertiary and quaternary effects of blast, any one injured soldier is likely to suffer a TBI caused by multiple mechanisms and thus studies to examine bTBI in humans will be inherently limited. This demonstrates the need for animal models of bTBI.

I have described the basic physics of MRI and how DTI can be used to identify WM damage with greater sensitivity than other modalities currently in use, thus explaining the rationale for its use in this study.

I have shown that endocrine dysfunction is a significant problem after nbTBI, where it causes symptoms that impact on physical and psychological wellbeing and why this is likely to be true in bTBI as well. This may represent an important therapeutic opportunity.

I have defined primary brain injury and the cellular processes that it initiates, leading to secondary brain injury (this will be expanded on in chapter 5). Importantly I have explained how axonal injury can lead to metabolic failure and neuronal death that in turn causes microglia to release cytokines which increase the blood brain barrier (BBB) permeability. This is the rationale for examining APP (as a marker of axonal injury) Iba1 (as a marker of inflammation) and Fibrinogen (as a marker of increased BBB permeability) in the porcine model.

The following 3 chapters are the results of the BIOSAP study. In Chapter 2, I use a human case study to illustrate the limitations of current standard imaging investigations to detect WM damage and the variability in outcome of apparently similar severity TBIs. In Chapter 3, I show that DTI can be used to compare the location and extent of WM damage in a blast injured population with a similarly injured non-blast population and importantly link this to cognitive dysfunction. In Chapter 4, I describe the increased incidence and pattern of endocrine dysfunction seen in the bTBI subjects when compared to the nbTBI group.

Chapter 5 is a summary of the results of the BIIPs. It can be considered separate, but parallel work, seeking to help determine if the BOP wave can cause TBI in battlefield conditions. I hope that the conclusions of this thesis will help draw together the different themes investigated and add to our understanding of bTBI.

2 Human Case Studies

In the introduction I described how bTBI is a significant problem for military populations and, whilst conventional imaging (CT and T1 and T2* MRI) are able to detect acute bleeds and large structural lesions, they are not as sensitive as DTI when assessing WM damage. In this chapter I use a human case study to illustrate the limitations of current standard imaging, when investigating WM damage and the variability in outcome of apparently similar severity TBIs.

2.1 Introduction

This chapter describes two cases of UK soldiers of similar age injured by IEDs whilst travelling in vehicles in Afghanistan. Both were classified as moderate-to-severe TBI on the basis of their post traumatic amnesia (PTA) duration. However, their long-term cognitive outcomes have been very different, and this was not explained by their initial neuroimaging assessment. Given the young age of troops and the limited availability of treatments to improve recovery, the impact of these long-term physical, cognitive, behavioural and psychological problems is a significant health burden.

The case histories are presented to illustrate the contribution that advanced MRI can make to the assessment of WM damage. Historically, clinical assessment has concentrated on identifying the location of focal contusions within the brain, but this has been found to be poorly predictive of outcome (Bigler 2000) partly because a key factor is DAI. Although pathophysiological studies demonstrate the importance of DAI (Adams 1991), it has proven difficult to identify using conventional neuroimaging techniques. Here we show how advanced MRI techniques, particularly DTI, can be used to provide clinically relevant information in the context of blast TBI.

2.2 Case 1

A 28-year old male soldier was injured by an IED whilst travelling in a vehicle. He was wearing full personal protective equipment. His GCS was 12/15 at the scene; he suffered multiple long limb fractures, a left sided pneumothorax and superficial lacerations. He experienced six weeks of PTA and described two weeks of retrograde amnesia. Subjectively he reported problems with long and short-term

memory, difficulty concentrating and speaking as well as an increased frequency of headaches, more emotional lability and dizziness. The soldier had persistent neurological abnormalities, and neuropsychological testing revealed impaired executive function as measured by the Trail Making Test, and the Colour Naming and Word Reading indices from the Delis Kaplan Executive Function System, and slow processing speed on a simple computerised choice reaction task. He initially returned to work in a very limited capacity, but has now been medically discharged from the army because of persistent cognitive problems. Details of the neuropsychological tests can be found in Appendix 1.

The duration of PTA and long-term disability are suggestive of a significant brain injury. However, conventional CT and MRI (T1) were normal (Figure 2-1(i)). Failure to find a neuroimaging abnormality produces uncertainty about the presence of brain injury. DAI results in small haemorrhages (microbleeds) in characteristic locations within the WM (Scheid 2003) which can be assessed using MRI techniques such as Gradient Recalled Echo (GRE) and Susceptibility Weighted Imaging (SWI) (Haacke 2014). In this patient, GRE showed one microbleed in the right occipital and one in the right frontal lobe. Although the presence of two microbleeds in different brain regions suggests underlyling DAI, the true extent of WM damage is unclear as it is possible to have DAI without the presence of microbleeds (non-haemorrhagic DAI) or for the extent of injury to be much greater than demonstrated by SWI (Kinnunen 2011).

Many of the limitations of microbleed imaging can be addressed by the use of DTI. This provides a robust and quantitative measure of DAI. In this first case, DTI provided evidence of significant and widespread WM abnormality. This is illustrated in Figure 2-1(iii), which shows average measurements of FA, from a number of large WM tracts, compared to a normative control data (59 controls, average age 30.6 years +/- 8.1). This automated region of interest approach provides a detailed assessment of WM damage in the individual patient, and demonstrates the presence of significant damage, with low FA in the superior longitudinal fasciculi (red columns >2.3 SD from the control group mean).

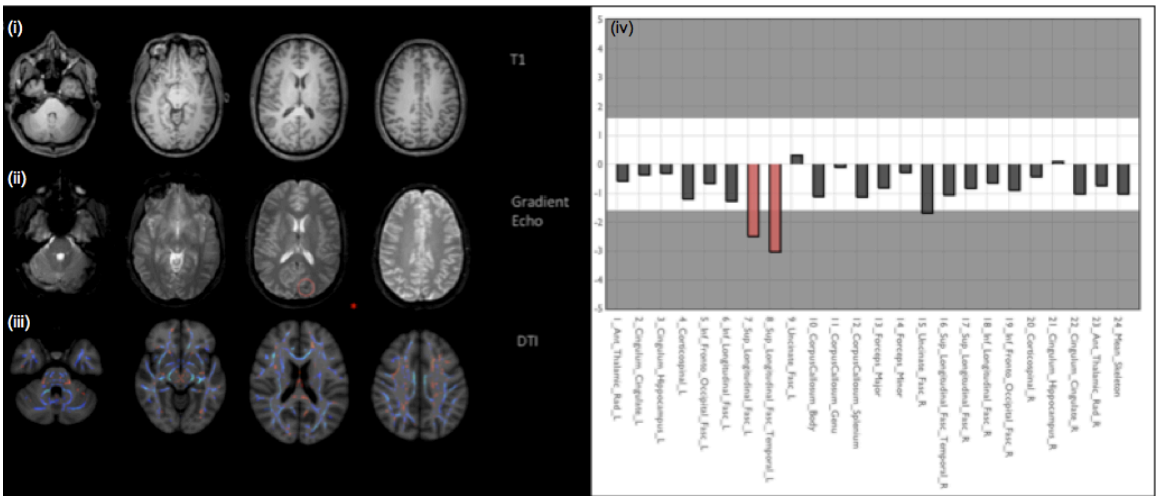


Figure 2-1. MR imaging results corresponding to Case 1

(i) Conventional (T1) imaging was normal (ii) GRE detected the presence of two microbleeds (iii) DTI measures for individual tracts (iv) Z-scores of the comparison between this soldier's WM tract FA and the control group. Red bars denote where that tract's FA value was > 2.3 standard deviations ($p<0.001$) from the control group mean. The central white area denotes the area of $Z < 1.64$ ($p > 0.01$) for the control groups FA. Analysis of the WM tracts revealed that Case 1 had significant damage in the superior longitudinal fasciculi.

2.3 Case 2

A 27-year old, male soldier was also injured by an IED, whilst travelling in a vehicle. He was wearing full personal protective equipment. His GCS was 13/15 at the scene; he suffered multiple long limb fractures as well as a left sided pneumothorax. He experienced four days of PTA and had one day of retrograde amnesia. He subjectively reported more difficulty balancing and increased emotional lability since the injury though no increased frequency of headaches, hearing loss or change in sleep pattern. Neuropsychological testing revealed only impaired associative learning and memory as measured by the People Test from the Doors and People Test. In contrast to our first case, he has made a good functional recovery; he was able to return to work and is currently studying for a Masters degree. As demonstrated with Case 1, conventional imaging with T1 demonstrated no evidence of brain injury Figure 2-2 (i). SWI showed a single microbleed Figure 2-2 (ii), providing some evidence of DAI. In contrast, the tract-based DTI assessment of WM damage showed no evidence of DAI, with all FA measurement within the normal range Figure 2-2 (iv).

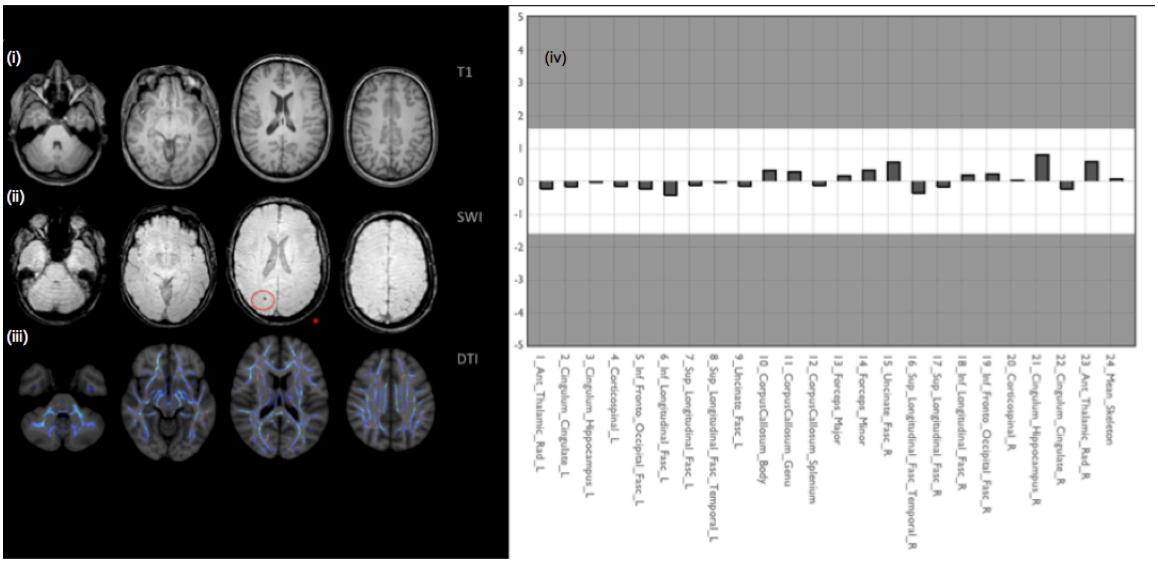


Figure 2-2. MR imaging results corresponding to Case 2

(i) Conventional (T1) imaging was normal and (ii) SWI detected the presence of one microbleed (iii) DTI measures for individual tracts (iv) Analysis of the WM tracts revealed that Case 2 did not have any significant WM damage.

2.4 Discussion

DAI is a key factor determining clinical outcome after TBI**.** Until recently, it has been difficult to study the location and the extent of DAI. CT and conventional MRI do not provide specific diagnostic information (Van Boven 2009), but microbleed imaging and DTI are more sensitive (Mac Donald 2007). The two cases of blast TBI presented here illustrate the highly variable clinical outcomes that are common after moderate/severe TBI, with neither conventional neuroimaging nor microbleed imaging using SWI providing an explanation for this difference. However, evidence for WM tract abnormalities was only seen in Case 1 using the advanced MR technique, DTI, which is increasingly being used in TBI as a research tool. In this soldier the technique demonstrated DAI, which is believed to account for his persisting and significant cognitive damage and ongoing disability.

DTI provides a measure of water molecule diffusion within the brain (Assaf 2008). Within the WM, the direction of this diffusion is strongly determined by the predominant orientation of axons. Following brain trauma, the organisation of axons is disrupted and results in a reduction in the direction of water diffusion. DTI is often analysed by fitting a tensor model to the acquired data. This allows the asymmetrical pattern of diffusion in the WM to be described quantitatively, as fractional anisotrophy (FA). Animal studies demonstrate that FA measurements from the WM become abnormal quickly after brain injury and evolve dynamically over time (Mac Donald 2007); hence DTI provides a way of sensitively studying DAI.

DAI causes disruption to the connectivity of the brain networks that support cognitive function, and this appears to be an important factor in the development of long-term disability (Sharp 2011, Bonnelle 2011). In the civilian population, abnormalities have been shown to be related to persistent cognitive and neuropsychiatric problems (Kinnunen 2011). These impairments are common and influence outcome (Whitnall 2006). As a result, being able to diagnose them accurately and identify this type of brain injury is crucial.

Soldiers exposed to blast often have persistent cognitive and neuropsychiatric problems (Tanielian 2008), but the cause is often unclear. Where there is no obvious brain injury on standard imaging, a purely psychiatric cause may be proposed. An important question is whether these problems result in part from the presence of

previously unrecognised DAI. Previous work has demonstrated the presence of DTI abnormalities following mild blast TBI (Mac Donald 2011). The two cases we discuss illustrate that DTI abnormalities can be present after more severe blast exposure, but also that there is variability in the amount of WM abnormality, which we believe is associated with the two very different clinical outcomes.

The cases shown here illustrate that DTI can provide clinically useful information in single cases of TBI. The challenge is to establish the optimal way of using this neuroimaging method in routine clinical practice, although there are a number of obstacles that need to be overcome. First, DTI is a quantitative neuroimaging method, that requires accurate processing once acquired, and this needs to be standardised. Second, the individual patient data must be compared to an appropriate control population, and this is a major current limitation. Finally, all controls and patients were scanned on a single scanner, allowing straightforward comparison, but methods need to be developed to allow comparison of DTI data acquired on different scanners.

In summary, this chapter reports the clinical and radiological findings in two soldiers who experienced blast TBI as a result of IEDs during the conflict in Afghanistan. Both soldiers were injured by blast, had a similar initial clinical presentation and normal CT and standard structural MR imaging. Despite the apparent similarities, Case 2 showed a good functional recovery while Case 1 reported persistent long-term cognitive and emotional problems. Their clinical outcomes were very different and we believe that this was due to the degree of DAI experienced by the soldiers. When we examined the white matters tracts using DTI, Case 1 showed significantly reduced FA in the superior longitudinal fasciculus. This level of DAI was not identifiable using standard structural MRI, and we have therefore shown how DTI can be used to identify WM damage in a single subject.

In the next two Chapters, I report the results of the BIOSAP study. In Chapter 3, we use DTI to examine the differences at the group level, in both extent and location of WM damage sustained as a result of bTBI versus nbTBI. In Chapter 4, we look at the incidence in endocrine dysfunction in a group of blast injured soldiers compared to a group of civilians with nbTBI.

3 White matter damage and cognitive dysfunction after blast traumatic brain injury

3.1 Introduction

This chapter reports the results of the neuroimaging component of the Blast Injury Outcome Study of Armed Services Personnel (BIOSAP), a study of the effects of blast injury in United Kingdom personnel injured whilst on operations in Afghanistan. Soldiers suffering a moderate or severe TBI secondary to blast exposure were investigated using advanced MRI, including diffusion tensor imaging. Scan results were compared to a matched cohort of civilian traumatic brain injury, taken from a large cohort study investigated using the same scanner.

This study extends previous studies investigating the long-term effects of bTBI in a number of important ways. By comparing patterns of brain injury across blast and non-blast TBI investigated with the same neuroimaging protocol, we are able to directly test the hypothesis that blast TBI produces a unique pattern of WM injury, with more damage within the posterior fossa. Building on previous work in nbTBI (Kinnunen 2011, Bonnelle, 2012, Bonnelle, 2011), this study investigated in detail the relationship between WM damage and persistent cognitive impairment after bTBI.

We tested a number of specific hypotheses:

a. that damage to the fornices correlates with associative memory performance;

b. that damage to frontal lobe connections correlate with impairments of executive function; and

c. that widespread damage to the WM correlates with impairments of information processing speed.

A voxelwise approach to the analysis of WM injury (tract based spatial statistics (Smith 2006)), which has a number of important advantages over a region of interest approach, was also used for this study of TBI. This approach allows the effect of

bTBI on all large WM tracts to be studied without making an *a priori* assumption about the location of injury (Kinnunen 2011).

3.2 Background

The recent conflicts in Iraq and Afghanistan have seen significant numbers of soldiers exposed to bomb blasts, often the result of improvised explosive devices. Up to 20% of the 1.64 million deployed US troops are estimated to have suffered a bTBI (Tanielian 2008), which has been termed the “signature” injury of the Iraq and Afghanistan conflicts (Benzinger 2009). Many soldiers exposed to blast have persistent cognitive and neuropsychiatric problems (Schneiderman 2008), but the causes for these problems are still poorly understood. Clearly there are direct effects of brain injury but other factors, such as psychiatric problems including PTSD, may interact with this (Schneiderman 2008). Some soldiers have focal brain injuries that are clearly visible on conventional CT or MRI investigations, but frequently these injuries do not predict clinical outcomes (Bigler 2001). Conversely, those with normal appearing scans can have persistent and disabling problems. This discrepancy leads to significant diagnostic uncertainty, and often results in an assumption that symptoms are primarily produced by psychiatric or other factors unrelated to a structural brain injury produced at the time of the blast (Bitonte 2016).

One possible explanation for the diagnostic confusion is the presence of unrecognised DAI. DAI can be produced by a variety of types and severities of TBI (Adams 1989, Blumbergs 1994), including blast exposure (Elder 2009). Long-distance WM tracts within the brain are vulnerable to the biomechanical effects of trauma. Diffuse multifocal WM injury can be produced by rapid changes in acceleration and deceleration, which impart shear, compressive and tensile strains to axons (Smith 2003, Johnson 2013). Axons usually remain intact, but long-lasting damage commonly occurs to the axolemma, disrupting axonal transport, and to the myelin sheath. DAI is almost universally present in cases of fatal brain injury (Gentleman 1995) and in nbTBI shows a characteristic distribution, with the corpus callosum and brainstem WM frequently affected (Adams 1989).

The function of damaged axons is impaired, resulting in a partial disconnection of the brain networks that are connected by these tracts (for review see Bonnelle 2011). In

the context of civilian TBI, DAI is an important predictor of long-term outcome (Adams 1989, Sidaros 2008). Therefore, DAI may contribute significantly to persistent disability after bTBI.

Conventional CT or MRI often under-estimates the extent of this WM injury after TBI (for review see Bonnelle, 2011). Advanced MRI techniques, in particular diffusion MRI, provide a sensitive way of identifying WM pathology (Alexander 2007). Water molecules tend to diffuse along the direction of large WM tracts, and this anisotropic diffusion can be quantified by diffusion tensor imaging (DTI) (Mori 2006). TBI commonly leads to abnormal DTI measures, such as reduced FA (Mac Donald 2007). These measures have been validated as a method of identifying axonal injury in animal models of both nbTBI (Mac Donald 2007) and bTBI (Calabrese 2014). Using a shock tube model in rats, Calabrese and colleagues showed areas of reduced FA, particularly within cerebellar WM, after double blast exposure. They performed DTI analysis using voxelwise methodology, and the results of histological analysis correlated well with DTI results. Significant reductions in FA after blast exposure were associated with severe and consistent evidence of axonal injury provided by silver staining (Calabrese 2014).

In humans, diffusion MRI has been widely used in the assessment of civilian TBI (Sidaros 2008, Kinnunen 2011) and has begun to be applied to study bTBI (Mac Donald 2011, Jorge 2012). MacDonald and colleagues investigated US military personnel within 90 days of mild bTBI (Mac Donald 2011). All had normal CT imaging, yet compared to non-injured soldiers, almost a third had abnormalities on DTI consistent with traumatic axonal injury, which persisted at follow-up 6 to 12 months later. Other studies have also shown areas of abnormally low FA following mild bTBI, with increasing abnormality associated with soldiers with longer PTA (Jorge 2012). Using a different MRI diffusion technique (High Angular Resolution Diffusion Imaging – HARDI), Morey *et al* also showed evidence for WM injury in mild TBI in veterans, which correlated with the degree of loss of consciousness (Morey 2013). However, this group was heterogenous with respect to the type of injury, with only around a third of soldiers having been exposed to blast. The results with diffusion MRI assessments of mild bTBI have not always been completely consistent, as Levin *et al.* did not demonstrate WM changes in veterans and service members with mild to moderate TBI despite symptoms and difficulty with verbal

memory (Levin 2010). However, this may have been because of the heterogeneity of the subjects: firstly, by looking at the mild end of the spectrum they may have included subjects without brain injury; secondly, subjects were recruited on the basis of self-reported injury severity which was not confirmed at the time of injury; and thirdly many soldiers were referred for persisting symptoms which may have in fact been PTSD.

An important issue is to what extent neuroimaging abnormalities produced by blast exposure are specific to the effects of a BOP wave or wind. It is very rare for soldiers to receive injuries from an isolated blast wave, as other types of secondary or tertiary injuries almost always occur (Mac Donald 2011). This means the neuroimaging abnormalities observed are likely to be a combination of a number of different injury mechanisms, so identifying any specific effects of the blast wave or wind is usually not possible. Computational modeling using a finite element model of the brain has been used to predict the effects of blast exposure (Taylor 2009). This model suggests that WM injury would be likely in regions not usually affected by other types of TBI, such as the cerebellum and its connections. Mac Donald and colleagues provide evidence that converges with the predictions of this computational modeling (Mac Donald 2011, Mac Donald 2014) suggesting that cerebellar peduncle damage may be a relatively specific effect of bTBI. However, studies have not usually included a control group consisting of non-blast TBI investigated in the same way, so it is unclear whether WM damage is specific to blast exposure, or due to other associated mechanisms of injury.

Although the location of WM injury is usually diffuse, damage to certain WM tracts produces impairment in the associated cognitive domain. This has previously been studied in civilian TBI. Previous work by our group has demonstrated that increasing damage within the fornix correlates with associative memory impairment (Kinnunen 2011), damage within the cingulum bundle with sustained attention impairment (Bonnelle 2011), and frontal lobe damage to a tract connecting nodes of the Salience Network correlates with impairments of executive function (Bonnelle 2012). Therefore, one would predict that the amount and distribution of DAI after bTBI would correlate with persistent cognitive impairment in veterans. Jorge and colleagues reported that the number of patches of low FA within the corpus callosum correlated with executive function in a group with mild bTBI (Jorge 2012). However,

a detailed assessment of the relationship between the location and severity of DAI, as measured by diffusion MRI and cognitive impairments in bTBI, has not previously been reported, hence the rationale for this part of the BIOSAP study.

3.3 Materials and Methods

Twenty soldiers with moderate to severe bTBI in the post-acute phase (20 males, mean age ± SD: 29.8 ± 5.9 years) were recruited. The nbTBI group consisted of 20 civilians taken from a civilian cohort and matched for age and brain injury severity (20 males, mean age ± SD 30.3 ± 7.6 years). An age matched group of 31 healthy controls (31 males, mean age ± SD 30.6 ± 6.7 years) were also recruited amongst Imperial College London laboratory workers and their social and professional contacts. Soldiers and civilians were matched for time since injury (for soldiers mean time ± SD 14.6 ± 5.9 months, for civilians mean time ± SD 12 ± 12.7 months).

Amongst the soldiers all injuries were as a result of exposure to explosive devices. Amongst the civilian group, injury was secondary to falls (42%), assaults (34%), road traffic accidents (19%), and sports related injuries (5%). Soldiers were identified by the author's monthly review of the academic department of military emergency medicine's (ADMEM) injury database. The civilians were recruited from the Imperial Healthcare traumatic brain injury service to which they had been referred because of the presence of functional impairments following their TBI. Access to these civilians has been previously reported (Kinnunen 2011, Baxter 2013).

All cases of military and civilian injury were categorised as moderate or severe based on the Mayo Classification System for Traumatic Brain Injury Severity, relating to the duration of loss of consciousness, the length of PTA, the lowest recorded GCS in the first 24 hrs and/or CT or MRI result (Malec 2007).

Exclusion criteria were as follows: penetrating brain injury, neurosurgery, except for intracranial pressure monitoring; a history of psychiatric or neurological illness prior to head injury; a history of previous TBI; anti-epileptic medication; current or previous drug or alcohol abuse; or contraindications to MRI. All participants gave written informed consent according to the Declaration of Helsinki; no subject without capacity could enter the study. The study was approved by the Hammersmith, Queen Charlotte's and Chelsea Research Ethics Committee.

3.3.1 Neuropsychological assessment

All participants completed a standardised neuropsychological test battery sensitive to cognitive impairment associated with TBI (Kinnunen 2011). The cognitive functions of specific interest were indexed by:

a. current verbal and non-verbal reasoning ability via the Wechsler Abbreviated Scale of Intelligence (WASI) Similarities and Matrix Reasoning subtests (Wechsler 1999);

b. associative learning and memory via the immediate recall score on the People Test from the Doors and People Test (Baddeley 1994);

c. the executive functions of set-shifting, inhibitory control, cognitive flexibility and word generation fluency via the Trail Making Test (Reitan 1958), alternating-switch cost index (time to complete alternating letter and number Trails B minus time to complete numbers-only Trail A) and two indices from the Delis–Kaplan Executive Function System (Delis 2001), namely the inhibition/switching minus baseline score from the Color–Word subtest (high scores indicating poor performance) and the total score on Letter Fluency; and

d. information processing speed via the median reaction time for accurate responses on a simple computerised choice reaction task (Prof. Jane Powell, Goldsmiths, UK, personal communication).

3.3.2 Structural imaging

Each patient had standard high-resolution T1 and gradient-echo (T2*) imaging to assess focal brain injury and evidence of microbleeds. MRI was performed on a Philips 3T Achieva scanner (Philips Medical Systems, The Netherlands) using a body coil. The T1 and T2*-weighted images were obtained prior to DTI. For DTI, diffusion-weighted volumes with gradients applied in 16 non-collinear directions were collected in each of the four DTI runs, resulting in a total of 64 directions. The following parameters were used: 73 contiguous slices, slice thickness = 2mm, field of view 224 mm, matrix 128 x 128 (voxel size = 1.75 x 1.75 x 2 mm^3), b value = 1000 and four images with no diffusion weighting (b=0s/mm^2). The images were registered

to the b=0 image by affine transformations to minimise distortion due to motion and eddy currents and then brain-extracted using the Brain Extraction Tool from the Oxford functional MRI of the brain (FMRIB) neuroimaging research facility Software Library image processing toolbox. FA maps were generated using the Diffusion Toolbox.

3.3.3 Diffusion tensor imaging data analysis

Voxelwise analysis of the FA was carried out using Tract Based Spatial Statistics (TBSS) in the FMRIB Software Library (vide supra). Image analysis using TBSS involved a number of steps:

a. non-linear alignment of each subject's FA images into common FMRIB58 FA template space (see Appendix 1);
b. affine-transformation of the aligned images into standard MNI152 1mm space;
c. averaging of the aligned FA images to create a 4D mean FA image;
d. thinning of the mean FA image to create a mean FA 'skeleton' representing the centre of all WM tracts, and in this way removing partial-volume confounders; and
e. thresholding of the FA skeleton at FA ≥ 0.2 to suppress areas of extremely low mean FA and exclude those with considerable inter-individual variability.

Non-parametric permutation-based statistics were employed using *Randomise* with threshold-free cluster enhancement and 5000 permutations. A statistically significant threshold of $p \leq 0.05$ was then applied on the results, to correct for multiple comparisons. Age was included as a covariate of no interest in all TBSS analyses (Smith 2006).

In civilian TBI it has previously been shown that there are increased DTI abnormalities in patients with microbleed evidence of DAI (Kinnunen 2011). Therefore we performed additional analyses on subgroups of patients with and without microbleeds. We also performed a more targeted region of interest (ROI) analysis of FA, which was informed by the results of previous work. ROI masks were

created in the MNI152 one mm space that were then applied to the aligned brain images, and mean FA assessed within this region for each subject. The regions investigated were the anterior and posterior internal capsule, cingulum, body, genu and splenium of the corpus callosum, cerebral peduncles, middle cerebellar peduncles, the orbito-frontal WM and uncinate fasciculi bilaterally (see Figure 3-1).

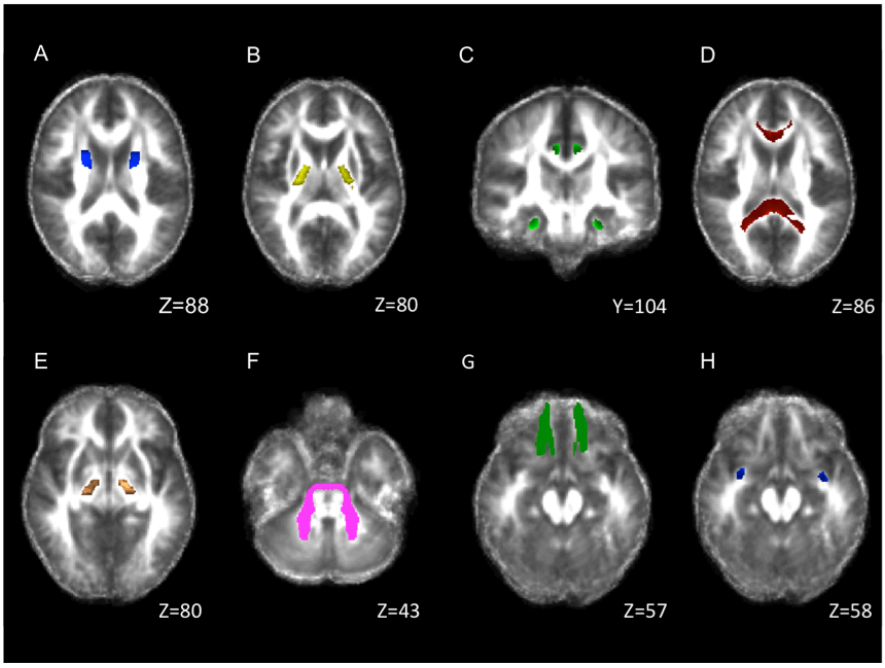


Figure 3-1. ROI Masks

Regions of interest used for determination of FA in both soldiers and civilians traumatic brain injury. Individual color masks overlaid onto group average FA map for soldiers with bTBI (n=19) registered into standard MNI space. (A) anterior internal capsule, (B) posterior internal capsule, (C) cingulum, (D) corpus callosum, (E) cerebral peduncles, (F) middle cerebellar peduncles (G) orbitofrontal WM, (H) uncinate fasciculi.

3.3.4 Analysis of WM structure and cognitive function

The relationship between WM structure and cognitive function was investigated within the framework of a general linear model in the FMRIB Software Library. The effect of group was modelled, allowing analysis of the relationship between WM structure and cognitive function across voxels. Overall correlations across both groups, correlations within each group and group interactions were examined. Analysis was focused on the neuropsychological domains previously investigated in civilian TBI (Kinnunen 2011).

As detailed in the introduction, analysis was carried out using:

a. WASI similarities and matrix reasoning to assess intellectual ability;
b. the People Test immediate recall to assess associative memory;
c. the Trail Making Test A and B as well as the Colour naming and Word reading to assess Processing speed;
d. the Trail Making Test Trails B minus A to assess the alternating-switch cost component of Executive function;
e. inhibition switching and inhibition switching minus a baseline of colour naming and word reading to assess cognitive flexibility as a measure of Executive function;
f. Letter Fluency as a measure of the word generation fluency component of Executive function; and
g. the choice reaction time as a measure of processing.

Permutation-based significance testing was carried out as described above. For illustrative purposes, FA values from the peak voxels of the significant clusters of interest were then extracted for each participant from their skeletonised images and plotted against the cognitive scores.

3.4 Results

3.4.1 Standard neuroimaging showed no significant difference between blast and non-blast groups

Standard T_1 MRI was normal in 70% of soldiers with bTBI, and in 40% of civilians with nbTBI. Gradient echo imaging showed intraparenchymal microbleeds, indicative of DAI in 45% of those suffering bTBI and 50% of the nbTBI group. Only 20% (4) of the bTBI group had evidence of focal damage on T1/Flair imaging. This was located mainly in the left frontal and temporal lobes (Figure 3-2). In contrast, 60% of the nbTBI group, had evidence of focal damage which was in a similar fronto-temporal distribution. There was no overlap in location of contusions between the two groups (Figure 2). Of the bTBI group, 15 showed no contusions compared with 8 of the nbTBI group. The mean volume of contusions in the bTBI group was 2.7cm^3,

compared to 5.3 cm^3 in the nbTBI group (p=0.07). MRI evidence of superficial siderosis was found in 20% of those with bTBI and in 36% of the nbTBI group.

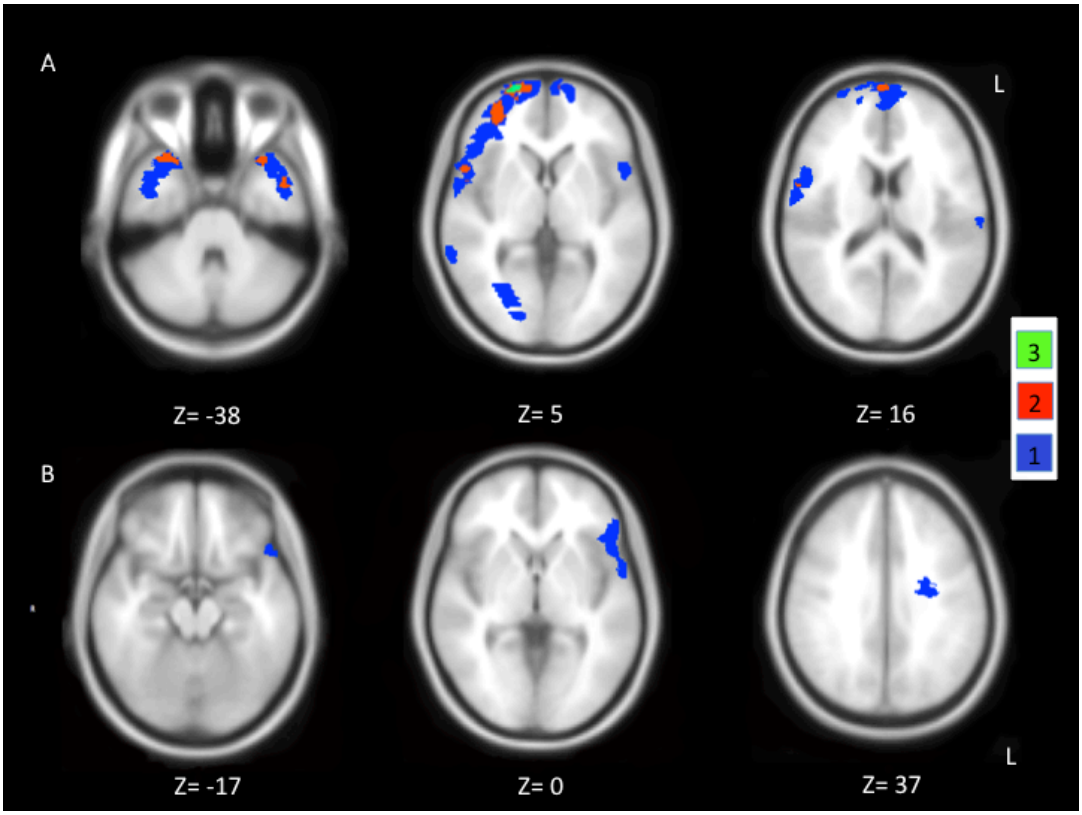


Figure 3-2. Location of contusions of (A) civilians with nbTBI and (B) soldiers with bTBI

The colour bar indicates the number of lesions at each site. Green indicates where a contusion was present in three subjects, red where a contusion was present in two subjects and blue where a contusion was found in one subject only.

3.4.2 The bTBI group showed impaired cognitive function relative to nbTBI and controls

Both TBI groups showed a pattern of cognitive impairment characteristic of TBI (Ponsford 1992). Compared to the healthy control group, soldiers showed cognitive impairments across a range of tasks including:

a. processing speed measured using the Trail Making Test A and B, naming/reading on the STROOP colour naming task and response speed on the choice reaction time task; and

b. impaired executive functioning assessed using the Trail Making Test B minus A, inhibition switching minus a baseline of colour naming and word reading, and the word generation fluency task (Table 3-1).

There was also a borderline impairment across the group in memory performance on a test of associative memory, the Doors and People test.

Similarly, the nbTBI group showed evidence of impairments in:

a. processing speed (Trail Making Test A and B, and naming/reading as measured by the STROOP colour naming task); and

b. impaired executive functioning assessed using the Trail Making Test B minus A, inhibition switching minus a baseline of colour naming and word reading and word generation fluency (Table 3-1).

The nbTBI group had higher average current intellectual ability as measured by the WASI test, although there was no significant difference in this estimate of IQ between the bTBI and controls.

Using standard neuroimaging, the bTBI group showed less damage than the nbTBI. Despite this, the bTBI group showed more impairment of cognitive function relative to the nbTBI group. The bTBI group showed worse:

a. processing speed (naming and reading speed on the STROOP word reading task;

b. executive functioning assessed with the Trail Making Test B minus A; and

c. impaired information processing speed tested on the choice reaction task (Table 3-1).

Table 3-1. Neuropsychological test results by group

Cognitive Domain	Cognitive Variable	bTBI	nbTBI	Controls	bTBI vs. nbTBI	bTBI vs. Controls	nbTBI vs. Controls
		Mean +/- SD	Mean +/- SD	Mean +/- SD			
Intellectual ability: verbal/non-verbal	WASI similarities	31.21 +/- 6.24	37.72 +/- 4.01	31.05 +/- 6.22	0.0003**	0.47	0.0002**
	WASI matrix reasoning	24.84 +/- 6.75	26.33 +/- 5.78	25.24 +/- 5.03	0.24	0.42	0.27
Memory: associative memory	People Test immediate recall	24.16 +/- 7.04	24.44 +/- 6.06	27.48 +/- 6.79	0.45	0.07	0.08
Processing speed: visual search/complex	Trail Making Test Trail A (s)	24.84 +/- 6.04	23.96 +/- 7.98	18.24 +/- 3.88	0.35	0.0001**	0.003**
	Trail Making Test Trail B (s)	49.79 +/- 13.74	60.36 +/- 30.66	42.41 +/- 11.98	0.09	0.04*	0.01*

Processing speed: naming/reading	Colour naming (s)	38.32 +/- 19.45	33.89 +/- 10.64	23.72 +/- 6.67	0.20	0.001**	0.0004**
	Word reading (s)	28.37 +/- 10.91	21.39 +/- 4.05	23.57 +/- 7.52	0.01*	0.06	0.14
Executive function: alternating-switch cost	Trail Making Test Trails B minus A (s)	24.95 +/- 11.96	36.39 +/- 27.76	24.17 +/- 10.46	0.05*	0.05*	0.03**
Executive function: cognitive flexibility	Inhibition/switching (s)	75.53 +/- 26.69	63.61 +/- 17.43	54.91 +/- 18.48	0.06	0.003**	0.07
	Inhibition switching minus a baseline of colour naming and word reading (s)	28.89 +/- 16.13	35.31 +/- 14	18.24 +/- 15.1	0.10	0.02*	0.0004**
Executive function: word generation fluency	Letter Fluency F+A+S total	37.79 +/- 11.36	40.61 +/- 11.32	28.67 +/- 16.04	0.23	0.02*	0.01*

Processing: choice reaction time	Choice reaction task median reaction time (ms)	0.48 +/- 0.14	0.42 +/- 0.06	0.40 +/- 0.05	0.05*	0.03*	0.29

Both bTBI and nbTBI performed worse than controls in several domains, showing a pattern of cognitive impairment typical after TBI. The bTBI group had worse processing speed, executive function and information processing speed.

*** p value is significant at <0.05**

**** p value is significant at <0.005**

3.4.3 Widespread WM abnormalities are present in both the blast and non-blast groups using advance imaging techniques

The bTBI group showed widespread evidence of WM damage (Figure 3-3). The comparison of soldiers with bTBI with the age-matched controls demonstrated evidence of WM disruption in the majority of the WM assessed, as indicated by a lower FA. There was a similar pattern of widespread WM damage in the nbTBI group.

In both injured groups, lower FA was found in both commissural inter-hemispheric fibres (the forceps minor and major and corpus callosum) and intra-hemispheric association fibres of the uncinate fasciculi, inferior and superior longitudinal fasciculi, inferior fronto-occiptal fasciculi and the cingulum bundle. Lower FA was also found in the projection fibres of the corticopontine and corticospinal tracts, as well as in the fornices, the anterior and posterior thalamic radiations, the anterior and posterior limbs of the internal capsule, the external capsule and the corona radiata.

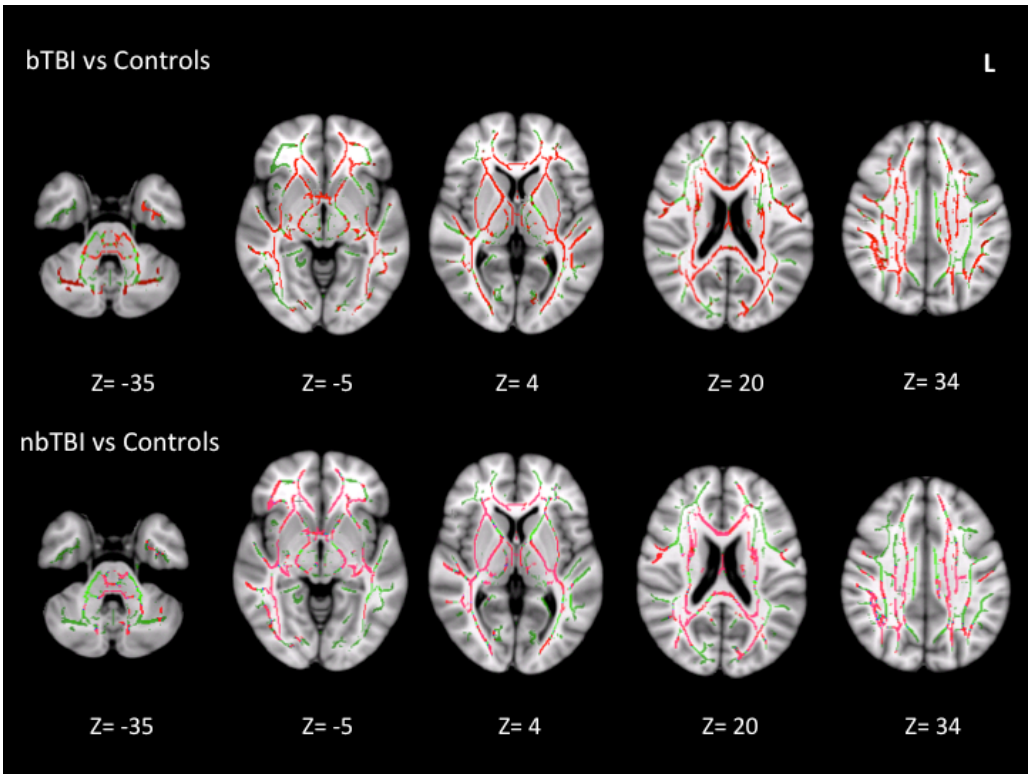


Figure 3-3. Widespread white matter disruption following traumatic brain injury

Axial slices show a comparison between the bTBI vs controls and nbTBI vs controls. The contrasts are overlaid on a standard Montreal Neurological Institute 152 T_1 1 mm brain and the mean fractional anisotropy skeleton (in green) with display thresholds set to range from 0.2 to 0.8. The results are thresholded at p≤0.05, corrected for multiple comparisons. Tracts in red indicate where FA is significantly lower in the bTBI or nbTBI group.

3.4.4 Evidence for increased WM damage in a subgroup of more severely injured bTBI soldiers compared to the nbTBI group

A direct whole-brain comparison between the bTBI and nbTBI groups did not reveal significant differences in FA. Recent work has reported lower FA in the middle cerebellar peduncles, the right orbito-frontal WM and the cingulum bundles in mild bTBI (Mac Donald 2011). Therefore the study went on to perform a targeted region of interest (ROI) analysis to investigate these specific regions, as well as a number of other regions that commonly show DAI after TBI. As expected for both TBI groups, FA in all regions studied was lower than in controls. A ROI analysis comparing the whole bTBI and nbTBI groups did not reveal significant differences in FA. However,

in patients with microbleeds, i.e. those with a high probability of DAI, there was lower FA in the anterior internal capsules and the middle cerebellar peduncles (p≤0.06 and p≤0.05 respectively) (Figure 3-4).

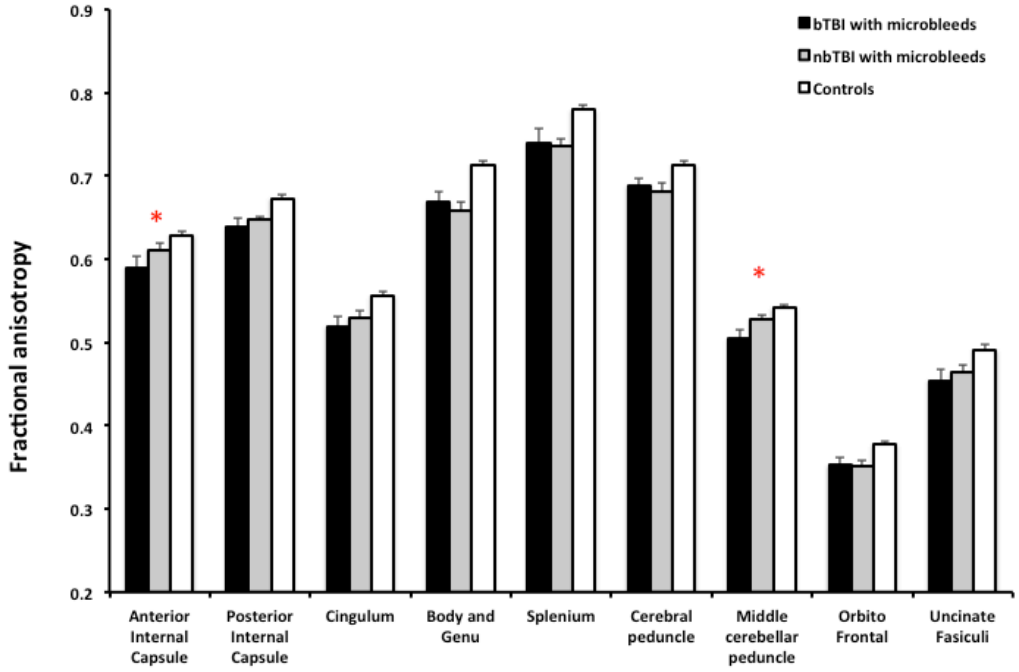


Figure 3-4. White matter damage in those soldiers and civilians with microbleeds

The graph shows the average FA within WM ROI for those with microbleeds in each of the groups (bTBI, nbTBI) and controls. There was significantly more WM damage in the Anterior Internal Capsule and Middle Cerebellar Peduncles in the bTBI vs. nbTBI groups.

*** indicates a significant p value**

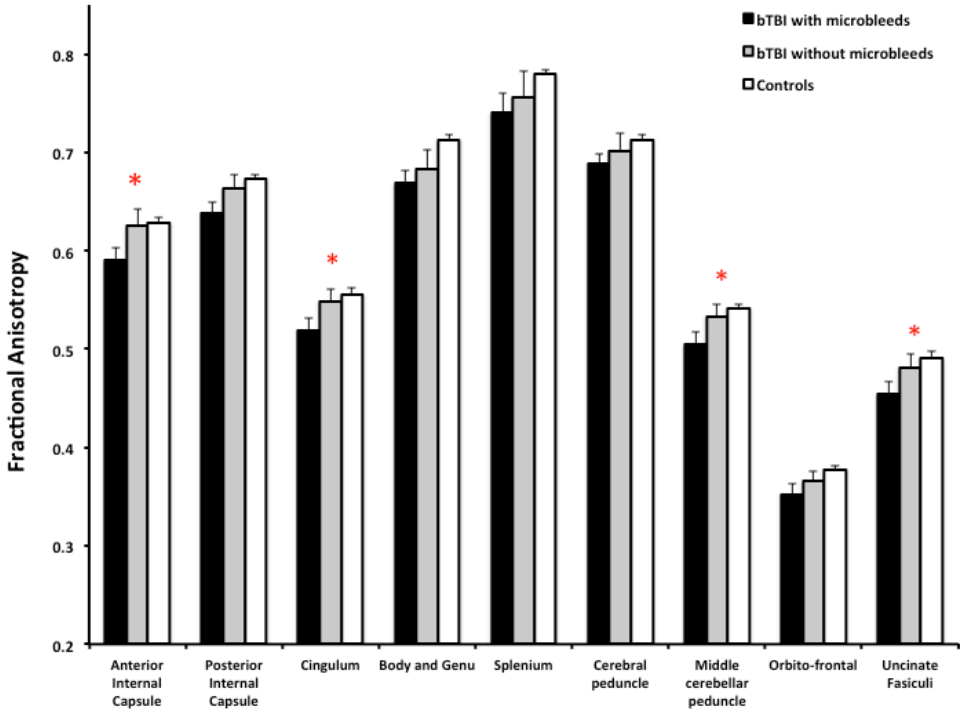


Figure 3-5. Amongst the bTBI group there was more widespread WM damage in those with microbleeds

The graph shows the average FA within WM ROI for those in the bTBI groups with microbleeds vs those without and controls.

*** indicates a significant p value**

3.4.5 More severe WM damage in patients with microbleed evidence of DAI

Within the bTBI group the study went on to compare patients with and without microbleed evidence of DAI, expecting that those with microbleeds would show more WM damage. A whole-brain comparison between the bTBI sub-groups showed no significant difference, which may have been because of small group sizes (n= 9 (microbleed) and 11 (non-microbleed). However, the ROI analysis showed lower FA in the uncinate fasciculi ($p \leq 0.09$) with a trend to lower FA in the anterior internal capsule, the cingulum and the middle cerebellar peduncles ($p \leq 0.065$, $p \leq 0.065$ and $p \leq 0.06$ respectively.

3.4.6 WM damage is associated with cognitive impairment in soldiers with blast TBI

3.4.6.1 Associative memory

Previous work in civilian TBI demonstrated evidence that WM damage was correlated with worse associative memory performance (Kinnunen 2011). In BIOSAPS, this research was extended by exploring this relationship in bTBI. The structure of a large part of the WM showed a strong correlation with memory function across all subjects. Average FA across the bTBI and nbTBI groups was significantly correlated with memory function, such that decreasing FA in a large number of WM tracts was related to worsening memory function (Figure 3-6A). Tracts demonstrating this relationship included the fornices, where we have previously reported the correlation, as well as the corticospinal tracts, the anterior thalamic radiations, the inferior longitudinal fasciculi, the inferior fronto-occipital fasciculi, the uncinate fasciculi, the corpus callosum, and the superior longitudinal fasciculi.

The structure of some parts of the frontal WM was more significantly correlated with memory function in the bTBI than the nbTBI group. A direct comparison of the strength of correlation in the two groups showed that parts of the right orbito-frontal WM, uncinate fasciculi and anterior corpus callosum were strongly related to memory function only in the bTBI group (Figure 3-6B). A direct comparison of the bTBI group with the control group also demonstrated a trend towards a stronger relationship between memory and FA in the fornices, the forceps minor, the forceps major, the stria terminalis, the cingulum, inferior fronto-occipital fasciculi, the corpus callosum and the corona radiata ($p<0.1$ cluster corrected threshold).

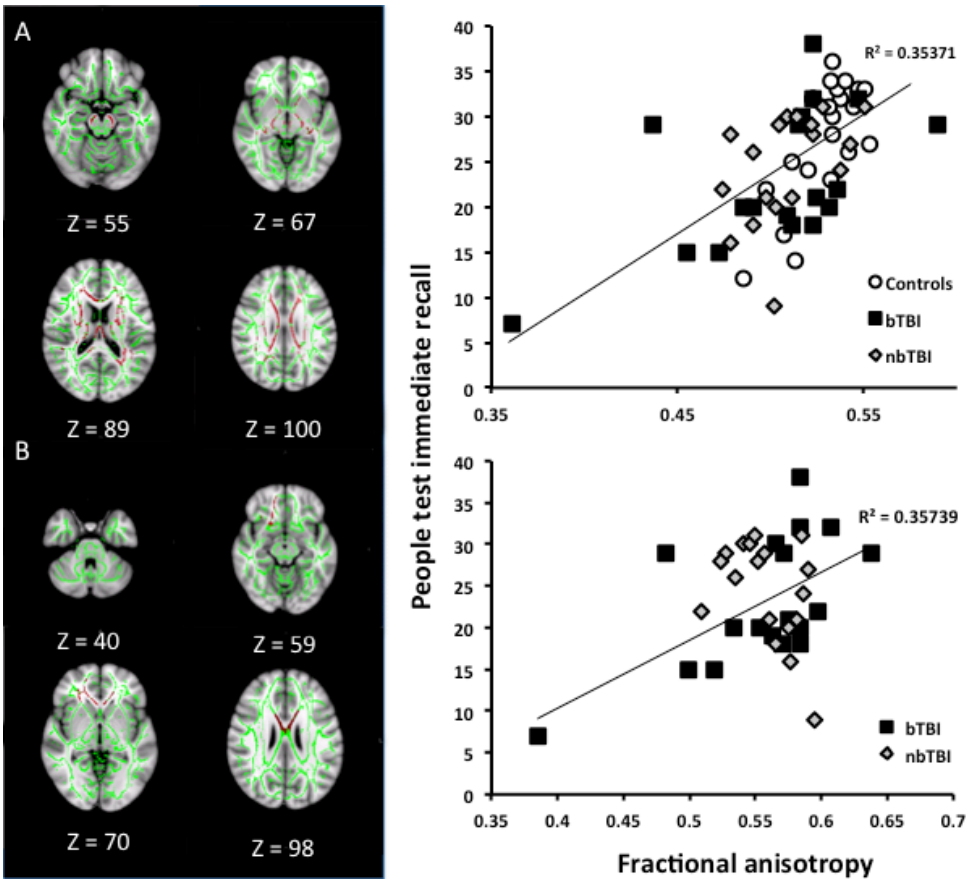


Figure 3-6A and B. The results of the TBSS regression analysis of associative memory (People test immediate recall) by FA across (A) both TBI groups and (B) bTBI group

(A) Areas where the average FA across both the bTBI and TBI groups was positively correlated with the People Test (PT) recall score. (B) Areas of frontal WM that were more strongly correlated with memory in the bTBI rather than the nbTBI group. The results are overlaid on a standard Montreal Neurological Institute 152 T_1 one mm brain and the mean FA skeleton (in green). For display purposes the result is displayed with a multiple comparisons threshold of p≤0.1. The graph shows individual data points in all groups for People Test recall score against FA in the peak voxel. A regression slope is shown.

3.4.6.2 Executive function

In the bTBI group, there was also a significant correlation between frontal WM structure and a measure of executive function (alternating switch cost from the Trail Making Test). Decreasing FA within the left orbito-frontal and transcallosal fibres was

associated with increasing switch cost (Figure 3-7). This relationship was not observed in the nbTBI or control subjects. Analysing all three groups together, there was no overall relationship between WM structure and executive function, and there were no areas where the relationship was significantly stronger for the bTBI group than nbTBI or controls in a direct comparison.

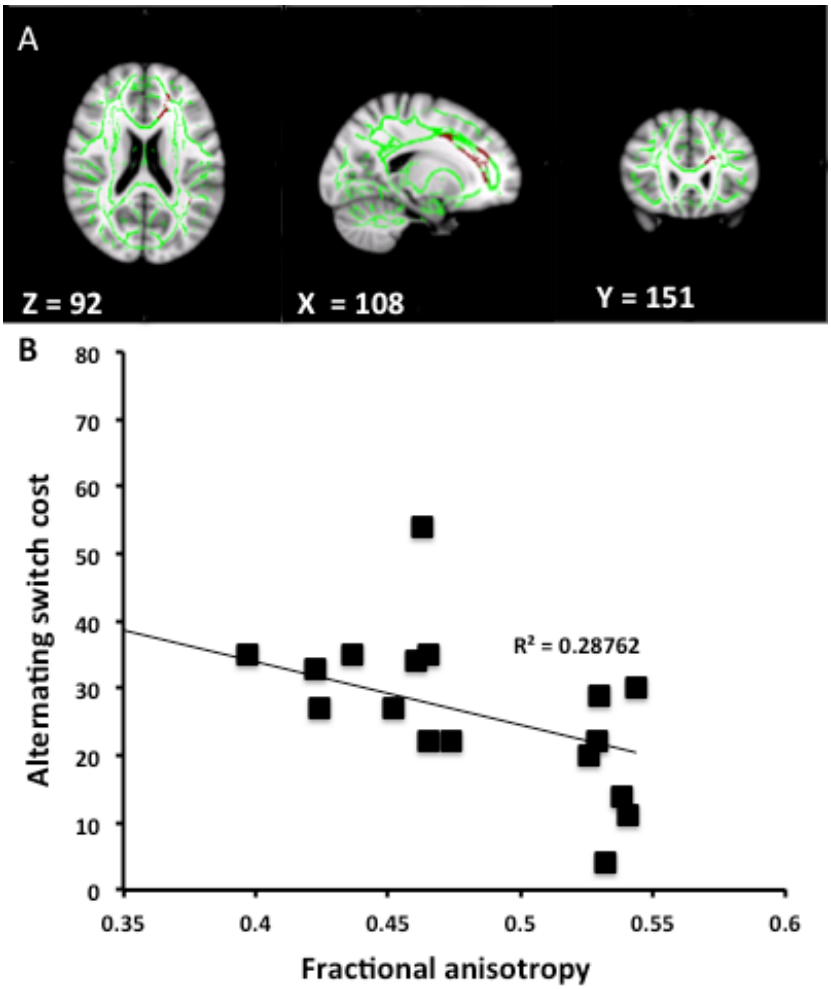


Figure 3-7. The results of the TBSS regression analysis of executive function (Trail Making Test B minus A) between the bTBI and control groups

(A) Shows the whole brain analysis with the areas in red being significant for interaction effect for FA. The results are thresholded at p≤0.01, corrected for multiple comparisons and overlaid on a standard Montreal Neurological Institute 152 T_1 one mm brain and the mean FA skeleton (in green). The graph illustrates a linear regression slope for the bTBI groups and individual data points for alternating switch cost against FA in the peak voxel of the interaction effect.

3.4.6.3 Information processing speed

At the voxel level, WM structure was not correlated with information processing speed, as measured by median reaction time for accurate responses on the Choice Reaction Task, either across the whole group or in separate group analyses.

3.5 Discussion

Presented here are the neuroimaging findings of the Blast Injury Outcome Study of UK Armed Services Personnel (BIOSAP). Soldiers who have suffered bTBI often have persistent cognitive problems despite often having normal standard brain imaging. This leads to considerable uncertainty about the cause of their symptoms and the correct treatment. There are several possible aetiologies for persistent neurological problems after trauma. These include primary psychiatric problems, such as PTSD, which has been the focus of much research into bTBI (Rosenfeld 2013). However, it is possible that structural brain injury produced by bTBI may not be identified by standard brain imaging, and our work provides evidence that DAI is common after moderate/severe bTBI, and may account for cognitive impairments that are prominent in this patient group. The bTBI patient group had lower FA, in large parts of their WM, providing evidence for widespread DAI that is not apparent on standard MRI or CT imaging. This study also provides evidence for a correlation between the amount of DAI and cognitive function, which was more prominent in bTBI than nbTBI. In addition, the work is a pioneering study investigating, for the first time, brain injury in UK troops exposed to blast, and complementing previous studies in US soldiers.

Recent work has focused on comparing soldiers exposed to blast with uninjured controls (Mac Donald 2011). This work provides clear evidence that bTBI can produce WM injury, but the degree to which the neuroimaging abnormalities observed are specific to blast exposure is uncertain. Explosive injuries cause a complex mixture of primary, secondary tertiary and quaternary injuries (Elder 2010b), and it is very rare for soldiers to have an isolated blast injury (Chapman 2014). Therefore, imaging abnormalities could result from non-blast mechanisms of injury such as head impacts or the rotational forces that often produce DAI in civilian TBI (Johnson 2013). By comparing our bTBI group with a matched group of nbTBI, we

are able to investigate the specificity of WM injury to blast exposure. The most striking aspect of these results is the similarity in widespread DTI abnormalities seen in both the bTBI and nbTBI groups. This suggests firstly, that there is widespread WM pathology present after moderate/severe bTBI; and secondly, that this is mainly not specifically related to blast exposure. This similarity is likely to reflect the complex mechanisms of injury that are present in most cases of bTBI. Soldiers are not only exposed to the effects of the primary blast wave and wind, but also to secondary and tertiary damage produced by fragments and debris propelled by the explosion, and the effects of the head impacting on other objects. This produces a mix of biomechanical effects that overlap with those seen in civilian injuries such as road traffic accidents, falls and assaults, which make up the majority of our nbTBI group.

Although patterns of WM injury were generally similar across bTBI and nbTBI, these results do provide some support for the proposal that the cerebellum is particularly vulnerable to the effects of blast exposure. In soldiers with microbleeds, i.e. those very likely to have underlying axonal injury, FA was lower within the middle cerebellar peduncles than in the nbTBI group. This builds on previous computational modeling by Taylor *et al.* who predicted that the blast pressure wave would cause focal injury in the posterior fossa and orbit-frontal WM and also by Brody and colleagues who have reported damage to the cerebellar WM in soldiers with a mixed mechanism of injury (Taylor 2009) (Mac Donald 2011), as well as in a small number (N=3) with isolated primary blast exposure leading to mild bTBI (Mac Donald 2014). In addition, Jorge *et al.* reported increasing WM damage after mild nbTBI was associated with increasing impairments of executive function (Jorge 2012). Brain metabolism measured using fluorodeoxyglucose positron emission tomography (FDG-PET) in 12 Iraqi veterans with post-concussion syndrome (PCS) following blast exposure showed hypometabolism in the cerebellum, brain stem and temporal lobe compared with 12 controls. Possible reasons that regions within the posterior fossa may be more at risk from injury by blast are that pressure wave interactions occur in these areas as a result of the shape and physical properties of the cranium and its contents, that the pressure wave may sensitise these areas of the brain to subsequent injury, and that the direction of the blast favours damage to these areas (Peskind 2011).

Previous work on patients with bTBI has tended to focus on examining a limited number of brain locations (regions of interest) (Mac Donald 2011). Although this is a sensitive method of identifying WM damage, it suffers from two main limitations. Firstly, TBI produces a complex pattern of DAI at various locations across individuals and so any attempt to decide "*a priori*" where to look is likely to fail to identify WM damage elsewhere in the brain. Secondly, as the cognitive functions commonly affected after TBI depend on the underlying WM connectivity, a ROI approach limits the analysis of the structural causes of cognitive impairment. To overcome these limitations, we have used Tract-based spatial statistics (TBSS), which is a voxel-based approach to analysing WM structure across the whole brain. It allows complex patterns of WM disruption to be identified and their relationship with cognitive dysfunction to be studied. Statistical calculations are performed at each voxel within the individual's WM "skeleton," allowing for a comprehensive analysis of tract structure to be performed. TBSS has been used to show the relationship between WM structure and cognitive function in other neurological conditions including civilian TBI (Bonnelle 2011, 2012).

Memory and executive function are commonly impaired following bTBI (Karr 2014). Results presented here show a strong relationship between the location of WM damage and cognitive dysfunction in these areas, suggesting that DAI may cause persistent symptoms produced by these problems. These findings build on previous work in nbTBI demonstrating a correlation between the structure of the fornix and memory function (Kinnunen 2011). It is likely that the diffuse injury seen in both blast and non-blast injury has affected distributed brain networks involved in supporting higher cognitive function. These results show a much stronger relationship between cognitive function and WM damage in the bTBI group, which in the case of executive function showed a specific relationship in the frontal WM regions. Given that computational models and human studies looking at mild blast traumatic brain injury have previously identified this region (Taylor 2009, Mac Donald 2011), this provides evidence that moderate/severe bTBI is likely to impair executive function through damage to frontal WM tracts.

Animal models confirm that reduced FA is a marker of axonal injury in both blast (Calabrese 2014) and nbTBI (Mac Donald 2007). In a mouse model of contusional brain injury, Mac Donald and colleagues showed that FA and axial diffusivity reduced

in the early hours following injury, reflecting axonal injury (Mac Donald 2007). Over time macrophage infiltration, tissue oedema and demyelination were accompanied by an increase in radial and mean diffusivity, with a persistently low FA. A similar relationship between reduced FA and axonal injury has been demonstrated in a rat model, albeit only after double blast exposure (Calabrese 2014). To date, most animal studies have been performed in rodents, and studies in larger animals such as pigs are important in providing information about the relationship between histology and DTI measures in brains that are more similar to humans (Cernak 2005). Another limitation of the animal work is that studies are performed in the acute or subacute phase after exposure, whereas in humans studies, imaging is typically performed longer from the initial injury. As the DTI changes evolve, this adds complexity to understanding the relationship between DTI and histology. However, studies to date have shown that FA remains relatively stable over time.

A possible limitation of human studies is that they are not able to measure the forces that the brain was exposed to and thus quantify the level of blast exposure. This criticism is particularly true of those studies that used self-reporting of blast exposure. Only including those who have suffered moderate to severe injury as a result of a single exposure to an explosion limits the impact of this problem. Estimates obtained (but not yet publishable) of the size explosive device used as well as the victim's proximity to it clearly demonstrate a significant blast exposure in each case. A possible limitation of the method of imaging analysis used is inaccuracy caused by registration of the subjects' brains into a common space. This is particularly important when structures are in close proximity to cerebrospinal fluid containing spaces and hence may be subject to partial volume effects. Using skeletonisation of the WM, focusing on the central portion of the tracts in relatively young patients who are less likely to have brain atrophy, limits the likelihood of sampling from adjacent ventricles. The selection process excluded soldiers who had suffered intracranial lesions causing a mass effect, and in fact the blast group had fewer and smaller volume contusions than the civilian controls.

To conclude, this study found widespread WM damage in soldiers exposed to blast when compared to uninjured controls. Using a region of interest approach, it was found that the anterior internal capsules and middle cerebellar peduncles showed more evidence of injury of borderline significance. In the context of similar findings by

computational models and human studies, this may indicate that these areas are more vulnerable to blast injury.

3.6 Summary

In this chapter I have shown that widespread WM abnormalities demonstrated by reduced FA were observed in blast injured soldiers, relative to controls. These were not visible on conventional brain imaging. A similar distribution of damage was observed in the non-blast TBI patient group, suggesting that most of the observed WM abnormalities are not specific to blast exposure. In patients with microbleed evidence of DAI, greater WM damage was observed in the posterior fossa, in keeping with previous work. A clear relationship was observed between WM pathology and both memory and executive function. Decreasing FA was correlated with increasing impairment in these domains, with parts of the frontal lobe WM showing a specific relationship with executive function in soldiers exposed to blast. These results provide evidence for widespread DAI in soldiers exposed to blast injury that may be missed by standard neuroimaging. Most of the changes are not specific to blast exposure, although the posterior fossa WM tracts may be particularly vulnerable to this type of injury. Persistent memory and executive function problems after blast may result from injury to the WM tracts as a result of DAI, and screening these patients using diffusion tensor imaging will add important diagnostic information.

In the next Chapter, I describe the prevalence of pituitary dysfunction in this bTBI population in comparison to a different civilian nbTBI population matched for age, injury severity and time since injury. I will then use neuropsychological tests as well as the MRI techniques described in the previous chapters to describe this group further.

4 Endocrine dysfunction, cognitive function and structural brain imaging in blast injury

4.1 Introduction

This chapter addresses the investigation of:

a. the prevalence and consequences of pituitary dysfunction following moderate-severe bTBI; and

b. potential associations with particular patterns of brain injury.

It describes a study in which 19 male soldiers who suffered a moderate or severe TBI secondary to blast exposure (bTBI), and 39 male controls with moderate-severe nbTBI, underwent comprehensive endocrine assessment between 2 and 48 months after injury. We then went on to explore the relationship between pituitary dysfunction, cognition and WM damage secondary to blast by performing structural brain magnetic resonance imaging, including DTI, and cognitive assessment on the bTBI group.

We hypothesized:

That bTBI would be associated with pituitary dysfunction and that DTI would reveal more WM damage in those soldiers with pituitary dysfunction after bTBI than without.

4.2 Background

The use of IEDs has characterised the Iraq and Afghanistan conflicts with bTBI described as a ‘signature injury’ (Benzinger 2009) of these wars. Blast injuries in Afghanistan have fatally wounded over 450 soldiers from the UK and 2,000 soldiers from the USA since 2001 (Chesser 2012). 19.5% of the 1.64 million troops from the USA deployed in both conflicts are estimated to have suffered a probable bTBI (Tanielian 2008). Soldiers are usually young meaning that the long-term impact of their physical, cognitive and psychological problems represents a significant health burden. There are currently no pharmaceutical treatments that improve recovery following TBI (Ruff 2012).

In non-blast TBI, GH deficiency (Schneider 2007) is a recognised cause of pituitary dysfunction. The reported prevalence of pituitary dysfunction following nbTBI varies between 2 and 68% (Schneider 2007, Kokshoorn 2011). This variability is due, in part, to differences in the injury severity of the subjects, their time since injury and the normal ranges and dynamic endocrine tests used (Schneider 2007, Kokshoorn 2011¸ Kokshoorn 2010). As hypopituitarism causes multiple symptoms that can impact on physical and psychological well-being as well as adverse metabolic consequences, that will impair recovery after TBI, hormone replacement represents a significant therapeutic opportunity (Salvatori 2005, Cherrier 2009, Molitch 2011, Bondanelli 2007). Before this work, one study had investigated the prevalence of pituitary dysfunction after mild bTBI (Wilkinson 2012). However, methodological issues including, the authors' reliance on basal hormone measurements, the definition of normal ranges, and the non-standard assessment of posterior pituitary function make their results difficult to interpret. The prevalence of pituitary dysfunction following moderate to severe bTBI was not known (Guerrero 2010).

As explained in earlier chapters, DTI is a sensitive MR technique that can assess the presence and severity of WM damage after TBI (Mac Donald 2007, Kinnunen 2011). TBI alters the pattern of water diffusion within WM resulting in the abnormal diffusion characteristics of measures such as FA. DTI abnormalities in several brain regions, including the orbitofrontal WM and the middle cerebellar peduncles, have been reported in soldiers following mild bTBI (Mac Donald 2011). We hypothesised that bTBI would be associated with pituitary dysfunction and that DTI would reveal more WM damage in those soldiers with pituitary dysfunction after bTBI than without.

This chapter reports further findings from the Blast Injury Outcome Study of Armed Forces Personnel (BIOSAP). It investigates the prevalence and associations of pituitary dysfunction in soldiers after moderate-severe bTBI compared to a control group of patients after nbTBI.

4.3 Methods

From the 20 soldiers that we recruited in the first study 19 bTBI patients were recruited into the endocrine study. Using the Academic Department of Military Emergency Medicine (Birmingham, UK) trauma database to identify soldiers from the

UK, injured between December 2009 and March 2012. This group represented 10.4% of the 183 UK soldiers who had survived a moderate-severe bTBI in Afghanistan during this 27 month period. In total, the war lasted 13 years, and a total of 453 UK personnel lost their lives. We compared this bTBI group with an age-matched and gender-matched control group of 39 patients who had suffered nbTBI. The nbTBI group were all identified and recruited from the Traumatic Brain Injury clinic at Charing Cross Hospital, London, UK. We included in the nbTBI group, all the patients assessed in the multi-disciplinary TBI clinic who met the inclusion and exclusion criteria and who were within the age range of the bTBI group. We recruited the nbTBI group between August 2009 and March 2012. We performed an identical endocrine assessment, to the bTBI group, as part of their routine clinical care. All subjects gave informed consent. This study received ethical approval from the Hammersmith, Queen Charlotte's and Chelsea Research Ethics Committee.

We aimed to recruit subjects who mainly had primary blast injury, and to minimise the influence of secondary, tertiary and quaternary injuries. The inclusion criteria for the bTBI group were a moderate-severe brain injury that was directly caused by a single blast exposure.

We excluded subjects from the bTBI group if they:

a. had haemorrhagic blood loss requiring a massive blood transfusion;

b. had an intracranial lesion causing a mass effect on acute imaging; or

c. had post-traumatic stress disorder (PTSD).

PTSD, in isolation from TBI, is associated with endocrine disturbance (Pervanidou 2010, van Liempt 2011). We diagnosed PTSD by an interview with a psychologist and, if they suspected the diagnosis, subsequent self-reported symptom ratings from the PTSD Checklist–Military (PCL-M) version derived from DSM-IV criteria (Wilkins 2011). Many of the soldiers with bTBI described: loss of memory of the event, anhedonia, social isolation, sleep disturbance, emotional lability and poor concentration. However, they did not report additional symptoms required for the diagnosis of PTSD. These symptoms include "recurrent and intrusive distressing recollections of the event; affecting their thoughts or perceptions," "behaving or feeling as though the traumatic event were recurring," and "physical reactions like

palpitations, shortness of breath or sweating when reminded of the past stressful experience" (Friedman 2011).

The inclusion criteria for both bTBI and nbTBI groups were:

a. male gender;
b. >2 and <48 months from a single TBI;
c. moderate-severe brain injury using the Mayo classification criteria (Malec 2007);
d. ongoing cognitive and/or psychological symptoms.

Exclusion criteria were:

a. diabetes mellitus;
b. pre-TBI history of psychiatric disorder;
c. current or previous drug or excess alcohol use;
d. reversed sleep-wake cycle; and
e. craniotomy following injury.

We excluded subjects who had had craniotomies to avoid difficulties in brain image registration that result from gross changes in the shape of the brain. Both bTBI and nbTBI subjects underwent clinical assessment and completed quality of life (QoL) and symptom questionnaires (see Supplementary Methods in Appendix 2). Additionally, we calculated their Abbreviated Injury Scores (AIS), and total Injury Severity Score (ISS).

4.3.1 Endocrine testing

We used the algorithm shown in Table 4-1 (see Supplementary Methods in Appendix 2) to define pituitary dysfunction. All patients had their basal serum anterior pituitary hormones measured followed by dynamic endocrine testing. Initial screening for growth hormone (GH) and adrenocorticotropic hormone (ACTH) deficiency was performed using the glucagon stimulation test (GST) (Leong 2001, Cegla 2012). We confirmed the diagnosis of GH deficiency with either second-line growth hormone releasing hormone (GHRH) Arginine or an insulin tolerance (ITT) test (Molitch 2011,

Colao 2009, Yuen 2009). The diagnosis of ACTH deficiency was confirmed with an ITT or metyrapone stimulation test, and a cortisol day curve (Cegla 2012, Grossman 2010). If subjects had symptoms of diabetes insipidus, they were investigated with a water deprivation test.

Table 4-1. Diagnostic algorithm for pituitary dysfunction

Pituitary Axis	1st Test	Confirmatory test
GH Deficiency	Glucagon Stimulation Test (Peak GH < 5µg/L)	GHRH-Arginine Test (GHD cut off based on age and BMI) or ITT peak GH <3 µg/L (Colao)
ACTH Deficiency	Glucagon Stimulation Test (Cortisol <350 nmol/L)	Metyrapone Stimulation Test (ACTH <60 ng/L and 11-DOC<200 nmol/L) or peak cortisol ITT <500nmol/L
Prolactin	Prolactin >375 mu/L	Repeat prolactin >375 mu/L AND Macroprolactin Negative
Gonadotrophin Deficiency	Non-elevated LH (1.7-12.0) and FSH (1.7-8.0) AND Testosterone < 10 nmol/L or if SHBG low (<15 nmol/L) FAI <30	Non-elevated LH (1.7-12.0) and FSH (1.7-8.0) AND 9 am Testosterone < 10 nmol/L or if SHBG low (<15nmol/L) FAI <30
TSH Deficiency	Non-elevated TSH (0.3-4.22) AND Free T4 <9.0 pmol/L or free T3<2.5pmol/L	Non-elevated TSH (0.3-4.22) AND Free T4 <9.0 pmol/L or free T3<2.5pmol/L
ADH Deficiency (Vasopressin)	Symptoms score	Water Deprivation Test

*FAI=Free Androgen Index =100x free testosterone titre/SHBG

4.3.2 Cognitive function assessment

Each soldier with bTBI completed a standardised neuropsychological test array that is sensitive to cognitive impairment following TBI (Kinnunen 2011). The tests looked at the cognitive domains of:

a. current verbal and non-verbal reasoning;

b. associative memory and learning;

c. executive functions; and

d. information processing speed (see Supplementary Methods in Appendix 2).

In addition, we used the quality of life assessment of growth hormone deficiency in adults (QoL – AGHDA). The AGHDA consists of 25 yes/no self-administered questions which looks at seven areas of concern in growth hormone deficiency, namely:

a. body image (fat distribution);

b. energy level;

c. concentration;

d. memory;

e. irritability;

f. strength and stamina; and

g. coping with stress.

A high AGHDA score indicates the patient suffers from many symptoms and thus a worse quality of life.

We also used the Beck Depression Inventory (BDI-II), which is a self-administered 21-question multiple choice assessment of depression. A high BDI-II score indicates a higher level of depression.

4.3.3 Structural brain imaging

We performed standard T1, gradient-echo (T2*) and SWI MRI on each subject in the bTBI group to look for focal brain injury, microbleeds, superficial siderosis, gliosis, contusions, as well as DTI. Most patients who were found to have pituitary dysfunction went on to have an MRI of the pituitary gland with gadolinium contrast to

look for more specific hypothalamic-pituitary abnormalities. Patients with nbTBI had radiological imaging as part of routine clinical practise which was usually a CT brain scan if required during the acute presentation or a standard T1/T2 brain MRI requested in the TBI clinic. DTI analysis of WM tracts combined tract-based spatial statistics (TBSS) and region of interest (ROI) approaches (FSL, FMRIB, Oxford, UK), focussing on regions previously shown to be sensitive to damage in bTBI and nbTBI (Figure. S1 and Supplementary Methods in Appendix 2) (Kinnunen 2011, Mac Donald 2011). We used this to perform a regional assessment of FA, which is a measure of axonal injury.

4.3.4 Statistical analyses

We compared the different groups (nbTBI vs. bTBI; and bTBI with pituitary dysfunction vs. bTBI without pituitary dysfunction) using Fisher's exact test for prevalence data, and unpaired Student t-test (FA and neurocognitive variables), or Mann-Whitney U test (other variables) for continuous data (SPSS v19.0). We defined significance as $p<0.05$. A group x ROI repeated measure ANOVA was performed to assess the overall effect of pituitary dysfunction on FA.

4.4 Results

4.4.1 Patient characteristics

All of the soldiers in the bTBI group had been injured by IEDs and had been wearing full personal protective equipment. They required immediate transfer to Camp Bastion for emergency medical treatment, and were repatriated to the UK within 48 hours. Detailed information about the blast exposure was known, but for operational security reasons, these are not reported. In the control nbTBI group, injuries were secondary to RTA (43%), assaults (32%), falls (23%) and sporting injuries (2%). Three subjects in the nbTBI group had multiple TBIs (one subject had a mild TBI from an RTA and a second from an assault while another had one mild TBI from a fall and another TBI of unknown severity from an assault).

The blast and non-blast TBI groups were matched well in most characteristics (Table 4-2). There were no significant differences in age, whole body injury severity (ISS), skull/facial fractures (15.8 vs. 15.4%) or the incidence of post-traumatic seizures (10.5 vs. 7.7%) between the two groups. The bTBI group had a longer period of PTA (median 5.5 days vs. 0.5 days, $p=0.01$), as well as more injuries requiring surgery to, or causing loss of function of, extracranial organs (57.9 vs. 7.7%, $p=0.002$), and more amputations (36.8 vs. 0%, $p<0.001$). The increased incidence of extracranial injury was in keeping with a higher proportion using strong opiates (47.3 vs. 7.7%, $p=0.001$). In the bTBI group, there was a significantly longer time interval between the TBI to performing endocrine function testing (median 15.2 vs. 5.8 months, $p=0.001$).

Table 4-2. Patient characteristics

Category	Max Score/ units	All nbTBI	All bTBI	p value	bTBI: No Pituitary Dysfunction	bTBI: Pituitary Dysfunction	p value
n		**39**	**19**		**13**	**6**	
Age at TBI	years	31.3 [22.5-35.7]	26.7 [26.1-30.9]	0.40	26.6 [24.6-30.6]	29.3 [25.8-36.6]	0.35
		17.2 - 44.8	19.0 - 43.5		19.0 - 36.3	25.0 - 43.47	
Age at testing	years	32.3 [23.1-36.7]	28.3 [26.8-32.2]	0.40	28.0 [25.3-31.4]	30.3 [27.4-38.3]	0.35
		19.9 - 45.1	19.6 - 44.7		19.6 - 37.6	26.3 - 44.7	
Time since TBI	months	5.8 [3.1-11.0]	15.2 [10.8-19.3]	**0.001**	1.27 [0.7-1.4]	1.5 [1.0-1.7]	0.35
		1.9-41.2	4.1 - 23.6		0.34 - 1.97	0.41 - 1.83	
ISS	75	25 [16-32]	33.0 [20.0-45.0]	0.17	24.0 [14.5-40.5]	35.5 [27.0-51.3]	0.21
		1-75	9-70		9-45	9-70	
AIS Head	6	5.0 [4-5]	4.0 [3.0-5.0]	**0.04**	4.0 [2.5-4.0]	5.0 [3.0-5.3]	**0.05**
		1-6	0-6		0-5	0-6	
AIS Chest	6	0 [0-0]	0 [0-2]	0.11	0 [0-3]	0 [0-2.3]	0.76
		0-6	0-4		0-4	0-3	
AIS Abdomen	6	0 [0-0]	0 [0-2]	**0.02**	0 [0-2]	0 [0-2.3]	0.96
		0-3	0-3		0-2	0.3	

Category	Max Score/ units	All nbTBI	All bTBI	p value	bTBI: No Pituitary Dysfunction	bTBI: Pituitary Dysfunction	p value
GCS	15	14.0 [6.0-14.0][a]	3.0 [3.0-14.5][b]	0.24	14.0 [3.0-15.0]	3.0 [3.0-3.0]	0.17
		3-15	3-15		3-15	3-3	
PTA	days	0.5 [0-7.3][c]	5.5 [0.8-22.8]	**0.01**	3.0 [0-19.3]	15.5 [6.3-31.5]	0.13
		0-42	0-84		0-84	4-42	
PTA>24 hrs		20 (51.3%)	13 (72.2%)	0.70	7 (58.3%)	6 (100%)	0.48
BMI **BMI**	(kg/m^2)	24.7 [22.4-29.4]	26.7 [24.5-28.9]	0.28	26.6 [24.5-28.7]	25.5 [22.4-32.0]	0.71
		17.0-33.4	21.7-33.7		23.6-29.4	21.7-33.7	
Limp Amputation		0 (0%)	8 (42.1%)	**0.0008**	6 (46.1%)	2 (33.3%)	0.99
Major Organ Damage		3 (7.7%)	11 (57.9%)	**0.002**	7 (53.9%)	4 (66.7%)	0.99
Skull/facial fracture		6 (15.4%)	3 (15.8%)	1.0	0 (0%)	3 (50.0%)	0.10
Opiate Use		3 (7.7%)	9 (47.3%)	**0.02**	6 (46.2%)	3 (50.0%)	1.00
Antidepressant Use		5 (12.8%)	9 (47.3%)	0.08	6 (46.2%)	3 (50.0%)	1.00
Seizures post TBI		3 (7.7%)	2 (10.5%)	1.0	1 (7.7%)	1 (16.7%)	0.99
Primary Hypogon		1 (2.6%)	4 (21.1%)	0.24	4 (30.8%)	0 (0%)	0.26

Category	Max Score/ units	All nbTBI	All bTBI	p value	bTBI: No Pituitary Dysfunction	bTBI: Pituitary Dysfunction	p value
adism							

All data expressed as median [interquartile range], range or n (%)

P values from Mann-Whitney U test or Fisher's exact test between groups.

Data available for a n=16, b n=9, c n=38, and due to amputations: f n=7, g n=4

For analgesic purposes only in h n=5 (12.8%), i n=5 (26.3%), j n=3 (23.1%), k n=2 (33.3%)

For depression itself in h n=0 (0%), i n=4 (21.1%), j n=3 (23.1%), k n=2 (16.7%)

On anti-epileptic drugs in l n=3, m n=1, n n=0, o n=1

p not due to trauma, q due to perineum trauma

4.4.2 Prevalence of pituitary dysfunction in bTBI and nbTBI cohorts

Six of the 19 soldiers with bTBI (31.6%) had anterior pituitary dysfunction compared to only one out of 39 (2.6%) subjects with nbTBI (p=0.004, Figure 4-1 and Table S1-S3 in Appendix 2). Two soldiers (10.5%) had monomeric hyperprolactinaemia (without secondary hypogonadism), one (5.3%) had isolated ACTH deficiency, two (10.5%) had isolated GH deficiency, and one (5.3%) had combined ACTH, GH and gonadotrophin deficiencies. The only pituitary dysfunction noted in one patient with nbTBI was isolated GH deficiency following a single TBI. No patients in either group had TSH deficiency or diabetes insipidus.

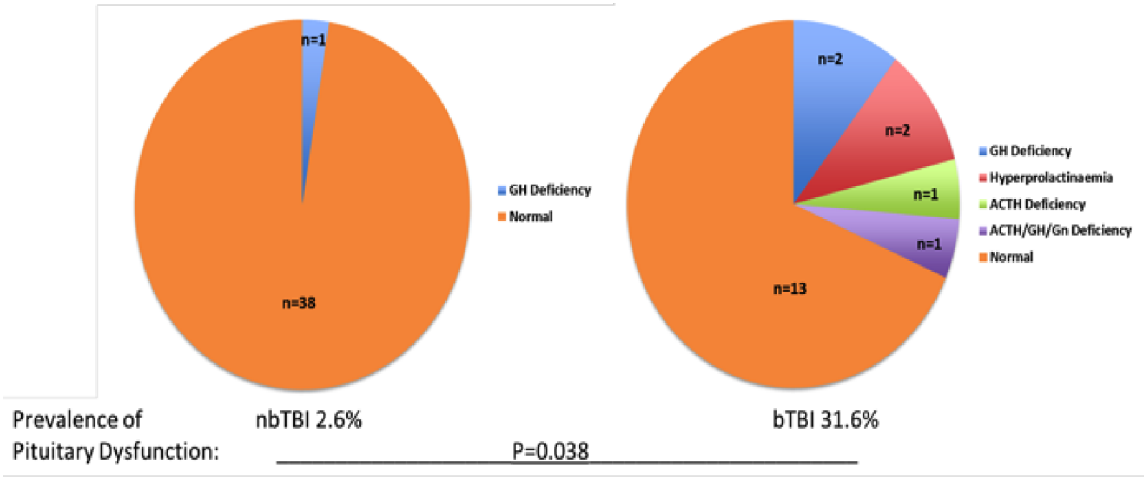


Figure 4-1. Prevalence of pituitary dysfunction in non-blast and blast traumatic brain injury

The greater prevalence of anterior pituitary dysfunction in subjects after (right) blast TBI than (left) non-blast TBI. No subjects had TSH deficiency or diabetes insipidus.

The three soldiers with GH deficiency had IGF-1 levels in the low normal range (see Supplemental Results, Table S2 in Appendix 2). The two soldiers with ACTH deficiency had normal early morning cortisol levels on their initial assessment of 287-292 nmol/L, 10.3-10.5 µg/dL (NR >150 nmol/L, >5.4 µg/dL) (see Supplemental Results, Table S3 in Appendix 2). However, on subsequent cortisol day curves, both subjects with ACTH deficiency had low cortisol levels (<100 nmol/L, 3.62 µg/dL) at either 0900 or 1200 h on a day curve consistent with the diagnosis (see Supplemental Results, Table S3 in Appendix 2). To reduce the risk of seizures we occasionally used the metyrapone test, instead of the gold standard ITT, to confirm test or exclude ACTH deficiency, and the findings were always compatible with the baseline or day curve cortisol levels. There was no history of hypotension, hypoglycaemia or hyponatraemia in any of the soldiers with ACTH deficiency.

We found primary hypogonadism due to perineal/testicular injury in four out of 19 soldiers with bTBI (21.2%); none of these subjects had pituitary dysfunction. All were already receiving testosterone replacement (see Supplemental Results, Table S1 in Appendix 2).

4.4.3 Comparison of bTBI with vs. bTBI without pituitary dysfunction

We did not find a significant difference in age at the time of TBI, the time since injury, ISS, abdominal AIS, BMI, prevalence of amputations, seizures, use of anti-depressants or prevalence of depression, or strong opiate use between bTBI patients with vs. without pituitary dysfunction (Tables 4-2, S6 and S7 in Appendix 2). We could not accurately assess the BMI in the eight soldiers with bTBI who had limb amputations, but on clinical examination none was obese.

In those soldiers with pituitary dysfunction, there were trends for higher AIS head injury scores (p=0.06), and longer duration of PTA (median 15.5 vs. 3.0 days, p=0.10) when compared to those without.

We diagnosed one soldier (M08) with multiple pituitary deficiencies. At the time of diagnosis, he was taking opiates, so both GH and ACTH deficiency were confirmed once these drugs had been discontinued using an ITT.

4.4.4 Neuroimaging results

The soldiers with pituitary dysfunction had a greater prevalence of skull/facial fractures when compared to those without (50% vs. 0%, p=0.02). We found contusional brain injury in three out of the six (50.0%) soldiers with pituitary dysfunction, compared to only one out of the 13 (7.7%) soldiers without pituitary dysfunction on MRI brain scans (p=0.07). One soldier with pituitary dysfunction had two contusions while the remainder had one contusion (Figure S2 in Appendix 2). The total volume of the contusion(s) was <10 cm^3 in all cases. The soldier without pituitary dysfunction had the smallest contusion volume.

There were no significant differences in the prevalence of extradural, subarachnoid or intraventricular haemorrhage, microbleeds, superficial siderosis or gliosis, between those soldiers with vs. without pituitary dysfunction (Table S4 in Appendix 2). We did not identify any hypothalamic-pituitary abnormalities on the MRI scans of any of the soldiers in the bTBI group, and there were no abnormalities seen on the dedicated MRI pituitary scans in the 4 with pituitary dysfunction (M01, M08, M10, M14).

In the bTBI group, we then investigated the association between WM damage and pituitary dysfunction. DTI analysis showed reduced FA in distinct regions indicating greater WM damage, in those soldiers with pituitary dysfunction compared to those without (p=0.14 effect of group, p=0.02 group x ROI interaction). We found significantly lower FA values within the cerebellum (p<0.05), and body/genu (p<0.05) and splenium (p=0.01) of the corpus callosum for those soldiers with pituitary dysfunction (Figure 4-2).

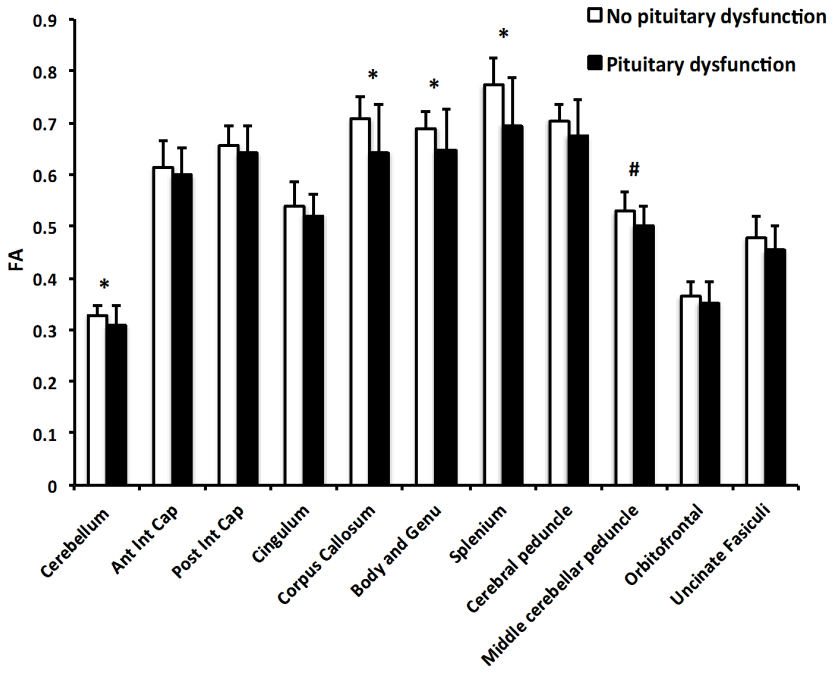


Figure 4-2. Pituitary dysfunction and WM damage in bTBI

Lower FA in a priori WM tract regions of interest in soldiers after blast TBI with pituitary dysfunction (black, n=6) compared to those without pituitary dysfunction (white, n=13). Data expressed as mean ± SD. *p<0.05 (unpaired t-test). Cap: Capsule

4.4.5 Symptoms, quality of life and cognitive function

Measured using the Nottingham Health Profile questionnaire, the bTBI group had significantly worse scores for physical activity and daily living problems than the control nbTBI group, consistent with their higher prevalence of polytrauma and amputations. There was no difference in measures of depression and emotional

well-being between bTBI and nbTBI (Table S5, Supplemental Results in Appendix 2).

In the bTBI group with pituitary dysfunction, there was a tendency towards worse measures of quality of life and symptom scores in several domains around emotional and social functioning, fatigue and mood compared to those without pituitary dysfunction; however, these did not reach significance (Table S5, Supplemental Results in Appendix 2).

There was significantly worse average current verbal intellectual ability in bTBI subjects with pituitary dysfunction than those without pituitary dysfunction, although there was no significant difference in their pre-injury measure of intelligence (Wechsler Test of Adult Reading). There was also significantly worse cognitive impairment in processing speed, verbal fluency and information processing in the bTBI group with pituitary dysfunction (Figure 4-3).

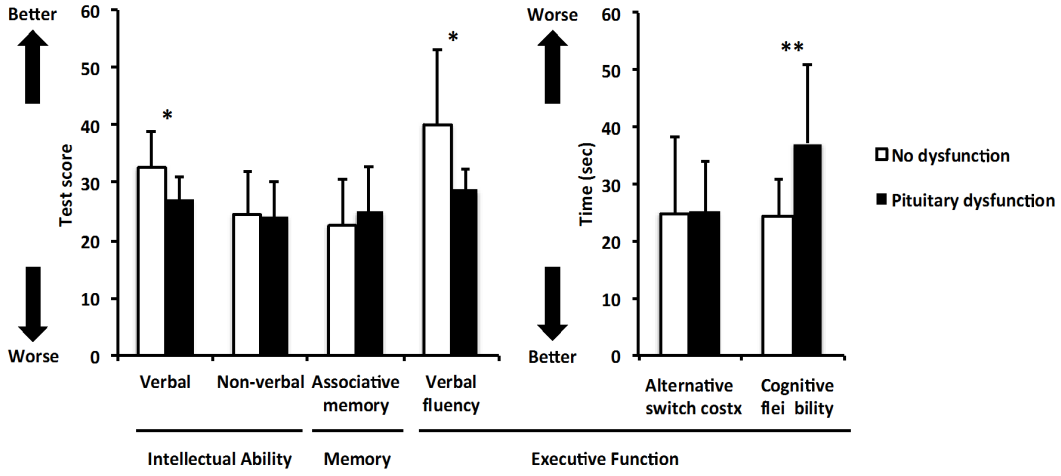


Figure 4-3. Pituitary dysfunction and cognitive function in bTBI

Worse cognitive function in soldiers after blast TBI with pituitary dysfunction (n=6) compared to those without pituitary dysfunction (n=13). Data expressed as mean ± SD. *p<0.05, **p<0.005 (unpaired t-test).

4.5 Discussion

This study demonstrates a high prevalence of pituitary dysfunction following moderate-severe blast TBI. Nearly one-third of soldiers with bTBI had anterior pituitary abnormalities. In comparison, just 2% of age- and gender-matched civilians with moderate-severe non-blast TBI had pituitary dysfunction. GH deficiency was the most common pituitary abnormality in bTBI, followed by hyperprolactinemia, ACTH and Gn deficiency. One patient had multiple hormone deficiencies.

We were careful to avoid over-diagnosis of pituitary dysfunction. We achieved this by using identical diagnostic algorithms in both groups, excluding the presence of macroprolactin, applying strict normal ranges for diagnosing testosterone and TSH deficiency, performing two stimulation tests to confirm ACTH or GH deficiencies, and adjusting for the confounders of age and obesity in diagnosing GH deficiency (Colao 2009). This enabled us to be confident in reporting the prevalence of pituitary dysfunction in both groups (Kokshoorn 2011¸ Kokshoorn 2010).

The results presented here suggest that all patients after moderate-severe bTBI should undergo endocrine assessment. Unlike TSH and gonadotrophin deficiency, GH and ACTH deficiency cannot be excluded or confirmed by basal IGF-1 or cortisol measurements and dynamic endocrine testing is required. The clinician must take into account contraindications, such as seizures, for use of the ITT, as well as the advantages and disadvantages of each test (specificity/sensitivity, age/obesity-adjusted normal ranges, resource implications, local expertise and drug availability) when choosing their investigations (Kokshoorn 2010, Cegla 2012, Yuen 2009).

Differences in age, gender and BMI did not explain the presence of pituitary dysfunction after bTBI. The observed difference in demographics would, if anything, have reduced the prevalence of pituitary dysfunction. As pituitary dysfunction can resolve over time, the longer time from injury to testing in the bTBI group compared to the nbTBI group should reduce the chance of over-diagnosis and certainly not increase the prevalence (Aimaretti 2005). Opiates can have complex neuroendocrine effects, including hypogonadotrophic hypogonadism, and potentially decreasing ACTH secretion but increasing GH secretion (Vuong 2010).

There was greater use of opiates in the bTBI as a whole compared to the nbTBI group, but the individual pituitary dysfunction seen in each soldier within the bTBI

group was not explicable by opiate use. The use of opiates and other medications does not explain these results.

Although there were a higher proportion of soldiers with bTBI and polytrauma than in the nbTBI group, there was no significant difference in the incidence of polytrauma between those with and without pituitary dysfunction in the bTBI group.

As discussed in earlier chapters blast can cause brain injury through several mechanisms. The BOP wave may generate shearing forces in the head that result in the primary injury; fragmentation from projectiles or debris causes the secondary injury; and the acceleration and deceleration forces that occur when a subject impacts nearby structures cause the tertiary injury (Cernak 2010, Goldstein 2012). Each of these different mechanisms could damage the hypothalamus, pituitary gland or stalk, resulting in damage to cell bodies or WM connections. Damage to the hypophyseal vessels could cause local superficial siderosis, and haemorrhage from extracranial polytrauma could cause hypovolaemic ischemic damage to the pituitary gland, similar to that seen in Sheehan's syndrome. The systemic inflammatory response seen following trauma may affect pituitary gland function (Brøchner 2009).

The imaging findings show increased damage in the corpus callosum and the posterior fossa of soldiers with pituitary dysfunction after bTBI. Injury to the corpus callosum occurs in DAI (Adams 1982) whilst WM damage in the posterior fossa has been previously demonstrated in mild bTBI (Mac Donald 2011). These findings suggest that pituitary dysfunction in bTBI is in part caused by increased severity of brain injury, as in nbTBI (Schneider 2007). The increased prevalence of skull and facial fractures, a trend for more cerebral contusions and longer period of PTA support this interpretation. These results do not provide clear evidence about the precise mechanism of hypothalamic-pituitary injury, nor was there a WM injury pattern that could predict pituitary dysfunction. There was no proof of focal damage to the hypothalamus or pituitary or superficial siderosis. However increased damage in the posterior fossa is indicative of a mechanism of injury unique to blast.

Our study looked at subjects with a single episode of moderate-severe bTBI. There is currently a lot of academic interest in repetitive mild bTBI, and we do not know if pituitary dysfunction is made worse following multiple injuries, this is a realistic concern as there is evidence that multiple bTBIs may worsen neurological deficits

(Ruff 2012). Our work builds on the previous study by Wilkinson *et al*. that suggested mild bTBI can produce endocrine disturbance (Wilkinson 2012).

Soldiers with bTBI and pituitary dysfunction had a trend for worse fatigue, emotional symptoms, social problems and mood. Worse underlying brain injury and their endocrine deficiencies may be the cause of these differences. These symptoms are well-recognised features of GH deficiency, and cortisol and testosterone deficiency are known to cause lethargy (Salvatori 2005, Cherrier 2009, Webb 2008). Similarly, the cognitive impairment we have demonstrated could be the result of either, or both, the more severe bTBI or the hormone deficiency (Kinnunen 2011, van Dam 2005, Bonnelle 2011).

The findings of this study led to changes in clinical management. The soldier with hypogonadotrophic hypogonadism received treatment with long-acting intra-muscular testosterone and both soldiers with ACTH deficiency commenced hydrocortisone replacement. We gave all three soldiers with GH deficiency replacement therapy, given their neuropsychological symptoms which had persisted for over one year despite replacement of other pituitary hormones.

At the time of writing, two of the three soldiers on GH replacement therapy had 12-month follow-up data. They both showed an improvement in their symptoms. The AGHDA score fell from 19 to 14 (out of 25), and BDI-II score from 36 to 18 (out of 63) in 1 (subject M14), and AGHDA from 14 to 3, and BDI-II from 20 to 16 in another (subject M08) during this period. This indicated an improvement in quality of life and reduction of symptoms of depression.

The soldiers with mild hyperprolactinaemia did not require treatment as secondary hypogonadism was absent.

In conclusion, this study demonstrated a high prevalence of anterior pituitary dysfunction after moderate-severe bTBI that was significantly greater than in a matched group of civilian nbTBI. This difference suggests that pituitary dysfunction is a particular problem after blast exposure. The imaging findings supported the hypothesis that soldiers with pituitary dysfunction would have more widespread WM injury than those without. The increased damage found in the posterior fossa of soldiers with pituitary dysfunction will continue to fuel speculation that the primary blast overpressure wave (BOP) may damage the brain through a unique mechanism

(Mac Donald 2011). Some of the worsened cognitive function and neuropsychological symptoms associated with pituitary dysfunction following bTBI improve with hormone replacement therapy, demonstrating the importance of diagnosis and treatment. There were no imaging findings that were diagnostic predictors of pituitary dysfunction in bTBI, and we recommend that all soldiers with moderate-severe bTBI undergo routine and comprehensive pituitary function testing during rehabilitation.

4.6 Summary

In this chapter, I have demonstrated that symptomatic pituitary dysfunction is prevalent after bTBI and represents a treatment opportunity. Although the MR imaging findings show widespread WM damage following blast similar to that seen in non-blast TBI, the findings also suggest that the posterior fossa is particularly vulnerable to blast injury. Our study selected soldiers who had mainly been injured by primary blast. However, it is likely that these soldiers also suffered secondary and tertiary effects, resulting in the complex pattern of injuries shown by the MR imaging. Given the nature of combat, it is difficult to identify soldiers who have been exposed only to primary blast, and not also its secondary and tertiary effects, and therefore isolate the effects caused by primary blast only. For this reason, we extended our study to an animal model so that we could simulate, isolate and study the primary blast effect.

The following chapter describes the method, results and discussion of this porcine study.

5 Blast injury in pigs

The previous chapters have shown that soldiers who suffer bTBIs have persisting cognitive symptoms and endocrine abnormalities. MR imaging revealed widespread WM abnormalities similar to those seen in nbTBI but with increased damage in the middle cerebellar peduncles suggestive of a mechanism of injury unique to blast.

The nature of combat means that soldiers will suffer a mixture of primary, secondary, tertiary and quaternary brain injuries making it difficult to study the effects of the BOP wave in isolation. We used a porcine model to assess the impact of a primary blast in the context of polytrauma. We performed histopathology to investigate structural changes, axonal degeneration and the early microglial immune response. We also used standard MR imaging and DTI techniques to assess WM damage. This study aimed to identify accurate and robust correlates between neuroimaging and histopathology findings, strengthening the use of neuroimaging as a reliable diagnostic tool in human blast injuries.

This work was in collaboration with DSTL Porton Down, Imperial College London and UCL. DSTL developed the porcine blast injury model and conducted the animal injury and resuscitation phases. I attended the experiments and retrieved the brains once the animals were sacrificed. I developed the imaging protocols with Marina Arridge at the Brain Imaging Centre at Imperial College London and performed the DTI analysis. With Professor Steve Gentleman, I co-supervised Ting Kwok perform the immunohistological preparation and I recorded and analysed the data with her.

5.1 Introduction

As discussed earlier in Chapter 1, IEDs have become a major contributor to mortality and morbidity in the conflicts in Afghanistan and Iraq. Following discharge, veterans often present with symptoms consistent with mild TBI (Terrio 2009, Okie 2005). While the neuropathology underlying this cognitive impairment is currently unknown, it has been linked to a condition called chronic traumatic encephalopathy (Goldstein 2012), previously known as dementia pugilistica, in which chronically activated microglia cause a tauopathy in axons. This topic is important as blast injuries

continue to be the main threat to troops around the world whilst survival rates of blast victims are improving (Penn-Barwell 2015).

5.1.1 Pathophysiology of TBI

To fully understand the histopathology results, it is necessary to describe what happens at a cellular level when an injury to an axon occurs. In the healthy brain, glutamate is produced by neurons and taken up by astrocytes. These astrocytes then convert the glutamate into glutamine and return it to the neurons where it is an alternative energy source. Injured neurons overproduce glutamate and, if they die, release glutamate into the extracellular space. When there is too much glutamate for the astrocytes to remove, it binds to neuronal receptors (such as NMDA) and induces an influx of Ca^{2+} and Na^{+} and an efflux of K^{+}. This ionic imbalance causes the cell membrane to depolarise. Intracellular Ca^{2+} levels rise leading to mitochondrial dysfunction, reduced ATP formation (see Figure 5-1), energy failure and ultimately cell death. Mitochondrial dysfunction leads to a release of reactive oxygen and nitric oxide species which cause oxidative stress and damage to membrane lipids, proteins and DNA. Free Ca^{2+} activates enzymes (calpains) that disrupt the axon's cytoskeletal filaments. This disruption causes impaired axonal transport and a build up of amyloid precursor protein (APP) (Rosenfeld 2012, Gentleman 1993).

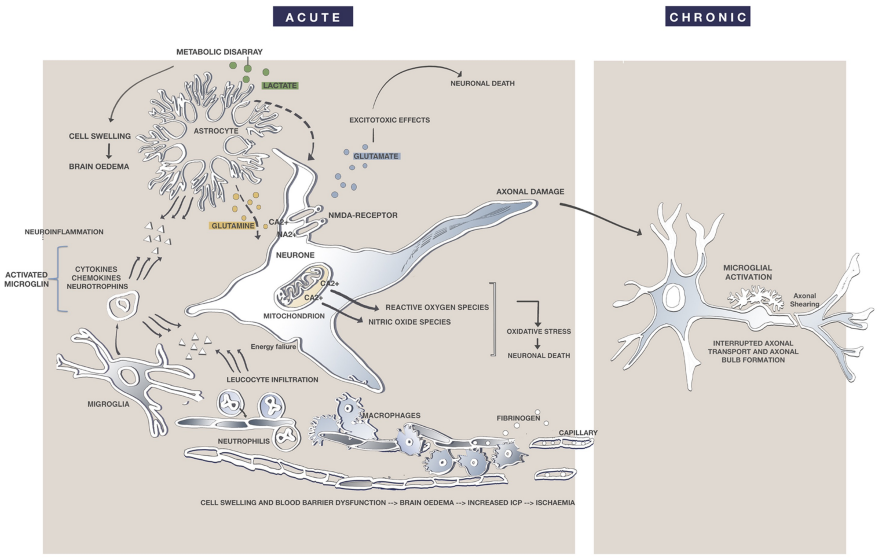


Figure 5-1. Pathophysiology of brain injury

Acute - The cycle of cellular events that occur when a neuron is injured and which leads to APP accumulating in the axons, microglial activation and fibrinogen leakage from blood vessels. Chronic - The activated microglia modulate tau metabolism leading to beta-amyloid plaque (different to APP accumulation) deposition and neurofibrillary tangles (McKee 2009).

Microglia are the immune cells of the central nervous system. Signals emitted from injured neurons activate microglia which then change shape (Figure 5-2). If there are dead cells present, the microglia become phagocytes. Activated microglia accumulate at the injury site and secrete inflammatory cytokines, chemokines that stimulate the migration of activated leucocytes into the brain. Infiltrated neutrophils maintain the immune response to injury, impairing the blood brain barrier's integrity which in turn leads to fibrinogen leakage into tissues, increased extracellular fluids, cell swelling and brain oedema. In the long term, for an unknown reason, in some individuals activated microglia remain in the brain and can cause chronic traumatic encephalopathy by modulating tau protein metabolism (Goldstein 2012). In this study, we looked for APP as a marker of axonal injury, fibrinogen as an indicator of

blood-brain barrier permeability and Iba1 (a microglia-specific calcium binding protein) to assess microglial morphology (Rosenfeld 2012, DeWitt 1995).

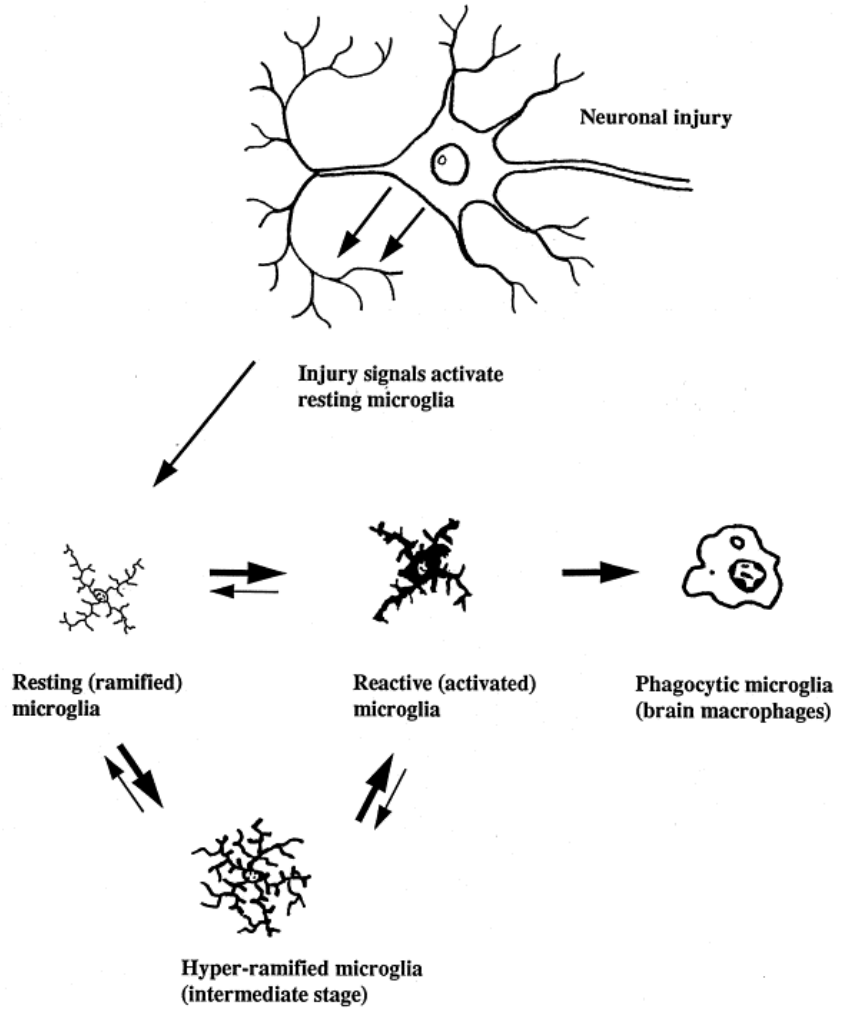


Figure 5-2. Functional plasticity of microglial (Streit 1999)

Injured or diseased neurons cause resting microglia to become activated by emitting injury signals. The degree of microglial activation varies with the severity of the neuronal injury. The mildest injuries may only cause hyper-ramification of microglia, but most types of neuronal damage will cause resting microglia to become reactive microglia. If neurons die, microglia transform into brain macrophages and remove the dead cells. If an injured neuron recovers, hyper-ramified and reactive microglia may revert to the resting form. Microglia-derived brain macrophages probably do not revert to the resting state, but may undergo cell death (Streit 1999).

5.1.2 Haemorrhagic shock and resuscitation

Isolated blast injury is very uncommon and it usually occurs in the context of polytrauma. Approximately 4% of soldiers suffered from both TBI and haemorrhagic shock (HS) (Okie 2005) in combat operations in Iraq and Afghanistan. The presence of HS is known to worsen the morbidity and mortality significantly from TBI (Wald 1993). The worsened morbidity and mortality seen in TBI with HS may be due to secondary ischaemic damage as well as the effect of the loss of cerebral autoregulation. The current treatment for soldiers and civilians suffering from both TBI and HS is the infusion of crystalloid fluids, such as saline to restore BP and tissue perfusion. However, there is some evidence that this may worsen cerebral oedema causing intracranial hypertension and a reduction of brain compliance (Teranishi 2012, Hariri 1993).

Our injury model was designed to replicate the effects of battlefield polytrauma and the journey from injury and first-aid (Role 1), through evacuation (Role 2) to a medical facility (Role 3) (Garner 2009). The term "Role" or "Echelon" is used by NATO to describe the stratification of tiers of medical support. Role 1 medical support is integrated into a unit and includes the capabilities for providing first aid and immediate lifesaving measures such as stopping the haemorrhage. Role 2 is typically provided at a larger unit level, usually Brigade size, though it may be provided farther forward, depending upon the operational requirements. In general, it provides evacuation from Role 1 facilities. Role 3 is at Division level and above. It incorporates additional resources, including diagnostic equipment such as CT scanners, as well as specialist surgical and medical capabilities (NATO 1997). The resuscitation strategies and timelines used in this study replicate these echelons of medical support.

5.1.3 The porcine model

Animal models examining pathological changes have improved understanding of the fundamental pathophysiology underlying blast trauma. However, findings from these studies cannot be readily translated to humans. Most animal studies of bTBI have used rodents (Xiong 2013). However, there are a number of limitations to using these types of animals. Rodent brains are smaller and have a porencephalic

structure; this limits the applicability of their findings to humans. The human brain has a gyrencephalic structure. The convolutions of the sulci and gyri will interact differently with any force acting on the brain and create a different pattern of injury.

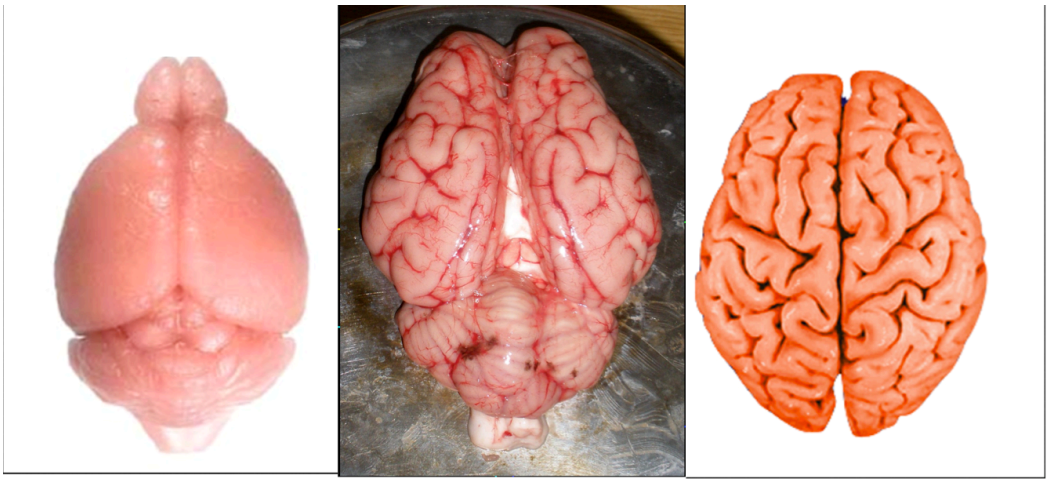


Figure 5-3. Comparison of rodent, pig and human brain

A porencephalic rodent brain and the gyrencephalic pig and human brain. The folds on the brain's surface will influence the transmission of energy and the location of the injury (adapted from Gholipour 2014, Heteroherent 2011).

In chronic traumatic encephalopathy following blast exposure, there is a predilection for injury at the base of the sulci, this illustrates the way that sulcal and gyral anatomy influence the location of damage (McKee 2014). Garner *et al*. (2009) developed a large-animal porcine model to address some of these limitations. Pigs have a gyrencephalic brain structure that is similar to the human and also have comparable glial-to-neuron ratios, myelin levels and water content. Also, experiments have shown that pigs' brain tissue is analogous to human brain tissue when assessed biomechanically (Thibault 1998, Manley 2006).

We used a porcine model developed by Garner *et al.* (2009) to investigate the structural and early immune effects of military blasts. We gave ten pigs a peripheral injury, exposed them to either sham or blast conditions, limiting the secondary and tertiary blast effects, before controlled haemorrhages. Both groups of pigs were then given normal saline corresponding to Role 1 care, prior to being assigned to one of

two resuscitation strategies. The early resuscitation group received packed red blood cells (PRBC) and fresh frozen plasma (FFP) one h after injury, corresponding to Role 2 care, these were continued in the late phase of the resuscitation (corresponding to Role 3). The late resuscitation group continued to receive crystalloid fluid to maintain BP whilst at Role 2 before receiving PRBC and FFP once at Role 3.

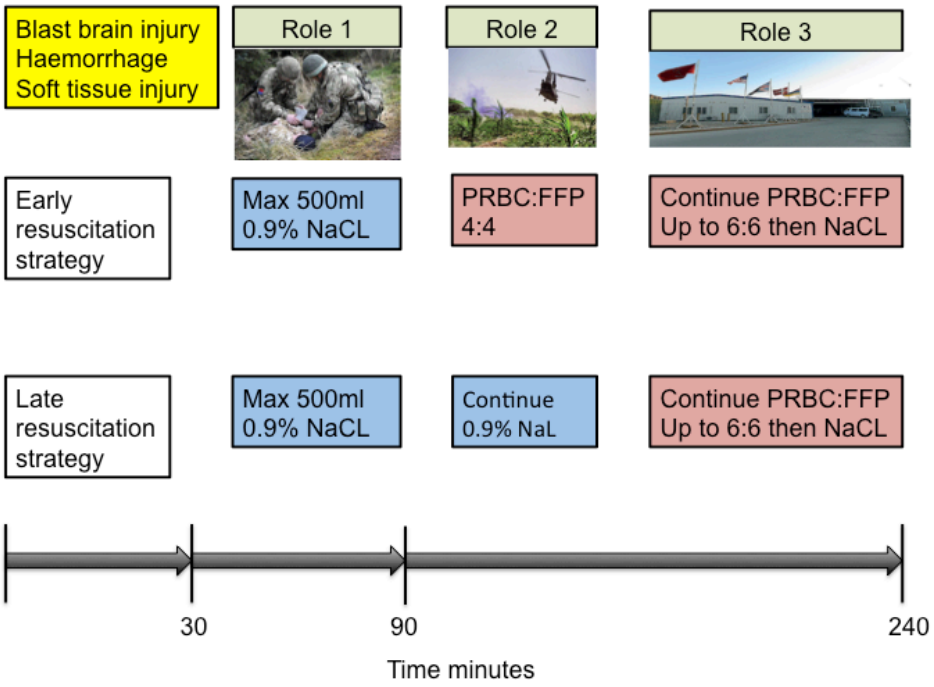


Figure 5-4. Timelines for fluid resuscitation in the early and late groups

An overview of the injury model showing the different fluids used by the early and late resuscitation strategies and their corresponding timelines. This model replicates the timelines to Role 1, Role 2 and Role 3 medical care as set out by NATO.

5.2 Methods

All blast experiments were conducted by the Defence Science and Technology Laboratory (Dstl) at Porton Down. Garner *et al.* 2009 provides a detailed account of the development of this injury model, which combines blast, controlled haemorrhage and a soft tissue injury in a reproducible animal model, in order to carry out detailed physiological testing.

The study was conducted on 10 terminally anaesthetised large white pigs in accordance with the Animal Scientific Procedures Act (1986). The pigs were anaesthetised with Isoflurane (5%) in O_2N_2O (FiO_2 0.3) followed by Alfaxan (Saffan^{TM}), before experimentation. Arterial blood and central venous pressures were recorded throughout the experiment via intravascular cannulation. The injury and resuscitation model was divided into three phases: the shock phase, the pre-hospital phase and the in-hospital phase, to realistically simulate the experience of an injured soldier.

5.2.1 Shock phase (Pre Role 1)

After a 60 min recovery period following induction of anaesthesia, blood gases and cardiovascular measurements were made and the animal was randomly allocated to receive blast or sham (non-blast) treatment. The animals were wrapped in a Kevlar blanket to protect from secondary and tertiary blast effects and positioned outdoors on a trolley 2.15 m from a cylindrical charge of EDC1S explosive (2.2 kg), which was detonated remotely.

Figure 5-5. Blast Rig

The animal is seen here, on the right, wrapped in a Kelvar blanket on a sliding rail, which protected it from secondary and tertiary injuries. The high explosive charge was placed on top of the tube on the left.

Animals subjected to the sham blast were treated identically but not exposed to the blast. All animals then received a haemorrhage of approximately 30% blood volume loss and blunt injury to the muscle of the right thigh. The animal was then left to enter a 30 min shock phase during which a capped amount of 500 ml saline was given to prevent cardiovascular collapse and maintain the hypotensive target.

5.2.2 Pre-hospital phase (Role 1)

The treatment groups diverged at this point, those in the early resuscitation strategy group received up to 4:4 units of PRBC:FFP, which had been both forward and back cross-matched to the recipients. Animals in the late-resuscitation strategy group received saline to the same hypotensive BP target. At this stage, oxygen was used (at least FiO_2 0.3) to maintain an arterial concentration of 98%.

5.2.3 In-hospital phase (Role 2+)

After a 60 min simulated pre-hospital resuscitation phase, animals in the late-resuscitation group then received fluid to a maximum of 6:6 PRBC:FFP to reach and maintain a normotensive BP target, while a similar BP target was also employed in the early-resuscitation group. This resuscitation was continued for a further 150 min by which time all animals were sacrificed humanely with an overdose of pentobarbital (150 mg/kg i.v) and the heads removed for further analysis.

5.2.4 Tissue preparation

The heads of the animals were immediately removed and the soft tissues and mandible were separated from the skull. The skull was perforated with a 1 cm cranial perforator in the frontal and occipital bones and diffusion fixed in 2% paraformaldehyde solution for two weeks. Perfusion and diffusion fixation are both

accepted methods for fixing whole brains. Perfusion fixation requires paraformaldehyde to be pumped continuously through the arterial supply to the head (Dyrby 2011) whilst diffusion fixation is performing by submerging the brain in paraformaldehyde for a predetermined period of time (Miller 2011).

Diffusion fixation was chosen as perfusion with paraformaldehyde would have invalidated the concurrent investigations into porcine physiology following trauma. In addition, the effectiveness of diffusion fixation has been demonstrated in larger, human brains. After two weeks, the brains were surgically extracted from the skulls and then examined for apparent external damage. They were then suspended in TechAgar and stored at 4°C and scanned in a 4.7 Tesla MRI scanner. We performed MR imaging on 8 of the 10 brains (five blast and three sham animals).

5.2.5 Immunohistochemistry

We used a standard haematoxylin and eosin (H&E) staining procedure. Antibodies against Iba1, APP and fibrinogen, had not previously been used with porcine tissue, so the protocol was derived using experiments with antigen retrieval techniques and exposure times (see Supplementary Methods in Appendix 3). A Consultant Neuropathologist blinded to the group and resuscitation strategy of the animal examined the slides for structural damage, microbleeds, axonal pathology and microglial activation.

5.2.6 H&E stain

We examined all of the slices for structural changes, including oedematous pathology, alterations in cell morphology, and ependymal stripping. We looked for the presence of perivascular oedema, denoted by fibrous cavities surrounding the vessels in several regions including the orbitofrontal WM, hippocampus, corpus callosum, pons, medulla and cerebellum.

5.2.7 Fibrinogen

We used the presence of fibrinogen immunoreactivity to assess BBB permeability. In healthy subjects, fibrinogen is observed only within the vasculature. Increased BBB

permeability leads to leakage of fibrinogen into the parenchyma, seen as a brown blush surrounding the vessel. We chose three standard sections throughout the brains and recorded all the cases of vascular leakage observed at 2 x magnification. We marked the presence and location onto a standardised outline of a porcine brain using graphics editing software (http://brainmuseum.org).

5.2.8 Amyloid Precursor Protein (APP)

We used APP to assess for the presence of axonal injury. When axons are injured axonal transport is interrupted and APP accumulates making the axon swell. We looked in the WM in the same three sections for each animal. We defined a focus as a distinct clustering of axonal bulbs and recorded their presence and location of the identified Foci onto a standardised outline of a porcine brain using graphics editing software (http://brainmuseum.org).

5.2.9 Iba 1

We stained the tissue with anti-Iba1 to observe changes in density and morphology of microglia. Semi-quantitative analysis of microglial profiles was performed to determine the locality and extent of the immunoreactive response. A severity scale of low (*), moderate (**) and severe (***) was set out, judged on intensity of clustering and degree of morphology change (Table 5-1), as shown in Figure 5-6.

Table 5-1. Severity scale of damage to microglia

Low (*)	Moderate (**)	Severe (***)
Microglia are mostly in a ramified state, with little retraction of processes and low density of cells	Microglia have slightly thickened and retracted processes but cells are evenly distributed, suggestive of early activation and little migratory response	Microglia have thickened and retracted processes, looking more like macrophages. Activated cells are often clustered indicative of widespread activation with proliferative and migratory responses

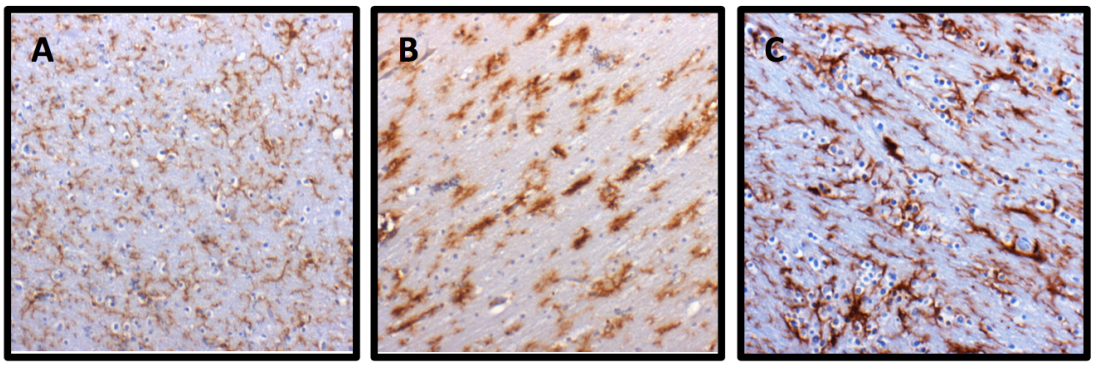


Figure 5-6. Visual impressions of the semi-quantitative rating of microglial activation: A) low (*); B) moderate (**); C) severe (***) 20 x magnification

5.2.10 Neuroimaging

We developed an *ex vivo* neuroimaging protocol (see Supplementary Methods in Appendix 3) to create high-resolution MPRAGE (T1) and gradient-echo (T2*) and DTI images to assess the extent of focal brain injury and haemorrhage. A Consultant Neuroradiologist, blinded to the pigs' blast injury status and resuscitation strategy, reviewed each of the standard structural scans (T1 and T2*sequences).

5.2.11 DTI analysis

Using a region of interest (ROI) approach we investigated FA within specified WM regions. We created ROI masks based on WM anatomy, in T1 space, for each animal. These regions were whole brain WM, the orbitofrontal WM, and the anterior internal capsule. These regions provide a representative measure of the degree of WM tract damage and are frequently disrupted by DAI (Mac Donald 2011). Informed by the histopathological results, we also created masks for the regions where we saw APP pathology. We extracted the mean FA value within the masks for each subject. SPSS was used to compare the mean FA in each of the regions between the blast and non-blast animals.

5.3 Results

5.3.1 Histopathology

5.3.1.1 Ependymal stripping

Stripping of the ependyma was identified in 4 of the six blast-exposed pigs, denoted by oedematous pathology underneath the ependyma (Table 1, Supplementary Figure 4 – see Appendix 3), with long fibrous attachments.

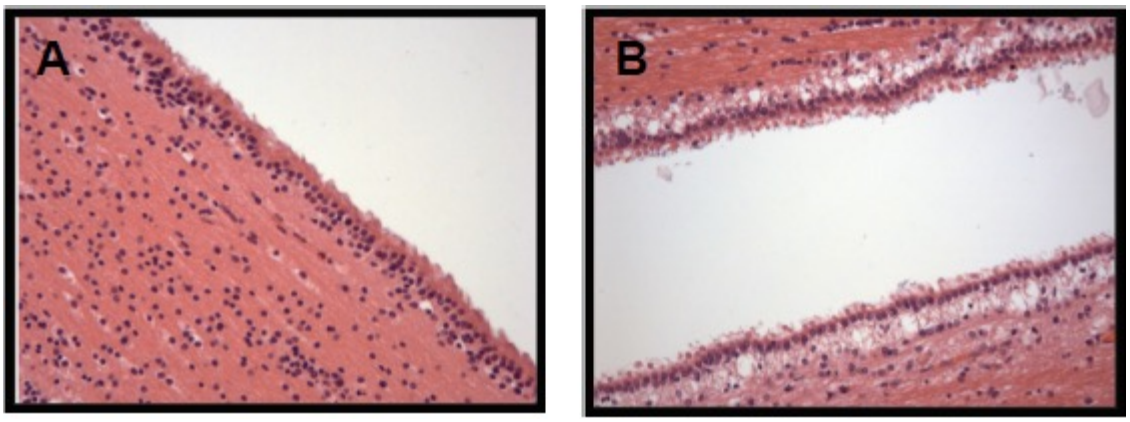


Figure 5-7. Ependymal stripping

(A) Normal ependymal compared with (B) Ependymal stripping

5.3.1.2 Hippocampal oedema

Two bTBI animals had hippocampal oedema that was not seen in the sham animals. One animal (B2) showed bilateral oedematous appearances in the dentate gyrus (DG) of the ventral hippocampi, and another (B10) had unilateral changes in the DG of the ventral hippocampus. In both animals with hippocampal oedema, there was associated microglial activation in the adjacent brain (Figure 5-8).

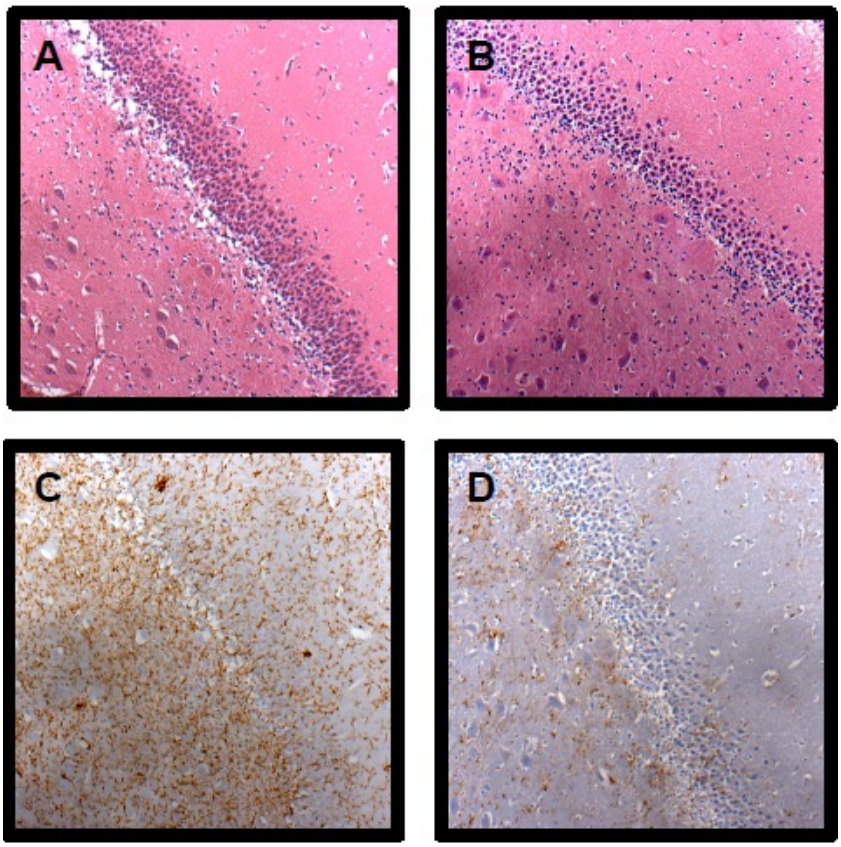


Figure 5-8. Hippocampal oedema with concurrent microglial activation

(A) and (C) are the slices from the same section through the hippocampus in pig B2. (A) H&E stained section showing fibrous structural pathology denoting oedema and (B) was stained with anti-Iba1 (brown colour) to show activation of microglia. (B) (D) Sections from animal B5 in which the oedema and microglial activation are not present.

5.3.1.3 Perivascular oedema

We observed perivascular oedema in both groups throughout the whole brain (Table 1, Supplementary Figure 4 – see Appendix 3). Although in five of the six blasted pigs there were no microbleeds found, there were several microbleeds (extravascular erythrocytes indicating haemorrhage) found in the medulla of one of the blasted animals (Supplementary Figure 1, Appendix 3). This extravasation was associated with fibrinogen leakage. Both bTBI and sham groups displayed widespread fibrinogen leakage. There was no discernible pattern to the leakage, with this abnormality seen throughout the brains of all the animals (see Figure 5-9).

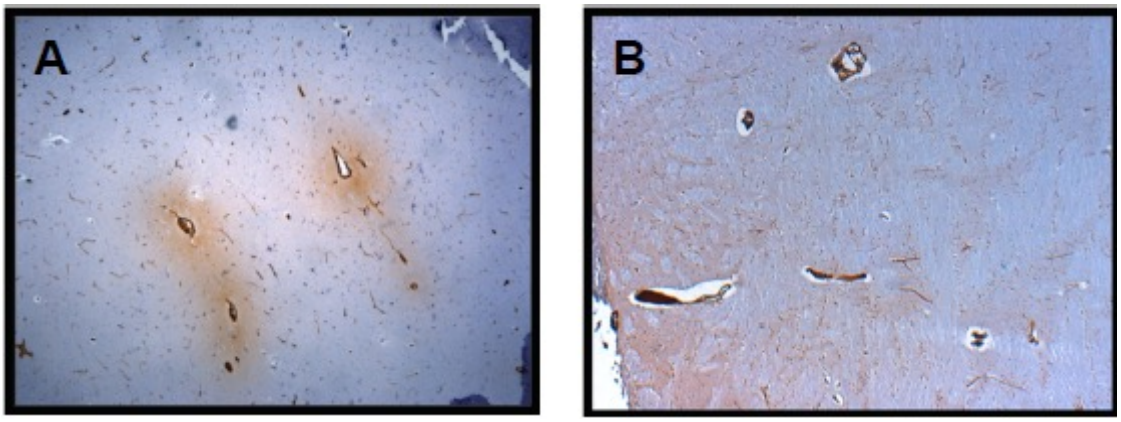


Figure 5-9. Fibrinogen leakage

(A) The brown blush around the blood vessel indicates fibrinogen leakage compared to (B) a typical vessel.

5.3.1.4 Amyloid Precursor Protein (APP)

All the pig brains, both blast and non-blast, displayed some APP immunoreactivity, with 8 out of 10 pigs showing widespread positive axonal varicosities. Axonal varicosities were mainly seen in the mid-coronal slice below the lateral ventricles, and in the internal capsule extending into the thalamus as shown in Figure 5-10 (also see Supplementary Table 2).

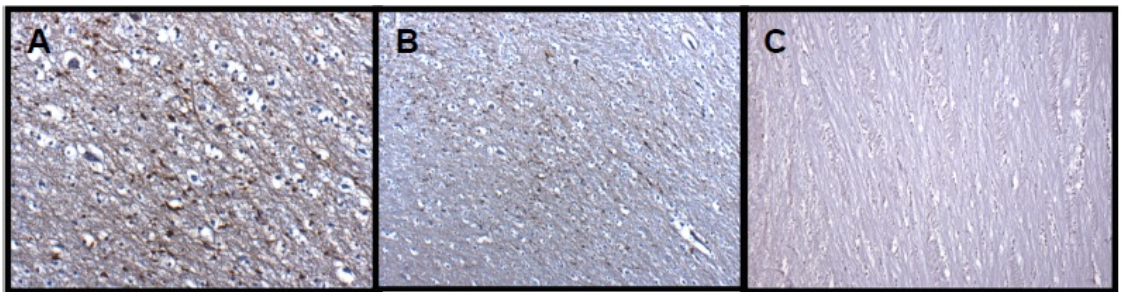


Figure 5-10. APP immunostaining at (A) 20x mag and (B) 10x mag compared to (C) normal WM without axonal injury

5.3.1.5 Iba 1

In animals exposed to a blast, there was evidence of focal microglial activation in areas of ependymal stripping as well as widespread activation of microglia in the sub-ependymal region (Figure 5-11). There was no evidence of sub-ependymal microglial activation in the sham animals. However microglial activation was seen in both bTBI and sham groups in other apparently undamaged parts of the brain, suggesting that a component of the injury model separate to blast caused microglial activation (Supplementary Table 3).

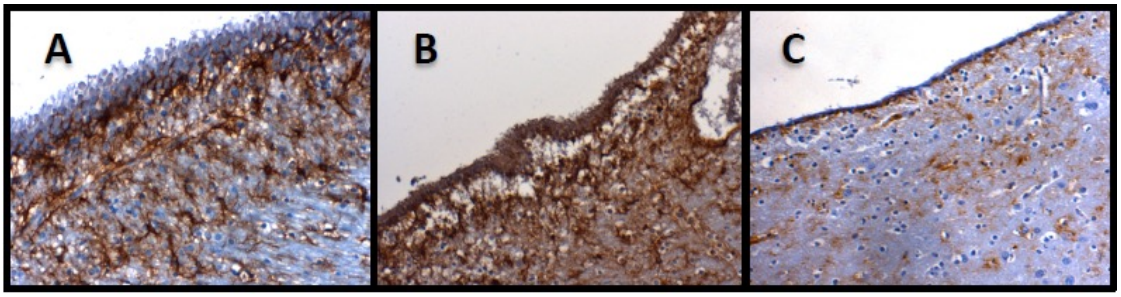


Figure 5-11. Microglial activation

(A) subependymal microglial activation and accumulation were seen in the blast pig (B1) without concomitant ependymal stripping; (B) subependymal microglial activation in the blast pig (B10) with ependymal stripping; (C) normal ependyma with ramified microglia.

5.3.2 Neuroimaging

The Gradient-echo and MPRAGE sequences showed no discernible difference between the two groups when reviewed (see Figure 5-12).

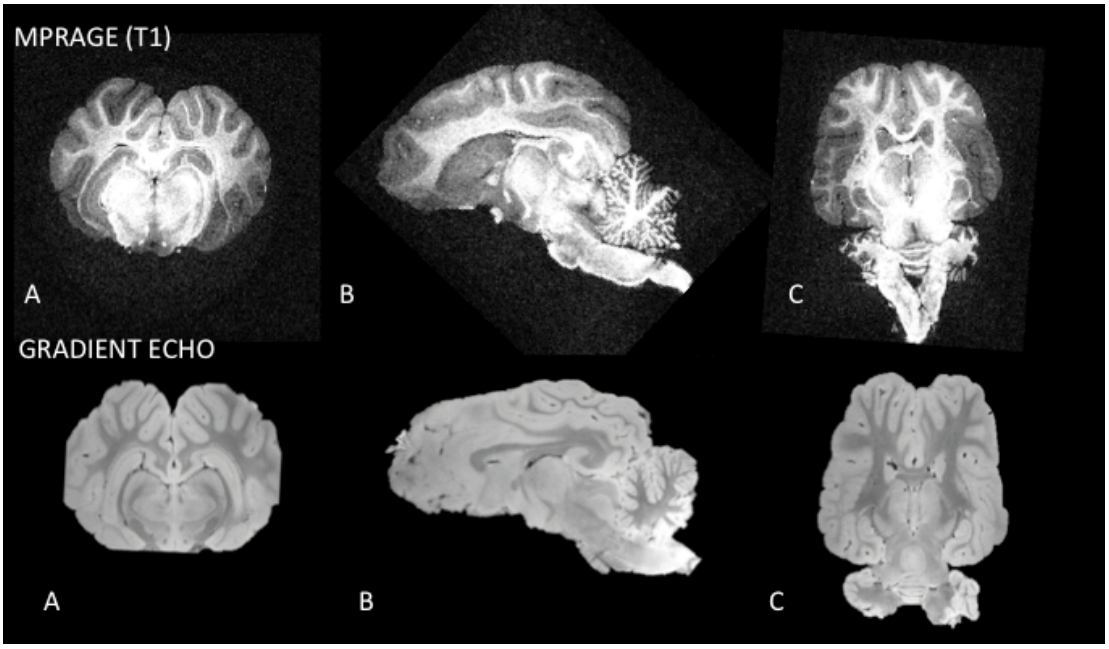


Figure 5-12. MR imaging in a pig brain

MPRAGE (T1) and Gradient Echo images of a pig brain in the (A) coronal, (B) saggital and (C) axial planes.

When the whole brain FA was compared for blast vs non-blast, we found a significantly lower FA in pigs with blast exposure (p=0.04), suggesting a difference in the WM integrity of the two groups (Table 5-2). However, no difference in FA was found between the two injury statuses in the ROI comparisons of the corpus callosum, the anterior internal capsule and the orbitofrontal WM (p=0.4, p=0.4 and p=0.2 respectively). Guided by the APP immunohistological results, we created ROI masks bilaterally in the internal capsule/thalamic areas. The more targeted analysis in this ROI yielded a significant FA difference between blast and non-blast brains, with a lower FA indicative of axonal injury being seen in the blast group when compared to the non-blast group (p=0.016) (see Table 5-2).

Table 5-2. Comparison of WM in blast vs. non-blast whole brain

ROI						
Injury status	**Pig**	**Whole brain**	**Corpus Callosum**	**Orbitofrontal WM**	**Anterior internal Capsule**	**Pathology led ROI**
Blast	B2	0.509	0.431	0.450	0.271	0.402
	B3	0.493	0.385	0.352	0.386	0.294
	B8	0.498	0.395	0.311	0.452	0.326
	B9	0.531	0.455	0.408	0.479	0.344
	B10	0.508	0.515	0.325	0.536	0.379
Non-blast	B5	0.531	0.434	0.329	0.434	0.399
	B6	0.514	0.398	0.365	0.409	0.424
	B7	0.539	0.493	0.342	0.445	0.480
Average blast FA		0.508	0.436	0.369	0.425	0.349
Average non-blast FA		0.528	0.442	0.345	0.430	0.434
t-test p-value		0.049	0.444	0.266	0.469	0.016

5.4 Discussion

The purpose of the blast injury in pigs study (BIPs) was to examine the effects of a primary BOP wave on the brain in a porcine model of polytrauma. The study has shown evidence that primary blast causes ependymal stripping with associated inflammation in the region of the lateral ventricles, helping to confirm that an isolated primary blast wave can cause brain injury. We found activation of the microglial cells throughout the brains of all animals raising important questions about the effects of polytrauma on the CNS and its treatment. Importantly the imaging results have confirmed that even at 4.7 Tesla, standard structural MR is not as sensitive to WM damage as DTI. More work is needed to develop DTI as a tool for use in trauma (see Recommendations Chapter).

5.4.1 Neuropathology

5.4.1.1 Ependymal stripping

We found evidence of ependymal stripping in the region of the lateral ventricles. There was oedematous change underlying the areas of stripping as well as early activation of microglial cells indicating that the injury happened while the animals were alive. This finding supports previous work that has shown microglia activation within six hours of injury (Hoogland 2015).

De Lanerolle *et al.* (2011) demonstrated periventricular axonal injury and astrocyte infiltration two weeks after blast exposure in a porcine model of mild blast TBI (de Lanerolle 2011). Similar to our study, de Lanerolle and colleagues did not observe any obvious injury such as haemorrhage in these animals. Other authors have shown an association between ependyma injury and localised microglial cell activation (Sarnat 1995). These findings suggest that the blast-induced ependymal damage we observed triggers early immune activation.

There are several proposed mechanisms by which blast could cause brain injury, including spallation, implosion, and inertial effects (Nakagawa 2011, Leung 2008). Spallation is the disruption that occurs between materials of differing densities. As the BOP wave travels between materials, the compression component is reflected at the material interface, leading to fragmentation of the denser material. Implosion occurs when gas bubbles in the tissue are compressed by the shockwave. The

tissues collapse as the gas re-expands following the wave passage, the surrounding tissue is damaged. While the BOP wave propagates, lighter density masses will accelerate more than denser ones, resulting in large stress forces at the interface. This is known as the inertial effect. As such, the most vulnerable organs affected in the blast are those with air/liquid interfaces, such as the auditory canals, lungs and abdomen (Elder 2010b, Champion 2009). Previous investigators have hypothesised that pressure waves could be transmitted through the CSF spaces of the brain and spinal canal (Courtney 2009, Bauman 2009). The ependymal stripping that we have observed at the interface between the CSF-filled ventricles and the ependymal of the lateral ventricles supports the pressure wave transmission theory.

5.4.1.2 Clinical implications of ependymal stripping

The ependymal lining of the lateral ventricles has a role in controlling the composition and production of CSF as well as providing a reservoir of neural stem cells that can proliferate and migrate to areas of nervous tissue injury (Johansson 1999). The apical surface of the ependymal cells of the central nervous system have been shown to absorb and regulate the composition of CSF and the tight junction between ependymal cells act as a semi-permeable barrier to nervous tissue. Modified ependymal cells form the choroid plexus that produces CSF. Damaged ependymal may no longer be able to regulate the transport of fluid, ions and small molecules causing hydrocephalus. Tearing of the ependymahas been shown to leave discontinuities that become filled with the processes of subventricular astrocytes and can lead to extensive gliotic nodules (Sarnat 1995). Gliosis may change the compliance of the ventricular wall also leading to hydrocephalus. At the time of injury, a discontinuity in the tight junctions between the ependymal cells may predispose to infection, and ependymitis and ventriculitis are known to have high mortality rates (Lu 1998, Berk 1980*)*. The loss of the neural stem cell reserve may have implications for neuroregeneration. Future research should be undertaken to determine if bTBI causes an ependymal injury in humans and if so whether there are higher rates of central nervous system infection and hydrocephalus. If future work confirmed ependymal injury, this would have significant implications for the design of personal protective equipment and the treatment of these injuries.

5.4.1.3 Hippocampal oedema

We also observed hippocampal oedema in two of the animals exposed to blast. This is in keeping with evidence from other studies which showed that the hippocampi are particularly susceptible to the effects of blast exposure (de Lanerolle 2011, Goldstein 2012, Miller 2015). Hippocampal injury is a well-documented consequence of nbTBI as well (Hicks 1993, Kotapka 1991). This vulnerability may be for several reasons: firstly, the hippocampus contains a large proportion of the CA1 fields of the cornu ammonis which are sensitive to trauma (Duvernoy 1988); secondly, the fronto-basal parts of the brain, which have extensive hippocampal projection fibres (Cavada 2000), are frequently damaged in moderate to severe TBI (Gennarelli 1998). This orbitofrontal damage may therefore result in transneuronal hippocampal cell death. In bTBI, damage to the hippocampi might be a direct result of the BOP wave, or could be secondary to hypoxia or impaired perfusion due to hypovolaemia. Previous studies looking at patients with hippocampal damage from epilepsy have found that they have poor memory (Addis 2007). Future research should be conducted to determine if these effects occur in trauma.

5.4.1.4 Perivascular oedema and generalised microglial cell activation

We saw perivascular oedema with fibrinogen leakage and widespread microglial activation in bTBI animals, but also in the sham group who had a soft tissue injury and IV fluid resuscitation but no exposure to blast. This suggests that these changes arose from another aspect of the injury model unrelated to the blast. Tissue oedema has been shown to occur in peripheral tissues following administration of IV fluids (Scallan 2010) and so it is possible that the changes we have observed are a result of the resuscitation strategy. Future research should be conducted to determine if the relationship between perivascular oedema and IV fluid resuscitation as this could potentially worsen TBI outcome by increasing cerebral oedema, intracranial hypertension and reducing brain compliance (Hariri 1993, Teranishi 2012).

Widespread microglial cell activation in both blast and sham groups is another interesting observation that resulted from an aspect of the model unrelated to blast exposure. Hoogland *et al.* 2015 conducted a systematic review of 51 animal studies and showed that peripheral inflammatory stimuli can cause microglial cell activation.

It is possible that inflammatory stimuli (cytokines) released by the soft tissue injury that the animals sustained activated the microglial cells in both groups.

5.4.1.5 Amyloid precursor protein

We found that both blast and sham pig brains showed early APP immunoreactivity, indicating that this result was not due to blast. However, there were some significant differences in the extent and location of reactivity seen between the two groups. The blasted pigs showed more extensive pathology in the orbitofrontal WM, the regions of the internal capsule and thalamus (Supplementary Table 2). This suggests that although blast is not the cause of the APP pathology it may exacerbate WM damage. De Lanerolle *et al.* (2011) noted similar pathology in pigs treated in a similar blast paradigm two weeks following a blast. Our finding of APP within four hours of blast is consistent with previous studies in nbTBI that has shown APP accumulation within three hours following injury (Sherriff 1994). The absence of a control group of pigs that had not received fluid resuscitation is an important limitation of the BIIPs study. The animals were sacrificed four hours after injury, if a longer survival time before sacrifice were possible, more APP accumulation may be detected, producing a clearer picture of the axonal injury. Future work should be conducted, comparing pig brains subjected to an isolated blast exposure and a group of normal pig brains to determine the role of the resuscitation strategy in APP pathology.

All histopathological analysis is subjective and, therefore, vulnerable to inter-observer variability and bias. In the BIIPs study, we limited our observations to describing the presence or absence of individual pathologies and using semi-quantitative rating scales to make the results as reproducible as possible.

5.4.2 Imaging

Even at 4.7 Tesla, standard structural imaging did not reveal any areas of damage in any of the brains. Using DTI, however, we observed that the blasted pigs had a lower whole brain FA than the sham animals. Areas of the brain found to have more APP accumulation drove this difference in FA. FA and APP are markers of axonal injury (Warner 2010, Zhu 2014) (Gentleman 1993) and our work supports these findings. The absence of injury on structural MRI supports the previously stated view

that standard structural imaging is not as sensitive as DTI when investigating WM damage and more work should be carried out to make DTI a readily available tool in the assessment of TBI. The imaging was performed on *ex vivo* brains and this may make the results difficult to translate into live human subjects. Also, the numbers of animals studied were small meaning that the findings need to be confirmed using a larger number of pigs and with a control group that had not received fluid resuscitation. High field strength MRI, using a 7 Tesla MRI demonstrates a hyperintense rim around the ventricles on FLAIR sequences (van Veluw 2015) and this may have a role in assessing ependymal integrity in the future.

Only one of the blasted pigs brains showed evidence of extravasation of erythrocytes, indicating haemorrhage in several areas of the medulla. These haemorrhages are analogous to microbleeds. Future work should be carried out to determine if there were factors, such as abnormal coagulation, that influenced this result.

5.4.3 Porcine model

We used a porcine model to examine the effects of blast on the brain because of similarities in gyral anatomy, glial-to-neuron ratios and the analogous behaviour of the tissues (Thibault 1998, Manley 2006). However there are significant differences in skull composition (Bauman 2009), size, shape and integrity (Nakagawa 2011), which mean the findings may be different in humans. Pigs have thicker skulls and a different hindbrain orientation as well as larger sub-arachnoid spaces (Manley 2006), which may absorb and reflect energy differently. The neck of a pig is much thicker than that of a human, meaning that the whiplash-like forces that act on the head will be greater in humans. Pigs have hypercoagulable blood in comparison to humans and haemodilution further modulates coagulation. Therefore, the resuscitation targets used in this model may not produce the same effect in humans (Calzia 2012). Finally for practical reasons, we chose to diffusion fix the brains rather than perform perfusion fixation. Diffusion of paraformaldehyde throughout the brain would not have been instantaneous and so the cellular changes that we have observed may have occurred later than four hours after the blast injury.

5.5 Summary

In summary, we studied the effects of primary blast exposure in a porcine model of polytrauma. We found that an isolated BOP wave produces ependymal stripping with associated microglial cell activation within four hours of injury, as well as hippocampal damage in a subgroup of blasted animals. Standard MR imaging did not identify any structural abnormalities which mean that these injuries may be unrecognised. DTI identified the internal capsule and thalamus as areas with lower FA indicating more axonal injury.

In the next chapter, I will bring together the results from the three experiments that make up this thesis and discuss the implications that they have on research in this field as a whole.

6 Discussion

6.1 Potential methodological limitations

6.1.1 Group size

A potential limitation of this study is the relatively small number of subjects. The analysis of WM integrity and its relationship with cognition described in Chapter 3 examined data from 20 soldiers, 20 civilians and 31 controls. These subject numbers are comparable to similar published work (Kinnunen 2011, Bonelle 2011). Furthermore, even with our small numbers, we were able to demonstrate a statistically significant effect of blast between the groups. This study was thus able to provide useful information about the impact of blast on the brain although, of course, larger numbers would have provided more accurate measures of the effect of blast. We excluded subjects with penetrating brain injury, previous neurosurgery; a history of psychiatric or neurological illness; previous TBI; anti-epileptic medication; drug or alcohol abuse; or contraindications to MRI, in order to better isolate the effects of primary blast thus reducing the number of potential subjects. At the time this work was conceived, there was no knowledge of the size of the effect that we were looking for and so a formal power calculation was not possible. Work published around the same time in nbTBI used numbers between 12 and 28 with a similar numbers of controls. We therefore chose to examine 20 soldiers with bTBI, 20 civilians who had suffered a nbTBI and 31 uninjured controls. We believed that this would generate data around the effect that could be used in power calculations in later studies.

In the porcine study, again the relatively small number of subjects is a limitation. Of the 10 porcine brains, we were only able to analyse DTI data on eight, due to data corruption on two of the brains.

6.1.2 Group selection

Inherent within any study comparing military and civilian populations is the potential limitation of group selection. Soldiers operating on the frontline are by definition young males with high levels of physical fitness. The environment in which they

operate is highly kinetic, and the physiological and environmental stresses that they experience are different to civilians. The civilians in contrast mainly came from north-west London, which is an affluent area and had a high level of education as demonstrated by their assessment of premorbid intelligence (see Chapter 3 and Appendix 1). We controlled for these group differences by matching the civilian TBI and control groups for sex and age, but there are certain differences in premorbid education, IQ, nutrition and social class that should be considered when interpreting our results.

6.1.3 Multiple comparison errors

The problem of undertaking multiple comparisons is that some fraction will be significant at the $p<0.05$ level due to chance alone. Thus in my study when measuring FA in the ~ 20,000 voxels in each brain, the differences observed which I determined to be statistically significant, might have resulted from the multiple comparisons made and led me into the error of rejecting the null hypothesis despite it being true. This potential error might also have resulted when investigating cognition differences between the groups.

To address this potential problem, we used threshold-free cluster correction in FMRIB Software Library (FSL) to correct for the number of possible independent observations in the spatially smoothed data. In FSL, the Gaussian Random Field Theory is used to implement a cluster-based correction for multiple comparisons. Another option would have been to use the Bonferroni correction. However, this assumes that there is no relationship between individual voxels (which there is in the case of WM tracts) and thus is very stringent, risking falsely rejecting real differences (a type II error). We did not correct for multiple comparison errors when analysing the cognition test results. This is a valid limitation but as this was an exploratory study with relatively small numbers we believe this is an accepted approach. Future analysis with larger numbers should be carried out correcting for this (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/TBSS).

6.1.4 Quantification of blast forces

As described in Chapter 1, the BOP wave rapidly dissipates energy as it expands from the centre of an explosion. Also, reflections from surrounding structures can cause amplification of the pressure wave leading to a wide variation in the pressure (Psi) experienced by subjects, depending on their proximity to the explosion, the presence of surrounding structures and the effectiveness of personal protective equipment. In the human study (BIOSAP) we could only approximate the size of the blast exposure and the individual contributions of primary, secondary and tertiary effects to the overall injury. This was the motivation for going on to conduct the animal work (BIIPs). Other published studies have used self-reporting of blast exposure (Mac Donald 2011), but this potentially includes soldiers who have not suffered the primary effects of blast. We attempted to control for this by only including soldiers with a moderate to severe bTBI secondary to a single blast exposure, and those that did not have lesions causing mass effect on imaging. Towards the end of the conflict in Afghanistan, some US troops were provided with helmets fitted with accelerometers that could quantify the forces they were exposed to if injured. However, I am not aware of any published work to date in the field of bTBI relating to this.

As described in Chapter 5, we used an existing model of porcine polytrauma opportunistically to investigate the effects of the BOP wave on WM integrity. This work is valuable in that it has shown that primary blast causes ependymal injury in this model. However, the fact that both blast and sham animals received a soft tissue injury and had different fluid resuscitation interventions means that further work needs to be carried out to isolate the effects of each of these.

As discussed in the previous chapter, the widespread fibrinogen leakage seen in both blast and sham animals, has highlighted a potentially deleterious effect of fluid resuscitation in bTBI. I address this further below.

6.2 Discussion of the results

6.2.1 The location of WM damage suggests a mechanism of injury unique to blast

Explosive injuries cause a complex mix of primary, secondary, tertiary and quaternary injuries (Elder 2010b), and it is very rare for soldiers to have an isolated primary blast injury (Chapman 2014). Therefore, imaging abnormalities could result from non-blast mechanisms of injury such as head impacts or the rotational forces that often produce DAI in civilian TBI (Johnson 2013). In this work, when the more severely injured sub-group was analysed, we saw more damage in the anterior internal capsule and middle cerebellar peduncles in the bTBI group. This supports previously theoretical work that predicted damage to these areas from direct stress wave coupling secondary to blast (Taylor 2009) and human studies that found injury in the middle cerebellar peduncles and orbitofrontal WM in mild bTBI (Mac Donald 2011) suggesting a mechanism of harm unique to blast.

6.2.2 WM damage and cognition

Results presented in Chapter 3 show a strong relationship between the location of WM damage and cognition (executive function and memory). Impaired executive function was associated **with** damage to the orbito-frontal WM, which is in keeping with current understanding about the role of the frontal lobes. Impairment in associative memory was correlated with **damage to** a large number of WM tracts; some like the fornices are known to have a role in memory whilst others, like the corticospinal tracts, are not. It is likely that the diffuse injury seen in both blast and non-blast injury has disrupted distributed brain networks that support higher cognitive function to produce these symptoms. Interestingly, the cerebellum that has traditionally been considered to be primarily dedicated to motor functions has been shown to have a role in cognition, especially in episodic memory (Andreasen 1999). The link between posterior fossa damage in bTBI and cognitive dysfunction as shown in my study is thus an area for further research.

6.2.3 DTI has identified previously unrecognised WM damage

Soldiers who have suffered bTBI often have persistent cognitive problems despite often having normal standard brain imaging and this can lead to uncertainty about the cause of their symptoms and the correct treatment. There are several possible causes for neurological problems after TBI, including functional causes. However, it is possible that the structural brain injury that we have demonstrated in bTBI may be missed using standard brain imaging. Our work using DTI which provides evidence that DAI is common after moderate/severe bTBI, may thus account for the cognitive impairments that are prominent in this patient group, possibly including psychiatric disorders such as PTSD. The bTBI patient group had lower FA, in large parts of their WM, providing evidence for widespread DAI that is not apparent on standard MRI or CT imaging.

6.2.4 Increased prevalence of endocrine dysfunction in bTBI

Our study found that nearly one-third of soldiers with bTBI had hypothalamic-pituitary axis dysfunction. The most common pituitary abnormality was GH deficiency followed by hyperprolactinemia, ACTH and Gn deficiency. Only 2% of the nbTBI group had hypothalamic pituitary axis dysfunction. The differences in age and BMI between the bTBI and nbTBI groups did not explain this difference. Although there was a longer time from injury to testing of the bTBI group, this difference would have, if anything, reduced the prevalence in this group as pituitary dysfunction can resolve over time (Aimaretti 2005). The data suggests that the increased prevalence of pituitary dysfunction is a result of a more severe head injury as seen by the increased prevalence of skull and facial fractures seen on CT and the increased damage to the corpus callosum and WM of the posterior fossa seen on DTI. Interestingly, the dedicated pituitary MRI scans did not show evidence of focal damage to the hypothalamus or pituitary or evidence of superficial siderosis, supporting the earlier statement that standard structural MRI misses a significant proportion of brain injury. In contrast to much of the current literature, we found that only 2% of age- and gender-matched civilians with moderate-severe non-blast TBI had pituitary dysfunction. The higher prevalence found in other studies may be secondary to the different diagnostic tests and the normal laboratory ranges used.

6.2.5 Porcine results

6.2.5.1 Ependymal stripping supports the theory that primary blast has a unique mechanism of injuring the brain

Histopathological examination of the pig brains revealed ependymal stripping in 4 of the 6 animals exposed to a blast. There was no evidence of ependymal stripping in any of the sham animals. This finding supports the theory that primary blast can cause injury by transmission of a transient pressure gradient through the CSF spaces of the brain with damage occurring at the fluid tissue interface. The ependymal damage was not visible on 4.7T MRI scanning reinforcing the point that even at this field strength MRI only detects a proportion of the whole injury. It is not surprising therefore that we did not see evidence of ependymal injury on 1.5T MRI in the soldiers with bTBI. The pig brain has a different hindbrain orientation to the human brain giving reason to think that primary blast would cause ependymal injury in a different location. In humans, the region around the fourth ventricle may be most vulnerable to a pressure wave transmitted through the spinal canal. Interestingly the middle cerebellar peduncles, which seem to be preferentially damaged by blast in humans, form the lateral boundaries of the fourth ventricle (http://radiopaedia.org/articles/fourth-ventricle).

6.2.5.2 Ependymal injury has important implications for neuroregeneration

Work by Johansson *et al.* presented evidence that some ependymal cells are neural stem cells that generate neurons that migrate to the olfactory bulb and can subsequently differentiate into astrocytes that have a role in scar formation in response to injury. If it is found that a similar ependymal injury occurs in humans as was seen in our porcine model there may be implications for human brain growth and recovery. For example, if the cellular mechanisms that direct the proliferation and differentiation of ependymal cells are understood it may be possible to reduce gliotic scarring by astrocytes and stimulate neurogenesis following injury (Johansson 1999).

6.2.5.3 Widespread microglial activation in both blast and sham animals

We found evidence of widespread microglial activation across the brains of both blast and sham animals evidenced by Iba 1 immunohistochemistry, indicating that this was caused by a component of the injury model separate to blast. Previous work by Hoogland *et al.* has shown that peripheral inflammatory stimuli (that is also observed in the systemic inflammatory response syndrome seen in trauma) can cause central immune activation of microglia (Hoogland 2015). Furthermore, there is now a substantial body of literature implicating activated microglial in the development of chronic traumatic encephalopathy. Our findings support this previous work and have implications for the investigation and treatment of CTE and repetitive mild TBI.

6.2.5.4 Fibrinogen leakage throughout the brains of both blast and sham animals

We saw perivascular oedema and increased BBB permeability throughout the brains of both blast and sham animals. This has important implications if the same is found in humans because oedema causes an increase in intracranial pressure that can lead to a decrease in cerebral perfusion pressure and worsens outcome following TBI (*Narayan 1981).* The systemic inflammatory response syndrome seen in peripheral trauma may increase BBB permeability and make the brain more susceptible to oedema. As crystalloid fluid resuscitation is common practice in much of the world, this represents a significant area for future research.

6.2.5.5 Amyloid Precursor Protein was found in both blast and sham animals

We found that both blast and sham pigs showed evidence of early APP immunoreactivity indicating that this result was not due to blast. However, there were differences in the location of APP accumulation between the groups with blasted animals showing more extensive activity in the orbitofrontal WM as well as in the internal capsule and thalamus.

In the final two Chapters, I outline my recommendations for the future research that is required to answer questions that arise from this work, as well as help to translate the findings contained here into clinical practice.

7 Conclusions

The BIOSAP and BIIPs projects have provided valuable information about the nature of TBI following exposure to a blast and its cognitive and endocrine consequences. Some of the results of this work are concordant and have allowed me to draw the conclusions stated below.

7.1 Standard structural MRI is not a sensitive technique to identify WM damage, and DTI provides more information

The majority (70%) of the standard structural MR scans performed in soldiers with bTBI were normal. However, a number of the soldiers had a range of cognitive symptoms that the presence of microbleeds alone did not predict. Also, pituitary MRI performed in those with endocrine dysfunction did not reveal evidence of hypothalamic or pituitary injury. Even using a 4.7T MRI, structural scans were not able to demonstrate the ependymal injury that we observed on histopathological examination of the pig brains. At the group level, DTI revealed widespread WM damage, supporting previous work that found standard MR is not sensitive to WM damage (Kumar 2009). In the human case studies chapter, we were able to demonstrate a method of using DTI on an individual basis to identify those with WM injury.

7.2 Explosive injuries cause a complex mix of primary, secondary and tertiary injury, however, in humans, primary blast appears to damage the middle cerebellar peduncles and anterior internal capsule preferentially

Explosions cause a heterogeneous mix of brain injuries, and many of the imaging changes result from non-blast mechanisms such as head impact and rotational forces. Our analysis of the more severely injured subset of soldiers who had suffered bTBI suggests that there is a unique mechanism of injury associated with blast that preferentially affects the middle cerebellar peduncles and the anterior part of the internal capsule. This finding supports previous human (Mac Donald 2011) and computational (Taylor 2009) work that identified these areas as vulnerable to blast injury.

7.3 There is a correlation between WM integrity and associative memory and executive function

We found a positive correlation between executive function and integrity of the left sided orbitofrontal and transcallosal WM. This finding supports previous work by our group that shows a relationship between damage to the WM of the frontal lobes and impaired executive function (Kinnunen 2011). We also found a positive correlation between damage to a large numbers of WM tracts, including the fornices and impaired associative memory. This finding reinforces prior work that showed cognitive impairment can result from disruption of brain networks (Bonnelle 2011), and gives weight to the idea that we should no longer only think about the brain in terms of regionalised function but also regarding network connectivity.

7.4 Extensive WM damage is associated with worse cognitive function and endocrine dysfunction

The BIOSAP study showed that widespread WM damage is associated with worse cognitive and endocrine function. This may be an indication of increased severity of injury.

7.5 High prevalence of hypothalamic-pituitary axis dysfunction in bTBI

There is a high prevalence (~30%) of hypothalamic-pituitary axis dysfunction in soldiers who have suffered moderate to severe bTBI which has implications for screening, treatment and follow-up of these individuals. In contrast we found a lower prevalence (2%) of endocrine dysfunction in the civilians with nbTBI than the current literature suggests. This finding leads us to question the recommendations to screen all patients who have suffered a moderate to severe nbTBI for hypothalamic-pituitary axis dysfunction.

7.6 Primary blast causes ependymal injury in a porcine model of injury

We found ependymal stripping in the region of the lateral ventricles in four of the six pig brains. Importantly it was associated with activation of microglia, indicating that the injury occurred *in vivo.* This finding supports the theory that primary blast can cause damage through the transmission of a transient pressure gradient that injures the brain at the fluid/brain interface.

7.7 Extracranial trauma is associated with widespread microglial activation

The results of the BIIPs project have shown that in a porcine model of bTBI, extracranial injury is associated with widespread microglial activation. This observation supports the findings of previous work (Hoogland 2015) that has shown peripheral inflammatory stimuli can cause microglial activation. If a similar mechanism exists in humans, it has important implications for brain research and possible disease management.

7.8 Our porcine model of injury produced widespread fibrinogen leakage of unknown aetiology

We saw extensive fibrinogen leakage in both the blast and sham groups. This suggests that the other variables (soft tissue trauma or fluid resuscitation) were responsible. The soft tissue trauma may initiate a systemic inflammatory response that alters blood-brain barrier permeability, or the resuscitation strategy may have a role in the development of perivascular oedema. The two mechanisms may co-exist. As it has important implications for the effect of resuscitation strategy on the outcome following TBI, this needs further research.

7.9 Further investigation needs to be carried out to determine the cause of the accumulation of APP

We found widespread accumulation of APP in both blast and sham animals; while some of its location can be explained by the WM injury we have identified using DTI, more work is required to understand why we observed APP accumulation in both groups of animals.

This chapter has outlined the conclusions that I have reached from the BIOSAP and BIIPs studies. In the next chapter, I discuss my recommendations for future research.

8 Recommendations for future research

8.1 Use a larger group size

To address the limitations that small numbers place on research future, we should seek opportunities to collaborate with other nations, including the United States, who have sustained greater numbers of injuries. Collaboration would require standardisation of data collection methods and MR scanning paradigms. Also, in the event of future conflicts, we should immediately set up a prospective study to look at the neurological effects of blast

.

8.2 Control for additional group factors

Although some of the issues around group selection in the military population are unavoidable, two variables have been identified in recent literature which should be controlled for in future work. Dunst *et al.* have shown that higher FA in the corpus callosum is associated with increased intelligence (Dunst 2014) whilst Takao *et al.* have shown a relationship between intracranial volume and DTI measures (FA and MD) (Takao 2011). We did not know of these relationships at the time of the BIOSAP study, but future work should aim to control for these variables.

8.3 Quantify the forces involved in an explosion

Knowledge of the size and nature of the forces that soldiers experience during an explosion will be immensely useful in the development of personal protective equipment. Accelerometers placed in the helmets of soldiers will provide valuable information but, by necessity, will be opportunistic; further animal work may provide the adequate information more promptly.

8.4 Use diffusion tensor imaging in bTBI

Most soldiers had normal structural MR imaging (T1, T2* and GRE) of the brain despite DTI revealing WM damage. Also, the dedicated pituitary MRI scans did not reveal any abnormalities in soldiers with hypothalamic-pituitary dysfunction. Future work should be carried out to develop DTI as a tool that can be used at the individual level to identify WM injury.

8.5 Assess all symptomatic soldiers who have suffered a bTBI

Given the association between extensive WM damage, worse cognitive and endocrine function and the fact that standard MRI does not reveal the true extent of injury, it may be appropriate to examine all soldiers with psychiatric or cognitive problems who have suffered a bTBI to determine if there is underlying WM damage.

8.6 Determine if there is a mechanism of injury unique to blast

The work carried out in the BIOSAP study should be extended to look at a larger group of soldiers with bTBI to determine if there is a mechanism of injury unique to blast, if so there may be implications for body armour design. There are several possible methods to investigate the theory that a transient pressure wave gradient is responsible for some of the injuries seen, including further animal work using primates or computational modeling of the brain.

8.7 Investigate symptomatic soldiers for hypothalamic-pituitary axis dysfunction

The high prevalence of hypothalamic-pituitary dysfunction seen in the bTBI group leads us to recommend screening of all symptomatic soldiers who have suffered a bTBI for endocrine dysfunction. Preliminary follow-up data has shown improvements in the assessments of quality of life in those soldiers on hormone replacement therapy, and we will follow their response to treatment.

Porcine

8.8 Determine if the ependymal injury seen in pigs occurs in humans

Given the potentially harmful effects on CSF circulation and the ability of the brain to recover from injury, humans should be investigated with a different strategy used thus far (such as high field MRI or CSF sampling) to determine if ependymal damage occurs following a blast.

8.9 Explore the long-term effect of activation of microglial cells

Given the implications for recovery from brain injury and development of chronic traumatic encephalopathy, the link between extracranial polytrauma and central activation of microglial cells needs to be explored in humans.

8.10 Determine the cause of the observed increase in permeability in the blood-brain barrier

Future work should isolate the effects of extracranial polytrauma, and IV fluid administration to determine the cause of the observed increase in permeability of the blood-brain barrier. This work may yield immediate results by enabling us to improve the resuscitation strategy used in TBI, potentially improving outcomes. At present, another doctoral student has taken over investigations using the porcine model; importantly they are looking at a control group of animals without a soft tissue injury or IV fluid administration. We hope that this will shed light on the observed changes in vascular permeability and determine the cause of the widespread amyloid precursor protein accumulation.

9 Appendix 1

In support of Chapter 3

A1.1 Neuropsychological assessment

I used a detailed neuropsychological battery that has previously been found to be sensitive to cognitive impairments following TBI (Kinnunen 2011, Bonelle 2012) to assess cognitive function in the bTBI group. Current verbal and non-verbal reasoning ability was assessed using the Wechsler Test of Adult Reading and the Wechsler Abbreviated Scale of Intelligence Similarities and Matrix Reasoning subtests (Wechsler, 1999). Verbal Fluency, Letter Fluency and Colour-Word (Stroop) tests, from the Delis-Kaplan Executive Function System, were used to assess cognitive flexibility, inhibition and set-shifting (Delis 2001). The Trail Making Test (A and B) was used to further assess executive functions (Reitan 2004). Working memory was assessed via The Digit Span subtest of the Wechsler Memory Scale-Third Edition (WMS-III) (Wechsler 1997). The Logical Memory I and II subtests of the WMS-III were used to measure immediate and delayed verbal recall. The People Test from the Doors and People Test battery was used as a measure of immediate and delayed associative learning and recall (Baddeley 1994).

Table 1. Cognitive domains and the neuropsychological tests used to assess them

Cognitive Domain	**Cognitive Subset**	**Test**
Intellectual ability	Pre-morbid intelligence	Wechsler Test of Adult Reading
	Verbal	WASI Similarities
	Non-verbal	WASI Matrix reasoning
Memory	Working memory	WASI Digit span

	Associative memory	People Test; Logical memory I and II (immediate recall)
		People Test; Logical memory I and II (delayed recall)
Executive function	Cognitive flexibility	D-KEFS Colour word interference test (Stroop)
	Alternating switch cost	D-KEFS Trail Making Test B minus A
	Word generation fluency	D-KEFS Letter fluency F+A+S total
Processing Speed	Visual search complex	D-KEFS Trail Making Test A (s) + Trail Making Test B (s)

A1.2 Delis-Kaplan Executive Function System

The Delis-Kaplan Executive Function System (D-KEFS) (Delis 2001) is a set of standardised tests for comprehensively assessing higher-level cognitive function. The tests were designed to assess mild brain damage and specifically frontal-lobe involvement (Swanson 2005). The complete D-KEFS comprise nine tests that measure a range of verbal and non-verbal executive functions. Subjects in this thesis were assessed using the D-KEFS Trail Making Tests A and B and the D-KEFS colour word interference test (Stroop) sub-tests to assess processing speed and mental flexibility. These two sub-tests are described below.

A1.3 Trail making tests A and B

The Trail Making Test (TMT) (Figure 1) tests frontal lobe function in particular the dorsolateral prefrontal cortex and the medial prefrontal cortex (Moll 2002). The test is administered in two parts, A and B: A tests visual scanning, numeric sequencing,

and visual-motor speed; whilst B assesses cognitive demands including visual-motor, visual spatial abilities, working memory and mental flexibility. A validated indicator of executive function can be obtained by subtracting the TMT B score form the TMT A score (TMTB – TMTA) (Sanchez-Cubillo 2009). The TMT A and TMT B are validated for use in organic brain injury (Reitan 2004). However, the test is biased towards individuals with higher education and previous work has found that education level affects TMT scores (Tombaugh 2004).

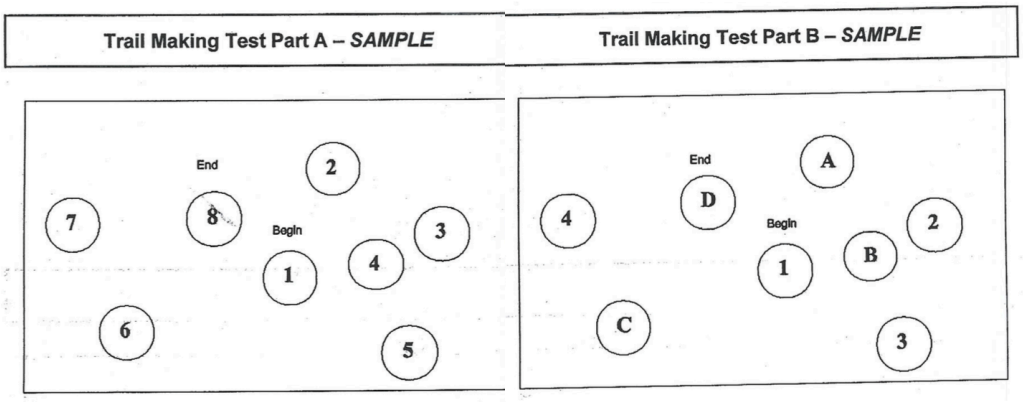


Figure 1. The Trail Making Test A and B

In part A of the test the subject must draw a line connecting the numbers in ascending order (i.e. 1-2-3-4). In part B the subject must draw a line connecting the numbers in ascending order whilst switching between drawling a line connecting letters, in alphabetical order (i.e. 1-A-2-B-3-C-4-D). The subject is instructed to perform the task as quickly and accurately as possible without lifting the pen from the paper. Errors are pointed out to the participants and adversely affect their scores.

A1.4 Colour-word interference test (Stroop test)

The colour-word interference test, also known as the Stroop test after the name of the effect that it is based on, evaluates inhibition and cognitive flexibility (Stroop 1935). The test measures a subject's ability to inhibit an over-learned verbal response (i.e. reading printed words) and to generate the conflicting response of naming the dissonant ink colours in which the words are printed (Figure 2). It was designed to evaluate both cognitive flexibility and ability to inhibit perseverative and unplanned impulsive verbal responses.

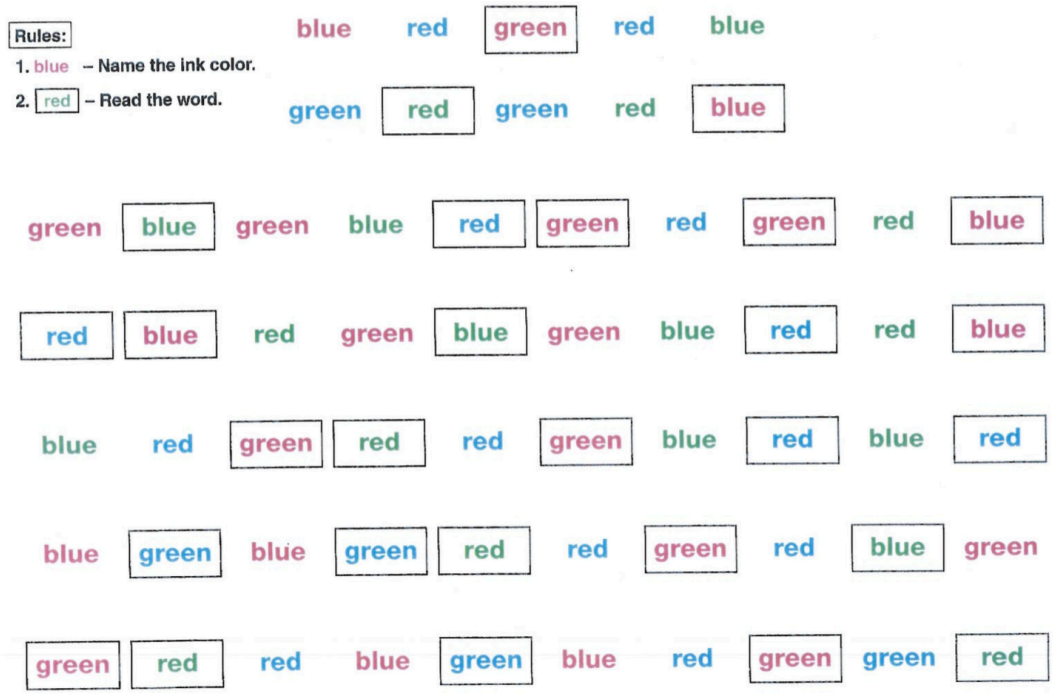


Figure 2. The Colour-word interference test

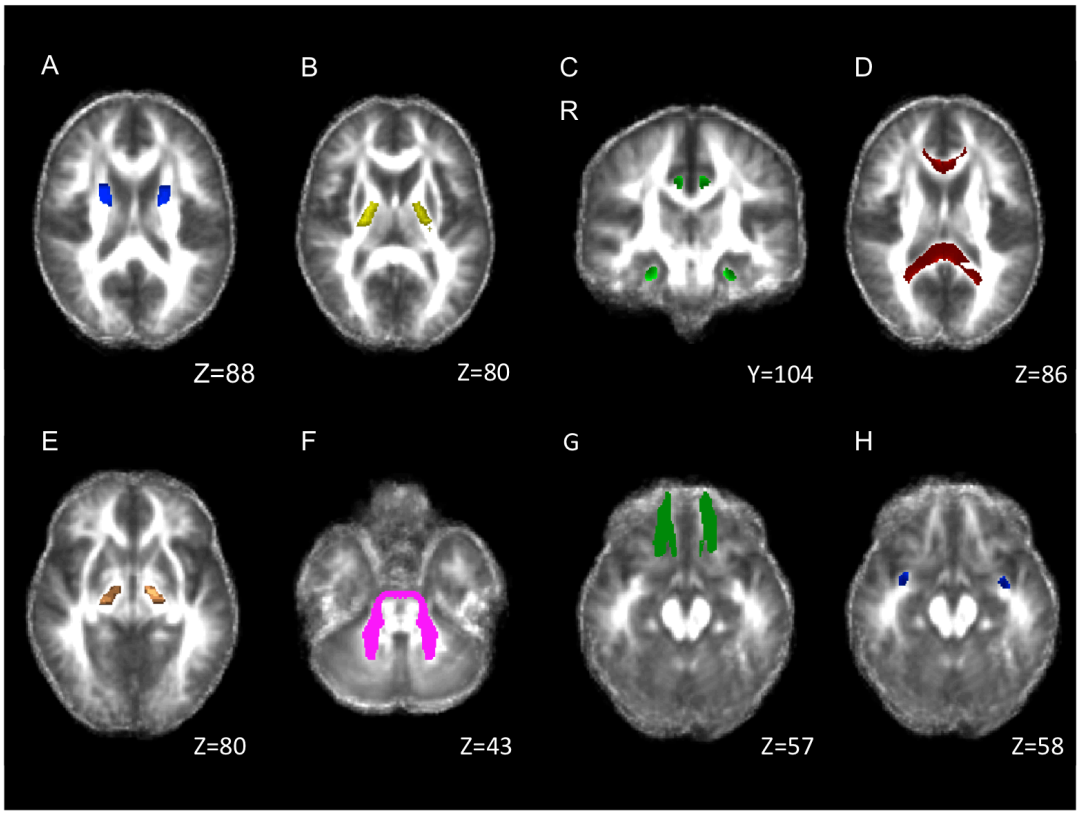
The Colour-word interference test is a timed test made of four parts. In the first, the subject is asked to name the colour, secondly they are asked to read the words, thirdly they must say the colour of the ink that the word is printed in (in the example on the first line above the correct answer would be red and blue) and finally they must switch between naming the colour of the ink and reading the word if a black box surrounds the word (in the example on the first line, third word along the correct answer is green).

10 Appendix 2

In support of Chapter 4

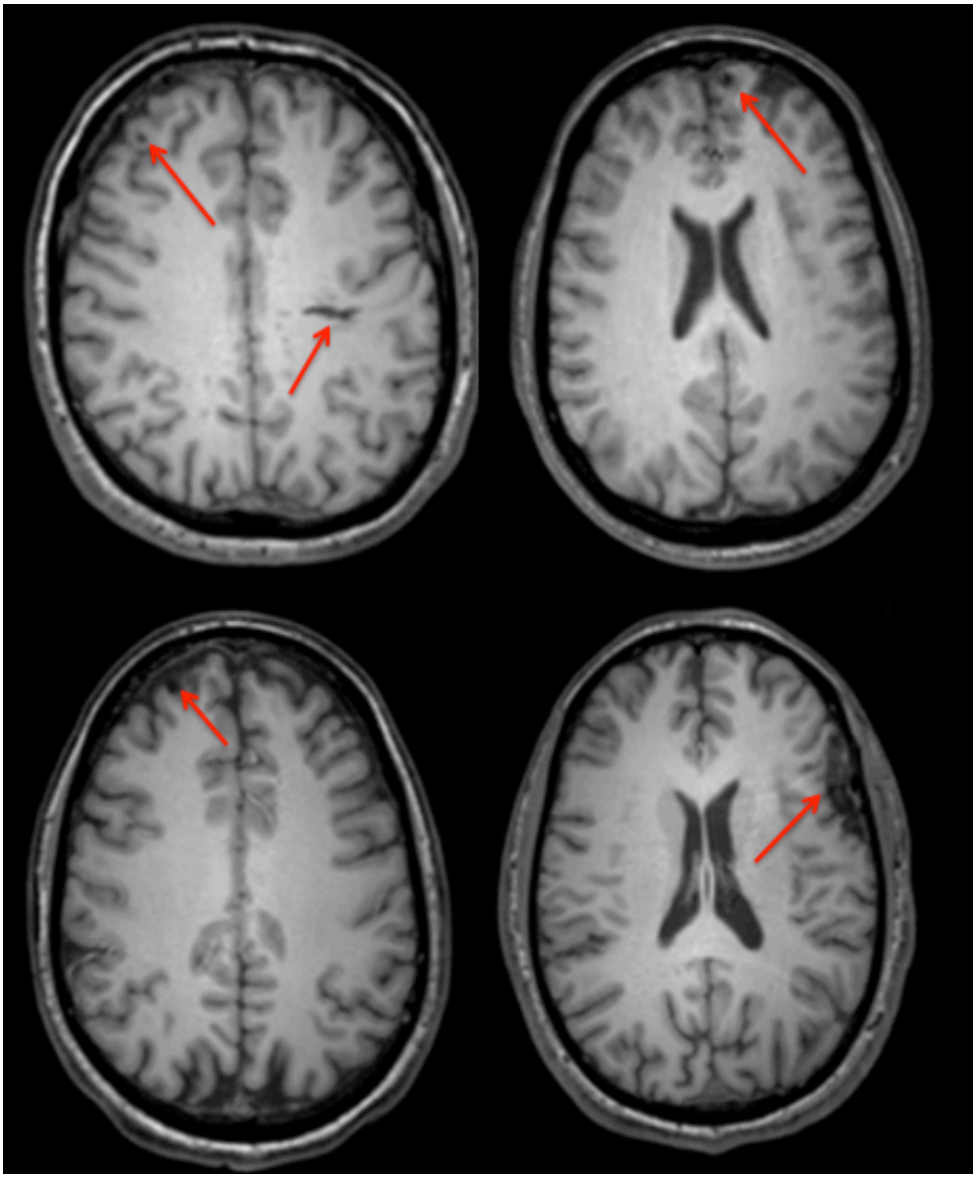
A2.1 Supplemental Figures

Figure S1. White matter tract regions of interest



Regions of interest (ROIs) used for determination of fractional anisotropy (FA) in soldiers after blast traumatic brain injury (bTBI). Individual colour masks overlaid onto group average FA map for soldiers with bTBI (n=19) registered into standard MNI space (using MNI coordinates). ROIs are: (A) anterior internal capsule, (B) posterior internal capsule, (C) cingulum, (D) corpus callosum, (E) cerebral peduncles, (F) middle cerebellar peduncles, (G) orbitofrontal WM, (H) uncinated fasciculi. FA was sampled from areas within a WM skeleton (not shown) produced by tract based spatial statistics (TBSS).

Figure S2. Intra-cerebral contusions following bTBI



High resolution T1 brain scans (axial sections) in subject space showing contusions (arrows) in soldiers after blast TBI (A) without pituitary dysfunction, and (B-D) with pituitary dysfunction. Total contusion volumes for these patients were: (A) 0.2, (B) 9.1, (C) 0.6, (D) 1.0 cm^3.

A2.2 Supplemental Tables

Table S1. Blast TBI Baseline Pituitary Function Results: Gonadotrophic/Thyroid/Prolactin Axes

Subjects (bTBI)	Summary Pituitary Dysfunction	GONADOTROPHINS						THYROID		PROLACTIN
		LH	FSH	Testosterone	SHBG	Free Androgen Index♯	Primary Hypogonadism	Free T4	Free T3	Prolactin
Units		IU/L	IU/L	nmol/L	nmol/L			pmol/L	pmol/L	mU/L
Normal Range		2-12	1.7-8.0	10.0-30.0	15-55	30-150		9.0-26.0	2.5-5.7	75-375
No Pituitary Dysfunction n=13										
M02	Nil	2.4	1.1	23.7	55.0	43.1	No	15.6	4.4	97
M04	Nil	4.7	3.9	21.0	33.0	63.6	No	12.2	5.7	146
M05	Nil	4.4	2.5	15.5	18.0	86.1	No	15.3	3.2	118
M09	Nil	1.2	4.8	39.5 *	18.0	219.4	Yes	13.4	5.8	219
M11	Nil	1.6	0.7	12.5	18.0	69.4	No	14.0	4.4	285
M12	Nil	18.5	36.4	6.2 *	10.0	62.0	Yes	15.5	3.5	177
M13	Nil	5.8	9.6	13.1	10.0	131.0	No	13.2	5.2	240
M15	Nil	40.3	60.3	11.6 *	18.0	64.4	Yes	14.0	6.6	136
M16	Nil	5.5	4.9	22.3	24.0	92.9	No	13.4	4.4	200
M17	Nil	4.7	3.3	25.2	39.0	64.6	No	16.9	5.2	131
M18	Nil	0.0	0.1	12.3*	19.0	64.7	Yes	18.5	4.8	312
M19	Nil	2.3	1.5	22.0	28.0	78.6	No	15.0	4.7	183
M20	Nil	3.8	3.6	28.8	32.0	90.0	No	13.2	4.6	330
Median [IQR] or n (%)		4.4 [2.0-5.6]	3.6 [1.3-7.3]	15.5 [0-23.0]	19.0 [18.0-32.5]	69.4 [64.0-91.5]	4 (30.8%)	14.0 [13.3-15.6]	4.7 [4.4-5.5]	183 [134-263]
Range		0-40.3	0.1-60.3	0-28.8	10.0-55.0	43.1-219.4		12.2-18.5	3.2-6.6	97-330
Pituitary dysfunction n=6										
M01	PRL	1.8	2.5	21.7	27.0	80.4	No	17.3	4.6	619
M03	ACTH	1.5	1.2	23.8	35.0	68.0	No	16.0	5.5	126
M07	GH	3.7	2.4	13.1	24.0	54.6	No	14.4	4.9	172
M08	ACTH/GH/Gn	1.3	1.8	2.0	18.0	11.1	No	15.5	4.3	199
M10	PRL	2.6	1.3	22.8	26.0	87.7	No	10.8	4.7	439
M14	GH	6.5	3.9	22.4	33.0	67.9	No	12.9	4.0	216
Median [IQR] or n (%)		2.2 [1.5-4.4]	2.1 [1.3-2.9]	22.1 [10.3-23.1]	26.5 [22.5-33.5]	68.0 [43.7-82.2]	0 (0%)	15.0 [12.4-16.3]	4.7 [4.2-5.1]	208 [161-484]
Range		1.3-6.5	1.2-3.9	2.0-23.8	18.0-35.5	11.1-87.7		10.8-17.3	4.0-5.5	126-619
P		0.37	0.28	0.42	0.42	0.47	1.00	0.90	0.70	0.47

Key:	
	Abnormal value
GH	Growth hormone deficiency
ACTH	ACTH deficiency
Gn	Secondary hypogonadism
PRL	Hyperprolactinaemia
*	On testosterone replacement
♯	Calculated by: 100 x total testosterone/SHBG
	Repeated elevated measurement

Abnormal values indicated by grey shading. * on testosterone replacement, # calculated from 100 x total testosterone/SHBG, a) to convert to µg/mL divide by 3.467,

b) to convert to µg/dL divide by 12.87, c) to convert to µg/dL divide by 15.36, d) remained elevated on repeat measurement with negative macroprolactin. P values from Mann Whitney U test or Fisher's exact test between groups.

Table S2. Blast TBI: Dynamic Growth Hormone results and IGF-1

Subjects (bTBI)	Summary Pituitary Dysfunction		GROWTH HORMONE / IGF-1 AXIS								
No Pituitary Dysfunction n=13			IGF-1 at GST	IGF-1 age related NR	IGF-1 (median of NR)	IGF-1 ratio to Median	Peak GH (GST)	IGF-1 at GHRH-Arginine Test	Peak GH (GHRH-Arginine Test)	GH cut off (GHRH-Arginine Test)*	Peak GH (ITT)
		Units	nmol/L	nmol/L	nmol/L	n/a	mcg/L	nmol/L	mcg/L	mcg/L	mcg/L
		Normal Range (NR)					>5				>5
M02	Nil		22.6	14.2-36.9	22.9	0.72	6.6	n/a	n/a	n/a	52.1
M04	Nil		58.0	15.2-42.8	25.5	1.84	13.1	n/a	n/a	n/a	n/a
M05	Nil		19.9	15.2-42.9	25.5	0.63	11.6	20.0	10.30	11.80	n/a
M09	Nil		29.0	18.3-62.8	33.9	0.62	1.0	18.7	17.20	15.60	n/a
M11	Nil		31.9	16.5-55.1	30.2	1.01	1.6	31.9	37.80	11.70	n/a
M12	Nil		38.1	15.2-42.8	25.5	1.21	0.6	38.1	13.50	11.80	n/a
M13	Nil		74.5	15.1-46.5	26.4	2.37	0.7	ND	17.30	15.60	n/a
M15	Nil		27.4	15.2-42.8	25.5	0.87	2.8	30.0	23.30	11.80	n/a
M16	Nil		31.3	14.2-36.9	22.9	0.99	3.2	29.8	27.00	8.10	n/a
M17	Nil		19.9	15.2-42.8	25.5	0.63	8.7	n/a	n/a	n/a	n/a
M18	Nil		17.9	15.2-42.8	25.5	0.57	2.7	19.9	10.00		n/a
M19	Nil		29.6	15.2-42.8	25.5	0.94	6.0	n/a	n/a	n/a	n/a
M20	Nil		17.2	15.0-39.9	24.4	0.55	8.0	n/a	n/a	n/a	n/a
		Median [IQR] or n(%)	29.0 [19.9-35.0]	n/a	n/a	0.87 [0.6-1.1]	3.3 [1.3-8.4]	29.8 [19.9-31.9]	17.3 [10.1-26.1]		n/a
		Range	17.2-74.5	n/a	22.9-33.9	0.6-2.37	0.6-13.1	19.0-38.0	1.0-38.0		
Pituitary dysfunction n=6											
M01	PRL		21.6	15.2-42.8	25.5	0.69	7.0	n/a	n/a		n/a
M03	ACTH		26.7	15.2-42.8	25.5	0.85	0.2	32.1	15.40		n/a
M07	GH		23.7	15.0-39.9	24.4	0.75	2.7	26.1	3.43		n/a
M08	ACTH/GH/Gn		16.1	13.1-34.7	21.3	0.66	0.1	16.6	4.26		0.18
M10	PRL		18.9	15.2-42.8	25.5	0.60	2.8	29.4	25.80		n/a
M14	GH		18.2	15.2-42.8	25.5	0.58	0.1	21.8	2.70		n/a
		Median [IQR] or n(%)	20.3 [17.7-24.5]	n/a	n/a	0.68 [0.60-0.78]	1.45 [0.1-3.9]	26.1 [19.2-30.8]	4.3 [3.1-20.6]		n/a
		Range	16.1-26.7	n/a	21.3-25.5	0.58-0.85	0.1-7.0	17.0-32.0	3.0-26.0		
		P	0.07	n/a		0.28	0.09	0.76	0.28		1.00

Key:	
	Abnormal value
GH	Growth hormone deficiency
ACTH	ACTH deficiency
Gn	Secondary hypogonadism
PRL	Hyperprolactinaemia
GST	Glucagon Stimulation Test
ITT	Insulin Tolerance Test
n/a	Not applicable
ND	Not Done
*	Or highest cut-off if BMI not calculable due to amputation

Abnormal values indicated by grey shading. a) to convert to ng/mL divide by 0.131, * using age and BMI normal ranges with BMI 25-30 kg/m^2 if not calculable due to amputation. P values from Mann Whitney U test between groups.

Table S3. ACTH-cortisol axis in bTBI

Subjects (bTBI)	Summary Pituitary Dysfunction		ACTH/CORTISOL AXIS								
No Pituitary Dysfunction n=13			Basal Cortisol (GST)	Peak Cortisol (GST)	Basal ACTH (GST)	Cortisol (Post Metyrapone)	ACTH (Post Metyrapone)	11-DOC (Post Metyrapone)	Basal Cortisol (ITT)	Peak Cortisol (ITT)	CBG
		Units	nmol/L	nmol/L	ng/L	nmol/L	ng/L	nmol/L	nmol/L	nmol/L	mcg/L
		Normal Range	150-500	>350		<200	>60????	>200	150-500	>450	27.1-52.3
M02	Nil		230	230.0	6.0	222	149	182.0	544	636	48.9
M04	Nil		283	378.0	27.4	123	247	244.5	n/a	n/a	n/a
M05	Nil		309	494.0	39.2	n/a	n/a	n/a	n/a	n/a	n/a
M09	Nil		335	389.0	15.3	n/a	n/a	n/a	n/a	n/a	n/a
M11	Nil		323	473.0	38.0	n/a	n/a	n/a	n/a	n/a	n/a
M12	Nil		192	478.0	17.2	n/a	n/a	n/a	n/a	n/a	n/a
M13	Nil		363	386.0	24.6	n/a	n/a	n/a	n/a	n/a	n/a
M15	Nil		376	497.0	ND	n/a	n/a	n/a	n/a	n/a	n/a
M16	Nil		427	427.0	92.7	n/a	n/a	n/a	n/a	n/a	n/a
M17	Nil		207	626.0	20.0	n/a	n/a	n/a	n/a	n/a	n/a
M18	Nil		409	533.0	27.0	n/a	n/a	n/a	n/a	n/a	n/a
M19	Nil		220	435.0	19.7	n/a	n/a	n/a	n/a	n/a	n/a
M20	Nil		420	420.0	28.6	n/a	n/a	n/a	n/a	n/a	n/a
		Median [IQR] or n(%)	323.0 [225.0-392.0]	435.0 [387.5-495.5	25.8 [17.8-35.7]	172.5 [123.0-172.5	198	213.3			
		Range	192.0-427.0	230.0-626.0	6.0-92.7]	123.0-222.0	149.0-247.0	182.0-244.5			
Pituitary dysfunction n=6											
M01	PRL		606	606.0	164.0	n/a	n/a	n/a	n/a	n/a	n/a
M03	ACTH		292	292.0	23.2	38	22	87.1	n/a	n/a	63.0
M07	GH		419	419.0	24.8	n/a	n/a	n/a	n/a	n/a	n/a
M08	ACTH/GH/Gn		287	287.0	18.7	n/a	n/a	n/a	110	268	74.0
M10	PRL		109	350.0	20.6	422	207	200.0	n/a	n/a	55.7
M14	GH		88	445.0	32.2	n/a	n/a	n/a	n/a	n/a	n/a
		Median [IQR] or n(%)	289.5 [103.8-465.8]	384.5 [290.8-485.3	24.0 [20.1-65.2]	230.0	114.5	143.60			63.00
		Range	88.0-606.0	287.0-606.0	18.7-164.0	38.0-422.0	22.0-207.0	87.1-200.0			55.7-74.0
		P	0.70	0.28	0.75	n/a	n/a	n/a	n/a	n/a	n/a

Abnormal values indicated by grey shading. To convert to ng/dL: divide a by 27.59, b by 28.86. P values from Mann Whitney U test between groups. ND: not done.

Table S4. Pituitary dysfunction and structural neuroimaging abnormalities in bTBI

	No pituitary dysfunction	Pituitary dysfunction	P
n	13	6	
Acute CT brain			
EDH	0 (0%)	0 (0%)	n/a
SDH	0 (0%)	0 (0%)	n/a
tSAH/IVH	0 (0%)	0 (0%)	n/a
Diffuse swelling	1 (7.7%)	1 (0%)	0.26
Study MRI brain			
Contusion	1 (7.7%)	3 (50.0%)	0.02
Siderosis	3 (23%)	1 (16.6%)	0.41
Microbleeds	7 (53%)	3 (50%)	0.50
Gliosis	0 (0%)	1 (16.6%)	0.06
Hypo-pituitary damage	0 (0%)	0 (0%)	n/a
MRI pituitary with contrast	ND	3 normal, 3ND	n/a

Data given as n (%). P values from Fisher's exact test between groups.

Table S5. Quality of life and symptom questionnaires in nbTBI and bTBI

Quality of Life Assessment		All nbTBI (n=38*)	All bTBI (n=18*)	bTBI: No Pituitary Dysfunction (n=12)	bTBI: Pituitary Dysfunction (n=6)	P no pit dys vs pit dys
Assessment of GH Deficiency in Adults (AGHDA)		9.5 [5.8-14.5][a]	16.0 [4.0-18.5][b]	14.0 [3.0-17.0]	17.5 [16.0-19.5]	0.10
Beck Depression Inventory Score (BDI-II)		11.0 [7.0-20.0][c]	21.5 [4.0-25.3]	11.5 [1.8-21.8]	24.5 [20.3-26.3]	0.08
Epworth Sleepiness Scale		7.0 [2.0-12.0][d]	7.0 [2.5-11.0]	6.0 [1.5-10.5]	10.0 [3.0-16.5]	0.25
Pittsburgh Sleep Index		n/a	9.0 [3.0-16.0]	4.5 [2.0-16.3]	12.0 [8.0-15.5]	0.37
NHP	Energy Levels	61.0 [0-100][e]	51.0 [0-82.0]	51.0 [6.0-100]	31.5 [0-76.0]	0.39
NHP	Pain	100 [52-100][e]	71.5 [47.5-90.3]	76.0 [49.0-86.0]	64.0 [45.5-100]	0.96
NHP	Emotional Reactions	80.0 [5.0-90.0][e]	67.5 [44.5-93.3]	85.0 [53.8-100]	46.0 [32.3-75.0]	0.10
NHP	Sleep	78.0 [27.0-100][e]	45.0 [45.0-100]	70.0 [6.8-100]	36.0 [0-68.3]	0.29
NHP	Social Isolation	100 [55.0-100][e]	79.0 [41.0-100]	100 [51.5-100]	71.0 [31.0-78.8]	0.13
NHP	Physical Activity	100 [78.2-100][e]	73.5 [58.0-89.0]	73.5 [58.0-86.8]	73.0 [39.3-100]	0.96
NHP	Average	78.0 [59.0-94.6][e]	58.5 [44.8-84.5]	71.5 [45.0-90.5]	51.5 [44.8-61.3]	0.25
NHP	Daily Living Problems (0-7)	2.0 [0-5.0][f]	4.5 [3.0-6.0]	4.5 [2.3-5.8]	4.5 [3.8-6.3]	0.62
SF-36	Physical functioning	85.0 [60.0-95.0][e]	52.5 [37.5-81.3]	52.5 [41.3-58.8]	60.0 [27.5-88.8]	0.82
SF-36	Role limitations due to physical health	12.5 [0-62.5][g]	12.5 [0-75.0]	25.0 [0-93.8]	0 [0-18.8]	0.10
SF-36	Role limitations due to emotional problems	67.0 [0-100][g]	67.0 [24.8-100]	83.5 [33.0-100]	50.0 [0-100]	0.49
SF-36	Energy/Fatigue	50.0 [35-60][e]	42.5 [33.8-66.3]	52.5 [36.3-70.0]	37.5 [26.3-47.0]	0.15
SF-36	Emotional well being	64.0 [52.0-80.0][e]	60.0 [51.0-81.0]	68.0 [53.0-83.0]	58.0 [31.0-66.3]	0.34
SF-36	Social functioning	63.0 [38.0-75.0][e]	50.0 [38.0-75.0]	56.5 [41.0-84.8]	44.0 [22.0-63.0]	0.18
SF-36	Pain	55.0 [33.0-88.0][e]	45.0 [30.5-70.5]	68.0 [35.5-75.5]	33.0 [23.0-58.8]	0.21
SF-36	Health change	50.0 [38.0-75.0][e]	32.5 [25.0-50.0]	25.0 [25.0-50.0]	45.0 [25.0-56.3]	0.49
SF-36	General health	50.0 [25.0-75.0][e]	50.0 [25.0-61.3]	50.0 [27.5-63.8]	40.5 [18.8-61.3]	0.55

All data expressed as median [IQR] Abbreviation NHP-Nottingham Health Profile, SF-36- Short Form 36 Health Survey, n/a-not available.

a Data available n=37 b Data available n=17 c Data available in 36 d Data available n=31 e Data available n=27 f Data available n=25 g Data available n=26

Table S6. Characteristics of soldiers with bTBI

Subjects	Age at TBI	Age at GST	Time since TBI	ISS	AIS Head	AIS Chest	AIS Abdo	GCS	PTA	BMI		PTA>24 hrs	Limb Amputation	Major organ damage	Skull/facial fracture	Opiate use	Antidepressant Use	Seizures Post TBI	Primary Hypogonadism
Units	Years	Years	Months						Days	kg/m2									
Maximum Score				75	6	6	6	15											
All nbTBI n=39	31.3 [22.5-35.7]	32.3 [23.1-36.7]	5.8 [3.1-11.0]	25 [16-32]	5.0 [4-5]	0 [0-0]	0 [0-0]	14.0 [6.0-14.0][a]	0.5 [0-7.3][a]	24.7 [22.4-29.4]	n (%)	20 (51.3%)	0 (0%)	3 (7.7%)	5 (12.8%)	3 (7.7%)	5 (12.8%)	3 (7.7%)	1 (2.6%)
	17.2 - 44.8	19.9 - 45.1	1.9-41.2	1-75	1-6	0-6	0-3	3-15	0-42	17.0-33.4									
All bTBI n=19	26.7 [26.1-30.9]	28.3 [26.8-32.2]	15.2 [10.8-19.3]	33.0 [20.0-45.0]	4.0 [3.0-5.0]	0 [0-2]	0 [0-2]	3.0 [3.0-14.5][b]	5.5 [0.8-22.8]	26.7 [24.5-28.9]	n (%)	13 (72.2%)	8 (42.1%)	11 (57.9%)	3 (15.8%)	9 (47.3%)	9 (47.3%)	2 (10.5%)	4 (21.1%)
	19.0 - 43.5	19.6 - 44.7	4.1 - 23.6	9-70	0-6	0-4	0-3	3-15	0-84	21.7-33.7									
P vs nbTBI	0.40	0.40	**0.001**	0.17	**0.04**	0.11	**0.02**	0.24	**0.01**	0.28		0.70	**0.0008**	**0.002**	1.0	**0.02**	0.08	1.0	0.24
bTBI No pituitary dysfunction																			
M02	36.3	37.6	15.2	20	4	0	2	3	1	25.4		No	No	No	No	Yes	Yes	No	No
M04	26.4	27.6	14.2	24	0	4	0	n/a	4	27.7		Yes	No	Lung/eye	No	No	No	No	No
M05	27.3	28.6	15.2	24	0	4	0	n/a	28	24.5		Yes	No	No	No	Yes	No	No	No
M09	19.0	19.6	6.7	45	4	0	2	n/a	4	n/a		Yes	Yes	Perineum	No	Yes	Yes*	No	Yes
M11	19.3	20.9	16.6	25	5	0	0	3	84	26.6		Yes	No	No	No	No	No	Yes	No
M12	30.2	30.5	4.1	33	2	0	2	n/a	0	n/a		No	Yes	Perineum	No	Yes	Yes*	No	Yes
M13	22.8	23.7	10.8	45	4	0	0	n/a	21	n/a		Yes	Yes	Eye/Skin	No	No	Yes*	No	No
M15	26.4	26.8	4.1	45	4	0	2	15	0	n/a		No	Yes	Eye/Skin/perineum	No	Yes	No	No	Yes
M16	34.7	36.7	23.7	24	4	2	0	15	0	28.7		No	No	No	No	Yes	Yes	No	No
M17	26.6	28.0	16.6	9	3	0	0	14	0	23.6		No	No	No	No	No	No	No	No
M18	26.7	28.3	20.2	36	4	4	2	n/a	14	n/a		Yes	Yes	Lung/colon/perineu	No	No	No	No	Yes
M19	26.6	27.7	13.6	9	3	0	0	n/a	n/a	n/a		n/a	Yes	Skin	No	No	No	No	No
M20	30.9	32.2	15.4	9	3	0	0	n/a	2	29.4		Yes	No	No	No	No	Yes	No	No
n=13	26.6 [24.6-30.6]	28.0 [25.3-31.4]	15.2 [8.4-16.8]	24.0 [14.5-40.5]	4.0 [2.5-4.0]	0 [0-3]	0 [0-2]	14.0 [3.0-15.0]	3.0 [0-19.3]	26.6 [24.5-28.7]	n (%)	7 (58.3%)	6 (46.1%)	7 (53.9%)	0 (0%)	6 (46.2%)	6 (46.2%)	1 (7.7%)	4 (30.8%)
	19.0 - 36.3	19.6 - 37.6	4.1 - 23.6	9-45	0-5	0-4	0-2	3-15	0-84	23.6-29.4									
Pituitary dysfunction																			
M01 (PRL)	30.0	30.4	4.9	33	5	0	0	3	4	21.7		Yes	No	No	Yes	Yes	Yes*	Yes**	No
M03 (ACTH)	25.0	26.3	15.9	70	6	0	3	3	17	n/a		Yes	Yes	Spleen/Liver	Yes	No	Yes*	No	No
M07 (GH)	34.3	36.2	21.9	38	5	3	0	3	7	26.7		Yes	No	Lung	No	No	No	No	No
M08 (ACTH/GH/Gn)	43.5	44.7	14.7	45	4	2	0	n/a	42	n/a		Yes	Yes	No	No	Yes	No	No	No
M10 (PRL)	28.5	30.1	19.3	33	5	0	2	n/a	28	24.3		Yes	No	Eye/Liver/lung	Yes	No	No	No	No
M14 (GH)	26.1	27.7	19.6	9	0	1	0	3	14	33.7		Yes	No	Skin	No	Yes	Yes	No	No
n=6	29.3 [25.8-36.6]	30.3 [27.4-38.3]	18.0 [12.0-20.4]	35.5 [27.0-51.3]	5.0 [3.0-5.3]	0 [0-2.3]	0 [0-2.3]	3.0 [3.0-3.0]	15.5 [6.3-31.5]	25.5 [22.4-32.0]	n (%)	6 (100%)	2 (33.3%)	4 (66.7%)	3 (50.0%)	3 (50%)	3 (50%)	1 (16.7%)	0 (0%)
	25.0 - 43.47	26.3 - 44.7	4.9 - 22.0	9-70	0-6	0-3	0.3	3-3	4-42	21.7-33.7									
P vs no pit dys	0.35	0.35	0.35	0.21	**0.05**	0.76	0.96	0.17	0.13	0.71		0.48	0.99	0.99	0.10	1.00	1.00	0.99	1.00

All data expressed as median [interquartile range] or n (%). P values from Mann Whitney U test or Fisher's exact test between groups.

Footnotes:

a Data available for n=16

b Data available for n=9

c Data available for n=38

*** For analgesic purposes**

**** On antiepileptics**

Table S7. Medications used by soldiers with bTBI

Subjects	Medications
No pituitary dysfunction (n=13)	
M02	Diclofenac, Sertraline, Tramadol
M04	C0-codamol
M05	Diclofenac, Tramadol
M09	Amitriptyline, MST, Nebido, Pregabalin
M11	None
M12	Amitriptyline, Diclofenac, Nebido, Pregabalin, Ranitidine, Sildenafil, Tramadol
M13	Amitriptyline, Baclofen, Pregabalin
M15	Amitriptyline, Nebido, Pregabalin, Tramadol
M16	Mirtazepine, Paracetamol, Pregabalin, Tramadol, Zopiclone
M17	None
M18	Nebido
M19	Diclofenac, Pregabalin, Ranitidine
M20	Sertraline, Zopiclone
Pituitary dysfunction (n=6)	
M01 (PRL)	Amitriptyline, Diclofenac, MST, Phenytoin
M03 (ACTH)	Amitriptyline, Erythromycin, Gabapentin
M07 (GH)	None
M08 (ACTH/Gn	Diclofenac, Lansoprazole, MST, Paracetamol, Pregabalin, Tramadol
M10 (PRL)	Betnovate ointment, Co-codamol
M14 (GH)	Amitripyline, Diclofenac, Fluoxetine, Mirazepine, MST, Paracetamol, Pregabalin, Salbutamol inhaler, Zopiclone

A2.3 Supplemental Results

A2.3.1 Non-pituitary endocrine diagnoses in bTBI and nbTBI cohorts

Other non-pituitary endocrine disorders were diagnosed in both groups. Primary hypogonadism due to perineum/testicular blast injury had been found in 4 out of 19 soldiers (21.2%), none of whom had pituitary dysfunction (Table 2 and S1). Although at the time of our assessment all these subjects were already on testosterone replacement, 3 had documented increased gonadotophins before its initiation (Table S1). One of these (M12) was under-replaced with testosterone at the time of assessment. A high prevalence of perineal blast injury has previously been reported in soldiers exposed to IED (Mossadegh 2012). One control patient with nbTBI had a pre-existing diagnosis of primary hypothyroidism, and another had previously undiagnosed primary hypogonadism of unknown cause unrelated to their nbTBI.

A2.3.2 IGF-I levels in bTBI patients with GH deficiency

IGF-I levels were within the normal range in all those soldiers with GH deficiency. When comparing those soldiers with bTBI who had GH deficiency (n=3) to those without GH deficiency (n=16), absolute IGF-I levels tended to be lower in those with than without GH deficiency (median [IQR] 18.2 [16.7-22.3] vs. 27.1 (19.9-31.6], p=0.11). However IGF-I relative to median of age-related reference range were similar between groups (0.66 [0.60-0.73] vs. 0.79 [0.63-1.00], p=0.40) (Table S1).

A2.3.4 Symptoms, quality of life and cognitive function

In our cohort of soldiers with bTBI, subjective symptoms included worsening of their memory (70%), changes in mood (70%), difficulty concentrating (65%), difficulty sleeping (55%), headaches (45%), and dizziness (30%).

Consistent with their higher prevalence of polytrauma and amputations, the soldiers with bTBI had significantly worse scores for physical activity (p=0.02) and daily living problems (p=0.04) from the Nottingham Health Profile (NHP) questionnaire, with a tendency for worse NHP pain scores (p=0.08) and change in health from the Short Form-36 (SF-36) QoL questionnaire 16 (p=0.06), than the control nbTBI group (Table S5). However there were no significant differences in measures of depression

and emotional well-being (from Beck Depression Inventory-II), NHP and SF-36 questionnaires) between the bTBI and nbTBI groups (p=0.30-0.71) (Table S5).

In the bTBI group, soldiers with pituitary dysfunction had trends towards worse measure of QoL and symptom scores in several domains compared to those without pituitary dysfunction (Table S5). Soldiers after bTBI with pituitary dysfunction had trends for higher AGHDA QoL score (p=0.10), worse scores for emotional reactions (NHP, p=0.10), social isolation (NHP, p=0.13), role limitations due to physical health (SF-36, p=0.10), energy/fatigue (SF-36, p=0.15), and social functioning (SF-36, p=0.18), and higher depression scores (BDI-II, p=0.10), though none had symptoms suggesting severe depression (all scores <28/63).

A2.3.5 Interpretation of metyrapone test

Although the metyrapone test is not a commonly used test for ACTH deficiency (Grossman 2010), it was only needed for the confirmatory diagnosis in one soldier (M03). Furthermore that subject also had very low cortisol levels throughout their day curve .50 nmol/L (.1.81 ƒÊg/dL) confirming the diagnosis of ACTH deficiency. The second soldier with ACTH deficiency (M10) failed their cortisol response to insulin-induced hypoglycaemia (peak 268 nmol/L), and also had low cortisol levels (<100 nmol/L, <3.62 ƒÊg/dL) at 1200h on their day curve supporting the diagnosis. Other soldiers who initially had low cortisol responses to glucagon stimulation, subsequently had ACTH deficiency excluded on the basis of normal responses to ITT (M02) or metyrapone test (M10), but both also had subsequent high basal morning cortisol levels (M02, M10, >400 nmol/L, 14.50 ƒÊg/dL). Previous studies comparing the metyrapone test to more commonly used tests for ACTH deficiency have demonstrated the metyrapone test to have specificity, sensitivity and concordance (accuracy) rates of 77-100%, 64-89%, 74-84% (n=17-32) and 86, 91, 87% (n=87) with the ITT and ACTH stimulation test respectively (Fiad 1994, Courtney 2000, Giordano 2008).

Furthermore in a recent audit of patients from our endocrine clinics suspected of having ACTH deficiency (n=24, excluding soldiers with bTBI from this study), we have found an overall 92% concordance rate between results of a metyrapone test,

and the ACTH stimulation test (n=12, normal response >480 nmol/L or 17.40 ƒÊg/dL, using alignment of the previous 550 nmol/L cut-off to the new Architect i2000 assay) or ITT (n=13) (unpublished observations). In this analysis, all patients failing the metyrapone test (n=5) also failed an ITT. The overall specificity for the metyrapone test in diagnosing ACTH deficiency was 100% and sensitivity was 71% (unpublished observations).

A2.4 Supplemental Methods

A2.4.1 Recruitment

Ethical approval was granted by the Ealing and West London Hospitals Research Ethics Committee. Studies were performed according to the Declaration of Helsinki and all soldiers gave informed written consent.

Inclusion of a military combat nbTBI group would have been a useful in addition to the civilian nbTBI group to control for active military service in an identical theatre. However in UK soldiers experiencing nbTBI in Afghanistan, the majority are due to gunshot wounds that are either fatal or complicated by penetrating brain injury often requiring surgery. The lower prevalence of military non-penetrating nbTBI, primarily due to RTAs, precluded endocrine assessment of a sufficient number of such soldiers to be included in this study.

Both bTBI and nbTBI subjects had clinical assessment, calculation of their AIS for each body region including brain, and total ISS (Baker 1974, Hawley 1996), and completed QoL and symptom questionnaires: Assessment of Growth Hormone Deficiency in Adults (QoL-AGHDA); Beck Depression Inventory-II (BDI-II); Nottingham Health Profile (NHP); Short Form 36 Health Survey (SF-36), Pittsburgh Sleep Quality Index and Epworth Sleepiness Scale (Hunt 1985, Buysse 1989, Johns 1991, Ware 1992, Beck 1996, McKenna 1999). Soldiers were excluded if they had needed massive blood transfusion so as to exclude pituitary dysfunction secondary to hypovolaemic shock (Stainsby 2006).

A2.4.2 Endocrine Testing

Endocrine assessment included baseline measurement of serum anterior pituitary hormones: TSH, free T4, free T3, prolactin, FSH, LH, testosterone (Abbott Architect Ci8200), ACTH, cortisol, GH, IGF-I (Immulite® 2000) and sex hormone binding globulin (SHBG). Free androgen index was calculated as 100 x total testosterone / SHBG.

A diagnosis of hyperprolactinemia was made on the basis of two consecutively raised prolactin readings (above upper reference range, Table 1) and a negative macroprolactin, an immunological artefact leading to misdiagnosis of

hyperprolactinemia (assessed by PEG precipitation) (Smith 2007). Subjects who met these criteria had MRI of the pituitary including gadolinium contrast to rule out an incidental pituitary tumour.

A diagnosis of gonadotrophin deficiency was made on the basis of a low morning testosterone <10 nmol/L (<2.9 ng/mL) with low or non-elevated LH (NR 1.7-12.0 IU/L) and FSH (NR 1.7-8.0 IU/L). If sex hormone binding globulin (SHBG) was low (<15 nmol/L), then FAI needed to be <30 for the diagnosis. Primary hypogonadism was defined as a low morning testosterone or FAI with elevated FSH and/or LH.

Growth hormone (GH) deficiency was defined as failure on 2 dynamic endocrine tests performed in the morning: (i) Glucagon Stimulation Test (GST) used as initial screening test and (ii) a confirmatory 2nd line test, either the GHRH-Arginine Test or an Insulin Tolerance Test (ITT). Similarly, a diagnosis of ACTH deficiency was made on the basis of failure on 2 dynamic endocrine tests performed in the morning: (i) a GST, and (ii) an ITT or an overnight Metyrapone Stimulation Test (MST). A 5 point Cortisol Day Curve (CDC) was also used to help confirm or exclude ACTH deficiency, and assess the need for maintenance hydrocortisone replacement as opposed to just during intercurrent illness.

An ITT was not routinely performed because of the prevalence of relative and absolute contraindications in this population. In our cohort 10.5% of soldiers after bTBI and 10.3% of controls after nbTBI had an absolute contraindication (history of seizures, ischemic heart disease, cardiac arrhythmias, abnormal ECG), whilst an additional 21.1% and 53.8% had a relative contraindication (intra-cerebral contusion, intra-cranial haemorrhage). If further confirmatory testing was required because of equivocal findings on the second dynamic test (e.g. difficulty calculating BMI in soldiers with amputations), and no contraindications were present, an ITT was carried out in addition to the glucagon test and GHRH-Arginine or metyrapone test. Diabetes insipidus was screened for on the basis of symptoms (polyuria and polydipsia) and measurement of paired random clinic urine and plasma osmolalities. If clinically indicated, a Water Deprivation Test was performed (n=6 controls with nbTBI, n=1 soldier with bTBI).

All dynamic endocrine tests were carried out in an in-patient facility at Charing Cross Hospital, London or St. Mary's Hospital, London. A summary of the algorithm used to define pituitary dysfunction is shown in Table 1.

A2.4.3 Glucagon Stimulation Test (GST)

Following an overnight fast, patients had basal blood samples. Glucagon (GlucaGen™, Novo Nordisk Pharmaceuticals, Crawley, UK 1 mg, or 1.5 mg if weight >90 kg) was administered intramuscularly. Blood samples for glucose, serum cortisol and GH were taken at 90, 120, 150 and 180 mins after glucagon administration from an IV cannula. The majority of subjects (89% soldiers and 70% controls) also had samples taken at 210 and 240 mins. An abnormal response was defined as a peak GH <5 μg/L and cortisol <350 nmol/L (<12.7 μg/dL) during the test (Yuen 2009, Cegla 2013). Subjects who failed to reach these thresholds underwent at least one additional confirmatory dynamic test.

The method for cortisol determination was changed in August 2010 from the Immulite® 2000 assay (Siemens) to a chemiluminescence immunoassay with the Architect i2000 (Abbott, UK). To assure comparability, quality controls and linear regression analysis were performed (data not shown) and results from the Immulite assay were aligned with the Architect i2000 assay. The Architect assay has coefficients of variation <10% for cortisol levels of 83–967 nmol/L (3.0-35.0 μg/dL).

A2.4.4 GHRH-Arginine Test

Following an overnight fast, patients had blood samples taken for GH and IGF-I measurement at 0 minutes. GHRH (Somatorelin, Ferring) 1 μg/kg was given as a bolus IV injection into one arm followed by the IV infusion of 0.5g/kg L-arginine monohydrochloride (Stockport Pharmaceuticals) as a 10% solution (30 g/300 mL up to a maximum of 30 g) in normal saline over 30 mins (Colao 2009). Further blood samples for GH estimation were taken at +30, 60, 90, 120 and 150 mins after the start of the arginine infusion.

GH cut offs to confirm GH deficiency varied according to age and BMI. For age groups 15-25 years old, 26-65 years old and older than 65 years, GH cut-offs were

respectively <15.6, <11.7, and <8.5 µg/L, <11.8, <8.1, and <5.5 µg/L, and <9.2, <6.1, and <4.0 µg/L, respectively, in lean (BMI <25.0 kg/m^2), overweight (BMI 25.0-30.0 kg/m^2) and obese (BMI >30.0 kg/m^2) subjects (Colao 2009). If amputations precluded accurate determination of BMI then cut-offs in the overweight range were used.

A2.4.5 Insulin Tolerance Test (ITT)

Following an overnight fast, basal blood samples were taken and IV insulin Actrapid (NovoNordisk) administered (0.15 U/kg). Blood samples were taken for GH, cortisol and glucose at 0, 30, 60, 90, and 120 mins. Blood glucose was also measured simultaneously. Once adequate hypoglycaemia (<2.2 mmol/L, <39.6 mg/dL) was achieved, hypoglycaemia was reversed with oral glucose and at least two further blood specimens were taken before test completion.

Abnormal cortisol response was defined as peak cortisol of <450 nmol/L (<16.3 µg/dL) providing adequate hypoglycaemia was achieved (using alignment of the previous 500 nmol/L cut-off to the new Architect i2000 assay). Severe GH deficiency was defined as a peak GH <3 µg/L (Plumpton 1969, Fish 1986, Molitch 2011).

A2.4.6 Cortisol Day Curve

Blood samples were taken from an IV cannula for serum cortisol estimation at 0900h, 1200h, 1500h, 1800 h and 2100 h (Immulite ® 2000 assay (Siemens) or Architect i2000 (Abbott, UK), and plasma ACTH at 0900h. Results helped confirm (cortisol <100 nmol/L or 3.62 µg/dL at 0900 or 1200h), or exclude (cortisol >400 nmol/L or 14.50 µg/dL at 0900h) ACTH deficiency, and assess the need for maintenance hydrocortisone replacement as opposed to just during intercurrent illness (Grossman 2010).

A2.4.7 Metyrapone Stimulation Test

Patients were given oral metyrapone (Metopirone™, Alliance Pharmaceuticals, Chippenham, UK) (30 mg/kg), at midnight with a snack, according to their body weight (<70 kg 2.0 g, 70-90 kg 2.5 g, >90 kg 3.0 g) (Steiner 1994, Cegla 2013). At

0900 h the following morning, blood samples were taken for serum cortisol, 11-DOC (Biosource, Oxford Biosystems, UK) and plasma ACTH (Immulite® 2000, Siemens). Hydrocortisone 10 mg was given orally to counteract hypocortisolism and the patients were discharged.

Metyrapone causes inhibition of 11 β-hydroxylase (used in the conversion of 11-deoxycortisol to cortisol) and cortisol suppression to <200 nmol/L (7.25 μg/dL) is the desired threshold to stimulate ACTH drive. Subjects were considered to be ACTH sufficient if 11-DOC was >200 nmol/L or, if the 11-DOC was unavailable, if ACTH >60 ng/L (Steiner 1994, Cegla 2013).

A2.4.8 Water Deprivation Test

This was carried out in two stages on non-fasted subjects (Vokes 1988). In Stage 1, patients drank no fluid from 0830-1630 h. Weight and urine volume (after urine passed and discarded at t=0) were recorded hourly. The test was stopped if >3% weight was lost. Urine specimens were taken for osmolality from the total hourly sample passes over 0830-0930 h (U1), 1130-1230 h (U2), 1430-1530 h (U3) and 1530-1630 h (U4). Blood samples were taken for osmolality and plasma sodium at 0900 h (P1), 1200 h (P2), 1500 h (P3) and 1600 h (P4).

In Stage 2, at 1630 h following the dehydration stage, Desmopressin (DDAVP 2 μg IM or 20 μg intra-nasally) was administered. Urine volumes were recorded and urine specimens for osmolality measurement were taken every hour until test completion at 2030 h.

Central diabetes insipidus was defined as plasma concentration to >300 mosmol/kg with inappropriately hypotonic urine (U3:P3 or U4:P4 ≤1.9) or urine osmolality <350 mosmol/kg. In addition, urine was required to concentrate to >150% of previous highest value following DDAVP administration.

A2.4.9 Neuropsychological Assessments

Each soldier completed a standardised neuropsychological test battery previously shown to be sensitive to cognitive impairment associated with TBI (Kinnunen 2011). The cognitive functions of specific interest were indexed by: (i) current verbal and

non-verbal reasoning ability via the Wechsler Abbreviated Scale of Intelligence Similarities and Matrix Reasoning subtests (Wechsler 1999); (ii) associative learning and memory via the immediate recall score on the People Test from the Doors and People Test (Baddeley 2011); (iii) the executive functions of set-shifting, inhibitory control, cognitive flexibility and word generation fluency via the Trail Making Test alternating-switch cost index (time to complete alternating letter and number Trails B - time to complete numbers only Trail A) and two indices from the Delis-Kaplan Executive Function System (Reitan 1958, Delis 2001), namely the inhibition/switching minus baseline score from the Colour-Word subtest (high scores indicating poor performance) and the total score on Letter Fluency; and (iv) information processing speed via the median reaction time for accurate responses on a simple computerised choice reaction task (Kinnunen 2011). The Wechsler Test of Adult Reading (WTAR) was also administered as a measure of pre-morbid intelligence (Green 2008).

A2.4.10 Structural Imaging

Each soldier had standard high-resolution T1 and gradient-echo (T2*) (1.75 x 1.75 x 2 mm^3) imaging to assess focal brain injury and evidence of microbleeds, superficial siderosis, presence and location of contusions and gross pituitary injury. All structural MR scans were reviewed by a single experienced consultant neuroradiologist. Contusion volume was calculated by converting the T1 images into standard 1 mm MNI brain space using FLIRT (FMRIB, University of Oxford, UK) and manually drawing a mask in the z plane.

MRI was performed on 3T Achieva scanner (Philips Medical Systems, Netherlands) using an 8 channel head coil. The T1 and T2*-weighted images were obtained prior to DTI. For DTI, diffusion weighted volumes with gradients applied in 16 non-collinear directions were collected in each of the four DTI runs, resulting in a total of 64 directions. The following parameters were used: 73 contiguous slices, slice thickness 2 mm, field of view 224 mm, matrix 128 x 128 (voxel size 1.75 x 1.75 x 2 mm^3), b value 1000 and four images with no diffusion weighting (b=0s/mm^2).

The images were registered to the b0 image by affine transformations to minimise distortion due to motion and eddy currents and then brain-extracted using Brain

Extraction Tool (Smith 2002) from the FMRIB Software Library image processing toolbox (Smith 2004, Woolrich 2009). FA maps were generated using the Diffusion Toolbox (Behrens 2003).

A2.4.11 DTI Analysis

DTI analysis used TBSS and non-parametric permutation based statistics for whole brain and ROI analysis (FMRIB software, FSL, University of Oxford, UK).

Voxelwise analysis of the FA, was carried out using TBSS in the FMRIB Software Library (Smith 2004, Smith 2006). Image analysis using TBSS involved a number of steps: (i) non-linear alignment of all subjects' FA images into common FMRIB58 FA template space; (ii) affine-transformation of the aligned images into standard MNI152 1mm space; (iii) averaging of the aligned FA images to create a 4D mean FA image; (iv) thinning of the mean FA image to create a mean FA 'skeleton' representing the centre of all WM tracts, and in this way removing partial-volume confounds; and (v) thresholding of the FA skeleton at FA 0.2 to suppress areas of extremely low mean FA and exclude those with considerable inter-individual variability. Non-parametric permutation-based statistics were employed using randomise with threshold-free cluster enhancement and 5000 permutations (Nichols 2002, Smith 2009). A threshold of $p < 0.05$ was then applied on the results, corrected for multiple comparisons. Age was included as a covariate of no interest in all TBSS analyses. ROI were defined using the John Hopkins University (JHU) WM atlas. We chose 10 areas that represented WM regions throughout the whole brain and have been shown to be damaged in nbTBI as well as mild bTBI (Kinnunen 2011, MacDonald 2011). These regions were: anterior and posterior internal capsules, cingulum, body/genu and splenium of the corpus callosum, cerebral peduncles, middle cerebellar peduncles, and uncinated fasciuli (Figure S1). In addition a cerebellum ROI mask was drawn manually and an orbitofrontal WM ROI mask made using the Washington University, St Louis criteria from the standard MNI152 1 mm T1 brain (Mac Donald 2011). A repeated measures ANOVA was performed to assess the overall significance effect of pituitary dysfunction on FA, including group, ROI and group x ROI interaction as independent variables, with post-hoc two-tailed t-tests for comparison of FA in individual ROIs between groups.

11 Appendix 3

In support of Chapter 5

Supplemental Methods

A3.1 Animal Blast Model

The study was conducted on 10 of terminally anaesthetised large white pigs in accordance with the Animal Scientific Procedures act 1986. Anaesthesia was induced with Isofluarane (5%) in O_2N_2O (FiO_2 0.3). The left common carotid artery was cannulated for arterial blood pressure monitoring and sampling for blood gas analysis. The left internal jugular brain was cannulated for saline and drug infusion. Once venous access had been obtained anaesthesia was continued with Alfaxan (Saffan^{TM}) and the Isoflurane discontinued. The right internal jugular vein was cannulated for recording the pulmonary arterial pressure, central venous pressure and mixed venous blood sampling using a balloon tipped arterial catheter. The left femoral artery art and vein were cannulated for late haemorrhage and resuscitation respectively. The bladder was cannulated and the spleen removed via a midline laparotomy. At the end of the surgery all surgical wounds were sutured. Arterial blood and central venous pressures were recorded throughout the experiment, the details of which were not provided for this project.

A3.2 Neuroimaging Data Acquisition

All MRI experiments were performed using 4.7T Direct Drive Agilent MR spectrometer with 40 Gauss/cm max gradient coil and Vnmr console (v0.1). We used 72 mm volume transmit/receive RF coil (m2m Imaging, Ohio,USA). The protocol included: localiser pilot for planning; FASTMAP shimming; anatomical 3D T1 weighted scan, gradient echo 3D scan for SWI, and Multi-slice, spin echo EPI with diffusion gradients for DTI. 3D T1K weighted scan was performed using MPRAGE sequence with the following parameters: FOV:80 x 80 x 90 cm transverse slab, matrix size: 256 x 256 x 192 (zero filled to 256), TR for invertion was 1.5 s, invertion time was TI=0.579 s; TE=2.08 ms. For SWI images, 3D gradient-echo sequences were acquired with the following parameters: same slab as for 3D T1-weighted; 80 x

80 x 90 cm transverse slab, matrix size 256 x 256 x 256; TR=30ms; TE=20 ms. Spin echo EPI with diffusion for DTI was acquired with the following: 60 transverse, 1.2 mm thick slices (acquired in two steps: 30 slices with 1.2 mm gap, and then again 30 slices with 1.2 mm gap but shifted by 1 slice, so that they could be combined to give continuous slices). FOV;80 cm x 80 cm; matrix 128 x 128; multishot spin echo EPI sequence; TE=30.0; TR=3.5s; 2 averages; 32 directions, b=0 and b=1000.

A3.3 Structural MRI and SWI analysis

The standard structural scans for each pig were reviewed by a consultant neuroradiologist, who was blinded to the pigs' blast injury status and resuscitation strategy. The clinical assessment of the scans involved a whole brain review of the MPRAGE and Gradient-echo sequences, as well as a second review, which looked at regions known to be predisposed to TBI damage: the orbitofrontal white matter, the corpus callosum, the hippocampus, brain-stem and cerebellum.

A3.4 Tissue preparation

Whole brain blocks were harvested coronally for the cortex and sagittally for the brainstem and cerebellum. The blocks were dehydrated with ethanol, washed with xylene and then embedded in paraffin wax. Immunocytochemistry was carried out on corresponding whole-brain sections throughout each brain. Serial cross-sections were cut to 6μm using a rotary microtome and then mounted on albumen coated glass slides to increase binding efficacy and then oven-dried at 60°C overnight. We mounted larger whole-brain slices on super mega (76 x 52 mm) slides, while the smaller slices were mounted on regular 25 x 75 mm slides.

All procedures were carried out at a constant room temperature unless otherwise indicated. We used Phosphate buffered saline solution (0.1 M PBS; pH 7.3) for all washes and dilutions. We dewaxed Slide-mounted tissue sections using two washes in xylene of 30 secs each and rehydrated through graded alcohol steps, in the order 100%, 100%, 90% and 70% industrial methylated spirits (IMS) for two mins each, before placing in distilled water.

We then incubated the tissue in 3% hydrogen peroxide (H_2O_2) in PBS for 30 mins to remove endogenous peroxidase activity, and then immersed it in distilled water.

A3.5 Haematoxylin and Eosin stain

The tissue was stained in Harris's Haematoxylin for eight mins then rinsed with tap water. We then differentiated them in 1% acid alcohol for 5 secs and blued in tap water. We applied a counterstain using 1% Eosin for 2 mins and differentiated in running tap water. The slides were then dehydrated by immersion in graded alcohol washes of 70%, 90% and 100% IMS for 5mins each, before being cleared in 2 washes of xylene for 5 mins each. Coverslips were then placed on top of the tissue using the xylene-based mountant, DPX, and left to dry overnight.

A3.6 Immunohistochemistry

For the immunohistochemistry staining for Iba1, APP and fibrinogen, the following methodology was used. Since the antibodies had not been previously used with porcine tissue, the appropriate protocols had to be derived by experimenting with various antigen retrieval techniques and exposure times. The concentrations were optimised for each polyclonal rabbit antibody using dilution series and human TBI tissue as a positive control.

To facilitate antigen retrieval, slides were treated with citrate buffer (pH 6.0) and heated in a steamer for 20 mins, then cooled in an ice bath before one wash in distilled water and three washes in PBS of 5 mins each.

The primary antibodies were prepared by diluting in primary diluent (490ml PBS, 1.5ml Triton X-100, 1.5 g sodium azide & 10 ml Bovine serum albumin (BSA)). Following the optimisation procedure, the resulting dilutions used: 1:12000 for the anti-fibrinogen antibody (Dako), 1:4000 for anti-Iba1 (Wako), 1:50000 for anti-APP (Millipore). The slides were blotted with paper towel at the back and sides to remove excess fluid and a circle was drawn around each section with a hydrophobic PAP pen to create a barrier to prevent applied fluids from leaving the slide. The slides were placed in moist incubation chambers. Tissue was covered with 150 µl (regular

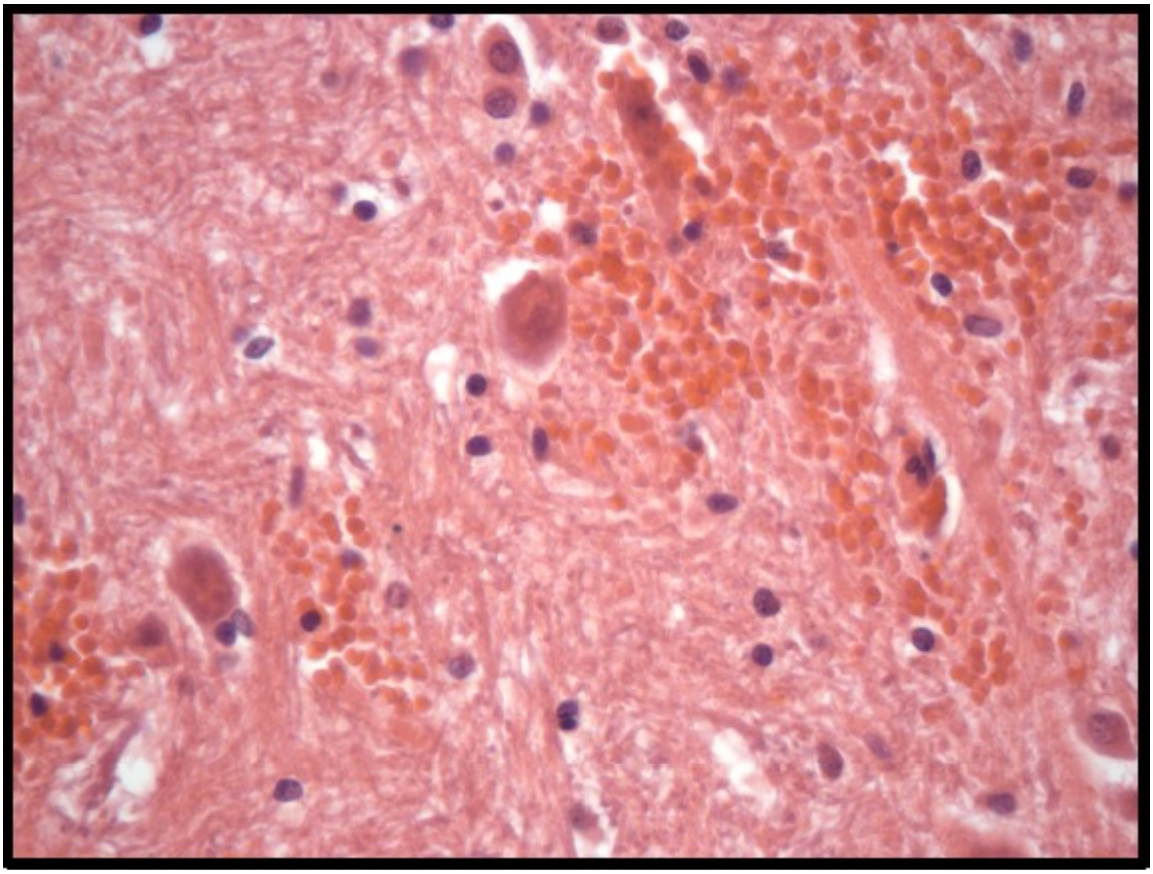
slide) and 450 µl (super mega slide) of the appropriate antibody and left overnight at 4°C.

Visualisation of the bound antibodies was performed using a Super Sensitive kit (BioGenex), which is based on polymer-HRP complex formation, according to the manufacturer's instructions. Briefly, the tissues were washed twice in PBS, incubated with for 20 mins with the enhancer, washed twice in PBS again, then incubated for a further 30 mins with the supersensitive reagent intensifier. The tissue was washed again using 3 PBS baths for 5 mins each before visualisation of the antigen of interest by exposure to a substrate of 0.1% 3-3'-diaminobenzidine-tetrahydrochloride (DAB) made up with DAB buffer. After 5 min, the reaction was terminated by rinsing twice in distilled water. Counterstain of Harris's Haematoxylin was applied for 3 secs before bluing the slides in running tap water. Finally, the slides were dehydrated, cleared and mounted with coverslips as described above.

A3.7 Histopathology analysis

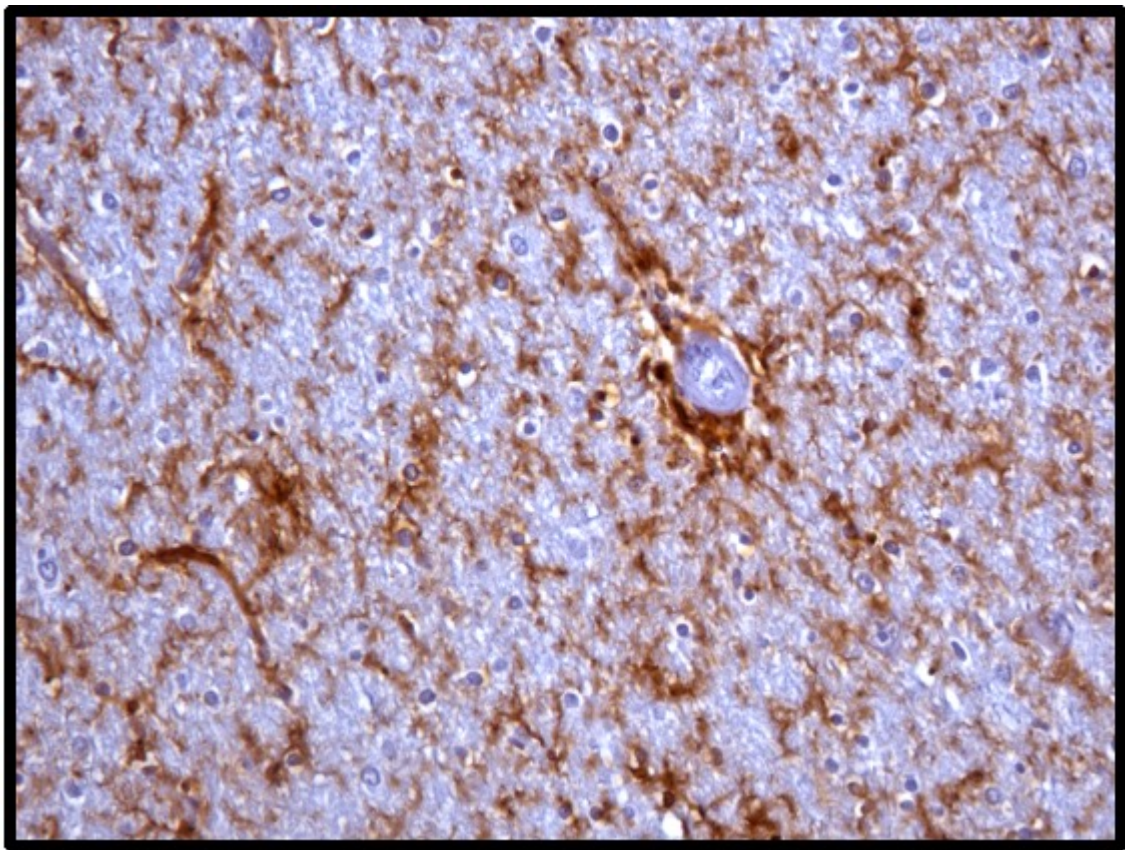
Analysis was performed examined using an Olympus BX50 microscope and photographs were obtained using an Olympus Vanox AHBT3microscope equipped with a MicroPublisher 3.3 RTV digital camera. The slides were assessed while blinded to the blast treatment and resuscitation strategy used on each pig and was examined for structural damage, microbleeding, axonal pathology and microglial activation. Various coronal and sagittal sections were chosen in order to inspect particular areas of interest: namely the orbitofrontal, parietal, hippocampal, cerebellar and brainstem regions.

A3.8 Supplementary Data

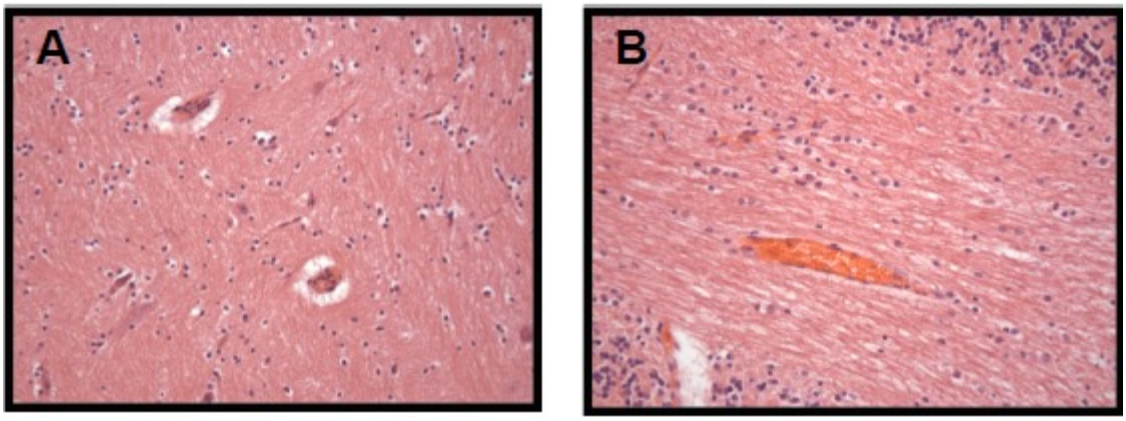


Supplementary Figure S1. H&E stain of the medulla of pig B2. Several areas were seen with extravascular erythrocytes

Supplementary Figure S2. H&E stain of the Hippocampus of pig B10. There is some evidence of oedematous character between the pyramidal cells and molecular layer



Supplementary Figure S3 – Iba1 immunostaining showing perivascular activation of microglia



Supplementary Figure S4 – example of perivascular oedema (A) compared normal vessels (B)

Supplementary Table S1. Summary of results from structural H&E stain

Pig		Ependymal Stripping	Hippocampal Oedema	Regions of the brain with Perivascular Oedema						
				Corpus Callosum	Orbitofrontal WM	Hippocampus	Occipital Lobe	Cerebellum	Pons	Medulla
Blast	B1	+	-	+	+	+	+	-	+	+
	B2	+	+	+	+	+	+	-	+	+
	B3	+	-	+	+	+	+	-	+	+
	B8	-	-	-	+	+	+	-	+	+
	B9	-	-	+	+	+	+	n/a	+	n/a
	B10	+	+	+	+	+	-	n/a	+	n/a
Non-blast	B4	-	-	+	+	+	+	-	+	+
	B5	-	-	+	+	+	+	-	+	-
	B6	-	-	+	+	+	+	-	+	+
	B7	-	-	+	+	+	-	-	+	+

***change is not extensive enough to rule as definite pathology**

n/a: the brainstem sections of pigs B9 and B10 were cut coronally through the pons rather than sagittally, thus the cerebellum and medulla were not assessed.

Supplementary Table S2. Summary of APP staining

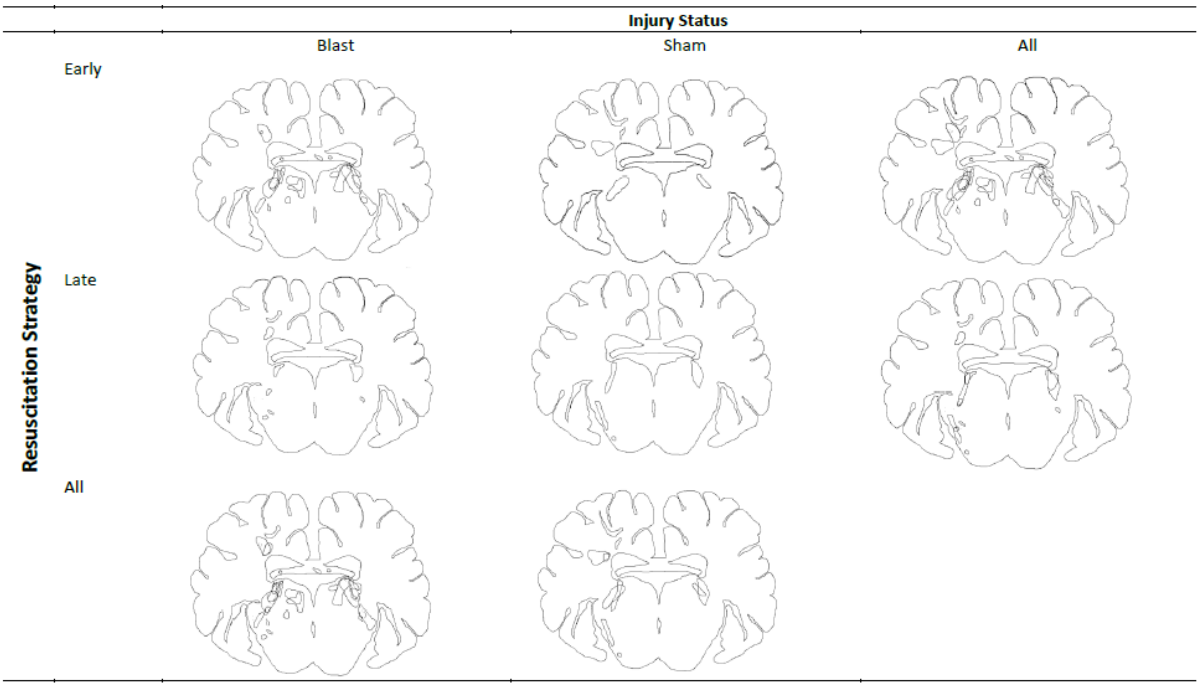
Pig		Orbital WM	Mid-frontal	Internal Capsule/ Thalamus	Parietal Cortical WM
Blast	B1	-	-	+	-
	B2	+	-*	+	+
	B3	-*	-	+	-
	B8	-*	-*	+	+
	B9	-*	-*	+	+
	B10	-	-*	-*	-
Non-blast	B4	-	-*	+	+
	B5	-*	-*	+	-*
	B6	-*	-*	+	+
	B7	-*	-*	-*	-

*isolated bulbs of accumulation observed

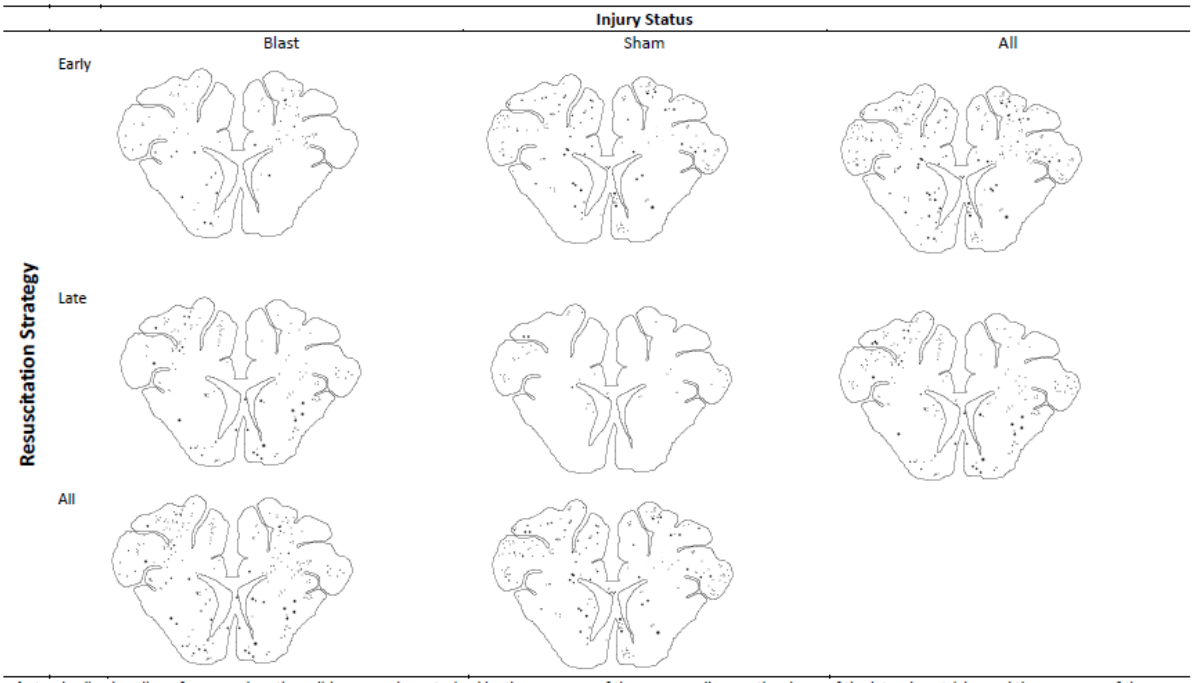
Supplementary Table S3. Summary of Iba1 immunoreactivity

Pig	Severity rating of activated microglia			Hippocampus	Basal ganglia	Corpus Callosum	General localisation
	Frontal	Mid-frontal	Parietal				
B1	**	**	**	+	+	+	PV, throughout WM
B2	**	**	**	+	+	+	Co-localised with APP pathology
B3	***	**	**	-	+	+	Cortical WM
B4	**	**	***	+	-	+	Cortical WM, PV
B5	*	**	**	-	-	+	Posterior cortical WM, co-localised with APP pathology
B6	*	*	**	-	+	+	Posterior lateral WM, co-localised with APP pathology
B7	**	**	**	-	-	+	PV, cortical WM
B8	**	**	**	-	+	+	Cortical WM, co-localised with APP pathology
B9	***	**	**	+	+	+	Throughout WM (esp main WM tracts), PV, co-localised with APP pathology
B10	***	***	***	+	+	+	Throughout WM, PV, co-localised with APP pathology

(-): no activation; (+): activation; Severity rating scale: (*): low; (**): moderate; (***): severe; PV: perivascular



Supplementary Figure S5. Schematic demonstrating the localisation of APP pathology mapped onto a standardised space



A standardised outline of a coronal section: slides were characterised by the presence of the corpus callosum, the shape of the lateral ventricles and the presence of the anterior striatum. The size of the marked dot coarsely denotes the size of vessel affected: small dots denote capillaries while larger dots are assumed to be arteriole/venules.

Supplementary Figure S6. Schematic demonstrating the localisation of fibrinogen leakage mapped onto a standardised space

12 References

Adams JH, Doyle D, Ford I, Gennarelli TA, Graham DI, McLellan DR. Diffuse axonal injury in head injury: definition, diagnosis and grading. Histopathology, 1989;15 (1): 49-59.

Adams JH, Graham DI, Gennarelli TA, Maxwell WL. Diffuse axonal injury in non-missile head injury. J Neurol Neurosurg Psychiatry. 1991;54(6):481-3.

Adams JH, Graham DI, Murray LS, Scott G. Diffuse axonal injury due to nonmissile head injury in humans: an analysis of 45 cases. Ann Neurol. 1982;12:557-563.

Adams JH. Head injury. In: Adams JH, Corsellis JAN, Duchen LW, eds. Greenfield's Neuropathology. 4th ed. New York: John Wiley and Sons; 1984: 85–124.

Addis DR, Moscovitch M, McAndrews MP. Consequences of hippocampal damage across the autobiographical memory network in left temporal lobe epilepsy. Brain. 2007;130(Pt 9):2327-42.

Aimaretti G, Ambrosio MR, Di Somma C, Gasperi M, Cannavò S, Scaroni C, Fusco A, Del Monte P, De Menis E, Faustini-Fustini M, Grimaldi F, Logoluso F, Razzore P, Rovere S, Benvenga S, Degli Uberti EC, De Marinis L, Lombardi G, Mantero F, Martino E, Giordano G, Ghigo E. Residual pituitary function after brain injury-induced hypopituitarism: a prospective 12-month study. J Clin Endocrinol Metab. 2005;90(11):6085-92.

Akiyama Y, Miyata K, Harada K, Minamida Y, Nonaka T, Koyanagi I, Asai Y, Houkin K. Susceptibility-weighted magnetic resonance imaging for the detection of cerebral microhemorrhage in patients with traumatic brain injury. Neurol Med Chir (Tokyo), 2009;49 (3): 97-99; discussion 99.

Alexander AL, Lee JE, Lazar M, Field AS. Diffusion tensor imaging of the brain. Neurotherapeutics. 2007;4(3):316-29.

Andreasen NC, O'Leary DS, Paradiso S, Cizadlo T, Arndt S, Watkins GL, Ponto LL, Hichwa RD. The cerebellum plays a role in conscious episodic memory retrieval. Hum Brain Mapp. 1999;8(4):226-34.

Andriessen TM, Jacobs B, Vos PE. Clinical characteristics and pathophysiological mechanisms of focal and diffuse traumatic brain injury. J Cell Mol Med. 2010;14(10):

2381-92.

Arfanakis K, Haughton VM, Carew JD, Rogers BP, Dempsey RJ, Meyerand ME. Diffusion tensor MR imaging in diffuse axonal injury. AJNR Am J Neuroradiol, 2002;23 (5): 794-802.

Armonda RA, Bell RS, Vo AH, Ling G, DeGraba TJ, Crandall B, Ecklund J,Campbell WW. Wartime traumatic cerebral vasospasm: recent review of combat casualties. Neurosurgery. 2006;59(6): 1215-25; discussion 1225.

Assaf Y, Pasternak O. Diffusion tensor imaging (DTI)-based white matter mapping in brain research: a review. J Mol Neurosci. 2008;34(1):51-61.

Babikian T, Freier MC, Tong KA, Nickerson JP, Wall CJ, Holshouser BA, Burley T, Riggs M. L, Ashwal S. Susceptibility weighted imaging: neuropsychologic outcome and pediatric head injury. Pediatr Neurol, 2005;33 (3): 184-94.

Baddeley A. Doors and people test: a test of visual and verbal recall and recognition. 2011. Thames Valley Test Company, Bury St Edmunds, Suffolk, UK.

Baddeley AD, Emslie H, Nimmo-Smith I. Doors and people test: a test of visual and verbal recall and recognition. Bury-St-Edmunds: Thames Valley Test Company; 1994.

Bains M, Hall ED. Antioxidant therapies in traumatic brain and spinal cord injury. Biochim Biophys Acta. 2012;1822:675–684.

Baker SP, O'Neill B, Haddon W Jr, Long WB. The injury severity score: a method for describing patients with multiple injuries and evaluating emergency care. J Trauma. 1974;14(3):187-96.

Bauman RA, Ling G, Tong L, Januszkiewicz A, Agoston D, Delanerolle N, Kim Y, Ritzel D, Bell R, Ecklund J, Armonda R, Bandak F, Parks S. An introductory characterization of a combat-casualty-care relevant swine model of closed head injury resulting from exposure to explosive blast. J Neurotrauma. 2009;26(6):841-60.

Baxter D, Sharp DJ, Feeney C, Papadopoulou D, Ham TE, Jilka S, Hellyer PJ, Patel MC, Bennett AN, Mistlin A, McGilloway E, Midwinter M, Goldstone AP. Pituitary dysfunction after blast traumatic brain injury: The UK BIOSAP study. Ann Neurol. 2013;74(4):527-36.

Bazarian JJ, Zhong J, Blyth B, Zhu T, Kavcic V, Peterson D. Diffusion tensor imaging detects clinically important axonal damage after mild traumatic brain injury: a pilot study. J Neurotrauma, 2007;24 (9): 1447-59.

Beck AT, Steer RA, Ball R, Ranieri W. Comparison of Beck Depression Inventories -IA and -II in psychiatric outpatients. J Pers Assess. 1996;67:588-97.

Behrens TE, Woolrich MW, Jenkinson M, Johansen-Berg H, Nunes RG, Clare S, Matthews PM, Brady JM, Smith SM. Characterization and propagation of uncertainty in diffusion-weighted MR imaging. Magn Reson Med, 2003;50 (5): 1077-88.

Benzinger TL, Brody D, Cardin S, Curley KC, Mintun MA, Mun SK, Wong KH, Wrathall JR. Blast-related brain injury: imaging for clinical and research applications: report of the 2008 St. Louis workshop. J Neurotrauma. 2009;26(12):2127-44.

Berk SL, McCabe WR. Meningitis caused by gram-negative bacilli. Ann Intern Med. 1980;93(2):253-60.

Bhattacharjee Y. Neuroscience. Shell shock revisited: solving the puzzle of blast trauma. Science. 2008;319(5862): 406-8.

Bigler E. The Lesion(s) in Traumatic Brain Injury: Implications for Clinical Neuropsychology. Archives of clinical neuropsychology 2000;16:1-39.

Bigler ED. Distinguished Neuropsychologist Award Lecture 1999. The lesion(s) in traumatic brain injury: implications for clinical neuropsychology. Arch Clin Neuropsychol. 2001;16(2): 95-131.

Bitonte R, Tribuzio B, Hecht K, DeSanto DJ. Mild Traumatic Brain Injuries were previously undiagnosable, and therefore treatment uncertain, and damages speculative. International Brain Injury Association. Available at: http://www.internationalbrain.org/mild-traumatic-brain-injuries-were-prev-undiagnosable-therefore-treatment-uncertain-and-damages/. Last accessed January 2016.

Blumbergs PC, Scott G, Manavis J, Wainwright H, Simpson DA, McLean AJ. Staining of amyloid precursor protein to study axonal damage in mild head injury. Lancet. 1994;344(8929):1055-6.

Bondanelli M, Ambrosio MR, Cavazzini L, Bertocchi A, Zatelli MC, Carli A, Valle D, Basaglia N, Uberti EC. Anterior pituitary function may predict functional and cognitive outcome in patients with traumatic brain injury undergoing rehabilitation. J Neurotrauma. 2007;24(11):1687-97.

Bonnelle V, Ham TE, Leech R, Kinnunen KM, Mehta MA, Greenwood RJ, Sharp DJ. Salience network integrity predicts default mode network function after traumatic brain injury. Proc Natl Acad Sci U S A. 2012;109(12):4690-5.

Bonnelle V, Leech R, Kinnunen KM, Ham TE, Beckmann CF, De Boissezon X, Greenwood RJ, Sharp DJ. Default mode network connectivity predicts sustained attention deficits after traumatic brain injury. J Neurosci. 2011;31(38):13442-51.

Bowen RA. Functional Anatomy of the Hypothalamus and Pituitary Gland. 2001 Available at: http://arbl.cvmbs.colostate.edu/hbooks/pathphys/endocrine/hypopit/anatomy.html. Last accessed July 2015.

Brain injury – the facts. Brain Injury Group. Available at: http://www.braininjurygroup.co.uk/living-with-brain-injury/brain-injury-the-facts/. Last accessed May 2015.

Brøchner AC, Toft P. Pathophysiology of the systemic inflammatory response after major accidental trauma. Scand J Trauma Resusc Emerg Med. 2009 ;17:43.

Buysse DJ, Reynolds CF, Monk TH, Berman SR, Kupfer DJ. The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. Psychiatry Res. 1989;28:193-213.

Calabrese E, Du F, Garman RH, Johnson GA, Riccio C, Tong LC, Long JB. Diffusion tensor imaging reveals white matter injury in a rat model of repetitive blast-induced traumatic brain injury. J Neurotrauma. 2014;31(10):938-50.

Calzia E, Huber-Lang M, Ignatius A, Radermacher P, Thiemermann AC. Modeling traumatic-hemorrhagic shock--nothing is simple and easy. Shock. 2012;38(6):685-6.

Cavada C, Compañy T, Tejedor J, Cruz-Rizzolo RJ, Reinoso-Suárez F. The anatomical connections of the macaque monkey orbitofrontal cortex. A review. Cereb Cortex. 2000;10(3):220-42.

CDC - Explosions and Blast Injuries - A Primer for Clinicians. Available at: http://www.cdc.gov/masstrauma/preparedness/primer.pdf. Last accessed July 2015.

CDC. Traumatic brain injury in the United States: A report to Congress. Atlanta, GA, Center for Disease Control and Prevention (2001).

Cegla J, Jones B, Seyani L, Papadoulou D, Wynne K, Martin NM, Meeran K, Chapman R, Donaldson M, Goldstone AP, Tan T. Comparison of the overnight metyrapone and glucagon stimulation tests in the assessment of secondary hypoadrenalism. Clin Endocrinol (Oxf). 2013;78(5):738-42.

Cernak I and Noble-Haeusslein LJ. Traumatic brain injury: an overview of pathobiology with emphasis on military populations. J Cereb Blood Flow Metab. 2010;30:255-266.

Cernak I, Vink R, Zapple DN, Cruz MI, Ahmed F, Chang T, Fricke ST, Faden AI. The pathobiology of moderate diffuse traumatic brain injury as identified using a new experimental model of injury in rats. Neurobiol Dis. 2004;17(1): 29-43.

Champion HR, Holcomb JB, Young LA. Injuries from explosions: physics, biophysics, pathology, and required research focus. The Journal of Trauma and Acute Care Surgery 2009;66 (5): 1468-77.

Chapman JC, Diaz-Arrastia R.Military traumatic brain injury: a review. Alzheimers Dement. 2014;10(3 Suppl):S97-104.

Chastain CA, Oyoyo UE, Zipperman M, Joo E, Ashwal S, Shutter LA, Tong KA. Predicting outcomes of traumatic brain injury by imaging modality and injury distribution. J Neurotrauma. 2009;26(8):1183-96.

Chavhan GB, Babyn PS, Thomas B, Shroff MM, Haacke EM. Principles, techniques, and applications of T2*-based MR imaging and its special applications. Radiographics. 2009;29(5):1433-49.

Cherrier MM. Testosterone effects on cognition in health and disease. Front Horm Res. 2009;37:150-62.

Chesser GS. Afghanistan Casualties: Military Forces and Civilians Congressional Research Service 2012;7:1-8.

Christman CW, Grady MS, Walker SA, Holloway KL, Povlishock JT. Ultrastructural

studies of diffuse axonal injury in humans. J Neurotrauma. 1994;11(2):173-86.

Colao A, Di SC, Savastano S, Rota F, Savanelli MC, Aimaretti G, Lombardi G. A reappraisal of diagnosing GH deficiency in adults: role of gender, age, waist circumference, and body mass index. J Clin Endocrinol Metab. 2009;94:4414-22.

Courtney A, Courtney M. A thoracic mechanism of mild traumatic brain injury due to blast pressure waves. Medical Hypotheses. 2009;72 (1): 76-83.

Courtney CH, McAllister AS, McCance DR, Hadden DR, Leslie H, Sheridan B, Atkinson AB. The insulin hypoglycaemia and overnight metyrapone tests in the assessment of the hypothalamicpituitary- adrenal axis following pituitary surgery. Clin Endocrinol (Oxf). 2000;53:309-12.

de Lanerolle NC, Bandak F, Kang D, Li AY, Du F, Swauger P, Parks S, Ling G, Kim JH. Characteristics of an explosive blast-induced brain injury in an experimental model. Journal of Neuropathology Experimental Neurology. 2011;70(11): 1046-1057.

Delis DC, Kaplan E, Kramer JH. Delis-Kaplan Executive Function System. 2001. The Psychological Corporation, San Antonio, Texas, USA.

DeWitt DS, Jenkins LW, Prough DS. Enhanced vulnerability to secondary ischemic insults after experimental traumatic brain injury. New Horizons (Baltimore, Md.). 1995;3(3): 376-383.

DeWitt DS, Prough DS. Blast-induced brain injury and posttraumatic hypotension and hypoxemia. Journal of Neurotrauma 2009;26 (6): 877-87.

DeWitt DS, Prough DS. Traumatic cerebral vascular injury: the effects of concussive brain injury on the cerebral vasculature. J Neurotrauma. 2003;20(9):795-825.

Dunst B, Benedek M, Koschutnig K, Jauk E, Neubauer AC. Sex differences in the IQ-white matter microstructure relationship: a DTI study. Brain Cogn. 2014;91:71-8.

Duvernay HM. The Human Hippocampus - An Atlas of Applied Anatomy 1988. Springer-Verlag Berlin Heidelberg GmbH.

Dyrby TB, Baaré WF, Alexander DC, Jelsing J, Garde E, Søgaard LV. An ex vivo imaging pipeline for producing high-quality and high-resolution diffusion-weighted imaging datasets. Hum Brain Mapp. 2011;32(4):544-63.

Edwards P, Arango M, Balica L Cottingham R, El-Sayed H, Farrell B, Fernandes J, Gogichaisvili T, Golden N, Hartzenberg B, Husain M, Ulloa MI, Jerbi Z, Khamis H, Komolafe E, Laloë V, Lomas G, Ludwig S, Mazairac G, Muñoz Sanchéz Mde L, Nasi L, Olldashi F, Plunkett P, Roberts I, Sandercock P, Shakur H, Soler C, Stocker R, Svoboda P, Trenkler S, Venkataramana NK, Wasserberg J, Yates D, Yutthakasemsunt S; CRASH trial collaborators..Final results of MRC CRASH, a randomised placebo-controlled trial of intravenous corticosteroid in adults with head injury-outcomes at 6 months. Lancet. 2005;365(9475):1957-9.

Elder GA, Cristian A. Blast-related mild traumatic brain injury: mechanisms of injury and impact on clinical care. Mt Sinai J Med. 2009;76(2):111-8.

Elder GA, Gama Sosa MA, De Gasperi R, Stone JR, Dickstein DL, Haghighi F, Hof PR, Ahlers ST. Vascular and inflammatory factors in the pathophysiology of blast-induced brain injury. Front Neurol. 2015;6: 48.

Elder GA, Mitsis EM, Ahlers ST, Cristian A. Blast-induced Mild Traumatic Brain Injury. Psychiatric Clinics of North America 2010b;33 (4): 757-81.

Facts About TBI in the USA. Brain Trauma Foundation. Available at: https://www.braintrauma.org/tbi-faqs/tbi-statistics/. Last accessed May 2015.

Fiad TM, Kirby JM, Cunningham SK, McKenna TJ. The overnight single-dose metyrapone test is a simple and reliable index of the hypothalamic-pituitary-adrenal axis. Clin Endocrinol (Oxf). 1994;40: 603-9.

Fish HR, Chernow B, O'Brian JT. Endocrine and neurophysiologic responses of the pituitary to insulin-induced hypoglycemia: a review. Metabolism. 1986;35:763-80.

Friedland D, Hutchinson P. Classification of traumatic brain injury. Available at: http://www.acnr.co.uk/2013/07/classification-of-traumatic-brain-injury/. Last accessed Jan 2016.

Friedman MJ, Resick PA, Bryant RA, Brewin CR. Considering PTSD for DSM-5. Depress Anxiety. 2011;28(9):750-69.

Fuller G, Bouamra O, Woodford M, Jenks T, Patel H, Coats TJ, Oakley P, Mendelow AD, Pigott T, Hutchinson PJ, Lecky F. Temporal trends in head injury outcomes from 2003 to 2009 in England and Wales. Br J Neurosurg. 2011;25(3): 414-21.

Garner J, Watts S, Parry C, Bird J, Kirkman E. Development of a large animal model for investigating resuscitation after blast and hemorrhage. World Journal of Surgery. 2009;33 (10): 2194-2202.

Gawande A. Casualties of war--military care for the wounded from Iraq and Afghanistan. N Engl J Med. 2004;351(24):2471-5.

Gennarelli TA, Graham DI. Neuropathology of the Head Injuries. Semin Clin Neuropsychiatry, 1998;3 (3): 160-75.

Gennarelli TA. The pathobiology of traumatic brain injury. Neuroscientist 1997;3:73–81.

Gentleman SM, Nash MJ, Sweeting CJ, Graham DI, Roberts GW. Beta-amyloid precursor protein (beta APP) as a marker for axonal injury after head injury. Neurosci Lett. 1993;160(2):139-44.

Gentleman SM, Roberts GW, Gennarelli TA, Maxwell WL, Adams JH, Kerr S, Graham DI. Axonal injury: a universal consequence of fatal closed head injury? Acta Neuropathol. 1995;89(6):537-43.

Gentry L. R. Imaging of closed head injury. Radiology, 1994;191 (1): 1-17.

Gentry LR, Godersky JC, Thompson B. MR imaging of head trauma: review of the distribution and radiopathologic features of traumatic lesions. AJR Am J Roentgenol, 1988;150 (3): 663-72.

Gholipour B. How the Human Brain Gets Its Wrinkles. August 2014. Available at: http://www.livescience.com/47421-human-brain-wrinkles.html. Last accessed Feb 2016.

Giordano R, Picu A, Bonelli L, Balbo M, Berardelli R, Marinazzo E, Corneli G, Ghigo E, Arvat E. Hypothalamus-pituitary-adrenal axis evaluation in patients with hypothalamo-pituitary disorders: comparison of different provocative tests. Clin Endocrinol (Oxf). 2008;68:935-41.

Glasgow Coma Scale. Available at: http://www.coma.ulg.ac.be/images/gcs_comments.pdf. Last accessed May 2015.

Goldstein LE, Fisher AM, Tagge CA, Zhang XL, Velisek L, Sullivan JA, Upreti C, Kracht JM, Ericsson M, Wojnarowicz MW, Goletiani CJ, Maglakelidze GM, Casey N, Moncaster JA, Minaeva O, Moir RD, Nowinski CJ, Stern RA, Cantu RC, Geiling J, Blusztajn JK, Wolozin BL, Ikezu T, Stein TD, Budson AE, Kowall NW, Chargin D, Sharon A, Saman S, Hall GF, Moss WC, Cleveland RO, Tanzi RE, Stanton PK, McKee AC. Chronic traumatic encephalopathy in blast-exposed military veterans and a blast neurotrauma mouse model. Sci Transl Med. 2012;4(134):134ra60.

Grady MS, McLaughlin MR, Christman CW, Valadka AB, Fligner CL, Povlishock JT. The use of antibodies targeted against the neurofilament subunits for the detection of diffuse axonal injury in humans. J Neuropathol Exp Neurol, 1993;52 (2): 143-52.

Green RE, Melo B, Christensen B, Ngo LA, Monette G, Bradbury C. Measuring premorbid IQ in traumatic brain injury: an examination of the validity of the Wechsler Test of Adult Reading (WTAR). J Clin Exp Neuropsychol. 2008;30:163-72.

Gregoire SM, Chaudhary UJ, Brown MM, Yousry TA, Kallis C, Jäger HR, Werring DJ. The Microbleed Anatomical Rating Scale (MARS): reliability of a tool to map brain microbleeds. Neurology. 2009;73(21):1759-66.

Grossman AB. Clinical Review: The diagnosis and management of central hypoadrenalism. J Clin Endocrinol Metab. 2010;95:4855-63.

Guerrero AF, Alfonso A. Traumatic brain injury-related hypopituitarism: a review and recommendations for screening combat veterans. Mil Med. 2010;175(8):574-80.

Gustavsson A, Svensson M, Jacobi F, Allgulander C, Alonso J, Beghi E, Dodel R, Ekman M, Faravelli C, Fratiglioni L, Gannon B, Jones DH, Jennum P, Jordanova A, Jonsson L, Karampampa K, Knapp M, Kobelt G, Kurth T, Lieb R, Linde M, Ljungcrantz C, Maercker A, Melin B, Moscarelli M, Musayev A, Norwood F, Preisig M, Pugliatti M, Rehm J, Salvador-Carulla L, Schlehofer B, Simon R, Steinhausen HC, Stovner LJ, Vallat JM, den Bergh PV, van Os J, Vos P, Xu W, Wittchen HU, Jonsson B, Olesen J. Cost of disorders of the brain in Europe 2010. Eur Neuropsychopharmacol, 2011;21 (10): 718-79.

Haacke EM, Xu Y, Cheng YC, Reichenbach JR. Susceptibility weighted imaging (SWI). Magn Reson Med, 2004;52 (3): 612-18.

Haughton V, Daniels DL, Hudetz A. The Cerebral Circulation: A Primer for

Neuroradiologists Initiating Functional Magnetic Resonance. International Journal of Neuroradiology 1998;4(5): 334-41.

Hariri RJ, Firlick AD, Shepard SR, Cohen DS, Barie PS, Emery III JM, Ghajar JB. Traumatic brain injury, hemorrhagic shock, and fluid resuscitation: effects on intracranial pressure and brain compliance. Journal of Neurosurgery. 1993; 79(3): 421-427.

Hartings JA, Wilson JA, Hinzman JM, Pollandt S, Dreier JP, DiNapoli V, Ficker DM, Shutter LA, Andaluz N. Spreading depression in continuous electroencephalography of brain trauma. Ann Neurol. 2014;76(5): 681-94.

Hawley A. Trauma management on the battlefield: a modern approach. J R Army Med Corps. 1996;142:120-5.

Hernandez-Ontiveros DG, Tajiri N, Acosta S, Giunta B, Tan J, Borlongan CV. Microglia activation as a biomarker for traumatic brain injury. Front Neurol. 2013;4: 30.

Heteroherent 2011. Available at: http://heteroherent.blogspot.co.uk/2011/03/blog-post_9028.html. Last accessed Feb 2016.

Hicks RR, Smith DH, Lowenstein DH, Saint Marie R, McIntosh TK. Mild experimental brain injury in the rat induces cognitive deficits associated with regional neuronal loss in the hippocampus. J Neurotrauma. 1993 Winter;10(4):405-14.

Hinzman JM, Andaluz N, Shutter LA, Okonkwo DO, Pahl C, Strong AJ, Dreier JP, Hartings JA. Inverse neurovascular coupling to cortical spreading depolarizations in severe brain trauma. Brain. 2014;137(Pt 11): 2960-72.

Hoogland IC, Houbolt C, van Westerloo DJ, van Gool WA, van de Beek D. Systemic inflammation and microglial activation: systematic review of animal experiments. J Neuroinflammation. 2015;12(1):114.

Howe LL. Giving context to post-deployment post-concussive-like symptoms: blast-related potential mild traumatic brain injury and comorbidities. The Clinical Neuropsychologist 2009;23 (8): 1315-37.

http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/TBSS

http://radiopaedia.org/articles/fourth-ventricle

http://www.inertproducts.com/. Last accessed Jan 2016.

Hunt SM, McEwen J, McKenna SP. Measuring health status: a new tool for clinicians and epidemiologists. J R Coll Gen Pract. 1985;35:185-8.

Hyder AA, Wunderlich CA, Puvanachandra P, Gururaj G, Kobusingye OC. The impact of traumatic brain injuries: a global perspective. NeuroRehabilitation, 2007; 22 (5): 341-53.

Injury Severity Score. Trauma.org. Available at: http://www.trauma.org/archive/scores/iss.html. Last accessed May 2015.

Johansson CB, Momma S, Clarke DL, Risling M, Lendahl U, Frisén J. Identification of a neural stem cell in the adult mammalian central nervous system. Cell. 1999;96(1):25-34.

Johns MW. A new method for measuring daytime sleepiness: the Epworth sleepiness scale. Sleep. 1991;14:540-5.

Johnson VE, Stewart W, Smith DH. Axonal pathology in traumatic brain injury. Exp Neurol. 2013;246:35-43.

Jorge RE, Acion L, White T, Tordesillas-Gutierrez D, Pierson R, Crespo-Facorro B, Magnotta VA. White matter abnormalities in veterans with mild traumatic brain injury. Am J Psychiatry. 2012;169(12):1284-91.

Karr JE, Areshenkoff CN, Duggan EC, Garcia-Barrera MA. Blast-related mild traumatic brain injury: a Bayesian random-effects meta-analysis on the cognitive outcomes of concussion among military personnel. Neuropsychol Rev. 2014;24(4):428-44.

Kinnunen KM, Greenwood R, Powell JH, Leech R, Hawkins PC, Bonnelle V, Patel MC, Counsell SJ, Sharp DJ. White matter damage and cognitive impairment after traumatic brain injury. Brain. 2011;134(Pt 2):449-63.

Kirkman E, Watts S, Cooper G. Blast injury research models. Philos Trans R Soc Lond B Biol Sci. 2011;366(1562): 144-59.

Kokshoorn NE, Smit JW, Nieuwlaat WA, Tiemensma J, Bisschop PH, Groote Veldman R, Roelfsema F, Franken AA, Wassenaar MJ, Biermasz NR, Romijn JA, Pereira AM. Low prevalence of hypopituitarism after traumatic brain injury: a

multicenter study. Eur J Endocrinol. 2011;165(2):225-31.

Kokshoorn NE, Wassenaar MJ, Biermasz NR, Roelfsema F, Smit JW, Romijn JA, Pereira AM. Hypopituitarism following traumatic brain injury: prevalence is affected by the use of different dynamic tests and different normal values. Eur J Endocrinol. 2010;162(1):11-8.

Kotapka MJ, Gennarelli TA, Graham DI, Adams JH, Thibault LE, Ross DT, Ford I. Selective vulnerability of hippocampal neurons in acceleration-induced experimental head injury. J Neurotrauma. 1991 Winter;8(4):247-58.

Kraus JF, Morgenstern H, Fife D, Conroy C, Nourjah P. Blood alcohol tests, prevalence of involvement, and outcomes following brain injury. Am J Public Health 1989; 79 (3): 294-99.

Kraus MF, Susmaras T, Caughlin BP, Walker CJ, Sweeney JA, Little DM. White matter integrity and cognition in chronic traumatic brain injury: a diffusion tensor imaging study. Brain. 2007;130(Pt 10):2508-19.

Kumar R, Husain M, Gupta RK, Hasan KM, Haris M, Agarwal AK, Pandey CM, Narayana PA. Serial changes in the white matter diffusion tensor imaging metrics in moderate traumatic brain injury and correlation with neuro-cognitive function. J Neurotrauma. 2009;26(4):481-95.

Langlois JA, Rutland-Brown W, Thomas KE. Traumatic brain injury in the United states: emergency department visits, hospitalizations, and deaths. Atlanta, GA: Centers for Disease Control and Prevention, National Center for Injury Prevention and Control (2006a).

Langlois JA, Rutland-Brown W, Wald MM. The epidemiology and impact of traumatic brain injury: a brief overview. J Head Trauma Rehabil, 2006b;21 (5): 375-78.

Lee LL, Galo E, Lyeth BG, Muizelaar JP, Berman RF. Neuroprotection in the rat lateral fluid percussion model of traumatic brain injury by SNX-185, an N-type voltage-gated calcium channel blocker. Exp Neurol. 2004;190: 70–78.

Leong KS, Walker AB, Martin I, Wile D, Wilding J, MacFarlane IA. An audit of 500 subcutaneous glucagon stimulation tests to assess growth hormone and ACTH secretion in patients with hypothalamic-pituitary disease. Clin Endocrinol (Oxf). 2001;54(4):463-8.

Leung LY, VandeVord PJ, Dal Cengio AL, Bir C, Yang KH, King AI. Blast related neurotrauma: a review of cellular injury. Mol Cell Biomech. 2008;5 (3): 155-68.

Levin HS, Wilde E, Troyanskaya M, Petersen NJ, Scheibel R, Newsome M, Radaideh M, Wu T, Yallampalli R, Chu Z, Li X. Diffusion tensor imaging of mild to moderate blast-related traumatic brain injury and its sequelae. J Neurotrauma. 2010;27(4):683-94.

Lu CH, Chang WN, Chuang YC, Chang HW. The prognostic factors of adult gram-negative bacillary meningitis. J Hosp Infect. 1998;40(1):27-34.

Mac Donald CL, Dikranian K, Bayly P, Holtzman D, Brody D. Diffusion tensor imaging reliably detects experimental traumatic axonal injury and indicates approximate time of injury. J Neurosci. 2007;27(44):11869-76.

Mac Donald CL, Johnson AM, Cooper D, Nelson EC, Werner NJ, Shimony JS, Snyder AZ, Raichle ME, Witherow JR, Fang R. Detection of blast-related traumatic brain injury in US military personnel. New England Journal of Medicine. 2011;364 (22): 2091-2100.

Mac Donald CL, Johnson AM, Wierzechowski L, Kassner E, Stewart T, Nelson EC, Werner NJ, Zonies D, Oh J, Fang R, Brody DL. Prospectively assessed clinical outcomes in concussive blast vs non-blast traumatic brain injury among evacuated US military personnel. JAMA Neurol. 2014;71(8):994-1002.

Maegele M, Engel D, Bouillon B, Lefering R, Fach H, Raum M, Buchheister B, Schaefer U, Klug N, Neugebauer E. Incidence and outcome of traumatic brain injury in an urban area in Western Europe over 10 years. Eur Surg Res. 2007;39 (6): 372-79.

Malec JF, Brown AW, Leibson CL, Flaada JT, Mandrekar JN, Diehl NN, Perkins PK. The Mayo Classification System for Traumatic Brain Injury Severity. Journal of Neurotrauma. 2007;24(9): 1417-24.

Manley GT, Rosenthal G, Lam M, Morabito D, Yan D, Derugin N, Bollen A, Knudson MM, Panter SS. Controlled cortical impact in swine: pathophysiology and biomechanics. Journal of Neurotrauma. 2006;23(2):128-39.

McCall JM, Braughler JM, Hall ED. Lipid peroxidation and the role of oxygen radicals in CNS injury. Acta Anaesthesiol Belg. 1987;38: 373–379.

McKee AC, Cantu RC, Nowinski CJ, Hedley-Whyte ET, Gavett BE, Budson AE, Santini VE, Lee HS, Kubilus CA, Stern RA. Chronic traumatic encephalopathy in athletes: progressive tauopathy after repetitive head injury. J Neuropathol Exp Neurol. 2009;68(7):709-35.

McKee AC, Robinson ME. Military-related traumatic brain injury and neurodegeneration. Alzheimers Dement. 2014;10(3 Suppl):S242-53.

McKenna SP, Doward LC, Alonso J, Kohlmann T, Niero M, Prieto L, Wiren L. The QoLAGHDA: an instrument for the assessment of quality of life in adults with growth hormone deficiency. Qual Life Res. 1999;8:373-83.

Menon DK, Schwab K, Wright DW, Maas AI. Position statement: definition of traumatic brain injury. Arch Phys Med Rehabil, 2010;91 (11): 1637- 40.

Miller AP, Shah AS, Aperi BV, Budde MD, Pintar FA, Tarima S, Kurpad SN, Stemper BD, Glavaski-Joksimovic A. Effects of blast overpressure on neurons and glial cells in rat organotypic hippocampal slice cultures. Front. Neurol. 2015;6:20.

Miller KL, Stagg CJ, Douaud G, Jbabdi S, Smith SM, Behrens TE, Jenkinson M, Chance SA, Esiri MM, Voets NL, Jenkinson N, Aziz TZ, Turner MR, Johansen-Berg H, McNab JA. Diffusion imaging of whole, post-mortem human brains on a clinical MRI scanner. Neuroimage. 2011;57(1):167-81.

Molitch ME, Clemmons DR, Malozowski S, Merriam GR, Vance ML; Endocrine Society. Evaluation and treatment of adult growth hormone deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab. 2011;96(6):1587-609.

Moll J, de Oliveira-Souza R, Moll FT, Bramati IE, Andreiuolo PA. The cerebral correlates of set-shifting: an fMRI study of the trail making test. Arq Neuropsiquiatr. 2002;60(4):900-5.

Moore L, Lavoie A, Camden S, Le Sage N, Sampalis JS, Bergeron E, Abdous B. Statistical validation of the Glasgow Coma Score. J Trauma. 2006;60(6):1238-43; discussion 1243-4.

Morales DL. Brain Contusion Imaging. November 2015. Available at: http://emedicine.medscape.com/article/337782-overview. Last accessed Jan 2016.

Morey RA, Haswell CC, Selgrade ES, Massoglia D, Liu C, Weiner J, Marx CE;MIRECC Work Group, Cernak I, McCarthy G. Effects of chronic mild traumatic

brain injury on white matter integrity in Iraq and Afghanistan war veterans. Hum Brain Mapp. 2013;34(11):2986-99.

Mori S, Zhang J. Principles of diffusion tensor imaging and its applications to basic neuroscience research. Neuron, 2006;51 (5): 527-39.

Moss WC, King MJ, Blackman EG. Skull flexure from blast waves: a mechanism for brain injury with implications for helmet design. Physical Review Letters. 2009;103 (10): 108702.

Mossadegh S, Tai N, Midwinter M, Parker P. Improvised explosive device related pelvi-perineal trauma: anatomic injuries and surgical management. J Trauma Acute Care Surg. 2012;73(2 Suppl 1):S24-31.

Muir KW. Glutamate-based therapeutic approaches: clinical trials with NMDA antagonists. Curr Opin Pharmacol. 2006;6: 53–60.

Murray CJ, Lopez AD. Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. Lancet 1997;349 (9063): 1436-42.

Nakagawa A, Manley GT, Gean AD, Ohtani K, Armonda R, Tsukamoto A, Yamamoto H, Takayama K, Tominaga T. Mechanisms of primary blast-induced traumatic brain injury: insights from shock-wave research. Journal of Neurotrauma. 2011;28 (6): 1101-19.

Narayan RK, Greenberg RP, Miller JD, Enas GG, Choi SC, Kishore PR, Selhorst JB, Lutz HA 3rd, Becker DP. Improved confidence of outcome prediction in severe head injury. A comparative analysis of the clinical examination, multimodality evoked potentials, CT scanning, and intracranial pressure. J Neurosurg. 1981;54(6):751-62.

NATO Logistics Handbook, Chapter 16: Medical Support, Role Support. Third Edition, October 1997. Available at: http://www.nato.int/docu/logi-en/1997/lo-1610.htm. Last accessed Feb 2016.

NCCAC (2007) (National Collaborating Centre for Acute Care) Head Injury: triage, assessment, investigation and early management of head injury in infants, children and adults, http://www.nice.org.uk/CG56.

NFL, ex-players agree to $765M settlement in concussions suit. Associated Press 2013. Available at: http://www.nfl.com/news/story/0ap1000000235494/article/nfl-explayers-agree-to-765m-settlement-in-concussions-suit. Last accessed Jan 2016.

Nichols TE, Holmes AP. Nonparametric permutation tests for functional neuroimaging: a primer with examples. Hum Brain Mapp. 2002;15:1-25.

No authors stated. Part 2: Prognosis in penetrating brain injury. J Trauma. 2001;51(2 Suppl):S44-86.

Nucifora PG, Verma R, Lee SK, Melhem ER. Diffusion-Tensor MR Imaging and Tractography: Exploring Brain Microstructure and Connectivity. Radiology, 2007;245 (2): 367-83.

Okie, S. Traumatic brain injury in the war zone. New England Journal of Medicine. 2005;352 (20): 2043-2047.

Ommaya AK, Gennarelli TA. Cerebral concussion and traumatic unconsciousness. Correlation of experimental and clinical observations of blunt head injuries. Brain 1974;97 (4): 633-54.

Ommaya AK, Hirsch AE. Tolerances for cerebral concussion from head impact and whiplash in primates. J Biomech, 1971; 4 (1): 13-21.

Patel HC, Bouamra O, Woodford M, King AT, Yates DW, Lecky FE. Trends in head injury outcome from 1989 to 2003 and the effect of neurosurgical care: an observational study. Lancet 2005;366 (9496): 1538-44.

Paterakis K, Karantanas AH, Komnos A, Volikas Z. Outcome of patients with diffuse axonal injury: the significance and prognostic value of MRI in the acute phase. J Trauma, 2000;49 (6): 1071-75.

Penn-Barwell JG, Roberts SA, Midwinter MJ, Bishop JR. Improved survival in UK combat casualties from Iraq and Afghanistan: 2003-2012. J Trauma Acute Care Surg. 2015;78(5):1014-20.

Pervanidou P and Chrousos GP. Neuroendocrinology of post-traumatic stress disorder. Prog Brain Res. 2010;182:149-160.

Peskind ER, Petrie EC, Cross DJ, Pagulayan K, McCraw K, Hoff D, Hart K, Yu CE, Raskind MA, Cook DG, Minoshima S. Cerebrocerebellar hypometabolism associated with repetitive blast exposure mild traumatic brain injury in 12 Iraq war Veterans with persistent post-concussive symptoms. Neuroimage. 2011;54 Suppl 1:S76-82.

Pettus EH, Christman CW, Giebel ML, Povlishock JT. Traumatically induced altered

membrane permeability: its relationship to traumatically induced reactive axonal change. J Neurotrauma. 1994;11(5):507-22.

Plumpton FS, Besser GM. The adrenocortical response to surgery and insulin-induced hypoglycaemia in corticosteroid-treated and normal subjects. Br J Surg. 1969;56:216-9.

Ponsford J, Kinsella G. Attentional deficits following closed-head injury. J Clin Exp Neuropsychol. 1992;14(5):822-38.

Povlishock JT, Christman CW. The pathobiology of traumatically induced axonal injury in animals and humans: a review of current thoughts. J Neurotrauma. 1995;12(4):555-64.

Povlishock JT, Katz DI. Update of neuropathology and neurological recovery after traumatic brain injury. J Head Trauma Rehabil. 2005;20 (1): 76-94.

Povlishock JT. Pathobiology of traumatically induced axonal injury in animals and man. Ann Emerg Med, 1993;22 (6): 980-86.

Prof. Jane Powell, Goldsmiths, UK. Personal communication.

Raghavendra Rao VL, Dhodda VK, Song G, Bowen KK, Dempsey RJ. Traumatic brain injury-induced acute gene expression changes in rat cerebral cortex identified by GeneChip analysis. J Neurosci Res. 2003;71: 208–219.

Reitan R. The validity of the Trail Making Test as an indicator of organic brain damage. Percept Mot Skills. 1958;8:276.

Reitan RM, Wolfson D. The Trail Making Test as an initial screening procedure for neuropsychological impairment in older children. Arch Clin Neuropsychol. 2004;19(2):281-8.

Ritzel 2008 – personal communication

Rosenfeld JV, Maas AI, Bragge P, Morganti-Kossmann MC, Manley GT, Gruen RL. Early management of severe traumatic brain injury. Lancet. 2012;380(9847):1088-98.

Rosenfeld JV, McFarlane AC, Bragge P, Armonda RA, Grimes JB, Ling GS. Blast-related traumatic brain injury. Lancet Neurol. 2013;12(9):882-93.

Ruff RL and Riechers RG. Effective treatment of traumatic brain injury: learning from experience. JAMA. 2012;308:2032-2033.

Ruff RL, Riechers RG 2nd, Wang XF, Piero T, Ruff SS. A case-control study examining whether neurological deficits and PTSD in combat veterans are related to episodes of mild TBI. BMJ Open. 2012;2(2):e000312.

Saatman KE, Duhaime AC, Bullock R, Maas AI, Valadka A, Manley GT; Workshop Scientific Team and Advisory Panel Members. Classification of traumatic brain injury for targeted therapies. J Neurotrauma. 2008;25(7): 719-38.

Salvatori R. Adrenal insufficiency. JAMA. 2005;294(19):2481-8.

Sánchez-Cubillo I, Periáñez JA, Adrover-Roig D, Rodríguez-Sánchez JM, Ríos-Lago M, Tirapu J, Barceló F. Construct validity of the Trail Making Test: role of task-switching, working memory, inhibition/interference control, and visuomotor abilities. J Int Neuropsychol Soc. 2009;15(3):438-50.

Sarnat HB. Ependymal reactions to injury. A Review. Journal of Neuropathology and Experimental Neurology. 1995;54(1):1-15.

Scallan J, Huxley VH, Korthuis RJ. Capillary Fluid Exchange: Regulation, Functions, and Pathology. San Rafael (CA): Morgan & Claypool Life Sciences; 2010. Integrated Systems Physiology: from Molecule to Function to Disease.

Scheid R, Preul C, Gruber O, Wiggins C, von Cramon DY. Diffuse axonal injury associated with chronic traumatic brain injury: evidence from T2*-weighted gradient-echo imaging at 3 T. AJNR Am J Neuroradiol. 2003;24(6):1049-56.

Schneider HJ, Kreitschmann-Andermahr I, Ghigo E, Stalla GK, Agha A. Hypothalamopituitary dysfunction following traumatic brain injury and aneurysmal subarachnoid hemorrhage: a systematic review. JAMA. 2007;298(12):1429-38.

Schneiderman AI, Braver ER, Kang HK. Understanding sequelae of injury mechanisms and mild traumatic brain injury incurred during the conflicts in Iraq and Afghanistan: persistent postconcussive symptoms and posttraumatic stress disorder. Am J Epidemiol. 2008;167(12):1446-52.

Segatore M, Way C. The Glasgow Coma Scale: time for change. Heart Lung. 1992;21(6): 548-57.

Sharp DJ, Beckmann CF, Greenwood R, Kinnunen KM, Bonnelle V, De Boissezon X, Powell JH, Counsell SJ, Patel MC, Leech R. Default mode network functional and structural connectivity after traumatic brain injury. Brain. 2011;134(Pt 8):2233-47.

Sharp DJ, Ham TE. Investigating white matter injury after mild traumatic brain injury. Curr Opin Neurol. 2011;24(6): 558-63.

Sherriff FE, Bridges LR, Sivaloganathan S. Early detection of axonal injury after human head trauma using immunocytochemistry for beta-amyloid precursor protein. Acta Neuropathol. 1994;87(1):55-62.

Sidaros A, Engberg AW, Sidaros K, Liptrot MG, Herning M, Petersen P, Paulson OB, Jernigan TL, Rostrup E. Diffusion tensor imaging during recovery from severe traumatic brain injury and relation to clinical outcome: a longitudinal study. Brain. 2008;131(Pt 2):559-72.

Silver JM, McAllister TW, Yodofsky SC, eds. Textbook of Traumatic Brain Injury. Arlington, VA:. Arlington, Va: American Psychiatric Publishing; 2005: 27-39.

Smith DH, Chen XH, Iwata A, Graham DI. Amyloid beta accumulation in axons after traumatic brain injury in humans. J Neurosurg. 2003;98(5):1072-7.

Smith DH, Meaney DF, Shull WH. Diffuse axonal injury in head trauma. J Head Trauma Rehabil. 2003;18(4):307-16.

Smith SM, Jenkinson M, Johansen-Berg H, Rueckert D, Nichols TE, Mackay CE, Watkins KE, Ciccarelli O, Cader MZ, Matthews PM, Behrens TE. Tract-based spatial statistics: voxelwise analysis of multi-subject diffusion data. Neuroimage. 2006;31(4):1487-505.

Smith SM, Jenkinson M, Woolrich MW, Beckmann CF, Behrens TE, Johansen-Berg H, Bannister PR, De LM, Drobnjak I, Flitney DE, Niazy RK, Saunders J, Vickers J, Zhang Y, De SN, Brady JM, Matthews PM. Advances in functional and structural MR image analysis and implementation as FSL. Neuroimage. 2004;23 Suppl 1:S208-S219.

Smith SM, Nichols TE. Threshold-free cluster enhancement: addressing problems of smoothing, threshold dependence and localisation in cluster inference. Neuroimage. 2009;44:83-98.

Smith SM. Fast robust automated brain extraction. Hum Brain Mapp. 2002;17:143-55.

Smith TP, Kavanagh L, Healy ML, McKenna TJ. Technology insight: measuring prolactin in clinical samples. Nat Clin Pract Endocrinol Metab. 2007;3:279-89.

Song SK, Sun SW, Ramsbottom MJ, Chang C, Russell J, Cross AH. Dysmyelination revealed through MRI as increased radial (but unchanged axial) diffusion of water. Neuroimage. 2002;17(3):1429-36.

Stainsby D, MacLennan S, Thomas D, Isaac J, Hamilton PJ. Guidelines on the management of massive blood loss. Br J Haematol. 2006;135:634-41.

Steiner H, Bahr V, Exner P, Oelkers PW. Pituitary function tests: comparison of ACTH and 11-deoxy-cortisol responses in the metyrapone test and with the insulin hypoglycemia test. Exp Clin Endocrinol. 1994;102:33-8.

Streit WJ, Walter SA, Pennell NA. Reactive microgliosis. Prog Neurobiol. 1999;57(6):563-81.

Stroop JR. Studies of interference in serial verbal reactions. Journal of Experimental Psychology. 1935;18:643-62.

Sugiyama K, Kondo T, Oouchida Y, Suzukamo Y, Higano S, Endo M, Watanabe H, Shindo K, Izumi SI. Clinical Utility of Diffusion Tensor Imaging for Evaluating Patients with Diffuse Axonal Injury and Cognitive Disorders in the Chronic Stage. J Neurotrauma. 2009;26(11): 1879-90.

Sundaramurthy A, Alai A, Ganpule S, Holmberg A, Plougonven E, Chandra N. Blast-induced biomechanical loading of the rat: an experimental and anatomically accurate computational blast injury model. J Neurotrauma. 2012 Sep;29(13): 2352-64.

Svetlov SI, Larner SF, Kirk DR, Atkinson J, Hayes RL, Wang KK. Biomarkers of blast-induced neurotrauma: profiling molecular and cellular mechanisms of blast brain injury. Journal of Neurotrauma. 2009;26 (6): 913-21.

Swanson J. The Delis-Kaplan Executive Function System - A Review. Canadian Journal of School Psychology. 2005;20(1/2):117-128.

Tagliaferri F, Compagnone C, Korsic M, Servadei F, Kraus J. A systematic review of

brain injury epidemiology in Europe. Acta Neurochir (Wien), 2006;148 (3): 255-68.

Takao H, Hayashi N, Inano S, Ohtomo K. Effect of head size on diffusion tensor imaging. Neuroimage. 2011;57(3):958-67.

Tanielian T and Jaycox HL. Invisible wounds of war: psychological and cognitive injuries, their consequences, and services to assist recovery. RAND Centre for Military Health Policy Research. 2008;1-499.

Tanielian T, Jaycox HL. Invisible Wounds of War. Psychological and Cognitive Injuries, Their Consequences, and Services to Assist Recovery. RAND Centre for Military Health Policy Research. 2008:1-499.

Taylor PA, Ford CC. Simulation of blast-induced early-time intracranial wave physics leading to traumatic brain injury. J Biomech Eng. 2009;131(6):061007.

Teasdale G, Jennett B. Assessment of coma and impaired consciousness. A practical scale. Lancet. 1974;2(7872):81-4.

Teranishi K, Scultetus A, Haque A, Stern S, Philbin N, Rice J, Johnson T, Auker C, McCarron R, Freilich D. Traumatic brain injury and severe uncontrolled haemorrhage with short delay pre-hospital resuscitation in a swine model. Injury. 2012;43 (5): 585-593.

Terrio H, Brenner LA, Ivins BJ, Cho JM, Helmick K, Schwab K, Scally K, Bretthauer R, Warden D. Traumatic brain injury screening: preliminary findings in a US Army Brigade Combat Team. The Journal of Head Trauma Rehabilitation. 2009;24(1):14-23.

Thibault KL, Margulies SS. Age-dependent material properties of the porcine cerebrum: effect on pediatric inertial head injury criteria. Journal of Biomechanics. 1998;31(12):1119-26.

Thornhill S, Teasdale GM, Murray GD, McEwen J, Roy CW, Penny KI. Disability in young people and adults one year after head injury: prospective cohort study. BMJ 2000;320 (7250): 1631-35.

Tombaugh TN. Trail Making Test A and B: normative data stratified by age and education. Arch Clin Neuropsychol. 2004;19(2):203-14.

Tong KA, Ashwal S, Holshouser BA, Nickerson JP, Wall CJ, Shutter LA, Osterdock

RJ, Haacke EM, Kido D. Diffuse axonal injury in children: clinical correlation with hemorrhagic lesions. Ann Neurol, 2004;56 (1): 36- 50.

Tong KA, Holshouser BA, Ashwal S. Evaluation of pediatric diffuse axonal injury using susceptibility weighted imaging (SWI) and MR spectroscopy. Proceedings of the ISMRM 14th Scientific Meeting and Exhibition. 2006 May 6–12; Toronto, Canada. Berkeley (CA): ISMRM; 2006.

Van Boven RW, Harrington GS, Hackney DB, Ebel A, Gauger G, Bremner JD, D'Esposito M, Detre JA, Haacke EM, Jack CR Jr, Jagust WJ, Le Bihan D, Mathis CA, Mueller S, Mukherjee P, Schuff N, Chen A, Weiner MW. Advances in neuroimaging of traumatic brain injury and posttraumatic stress disorder. J Rehabil Res Dev. 2009;46(6): 717-57.

van Dam PS. Neurocognitive function in adults with growth hormone deficiency. Horm Res. 2005;64 Suppl 3:109-114.

van Liempt S, Vermetten E, Lentjes E, Arends J, Westenberg H. Decreased nocturnal growth hormone secretion and sleep fragmentation in combat-related posttraumatic stress disorder; potential predictors of impaired memory consolidation. Psychoneuroendocrinology. 2011;36(9):1361-9.

van Veluw SJ, Fracasso A, Visser F, Spliet WG, Luijten PR, Biessels GJ, Zwanenburg JJ. FLAIR images at 7 Tesla MRI highlight the ependyma and the outer layers of the cerebral cortex. Neuroimage. 2015;104:100-9.

Vink R, Nimmo AJ. Novel therapies in development for the treatment of traumatic brain injury. Expert Opin Investig Drugs. 2002;11(10):1375-86.

Vokes TJ, Robertson GL. Disorders of antidiuretic hormone. Endocrinol Metab Clin North Am. 1988;17:281-99.

Vuong C, Van Uum SH, O'Dell LE, Lutfy K, Friedman TC. The effects of opioids and opioid analogs on animal and human endocrine systems. Endocr Rev. 2010;31(1):98-132.

Wald SL, Shackford SR. The effect of secondary insults on mortality and long-term disability after severe head injury in a rural region without a trauma system. The Journal of Trauma and Acute Care Surgery. 1993;34(3): 377-382.

Ware JE, Sherbourne CD. The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection. Med Care. 1992;30:473-83.

Warner MA, Marquez de la Plata C, Spence J, Wang JY, Harper C, Moore C, Devous M, Diaz-Arrastia R. Assessing spatial relationships between axonal integrity, regional brain volumes, and neuropsychological outcomes after traumatic axonal injury. J Neurotrauma. 2010;27(12):2121-30.

Webb SM. Measurements of quality of life in patients with growth hormone deficiency. J Endocrinol Invest. 2008;31:52-55.

Wechsler D. WASI: Wechsler Abbreviated Scale of Intelligence, 1999. The Psychological Corporation, San Antonio, Texas, USA.

Wechsler D. Wechsler Memory Scale- Third Edition: Administration and Scoring Manual. San Antonio: TX: Psychological Corporation, 1997.

Whitnall L, McMillan TM, Murray GD, Teasdale GM. Disability in young people and adults after head injury: 5-7 year follow up of a prospective cohort study. J Neurol Neurosurg Psychiatry. 2006;77(5):640-5.

Wilkins KC, Lang AJ, and Norman SB. Synthesis of the psychometric properties of the PTSD checklist (PCL) military, civilian, and specific versions. Depress Anxiety. 2011;28:596-606.

Wilkinson CW, Pagulayan KF, Petrie EC, Mayer CL, Colasurdo EA, Shofer JB, Hart KL, Hoff D, Tarabochia MA, Peskind ER. High prevalence of chronic pituitary and target-organ hormone abnormalities after blast-related mild traumatic brain injury. Front Neurol. 2012;3:11.

Woolrich MW, Jbabdi S, Patenaude B, Chappell M, Makni S, Behrens T, Beckmann C, Jenkinson M, Smith SM. Bayesian analysis of neuroimaging data in FSL. Neuroimage. 2009;45 Suppl 1:S173-S186.

Wrathall JR, Benzinger T, Brody DL, Cardin S, Curley K, Mintun M. Blast-related Brain Injury: Imaging for Clinical and Research Applications Report of the 2008 St. Louis Workshop. Journal of Neurotrauma; 2011:1-62.

Xiong Y, Gu Q, Peterson PL, Muizelaar JP, Lee CP. Mitochondrial dysfunction and calcium perturbation induced by traumatic brain injury. J Neurotrauma. 1997;14: 23–34.

Xiong Y, Mahmood A, Chopp M. Animal models of traumatic brain injury. Nature reviews Neuroscience. 2013;14(2):128-42.

Yaghmai A, Povlishock J. Traumatically induced reactive change as visualized through the use of monoclonal antibodies targeted to neurofilament subunits. J Neuropathol Exp Neurol, 1992; 51 (2): 158-76.

Yuen KC, Biller BM, Molitch ME, Cook DM. Clinical review: Is lack of recombinant growth hormone (GH)-releasing hormone in the United States a setback or time to consider glucagon testing for adult GH deficiency? J Clin Endocrinol Metab. 2009;94(8):2702-7.

Zhu D, Zhang T, Jiang X, Hu X, Chen H, Yang N, Lv J, Han J, Guo L, Liu T. Fusing DTI and fMRI data: a survey of methods and applications. Neuroimage. 2014;102 Pt 1:184-91.

Ziebell JM, Morganti-Kossmann MC. Involvement of pro- and anti-inflammatory cytokines and chemokines in the pathophysiology of traumatic brain injury. Neurotherapeutics. 2010;7: 22–30.