

**Neuroprotection by Noble Gases and Other Drugs in
Models of Trauma**

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Statement of originality

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Abstract

Traumatic Brain Injury (TBI) is a debilitating and complex condition that consists of a 'primary' injury followed by a 'secondary' injury, which develops over time. Much of the morbidity occurs due to the secondary injury, for which there are currently no approved pharmacotherapies. This thesis is structured as a study of models of TBI and an investigation of drugs to treat the condition.

First, an injury quantification methodology was developed in an *in vitro* model of TBI using organotypic hippocampal slice cultures and propidium iodide fluorescence. Second, 'combination therapies' with the noble gases xenon and argon, progesterone and meloxicam to modulate multiple secondary injury mechanisms were explored. 25% argon and 3 μ M, 10 μ M and 25 μ M progesterone are additively protective at 72 hours. Co-administering 25 μ M progesterone with 25% argon (29 $\pm$ 6% protective) abolished injury. Co-administering 50% argon (41 $\pm$ 5% protective) synergistically abolished injury with 3 μ M progesterone and additively abolished injury with 10 μ M and 25 μ M progesterone. 25% xenon and 3 μ M, 10 μ M and 25 μ M progesterone were additively protective at 72 hours. Co-administering 25 μ M progesterone with 25% xenon (44 $\pm$ 5% protective) abolished injury. 25% argon in combination with 0.1 μ M and 1 μ M meloxicam works additively. Neuroprotection by 25% argon doubled when co-administered with 1 μ M meloxicam. 25% xenon and 1 μ M meloxicam are additive, abolishing injury at 72 hours unlike sub-maximally effective 25% xenon.

Third, experiments to calibrate a controlled cortical impact (CCI) model of *in vivo* TBI were conducted. Contusion volume was measured and the expression of inflammatory cytokines was quantified in blood and brain samples at different time points. Finally, a *Drosophila melanogaster* model of polytrauma was developed as a high throughput assay. *Drosophila* are more sensitive to trauma in the early phase of their circadian day. Hypothermia treatment (4 $^{\circ}$ C) increased survival to the level of uninjured shams at 24 hours and more than doubled survival over 14 days.

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List of Abbreviations

AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate
ANOVA	analysis of variance
APC	antigen presenting cells
AQP4	aquaporin 4
Ar	argon
Atm	atmospheres
BBB	blood brain barrier
CA1	portion of hippocampal formation
CBF	cerebral blood flow
CCI	controlled cortical impact
CNS	central nervous system
COX	cyclooxygenase enzyme (COX-1 and COX-2 isoforms)
CSF	cerebrospinal fluid
CT	computerised tomography
DAI	diffuse axonal injury
DAMPs	danger-associated molecular patterns
DMSO	dimethyl sulfoxide
DOC	desoxycorticosterone
EEG	electroencephalogram
eGOS	extended Glasgow Outcome Scale
ELISA	enzyme linked immunoabsorbent assay
ERK	extracellular regulated signal kinase
FPI	fluid percussion injury
GABA	γ -Aminobutyric acid
GCS	Glasgow coma scale
GFAP	Glial fibrillary acidic protein
GPCR	G-coupled protein receptors
ICP	intracranial pressure
IFN γ	interferon γ
IL	interleukin family (IL-1, IL-1 β , IL-6, IL-8, IL-10, IL-12)
IV	intravenous
K-M	Kaplan Meier

KC	keratinocyte chemo-attractant
MAC	minimum alveolar concentration
MCP-1	monocyte chemo-attractant protein 1 (also known as CCL-2)
MEK-ERK	mitogen activated protein kinase pathway (originally called ERK – extracellular signal regulated kinase)
MIP	macrophage inflammatory protein (MIP-1 α and MIP-2)
MLX	meloxicam
NMDAR	N-methyl-D-aspartate receptor
NO	nitric oxide
NSAID	non-steroidal anti-inflammatory drug
OGD	oxygen glucose deprivation
OHCA	out of hospital cardiac arrest
OHSC	organotypic hippocampal slice cultures
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PI	propidium iodide
PR	progesterone receptor
PRG	progesterone
RCT	randomised control trial
ROS	reactive oxygen species
SAH	subarachnoid haemorrhage
SDH	subdural haematoma
SEM	standard error of the mean
SIRS	systemic inflammatory response syndrome
TASK-3	TWIK-related acid-sensitive K ⁺ channel 3
TBI	traumatic brain injury
TGF- β	transforming growth factor β
TNF	tumour necrosis factor (previously TNF- α)
TREK	two-pore-domain potassium channel
Xe	xenon

1 Chapter 1: Introduction

1.1 Traumatic Brain Injury

1.1.1 Definition

According to the World Health Organisation, Traumatic Brain Injury (TBI) is predicted to surpass many diseases and become the major cause of death and disability by the year 2020[1]. The most recent consensus on the definition of TBI was put forth by The Demographics and Clinical Assessment Working Group of the International and Interagency Initiative toward Common Data Elements for Research on Traumatic Brain Injury and Psychological Health in 2010[2]. TBI is defined as “an alteration in brain function or other evidence of brain pathology, caused by an external force”[2]. An alteration in brain function at the time of injury may consist of a loss of or a decreased level of consciousness, any anterograde or retrograde amnesia, neurologic deficits or any alteration in mental state[2]. A dynamic and heterogeneous disorder, TBI is distinguished from other acquired brain injuries due to cerebrovascular, neoplastic or neurodegenerative conditions because of the requirement of an external force. A ‘primary injury’, caused due to an external force, is followed by a ‘secondary injury’ that develops in the hours and days following the insult[3].

1.1.2 Epidemiology and Disease Burden

TBI is considered a ‘silent epidemic’ as society is often unaware of the magnitude of the problem[4]. However, TBI is currently a leading cause of death and disability in people under the age of 45 years[5]. Equally concerning is the increase in incidence among the elderly population. Individuals over 65 years have the highest rates of hospitalisations, deaths and poor outcomes following TBI[6].

Furthermore, consistent implementation of evidence-based guidelines for TBI management has been a challenge. Even though these were introduced in 1995[7], the heterogeneity of injury (eg. diffuse or focal, penetrating or non-penetrating, differences in severity and location) has prevented consistent uptake. Moreover, differences in healthcare systems, the lack of formal injury surveillance and ‘trauma registries’ make it challenging to directly compare the incidence of TBI globally. The true magnitude of the burden resulting from TBI

is often underestimated worldwide. Nonetheless, the global incidence of TBI is rising. Broadly, the incidence of all TBI injuries (mild, moderate and severe in nature) is currently estimated at 579, 344 and 235 per 100,000 people annually in the US, Asia and Europe respectively[8]. Studies suggest that the majority of civilian TBI injuries (60%) occur as a result of road traffic incidents, followed by falls (20 – 30%) and violent assaults (10%)[9].

Although the deaths from severe TBI have reduced from 50% to 25% over the past 40 years[10], people with lifelong disabilities have functional impairments that impede their quality of life, leading to substantial financial burdens. In the US alone, the estimated annual cost burden due to TBI is US\$60 billion, \$50 billion of which is attributed to productivity losses[11], while in Europe it was estimated to be \$36 billion in 2010[12]. Low and Middle Income Countries are particularly vulnerable to risk factors for TBI due to increasing motorisation and urbanisation associated with rapid economic growth. However, they often do not have the health care capacity to deal with associated outcomes. Despite these alarming figures, effective TBI treatment represents an unmet clinical need within an already severely underfunded field of brain research[5,13].

1.2 Pathophysiology of TBI

1.2.1 Primary Injury

Primary injury is a direct result of external mechanical forces, which may include but are not limited to the head striking or being struck by an object, rapid acceleration or deceleration movement rotational forces, blunt impact, penetration by a foreign object and exposure to forces generated by blast waves associated with an explosion.

Often accompanied by skull fracture, the primary injury deforms the brain tissue directly damaging neurons, glial cells, axons, dendrites and blood vessels in a diffuse or focal pattern. This damage initiates a dynamic series of events, which disrupts normal brain function through a variety of inflammatory, cellular, excitotoxic, mitochondrial and metabolic processes[14]. The initial damage from a macroscopic perspective may include haematomas (intra-cerebral and extra-cerebral); diffuse swelling and the shearing of white matter tracts. Focal contusions are the most common traumatic lesion and usually arise from an impact or contact type injury[5].

At a cellular level, early damage (within minutes to hours of the traumatic event) often stems from an ischaemic cascade and may include opening of ion channels, shearing of blood vessels, microporation of membranes and conformational changes to proteins. In contact type injuries, energy from the impact is absorbed from the skull, leading to depressed skull fracture with more superficial brain damage[5]. A primary injury can be prevented through the use of protective equipment, but once trauma occurs, it cannot be reversed.

1.2.2 Secondary Injury

The secondary injury component of a TBI is often considered the major burden of injury as the majority of neurologic damage and morbidity is the result of events that occur in the hours and days following trauma. However, secondary injury events do have the potential to be attenuated[15]. Different primary injuries may initiate different pathological cascades, which vary in extent and duration. Figure 1.1 represents a broad overview of secondary injury pathways, which may act synergistically and concurrently.

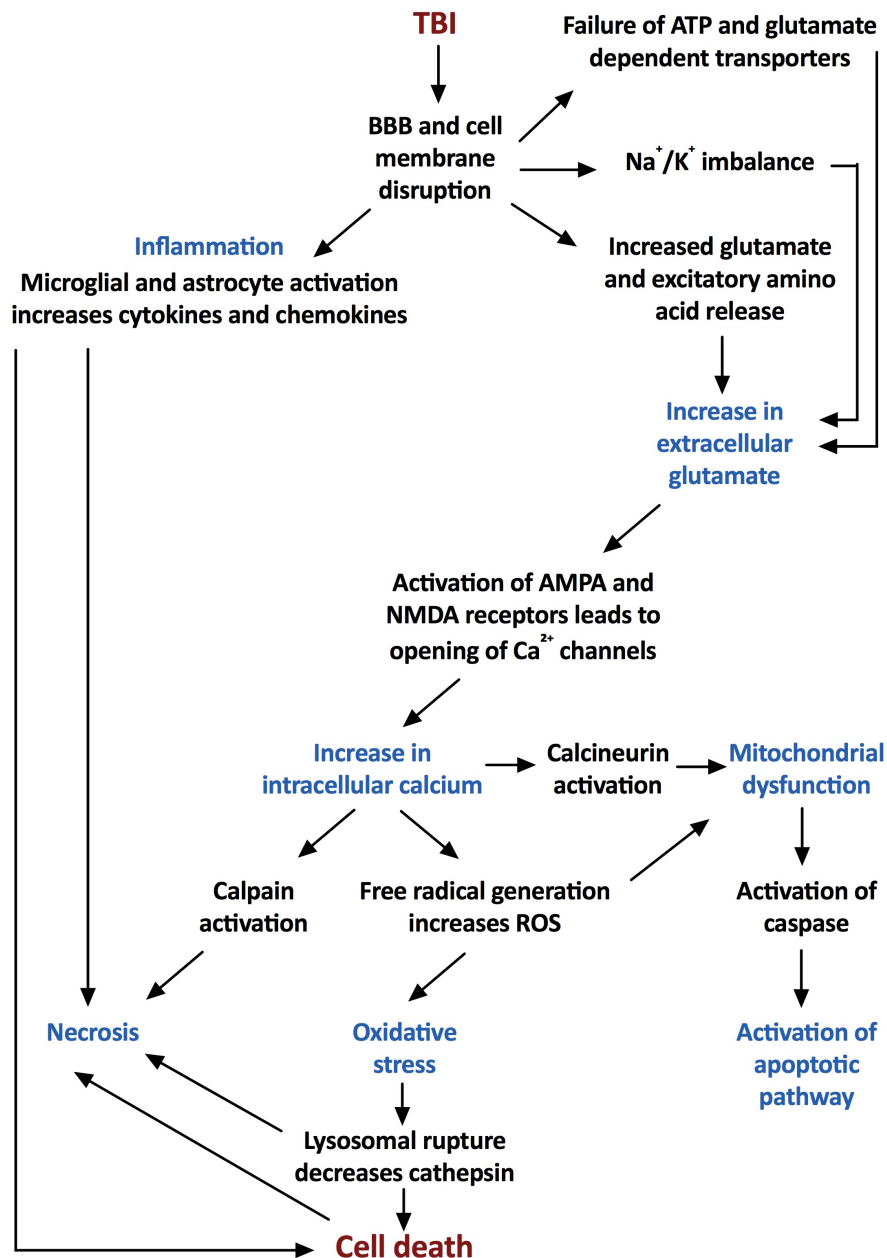


Figure 1.1: Secondary injury events that over time (hours to weeks) culminate in cell death. Adapted from Walker *et al.*, 2013[16] and McAllister *et al.*, 2011[17]; BBB: blood-brain barrier, ROS: reactive oxygen species, AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate, NMDA: *N*-methyl-D-aspartate

Secondary injury mechanisms = include neurotransmitter release, oxidative stress, free radical generation, mitochondrial dysfunction, inflammatory responses and activation of caspase and calpain dependent cell death pathways[5,18,19]. Within hours of the primary injury event, vasogenic and cellular fluid accumulates in the brain. This increases intracranial pressure (ICP), causing cerebral oedema. Brain swelling and neurotransmitter release, which occurs early on after the primary insult, continue for several days. Along with high ICP, free radical and calcium mediated damage; it is a major cause of necrotic cell death. Apoptotic

pathways are also activated due to early gene activation and the release of pro-apoptotic molecules like caspases[20]. The roles of excitotoxicity and inflammation are discussed further in the next sections, as they are the most relevant to the current study.

1.2.3 Neurobiology of excitotoxicity following TBI

Excitotoxicity is a process of intracellular events, initiated by excessive stimulation of neurotransmitters, which leads to intracellular calcium overload. Glutamate, a major excitatory neurotransmitter in the mammalian CNS, is important for neural development, synaptic plasticity and learning and memory under physiological conditions. Many studies have reported glutamate mediated excitotoxicity as a key factor in secondary injury development[21,22]. Neurons as well as oligodendrocytes are vulnerable to glutamate receptor activation[14]. Evidence for the increased concentration of extracellular glutamate following TBI comes from both clinical and laboratory observations. Large increases in extracellular glutamate proportional with injury severity have been demonstrated in microdialysis studies in rodents, following experimental TBI[21,22]. Moreover, analysis of glutamate in cerebrospinal fluid (CSF) has reported elevated glutamate levels beyond 1 week after injury. Microdialysis studies in patients who presented with severe TBI have shown elevated extracellular glutamate in 75% of the cohort. Although glutamate levels normalised in 60% of these patients over 120 hours, persistent elevations of extracellular glutamate were linked with higher mortality and poor functional outcome[23–25]. Glutamate is thought to be the primary contributor to triggering excitotoxic events because of its potent effect on ionotropic receptors – primarily *N*-methyl-D-aspartate (NMDA) receptors but also kainate and calcium permeable α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors.

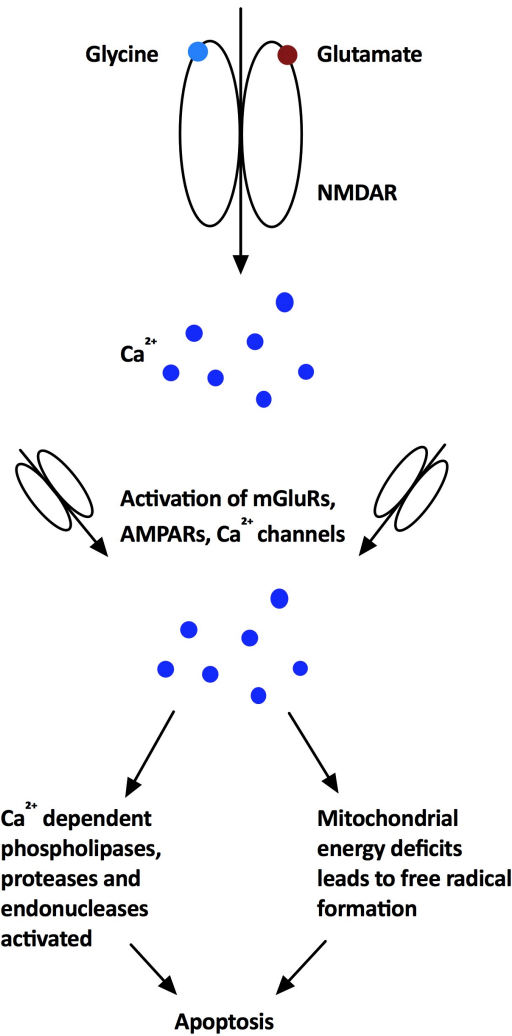


Figure 1.2: Downstream excitotoxic events following TBI injury[20,26]; mGluRs: metabotropic glutamate receptors

NMDA receptors are tetra-heteromeric structures, permeable to calcium, sodium, potassium, and zinc. Under normal physiological conditions and at a resting membrane potential, magnesium blocks the channel pore. The magnesium block is voltage dependent and is relieved at depolarised potentials. The binding of glutamate, co-agonist glycine and removal of the magnesium block activates the receptor. Figure 1.2 shows the downstream excitotoxic events following TBI. Immediately following injury, TBI produces a widespread membrane depolarisation at the site of impact[27]. The release of glutamate and other amino acids results in over-activation of NMDA receptors[28]. The stretching of neuronal membranes is believed to create micropores that allow for a sodium influx, which in turn leads to an ionic shift. The membrane potential becomes depolarised, opening voltage-gated calcium channels and triggering the further release of neurotransmitters, resulting in a positive feedback loop[20]. An influx of calcium leads to postsynaptic depolarisation and neuronal action

potentials. Calcium influx is exacerbated by alterations in AMPA receptor subunits, which are caused due to reduced AMPAR sensitivity at the postsynaptic level leading to AMPAR activation. The loss of GluR2 subunits makes AMPA receptors calcium permeable[29]. Postsynaptic density scaffold proteins like PSD-95, Homer and Shank have also been shown to mediate postsynaptic signal transduction and modulate cell death following TBI. These changes, along with group 1 metabotropic glutamate receptors which are additionally activated by elevated extracellular glutamate, contribute to the opening of more voltage gated calcium channels and further increase calcium influx[30]. Additionally, the reuptake of extracellular glutamate by astrocytes is impaired which also amplifies excitotoxicity[31]. Thus, overstimulation of glutamate receptors results in an overload of intracellular calcium and sodium in postsynaptic neurons, which is further enhanced under ischaemic conditions[27].

The resulting increase in intracellular calcium triggers multiple biochemical cascades. Calcium dependent phospholipases (eg. calcineurin), proteases (eg. calpains and caspases), and endonucleases are activated, which in turn degrade lipids, proteins and nucleic acids. In the mitochondria, calcium sequestration leads to energy deficits, free radical formation and apoptosis. Ultimately, programmed cell death is initiated, resulting in the loss of neurons and synapses. Cortical structures like the hippocampus are specifically vulnerable to neuronal damage due to excitotoxicity after moderate and severe TBI, in part due to the high density of NMDA receptors[16,18,32].

1.2.4 Neurobiology of Inflammation following TBI

Inflammatory processes have long been viewed as important in TBI development, with several studies having reported evidence for acute and chronic inflammatory processes in models of TBI[33]. The CNS is often considered ‘immunologically privileged’ as immune cells are not permeable to the blood-brain barrier (BBB). However, it is well established that TBI elicits an inflammatory response in the brain, albeit the response is not well characterised. It was previously believed that inflammation is detrimental to the injured brain. However, there is now evidence to suggest beneficial roles of a regulated inflammatory response and detrimental effects of excessive or sustained inflammation[34,35]. Figure 1.3 is a graphical overview of the inflammatory cascade post TBI.

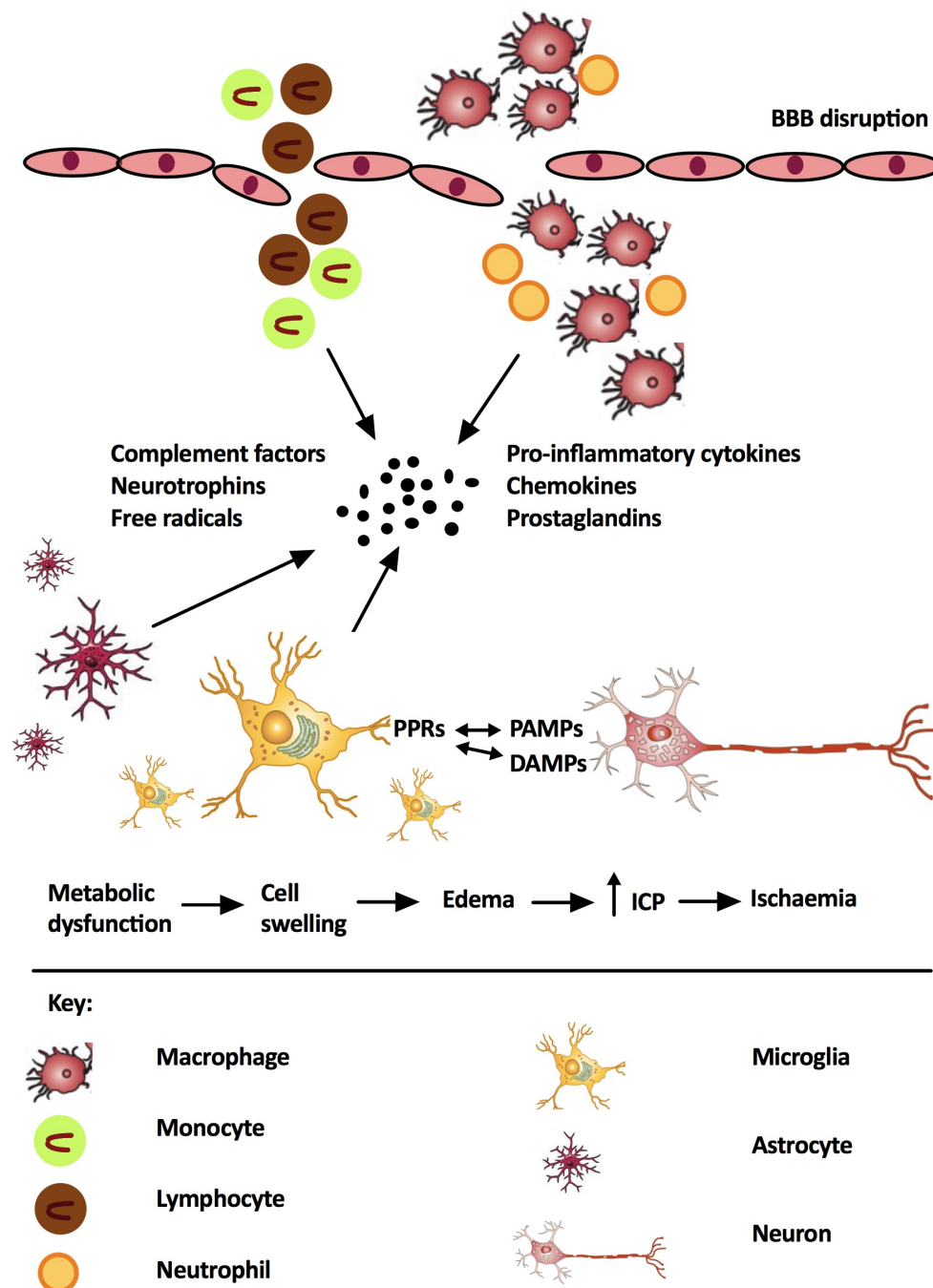


Figure 1.3: An overview of the basic inflammatory cascade post TBI based on Aertker *et al.*, 2015 and Rosenfeld *et al.*, 2011[36,37]. BBB: blood brain barrier, PPRs: pattern recognition receptors, PAMPs: pathogen associated molecular patterns, DAMPs: danger associated molecular patterns, ICP: intracranial pressure

Post-traumatic cerebral inflammation is characterised by an upregulation and secretion of cytokines and chemotactic cytokines (chemokines), leukocyte recruitment and glial activation. In the acute phase following a traumatic insult, shear forces and damage to endothelial cells due to generation of ROS cause the BBB to break down. As a result, circulating macrophages, lymphocytes, monocytes and neutrophils infiltrate the CNS parenchyma[14] (Figure 1.3). Animal models of brain trauma as well as clinical studies have

reported evidence for the accumulation of haematogenous immune cells in the parenchyma, which release free radicals, prostaglandins, complement factors and pro-inflammatory cytokines. These mediators induce the expression of chemokines and cell adhesion molecules along with mobilising immune and glial cells to the site of injury[38].

Clinical and experimental studies have provided evidence for the activation of microglia, the principle mediators of the adaptive immune response, to the damaged tissue[34,39–41]. Microglia originate in the bone marrow and facilitate communication between the brain and the immune system. Microglia become activated within minutes following TBI and coordinate the innate immune response by rapid proliferation and migration to the site of injury[35,42]. Here, they phagocytose cell debris and express pattern recognition receptors (PRRs) as inflammasomes to respond to pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) from damaged cells (Figure 1.3). They also act as antigen presenting cells (APCs) to T-lymphocytes as part of the adaptive immune response[43], releasing pro-inflammatory cytokines and chemokines along with neurotoxic products like nitric oxide (NO), superoxides and reactive oxygen/nitrogen species[44]. Their alternative function, with regards to neuro-restoration, is the removal of cell debris, which is necessary to dampen inflammatory processes and promote tissue repair. Three phenotypic states of microglia have been identified: (1) resting, ramified; (2) activated but non-phagocytic or antigen presenting cell like (found in areas of CNS inflammation) and (3) reactive – phagocytic microglia which are present around areas of trauma.

Pro and anti-inflammatory cytokines are polypeptides that are barely detectable in healthy tissue but are rapidly up regulated in physiologically stressful situations including TBI[45]. Their other functions also include intercellular communication and tissue homeostasis[46,47]. In the CNS, cytokines may be secreted from neurons as well as glia. Neuronally derived cytokines mediate cellular communication whereas cytokines originating from glial cells are involved in neuronal growth, survival and repair. They are additionally implicated in chronic pathological changes following neurodegeneration[48]. Chemokines are a sub-group of inflammatory mediators implicated in leukocyte activation and migration, neural embryogenesis, and host defence. They may be secreted by both glia and neurons, which also express chemokine receptors. α -chemokines act as neutrophil and lymphocyte chemo-attractants (examples include macrophage inflammatory protein (MIP)-2, interleukin (IL)-8 and keratinocyte chemo-attractant, (KC)), whereas β -chemokines are chemotactic for

monocytes and T cells (MIP-1 α and monocyte chemo-attractant protein (MCP)-1)[49,50]. Microglia can polarize into two major subtypes, M1 and M2. The M1 or ‘classical pro-inflammatory’ subtype is identified by high levels of the cytotoxic chemokines interferon- γ (IFN- γ), tumor necrosis factor (TNF), IL-6, IL-1 β , IL-12 and low levels of anti-inflammatory IL-10. Damage *via* NO and ROS is initiated and leakage of these inflammatory mediators into circulation causes systemic inflammatory response syndrome (SIRS), which worsens secondary injury[51]. The M2 or ‘alternatively activated’ subtype dampens inflammation, clears cellular debris like amyloid plaques and produces low levels of TNF, IL-1, IL-12, but high amounts of IL-10 and transforming growth factor (TGF)- β [52].

Over time, the BBB restores itself *via* repair mechanisms; however, there is a period of time in which the endothelium at sites of leakage is permeable to small molecules[53]. This leaves a window of time open to both prolonged altered neuronal function as well as an opportunity for drugs, which are normally blocked by the BBB, to reach sites of damage. Moreover, with the relatively recent discovery that inflammatory cells can participate in both neurodegenerative and repair processes following TBI, they have become an increasingly important target to understand secondary injury mechanisms and test neuroprotective agents.

1.3 Managing a traumatic brain injury

1.3.1 Current clinical interventions

TBI patients typically present with ‘polytrauma’, which may include numerous other injuries to the body, due to the accidental and/or violent nature of events that result in the primary injury. The emergency care team in the hospital must contain these injuries alongside managing the TBI. Broadly, the medical management of TBI patients includes specialised pre-hospital care, clinical intensive care and in some cases, long-term rehabilitation[54].

The main goals of pre-hospital care are to prevent hypoxia and hypotension as these exacerbate secondary brain damage[55,56] and are associated with poor outcome. Minimising the development of secondary injury begins with the early identification of severe brain injury and accurate assessment and treatment at the scene of the accident. The pre-hospital triage for TBI patients based on the *Guidelines for the Prehospital Management*

of Traumatic Brain Injury[57] recommend endotracheal intubation with assisted ventilation. Shock, defined as having a systolic blood pressure of less than 90mmHg, should be identified and treated by restoring blood pressure with intravascular fluids. Patients are then transported to local hospitals or specialised trauma centres based on the severity of their injuries[15].

Once at the hospital, TBI patients are assessed on injury type (closed, penetrating, crash or blast), structural damage or developing intracranial haematomas (using a CT scan), clinical severity on the basis of level of consciousness (using the Glasgow Coma Scale, GCS) and injury severity (using an abbreviated injury scale for six body regions including thorax, abdomen, pelvis, spine, extremities, skin and head/neck). The GCS is a graded scale that assesses the depth and duration of impaired consciousness and coma[58] and measures three aspects of behaviour – motor responsiveness, verbal performance and eye opening. While the GCS was once a universal system in a neurosurgical unit, its predictive value is now being questioned. More aggressive pre-hospital interventions involving early sedation and intubation prevents eye evaluation, verbal and motor assessment and influences the overall score[59,60]. Also, the GCS does not directly assess brainstem responses and indicates the neurological status only at the time of the test[60]. Neuroimaging using a CT scan focuses on the presence or absence of a mass lesion and diffuse injuries are differentiated by increased ICP. However, a CT scan cannot specify the type of mass lesion (eg. epidural or subdural), cannot detect ischaemic damage and diffuse axonal injury (DAI) and only captures momentary snapshots of a dynamically evolving TBI[5].

Intensive care management is aimed at limiting on-going damage through reducing elevated ICP and brain swelling. Patients with haematomas require neurosurgery. As with general intensive care guidelines, optimum oxygenation, perfusion, glycaemic control and temperature homeostasis are indicated[5]. Elevated ICP is managed using sedation and artificial ventilation. In some cases osmotherapy, involving the rapid infusion of mannitol to create an osmolar gradient and improve focal cerebral blood flow (CBF), is also used[5] but for short durations. In cases where hypertension persists, a decompressive craniotomy is required, but only 30% of patients require this[15]. Following emergency intensive care, TBI patients are monitored for progressive damage, which often develops without clear clinical signs. Neuroworsening is defined as the development of progressive lesions and deterioration of the GCS score and is reported in approximately 30 – 40% of patients. ICP monitoring is important and more advanced techniques include CBF measurements, cerebral oxygenation,

microdialysis and continuous electroencephalogram (EEG) monitoring to detect seizure like activity which can occur in 15 – 18% of patients with moderate to severe brain injury[5].

Ultimately however, medical care is limited for TBI patients because current treatment is supportive and aims to stabilise physiological parameters. There are currently no clinically proven effective pharmacological agents which can limit pathophysiological cascades in secondary injury or enhance repair following a TBI[5,14,37,54,61].

1.3.2 Current status of neuroprotection for TBI

Over the last 30 years, neuroprotection studies for TBI have focussed on arresting the development of secondary injury and/or improving outcomes. However, so far drug treatments have not displayed an improved outcome in clinical TBI, reflecting the poor translation between experimental, pre-clinical and clinical studies[62]. In fact, over 40 candidates that entered phase III clinical trials failed to show significance for their primary endpoint[54,62–64] (for a detailed list of these compounds, refer to Doppenberg *et al.*, 2004[65] and Schouten, 2007[54]) Besides intrinsic toxicity and failure to reach the site of action, a number of reasons for this have been identified which include: (1) an inadequate understanding of secondary injury mechanisms; (2) inadequate testing in different pre-clinical TBI models; (3) differences in strain, species, genders and ages of organisms used in pre-clinical testing; (4) lack of pharmacokinetic data and studies on therapeutic concentrations of drugs being studied; (5) lack of animal models that simulate systemic injuries; (6) use of heterogeneous patient populations in clinical trials; (7) lack of power due to inadequate sample size and (8) inadequate measurements of a variety of functional outcomes, molecular and neuroimaging biomarkers for clinical TBI[35]. While some of these factors are common reasons why drugs in general fail clinical trials, the majority of factors listed are actually consequences of the heterogeneous nature of TBI and its multifaceted clinical effects.

As excitotoxicity is heavily implicated in the spread of secondary injury following TBI and ischemia, an obvious drug target is the NMDA receptor. Considerable work went in to designing NMDA antagonists like Selfotel, MK-801, Cerestat and Gavestinel, and while these drugs were successful *in vitro*, they failed clinical trials. Besides generic reasons for failure and their psychotropic effects, conventional NMDA receptor antagonists abolish

glutamatergic excitation completely, making them neurotoxic and unsuitable for long-term use. It is well established that synaptic transmission mediated by NMDA receptors is essential for neuronal survival and development. Blocking NMDA receptors triggers a cascade that leads to apoptosis in the long term[66]. While excessive glutamate release immediately following injury is neurotoxic, several groups have independently demonstrated the plasticity of glutamatergic axons and formation of new; recurrent excitatory circuits post the acute phase of trauma[67]. Over time, glutamate resumes its normal physiological role and mild increases may be nootropic and implicated in neuronal regeneration[54,68,69]. Therefore, a reversible NMDA antagonist may be better as a neuroprotectant for TBI.

Moreover, where anti-inflammatory pathways are concerned, a number of anti-inflammatory drugs have been found to inhibit post-traumatic neuroinflammation, thus appearing as promising pre-clinical drug candidates. A number of these drugs are not originally classified as traditional anti-inflammatory drugs, for example, some are hormones, transcription factors and antimicrobial agents with anti-inflammatory action. These include but are not limited to minocycline, peroxisome proliferator-activated receptor agonists (PPAR), statins, oestrogen and progesterone[35,39,54,70]. For a complete review of anti-inflammatory drug candidates as neuroprotectants for TBI, refer to Bergold *et al.*, 2016[71]. One factor to be considered that is unique to the development of anti-inflammatory drugs as neuroprotectants for TBI is that inflammation is known to be a double-edged sword in TBI. Early anti-inflammatory actions encourage a dampening of the deleterious immune response but prolonged anti-inflammatory actions discourage neurorestoration[54]. A better pre-clinical understanding of the efficacy of anti-inflammatory drugs in different models of TBI and their therapeutic time window will aid the development of more relevant and effective anti-inflammatory neuroprotectants.

Other emerging neuroprotective therapies that are undergoing pre-clinical research and some of which have entered clinical trials recently include erythropoietin, immunophilin ligands (e.g. MPTP suppressors; cyclosporine A), bradykinin antagonists, nitric oxide and inhibitors of nitric oxide synthases, cell cycle inhibitors (eg. caspase inhibitors), substance P antagonists, diketopiperazines and sulfonylurea receptor 1 (SUR1)-regulated NC_{Ca-ATP} channels[35,39,54,70]. For a detailed review of emerging therapies, refer to Kochanek *et al.*, 2015[72].

1.3.3 Combination therapy as a strategy for TBI

Historically, TBI trials have focussed on treatments designed to act against single injury mechanisms[73]. However, as secondary injury is multifaceted[20,74], it is unlikely that a single agent can address all of the secondary injury mechanisms and their interconnectedness[39]. Therefore the concept of ‘combination therapies’, neuroprotective combinations that focus on modulating multiple mechanistic pathways, is gaining traction[75]. The success of drug combinations that enhance drug exposure or work in a complementary manner in cancer and human immunodeficiency virus have fuelled an interest in examining the potential of combination strategies for TBI[72]. Albeit an intuitive idea to focus on multiple pathways in a disorder as heterogeneous and complex as TBI, combination therapy strategies have received little attention thus far. In fact, a PubMed search conducted this year revealed only 37 studies that examined combination therapies for TBI and fit the criteria of a ‘traumatic’ brain injury based on validated models[76–79]. Only studies that reported ‘complete’ combination studies (i.e., where the effect of each therapy was established individually first) and where treatment was provided post trauma were included[80]. However, the idea has certainly gained more attention since a US convention on combination therapies for TBI in 2008[81]. One key recommendation was that prospective treatments should act on complementary rather than single targets[75,80,81]. Thus, drugs that have shown beneficial effects on their own and that may provide additional benefit when combined or that have modest effects on their own but may act synergistically to ameliorate a beneficial effect in combination opens a new window of opportunity for the future of TBI treatment.

1.4 Noble gases and drugs as treatments for TBI

1.4.1 Xenon as a neuroprotectant in TBI

1.4.1.1 Discovery

The ‘noble’ gas xenon is the 54th element in the Periodic Table and exists as a monoatomic gas with a full outer shell of electrons. First discovered in 1898 by Travers and Ramsay, xenon derives its name from the Greek word for ‘stranger’ because it represents no more than $8.75 \times 10^{-6}\%$ of atmospheric gases. Xenon exists naturally as nine isotopes of which ^{132}Xe is the most abundant, with a mean atomic mass of 131.293. Xenon’s biological effects were

first reported by Lawrence and colleagues in 1946, who observed that xenon (40% and above) and oxygen mixtures produced narcotic effects in mice[82]. Following this, xenon's anaesthetic effects in rodents and humans was reported by Cullen and colleagues in 1951[83]. Xenon has since been used in anaesthetic practice for more than 50 years and is manufactured by fractional distillation of air.

1.4.1.2 Clinical Profile and Pharmacokinetics

The published minimum alveolar concentration (MAC) values for xenon are between 63%atm[84] and 71%atm[83]. Xenon's favourable pharmacokinetic properties resemble many elements of an ideal anaesthetic. These include fast induction and emergence independent of anaesthetic duration due to its low blood-gas partition coefficient of 0.115 and low solubility in lipids with an oil/gas partition co-efficient of 1.9[85]. Xenon's blood-gas partition coefficient is significantly lower than those of other inhalational anaesthetics like nitrous oxide, sevoflurane and desflurane[86]. A large multi-centre trial by the Xenon Study Group reported rapid emergence from xenon anaesthesia, which may be advantageous in a clinical setting. Xenon is also regarded as a 'cardiostable' anaesthetic, causing no change in heart rate or mean arterial pressure[87]. In fact, 70%-inhaled xenon was shown to be cardioprotective in an *in vivo* rabbit model of coronary artery occlusion, reducing infarct size after regional ischaemia. This would be advantageous to TBI patients experiencing polytrauma[88]. Moreover, xenon is known to provide analgesic effects[89] and possesses no toxic, carcinogenic, mutagenic, embryotoxic, teratogenic or allergenic effects[86,90]. Xenon is inhaled and excreted *via* the lungs, with no modification by renal or hepatic systems, which makes its use versatile in critical care[91].

1.4.1.3 Proposed mechanisms of action

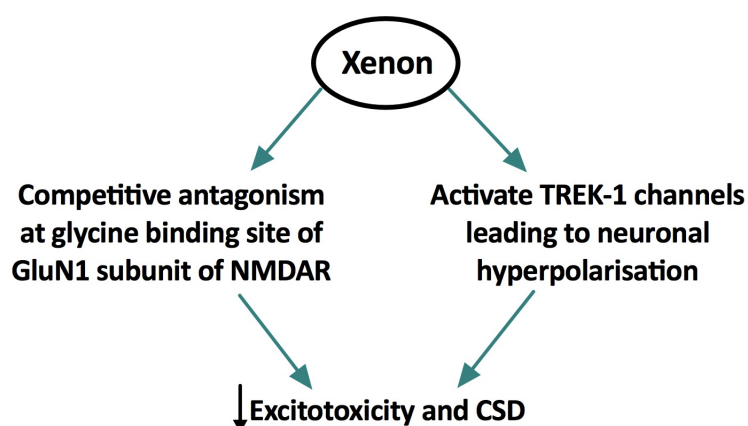


Figure 1.4: Proposed mechanisms of action of xenon[92–94]; CSD: cortical spreading depression

The full electron shell of xenon can be polarised by surrounding molecules causing the induction of a dipole, which enables interactions with proteins through Van Der Waals forces. These weak ‘induced dipole-dipole attraction’ forces are central to xenon’s ability to exert a pharmacological effect[95]. Thus, xenon, although classified as an ‘inert’ gas, is known to exert its biological effects *via* two mechanisms (Figure 1.4). Firstly, xenon is thought to act post-synaptically and exert its anaesthetic effect by potent non-competitive inhibition of NMDA receptors. Franks and colleagues were the first to demonstrate that the application of 80% xenon reduced NMDA evoked currents by 60% in hippocampal neurons[92,96]. Following this, the same group showed, using electrophysiology techniques, that xenon exerts its effects *via* competitive antagonism at the glycine-binding site of the GluN1 subunit of the NMDA receptor[97]. It was proposed that xenon binds the open conformation of the glycine binding domains and prevents domain closure, thus preventing NMDA receptor channel opening. Xenon inhibits NMDA receptors more potently at low glycine than at high glycine concentrations, which is consistent with competitive antagonism at the glycine site. Lineweaver Burk analysis revealed an additional non-competitive component of xenon’s mechanism of action[97]. Further electrophysiological analysis showed that interactions between xenon and the aromatic ring of the phenylalanine 758 amino acid were crucial for xenon binding[98]. A second mechanism of action involving two-pore domain potassium channels has been proposed for xenon. These channels are known to modulate neuronal excitability to exert their anaesthetic action[99–101]. Xenon was shown to activate TREK-1 but not TASK-3 channels. TREK-1 activation leads to neuronal hyperpolarisation which in turn dampens cellular excitability[94]. Finally, it was

shown that xenon has no effect on N-type voltage gated calcium channels[96] and little effect on GABA_A receptors[92] or non-NMDA glutamatergic receptors[102].

1.4.1.4 Xenon and TBI

Over the last decade, xenon has been shown to be neuroprotective in two *in vitro* models of TBI[93,103] and in several models of ischemic[104–107] brain injury. Coburn and colleagues[103] showed that 75% xenon provided significant neuroprotection (over 50%) compared to control traumatic injury. More recently, Harris and colleagues[93] showed 50% xenon abolished the development of secondary injury in a similar model of focal TBI. Xenon appears to prevent development of secondary injury after ischaemic injury[104] and TBI[93], *via* inhibition at the NMDA receptor glycine site[92]. Xenon has also been shown to be effective in an *in vivo* model of blunt trauma[108], where it improved neurological outcome and reduced contusion volume with a therapeutic window of up to 3 hours. Significant improvements in locomotor function were also noted 1 month after trauma.

In terms of clinical trials, xenon has been evaluated in phase II and III trials for ischaemic but not traumatic brain injuries. Results from the TOBY Xe study in 2013 showed that subanaesthetic doses of xenon have anticonvulsant effects in infants with asphyxia encephalopathy[109]. Xenon was also recently tested in combination with hypothermia and results showed that delayed xenon application (within 6 hours) was safe but unlikely to enhance the neuroprotective effect of hypothermia (33.5⁰C) following perinatal asphyxia[110]. A more recent study that evaluated xenon's ability to protect against white matter brain damage following out of hospital cardiac arrest (OHCA) showed that inhaled xenon combined with hypothermia (33⁰C) was more effective than hypothermia alone at reducing white matter damage but no significant difference was found in neurological outcomes or mortality at 6 months[111]. These studies are small and the results will need to be confirmed in larger cohorts to determine whether xenon can translate into clinical benefits.

1.4.1.5 Xenon in combination with other treatments for TBI

Although xenon has not been tested with another pharmacological agent as a combination therapy for TBI, it is an attractive candidate because of its 'ideal anaesthetic' properties discussed in section 1.4.1.2. In fact, xenon and hypothermia combination therapy has been explored in neonatal asphyxia[112], rodent models of ischemia/hypoxia[106,113,114] and

pig models of cardiac arrest[115], for example, Fries and colleagues have shown that the administration of 70% xenon for 1 hour (a short duration) during mild hypothermia at 33°C for 16 hours was protective following prolonged cardiac arrest in pigs[116]. In addition an important point to consider is the cost of xenon. Currently, 1 litre of xenon costs approximately \$20[117] and needs to be administered in a special closed circuit delivery system[86]. The high costs associated with xenon have limited its use so far as an anaesthetic and will prove to be a hurdle when translating xenon therapy for widespread use in clinical TBI. However, combination therapies that use lower concentrations of xenon with other drugs offer a route to lower costs whilst gaining clinical benefits.

1.4.2 Argon as a neuroprotectant in TBI

1.4.2.1 *Discovery*

Argon, the 18th element of the periodic table, is another member of the noble gas family. It was first discovered in 1785 by Cavendish[118] but was classified as a new element by Rayleigh and Ramsay in 1894[119]. Argon has a relative atomic mass of 39.948 and is the third most abundant gas in the atmosphere, at 0.934%atm. Similar to xenon, argon's inability to form strong covalent bonds with other elements means that it lacks chemical reactivity, but in fact was first identified as having biological effects in the 1930's, when its narcotic properties under hyperbaric circumstances were described[120]. Argon is extracted by fractional distillation of liquefied air and is less expensive than xenon due to its abundance in the atmosphere[121].

1.4.2.2 *Clinical Profile and Pharmacokinetics*

Little is known about argon's clinical profile as studies thus far have focussed on its organoprotective and neuroprotective effects *in vitro* and *in vivo*, rather than in the clinic. However, it is known that under normobaric conditions, argon has no anaesthetic or haemodynamic effects[122]. It was proposed that argon produces a strong narcotic effect at pressures higher than 10atm in the body[82,123].

1.4.2.3 Proposed mechanisms of action

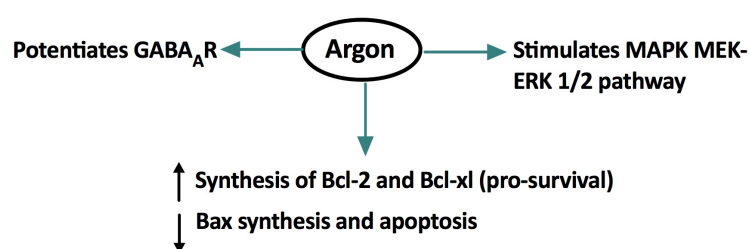


Figure 1.5: Proposed mechanisms of action of argon[124]

In contrast to xenon, very little is known about the mechanism of action of argon. The three most widely explored mechanisms are the Bcl-2 and Bcl-xl pro-survival pathways, GABA_A receptor and MEK-ERK1/2 pathway mediated neuroprotection (Figure 1.5). One study found that argon attenuated NMDA induced neuronal damage in an *in vitro* model of ischemia[121], however, Harris and colleagues[93] showed that while argon is protective in a model of *in vitro* TBI, it does not act *via* the NMDA pathway. They also showed, through electrophysiological methods, that argon does not act *via* TREK-1 potassium channels. Another group showed that argon does not act *via* K_{ATP}-channel opening[125]. Abraini and colleagues proposed that the GABA_A receptor and benzodiazepine site but not the GABA_B receptor might be involved by studying the narcotic potency in rats[126]. However, this study used an indirect method using the effects of antagonists that might have other systemic effects. An *in vivo* study looking at neonatal asphyxia proposed that argon may act *via* increased post-survival protein synthesis of Bcl-xL, a protein in the apoptotic pathway: Bax, Bcl-2 and Bcl-xL[127]. The use of microglial, primary neuronal and astroglial cultures in models of excitotoxicity indicate the involvement of the ERK1/2 pathway[128], but further studies are needed to address the implication of ERK1/2 activation mediated by argon. Further work is required to elucidate the mechanism of argon neuroprotection unambiguously.

1.4.2.4 Argon and TBI

In recent years, the neuroprotective and organoprotective effect of argon has been investigated using *in vitro* and *in vivo* models, predominantly in stroke and ischaemic models and to a lesser extent, in TBI models. Jawad and colleagues found that 75% argon was protective in a model of oxygen glucose deprivation (OGD) of ischemic injury *in vitro*[121]. Loetscher and colleagues examined found that concentrations of argon ranging from 25 to 74% in argon-oxygen mixtures were protective in both OGD and TBI models using

organotypic hippocampal slices *in vitro*. They found dose dependence of argon's neuroprotective effect in the *in vitro* TBI model and that delayed argon administration was still neuroprotective[129]. Harris and colleagues found that 50%atm argon was able to attenuate the development of injury, although they did not observe as great a reduction as found by Loetscher and colleagues[93]. In addition, argon has been assessed in *in vivo* models of neonatal asphyxia[127], middle cerebral artery occlusion[130] and cardiac arrest induced neurological damage[131] in rats. Concentrations of argon in the range of 50-70%[130,132] administered even after a 3-hour delay[131], have been shown to be neuroprotective. However, the effect of argon in TBI models *in vivo* has not been evaluated so far.

1.4.2.5 Argon in combination treatment for TBI

To date, there has been only two studies that have examined the potential of argon combined with hypothermia (33°C), but not with another pharmacological agents, in an *in vitro* and *in vivo* models of hypoxic-ischaemic encephalopathy[133]. Zhao and colleagues concluded that argon works synergistically with hypothermia to confer neuroprotection in their model[133]. In another *in vivo* model of hypoxia ischemia, inhaled 45 – 50% argon combined with hypothermia (33°C) improved brain energy metabolism, increased EEG recovery and reduced cell death[134]. Unlike xenon, argon lacks anaesthetic/sedative properties at atmospheric pressure, which makes it more versatile for use in situations where sedation is not required. It is more readily available than xenon and 100-fold more cost effective[135]. Thus, if in combination with other drugs, argon proves as effective as xenon administered alone, then argon is an attractive candidate for combination therapy in TBI.

1.4.3 Progesterone as a neuroprotectant in TBI

1.4.3.1 Discovery

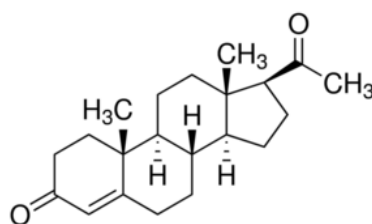


Figure 1.6: Chemical structure of progesterone (taken from Sigma Aldrich catalogue)

Progesterone (pregn-4-ene-3,20-dione, also known as P4) is a 21-carbon endogenous steroid hormone synthesized in the gonads, placenta and adrenal glands. Figure 1.6 shows the chemical structure of progesterone. Similar to neurosteroids, progesterone is produced in the brain, where it exceeds peripheral plasma levels by more than 5-fold[136]. While originally thought to be only a sex hormone of women, it is also synthesized in men[137] and there do not appear to be sex differences in brain levels[136,138]. Allen originally described the molecular formula of progesterone in 1933. However, progesterone was synthesized from diosgenin extracted from the plants *Dioscorea tokoro* and *Dioscorea mexicana* only in the mid 1940s[139], allowing it to be used in hormonal therapies. Pre-clinical and clinical studies have demonstrated that progesterone is more than simply a reproductive hormone[64,140–143]. Progesterone's neuroprotective effect was first described by Attella and colleagues in 1987, when they demonstrated reduced oedema in pseudopregnant rats after frontal cortex lesions when compared to control cycling rats. Following this discovery, progesterone has been shown to have neuroprotective effects in a variety of CNS injury models including TBI, Alzheimer's disease, multiple sclerosis, peripheral nerve injury, stroke and diabetic neuropathy[140].

1.4.3.2 Clinical profile and pharmacokinetics

Progesterone has an established clinical profile and several features that make it an attractive drug candidate for TBI. First, its pharmacokinetics, safety and adverse effects profile is well established. It has a wide therapeutic window, and a single bolus dose given up to 24 hours post injury has been shown to reduce cerebral oedema[144]. Progesterone has several established routes of administration including transdermal, sublingual, oral and intramuscular however in a clinical scenario involving a TBI, intramuscular administration would be the most appropriate as it ensures achievement of adequate plasma levels and monitoring by

serum concentration measurements. Progesterone has a relatively short half-life of only 5 minutes and is circulated in the bloodstream bound to cortisol-binding globulin (10%) and serum albumin[145]. Progesterone is known to rapidly cross the blood-brain barrier and equilibrate with the plasma within 1 hour of administration[146–148]. It is metabolised *via* renal 21-hydroxylation to the potent mineralocorticoid desoxycorticosterone (DOC)[145].

1.4.3.3 Proposed mechanisms of action

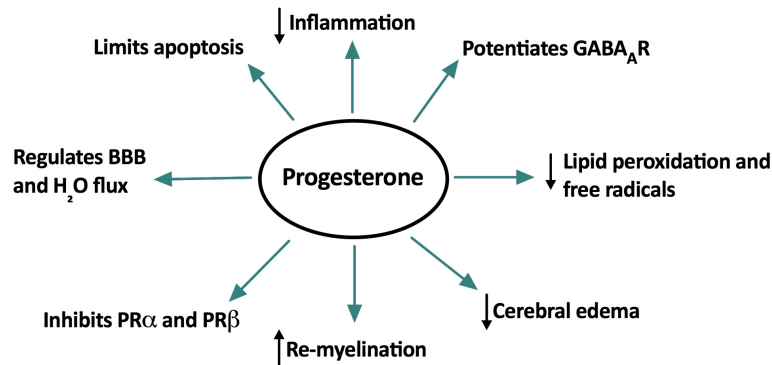


Figure 1.7: Progesterone is a pleiotropic drug which can (1)downregulate the inflammatory cascade and release of cytokines, (2) potentiate GABAergic signalling, (3) reduce lipid peroxidation and the release of free radicals, (4) decrease cerebral oedema and modulate brain water levels *via* aquaporin channels, (5) promote myelination, (6) inhibit progesterone receptors and (6) decrease apoptosis[149–151]

Progesterone is a pleiotropic compound that targets a number of different pathways (Figure 1.7). These will be reviewed briefly with respect to its actions following a TBI. Progesterone’s ‘classic’ mechanism of action is *via* the progesterone receptor (PR, also known as the sigma receptor), which is a nuclear transcription factor that regulates transcription through progesterone response elements in the promoter region of target genes. Progesterone receptors are widely distributed in both the adult and developing brain[152] in two major isoforms – membrane bound (m)PR- β and the N-terminally truncated version, (m)PR- α . The PR- α subtype, expressed by neurons, is widely distributed in male and female mouse and rat brain tissue, and is particularly concentrated in the hippocampus. Interestingly, Meffre and colleagues noted that after TBI (bilateral contusions to the medial prefrontal cortex) in rats, (m)PR- α is expressed on microglia, astrocytes and oligodendrocytes. They proposed that this plays a role in mediating the modulatory effects of progesterone after TBI, particularly in inflammation, ion and water homeostasis and myelin repair[153].

Moreover, several studies have investigated reduction in oedema as a mechanism of action of progesterone. Roof and colleagues reported a concentration dependent reduction in both

vasogenic and cytotoxic oedema with progesterone treatment after TBI[150,154]. A few possible underlying mechanisms for this have been proposed. Guo and colleagues suggested that progesterone might decrease levels of the water channel membrane protein aquaporin 4 (AQP4), which lowers cerebral oedema by inhibiting active ion uptake through the $\text{Na}^+/\text{K}^+/\text{ATPase}$ pump[155]. This would also modulate vasopressin levels, reduce neurogenic inflammation and prevent vessel growth associated with a leaky BBB[156]. Other groups have suggested that progesterone is neuroprotective *via* a reduction in lipid peroxidation through inhibiting free radical formation and by upregulating antioxidant enzymes like superoxide dismutase, which enhances the efficiency of scavengers that eliminate ROS[157,158]. This helps stabilise the BBB. Moreover, several groups have reported a modulation in cytokine release and inhibition of immune cell activation and migration as a mechanism for progesterone neuroprotection following TBI[159–161]. In an *in vivo* model of bilateral prefrontal cortical contusion injury, progesterone attenuated the release of IL-1 β and TNF[162]. In another model, progesterone decreased injury induced complement factors 3 and 5, CD24 and 74 and macrophage inducing factor 1. Progesterone's ability to dampen neuronal excitotoxicity by blocking calcium channels and upregulating GABA has also been reported[163–165]. Another suggested mechanism for progesterone is re-myelination by increasing myelin and brain derived neurotropic factor production[142,151,166]. In a rat model of TBI, progesterone increased nerve growth factor expression in the brain, which led to axonal growth, inhibition of neuronal apoptosis, reduced cholinergic cell death and prevention of neuronal loss[167]. Finally, progesterone has been reported to modulate mitochondrial function *via* inhibition of mitochondrial specific pro-apoptotic enzymes like cytochrome c, activated caspase 3 and upregulation of anti-apoptotic Bcl2 and ERK, which helps maintain cell integrity and promote survival[151,160].

1.4.3.4 Progesterone and TBI

Surprisingly, to date there have been no studies investigating progesterone in *in vitro* models of TBI. However, more than 50 studies investigating progesterone in animal models of TBI have been conducted alongside a several studies that have investigated progesterone in models of ischemia and intracerebral haemorrhage induced injury[70]. Besides mechanistic effects described above, progesterone has improved functional outcomes in a number of TBI models[160,168]. These include behavioural measures like locomotor activity[169–171], spatial learning and memory[144,170,172,173], anxiety[169], motor and cognitive

performance[174]. All of these results have contributed to progesterone being tested in clinical trials for TBI.

Progesterone showed promise in two Phase II randomised controlled trials (RCT) following blunt trauma. In the first trial, patients with a GCS score of 4-12 post resuscitation who received progesterone (0.71mg/kg loading dose followed by 0.5 mg/kg IV and five 12-hour IV infusions) showed a mortality rate that was over 50% lower than placebo treated participants at 30 days[175]. In the second Phase II RCT, patients with a GCS score<8 following severe blunt trauma who received progesterone (1mg/kg intramuscular loading dose within 8 hours of trauma and then once every 12 hours for 5 days) had significantly improved Functional Independence Measure scores and extended Glasgow Outcome Scale (eGOS, not to be confused with GCS) scores compared to healthy controls[176]. These results lead to progesterone being tested in two Phase III RCTs - SyNAPSe (NCT00822900) and ProTECT III (NCT01143064). These multi-site Phase III RCTs investigated IV progesterone in moderate and severe acute (GCS<8), and severe (GCS 4 – 12) TBI respectively. In the SyNAPSe trial, 1195 patients were treated with continuous IV progesterone (0.71mg/kg loading dose followed by 0.5mg/kg/h for 5 days)[177] and in the ProTECT III trial, 882 patients were treated with continuous IV progesterone (0.71mg/kg loading dose followed by 0.5mg/kg/h for 3 days and a 24-hour infusion taper of 0.12mg/kg/h)[178]. The results of SyNAPSe and ProTECT III have recently been published and neither showed a benefit of progesterone over placebo, as measured by eGOS scores 6 months after injury. Scientists have cited several reasons for this failure[179,180] (for a detailed review see Schumacher *et al.*, 2016[180]).

1.4.3.5 Progesterone in combination therapy for TBI

One compelling reason cited for the failure of phase III clinical trials is heterogeneity of TBI and the inability of single agents, even progesterone, to address all of the secondary injury cascades. Schumacher and colleagues suggest that the effects of progesterone are modulated by many factors, both internal and external, as it acts in combination with other signalling pathways[180]. Combination therapies offer an opportunity to further enhance the pleiotropic effects of progesterone in TBI. Specifically, it would be advantageous to combine progesterone with agents that act on complementary pathways. In particular, drugs that act on excitatory mechanisms and inhibit calcium influx, inhibit mitochondrial cascades, protect the

intracerebral vasculature, enhance free radical scavenging and protect against DAI would be particularly useful strategies[81]. Progesterone has already proven its efficacy in phase II clinical trials. Building on the wealth of literature on progesterone's effects as a single agent with potential combination strategies may allow us to gain more from its pleiotropic nature as a neuroprotectant.

1.4.4 Meloxicam as a neuroprotectant for TBI

1.4.4.1 *Discovery*

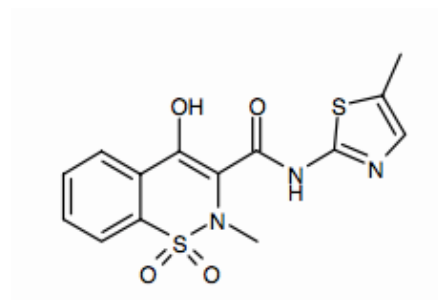


Figure 1.8: Chemical structure of meloxicam[181]

Meloxicam (4-hydroxy-2-methyl-N-(5-methyl-2-thiazolyl)-2H-1,2-benzo-thiazine-3-carboxamide 1,1-dioxide) is a newly synthesised enolcarboxymide non-steroidal anti-inflammatory drug (NSAID). Developed by Boehringer Ingelheim (Mobic™), meloxicam is approved for the treatment of osteoarthritis in the US and is registered for the treatment of rheumatoid arthritis and ankylosing spondylitis amongst other musculoskeletal conditions in other countries. Its chemical structure is shown in Figure 1.8. Meloxicam only recently began to gain attention as a neuroprotectant, when it was discovered that NSAIDs are able to modify the response to cerebral ischemia by inhibiting the cyclooxygenase 1 (COX-1) and COX-2 enzyme isoforms[182]. Following this it has been tested in *in vitro* and *in vivo* models of ischaemic injury and in two *in vivo* models of TBI.

1.4.4.2 *Clinical Profile and pharmacokinetics*

The clinical and drug safety profile of meloxicam has been extensively studied. Meloxicam is metabolised *via* the cytochrome P450-2C pathway to four pharmacologically inactive metabolites. The mean half-life is 19.5 – 23.4 hours and the drug is excreted *via* biliary and enteral secretion. Meloxicam circulates as 99% bound to plasma protein, in particular albumin. Routes of administration include oral, oral suspension and intramuscular injection.

The drug is known to have a good safety profile as it has been studied in several trials for musculoskeletal conditions[181].

1.4.4.3 Proposed mechanisms of action

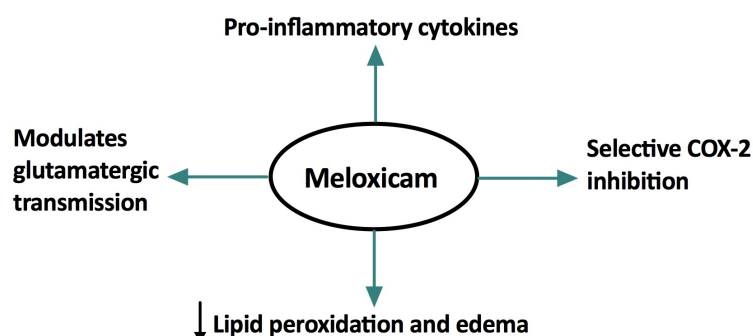


Figure 1.9: Proposed neurprotective mechanisms of meloxicam following TBI[183–185]

Meloxicam, like other NSAIDs, works by inhibiting prostaglandin synthetase *via* inhibition of the COX enzyme. COX regulates the metabolism of arachidonic acid and prostanoid synthesis, which is carried out by tissue-specific isomerases into prostaglandin (PG) E₂, PGI₂ (prostacyclin), PGD₂, PGF_{2a} and thromboxane A₂. Prostaglandins produce ROS and free radical which damage neural membranes, white matter and tight junctions following TBI. COX-1 and COX-2 are two isoforms of the COX enzyme, both of which are constitutively expressed in the brain and are essential for neuronal function and plasticity. COX-1 is the constitutive COX enzyme, which induces prostacyclin synthesis, leading to anti-thrombogenic effects and gastric mucosal repair. COX-2 is an inducible enzyme, which becomes upregulated over time following TBI, especially in the hippocampus[186]. It is induced in fibroblasts and macrophages by cytokines and pro-inflammatory stimuli[187,188]. COX-2 products play a role in secondary responses, which increase ICP, vasospasm and ischemia following TBI. Meloxicam is COX-2 selective, and inhibits COX-2 to the same extent as traditional NSAIDs (77.5%) but inhibits COX-1 less (53.3%). Preferential COX-2 inhibition leads to a better GI and safety profile. Meloxicam also has analgesic properties. Figure 1.9 shows the proposed mechanisms of action of meloxicam following TBI.

1.4.4.4 Meloxicam and TBI

The inhibition of COX as a therapeutic strategy for TBI is based on evidence for the implication of COX in the inflammatory cascade post TBI[183,186] and meloxicam has been shown to inhibit pro-inflammatory cytokines like IL-1 β , IL-6 and TNF[184]. COX-1 and

COX-2 inhibitors have been tested in *in vivo* models of TBI. In addition, there has been some evidence for the ability of COX enzymes to modulate glutamatergic transmission, thus interfering with glutamate induced cell death. COX-2 expression is increased in brains from animals subjected to experimental injuries involving excitotoxicity[189]. Selective COX-2 inhibition has been shown to reduce cortical brain damage caused by injection of NMDA and hippocampal oxidative damage caused by injection of kainate[190,191] in a model of ischaemic brain injury. Thus, the inhibition of COX enzymes plays a dual role – anti-inflammatory and anti-excitotoxic, in protection after a TBI.

To date, there have been no *in vitro* studies of meloxicam in TBI models. However, in an OGD model in rat organotypic hippocampal slices, meloxicam reduced cell death and modified the transcription of vesicular glutamate transporters. Llorente and colleagues suggest that this leads to a different stoichiometry of NMDA and AMPA receptors and thus lowers excitotoxicity induced by the OGD insult[192]. There have been two recent studies of meloxicam in *in vivo* models of blunt trauma. Hakan and colleagues reported a decrease in lipid peroxidation and oedema at 48 hours, restored glutathione and preserved BBB integrity at 24 and 48 hours. They also reported an improvement in neurological examination scores[185]. In another study, meloxicam reduced inflammation but did not alter oedema or functional outcome[193]. There have been no clinical trials for TBI with meloxicam.

1.4.4.5 Meloxicam in combination therapy for TBI

Based on pre-clinical studies, meloxicam has been shown to reduce excitotoxicity and inhibit pro-inflammatory cytokines and prostaglandins. There have also been mixed results concerning oedema[71]. Therefore, while meloxicam appears to elicit beneficial anti-inflammatory effects and may be linked with a decrease in excitotoxicity post experimental TBI, there is insufficient research to prove its efficacy as a single agent therapeutic in terms of preventing tissue damage and improving functional impairments post human TBI. Testing meloxicam in combination with drugs that elicit complementary effects, for example more potent NMDA antagonists, may augment its protective function.

1.5 Pre-clinical models of TBI

In view of the heterogeneous clinical manifestations of TBI and because the development of secondary injury is attributed to multiple mechanisms, an advantage of *in vitro* and *in vivo* TBI models is the study of more than one mechanism or effect. *In vivo* models simulate clinical outcomes of TBI and are useful to test the efficacy of treatment within a physiological context. Besides closely simulating many of the pathological processes that occur following human TBI, advantages of *in vivo* models include control of the biomechanical nature of injury, physiological monitoring and pre-clinical validation of treatment. However, the complexity means that it is difficult to monitor the real time progression of injury, isolate a particular pathway to study the mechanism of a novel treatment and control biomechanics of the injury at a cellular and tissue level[194]. On the contrary, *in vitro* models allow for the isolation of a particular tissue/pathway of interest, precise control of the extracellular environment, repeated access to the tissue for real time monitoring and have lower associated costs. Pathobiology can be examined within specific regions of interest and the biomechanics of injury can be controlled to determine the effects of different injury severities[195]. These factors make *in vitro* models particularly useful for studying the mechanisms of injury and action of neuroprotective agents. Notwithstanding, both *in vitro* and *in vivo* models complement each other and are both necessary to validate findings.

1.5.1 Organotypic Hippocampal Slices as an *in vitro* model of TBI

Organotypic cultures, unlike primary dissociated cell cultures, are slices of tissue that are cultured whole, in a three-dimensional system where the main architecture of the cells is preserved. Organotypic hippocampal slice cultures (OHSC) retain many of the *in vivo* properties and connective organizations found *in situ* whilst being accessible to experimental manipulations and allow long-term survival of the tissue. OHSC offer a significant advantage over cell and tissue culture systems (eg. primary cell cultures and dissociated primary cultures of neurons) because *in vivo* interconnections and heterogeneity are maintained.

Organotypic brain slices have several advantages but also some disadvantages. Components of synaptic function (including pre- and postsynaptic components, synaptophysin, and a postsynaptic adhesion protein) are expressed at steady state levels in slices for at least 30 – 60

days[196]. They also exhibit properties of *in vivo* tissue in the absence of extrinsic input, including histological and morphological organization[197–200], synapses and spine morphology which mature over time, protein expression and long term potentiation, allowing the study of synaptic plasticity[196]. Some examples of synaptic maturation that have been noted in OHSC are astrocyte maturation, high synaptic density and the presence of active synaptic vesicles. Patterns of protein expression and gene regulation of the adult hippocampus have also been noted in hippocampal slices[201]. Although a drawback is the lack of conventional vasculature in slices, at the cellular level, organotypic slices have been shown to contain brain capillaries stained positive for laminin, which is a basement membrane marker that stains vascular structures of the brain. These capillaries were shown to survive well although they lacked conventional blood flow[202,203]. Another disadvantage is the loss of target innervation due to axotomy, which promotes neuronal death and causes some reorganisation of the tissue[204].

In our laboratory, OHSC from five to six day old C57BL/6 mice pups are prepared using the Stoppini[205] technique. Experiments are conducted after slices have been in culture for 11-14 days to allow stabilisation of intrinsic axonal connections and ensure that synaptic connections are not activated by the endogenous release of glutamate or calcium and that reactive astrogliosis is limited[204]. Extended periods of culturing (more than 7 days) clears dead cells, repopulates denuded dendritic fields and re-establishes a quiescent state leaving a thin layer of healthy tissue[195]. When studying traumatic injury, it is particularly important that the slices are in a resting, non-activated state before trauma is inflicted. Although a limitation of the model is that the cultures are prepared from young animals, some developmental maturation does take place during the culture period, albeit at a slower pace than *in vivo*[195]. Also, brain tissue from young animals has been correlated with better survival due to increased resistance to mechanical trauma during the dissection process and a high degree of plasticity[206]. The equipment used to model TBI *in vitro* simulates a focal contusion injury using a weight drop model, which is a common *in vitro* model of TBI[207]. The model allows for a controlled reproducible injury to be monitored over time. Adjusting the impact force and duration of the impact can control the severity of the impact. This model has been used to model TBI previously. The neuroprotective effects of various compounds has been investigated such as xenon and helium[103], argon[129], levosimendan – a novel inodilator[208] and erythropoietin[209], amongst others. Overall, it is a robust model of *in vitro* TBI.

1.5.2 Controlled Cortical Impact *in vivo* model of TBI

In a clinical context, ‘focal’ or ‘blunt’ TBI is used to describe a spectrum of pathologies including intracerebral/intracranial hemorrhage and cortical contusion, regardless of the biomechanical nature of the primary injury. The Controlled Cortical Impact (CCI) model is one of the most widely used models of blunt TBI *in vivo* and, alongside the fluid percussion injury and weight-drop acceleration models, is used to study the complex molecular cascades initiated by blunt TBI.

First classified by Dixon and Lighthall in 1991[76,210], the CCI model has been used in ferrets[210], rats[76], mice[211], swine[212] and monkeys[213]. It mimics several pathological features of TBI that are seen in humans including contusion, cortical tissue loss, acute subdural haematoma, axonal injury, concussion, blood brain barrier disruption, hemorrhage and even coma[76,78,211]. Contusions typically appear hemorrhagic and necrotic, causing post-traumatic vasogenic cerebral edema and neuronal loss or axonal pathology beyond the contusion site. To create the injury, a pneumatic or electromagnetic impact device is used to drive a rigid impactor tip onto the exposed intact Dura of the brain, causing deformation of the underlying cortex[76]. The impact is delivered to the intact Dura through a unilateral craniotomy, usually between Bregma and Lambda on either side of the skull. The damage caused by the impact may extend all over the ipsilateral hemisphere and to the contralateral hemisphere[214]. Furthermore, whether the craniotomy is sealed following the impact affects the outcome, and is usually sealed so that it models elevated ICP.

The CCI model offers several advantages over other injury models. Firstly, it is highly reproducible and controllable. Parameters like the velocity, impact depth, dwell time, diameter of the impactor tip and stereotaxic location of the impact can be standardised and controlled. Additionally, when compared with other models that use gravity driven devices, the CCI model carries no risk of a rebound injury[215]. Increasing the cortical deformation and impact velocity increase the severity of histopathological outcomes. CCI injury also leads to functional deficits like cognitive impairments – performance on the Morris water maze test has been related to both the deformation depth and velocity of the impact[77], sensorimotor and emotional deficits. It has also been shown by various groups that tissue loss and histopathological features produced by CCI results in atrophy that persists for up to 1 year

post trauma, which is of clinical relevance to human TBI that has been associated with progressive neurodegenerative disorders[40,216–218]. Although the CCI model setup is costly and complex, it allows the collection of physiological data and monitoring similar to an intensive care unit and may therefore facilitate translation of animal data into clinical practice.

1.5.3 Pre-clinical models and combination therapies

Compared to *in vivo* models, *in vitro* models offer a low-cost and high-throughput screening platform to evaluate combination therapies. The weight drop model with OHSC in particular is able to reproduce tissue distortion of blunt TBI and can activate some of the same secondary injury mechanisms. A wide range of timing and dosing parameters can be explored. The use of *in vitro* models is also faster as they cut the need to gain the regulatory and ethical approval required in animal studies. Overall, for proof of principle studies, *in vitro* models offer a realistic screening platform to test combination therapies. The results from *in vitro* models can then be tested in *in vivo* models where dosing, safety, pharmacokinetic properties of the combination alongside functional outcomes can be optimised, with the best combinations put forth for further clinical testing[81].

1.5.4 High throughput models for TBI

The traditional drug discovery process is long-winded and can take several millions of dollars and over a decade to identify a single promising lead compound. Even then, the success of bringing this drug to the market is not guaranteed. Although the development of animal models of TBI has exponentially enhanced knowledge of pathophysiology, as reviewed in this chapter, pre-clinical evaluation of drugs for TBI can be lengthy and expensive. Traditional high-throughput drug screening (HTS) approaches based on cell culture and biochemical assays are useful and necessary to understand mechanisms of drug action and for proof of concept studies. To complement these, the primary screening of drugs directly in whole animals with relevant systems interacting with each other would increase the validity of drugs tested in *in vitro* models.

TBI research would greatly benefit from a HTS assay. The fruit fly *Drosophila melanogaster* has been used for over a 100 years as a highly tractable model organism in human diseases and has already proved useful in the study of neurodegenerative disorders, memory and

sleep[219]. *Drosophila* offers several unique advantages to the study of TBI. Firstly, large cohorts of animals can be used more rapidly and inexpensively (compared to rodents) to examine relationships between injuries of different biomechanical nature and the development of outcomes. *Drosophila* lines are easy to obtain and have a low cost of maintenance and propagation. Secondly, injury development can be examined over the entire lifespan of a fly that is otherwise expensive and challenging to do using rodents. Further, molecular tools may be used to explore different injury pathways in a high throughput and efficient manner. Using *Drosophila* enables scientists to take advantage of the sophisticated genetics tools available in addition to being able to mimic conditions to study metabolic stability and toxicity in whole organisms.

In terms of biology, *Drosophila* has several features that make it an attractive model to use. The fly genome has been completely sequenced and encodes for over 14,000 genes. Nearly 75% of disease related genes in humans are suggested to have functional orthologs in the fly[220,221]. At the level of nucleotides and protein sequences, flies share approximately 40% identity with mammals between homologs and 80 – 90% in conserved functional domains. The fly has a relatively short lifecycle and the adult fly is suggested to be a sophisticated and complex organism, ‘not unlike higher organisms’ with structures that perform functions equivalent to those of the mammalian heart, kidney, lung, gut and reproductive tract[222]. The fly brain is complex, with more than 100,000 neurons which form discrete circuits and model complex behaviours including sleep, learning and memory, feeding, courtship, aggression and navigation[222]. It is encapsulated by an exoskeleton known as the cuticle, which functions similarly to the human cranium. Inelastic in nature, its function is to encapsulate the brain, provide protection from the environment and maintain structure of the head. Thus, a jolt to the head or proboscis would most likely cause the brain to deform against the cuticle in a similar manner to a human or rodent brain deforming against the cranium. Katzenberger and colleagues[223] first explored the potential of flies to model TBI. Developing this concept and model further could lead to additional insights for TBI research.

1.6 Aims and hypotheses

1.6.1 Aims

This thesis is structured as a broad study of models of TBI and an investigation of drugs (both individually and in combination) to treat the condition. After reviewing the literature it is clear that there is a need to explore more different and multifactorial strategies to treat TBI. Noble gases present a viable option as combination treatments and these are explored in combination with the pleiotropic drug progesterone and the anti-inflammatory drug meloxicam in an *in vitro* model of TBI. Chapter 3 describes a series of experiments to develop an injury quantification methodology in an *in vitro* model of TBI. This is followed by a study of noble gases, drugs and combination therapies to treat TBI in chapter 4.

Moreover, from the literature, it is evident that both *in vitro* and *in vivo* models are required in tandem to assess the efficacy of neuroprotective agents. *In vivo* models and in particular, the CCI model of injury has been suggested to simulate many of the clinical outcomes of TBI. The results of neuroprotective efficacy obtained *in vitro* can be tested *in vivo* to validate findings. Thus, chapter 5 presents work undertaken to develop and characterise a CCI model of injury.

Finally, as pre-clinical evaluation of drugs for TBI has been shown to be a lengthy and expensive process, a *Drosophila melanogaster* model of TBI was set up as a HTS assay. Chapter 6 describes a series of experiments designed to establish a model of polytrauma and test neuroprotection with hypothermia, a treatment that is currently gaining attention in TBI research.

1.6.2 Hypotheses

1. I will test the hypothesis that progesterone and meloxicam are neuroprotective following TBI *in vitro*
2. If progesterone and meloxicam are found to be neuroprotective, I will test the hypothesis that combination treatment with xenon and progesterone and combination treatment with xenon and meloxicam is more effective than a concentration of xenon which is sub-maximally effective following TBI *in vitro*

3. I will test the hypothesis that combination treatment with argon and progesterone and combination treatment with argon and meloxicam is more effective than argon administered alone following TBI *in vitro*
4. I will test whether combination treatment with noble gases and the anti-inflammatory drugs is more effective than the drugs acting alone and whether the combined protective effect with noble gases is additive or synergistic, following TBI *in vitro*
5. I will set up an *in vivo* model of CCI injury and test whether contusion volume varies with severity of the injury
6. I will test whether pro-inflammatory cytokines CCL-2, TNF and IL-6 are up-regulated in the acute time period up to 24 hours post injury and whether up-regulation is correlated with injury severity
7. I will establish a *Drosophila* model of traumatic injury and test the hypothesis that hypothermia is protective and improves survival.

2 Chapter 2: Materials and methods

2.1 Modelling traumatic brain injury *in vitro* using organotypic hippocampal slice cultures from mice

2.1.1 Subjects and ethical approval

Hippocampal slices were obtained from five to six day old C57BL/6 mouse pups and cultured using the air-liquid interface technique of Stoppini[205] *et al.* with some modifications. Female mice with litters were purchased from Charles River, UK. All experiments were performed after approval of the Ethical Review Board, Imperial College London and in accordance with the United Kingdom Animal Scientific Procedures Act, 1986 (project license: 70/7466).

2.1.2 Set up of the laboratory for dissection

Organotypic cultures are sensitive to changes in the environment and as the slices were cultured for a relatively long period of time (11 – 14 days), it was important that they were manipulated at all stages from the dissection procedure through their maintenance over the culture period in as sterile an environment as possible. It was found that a low temperature during the dissection and sterility at every step were critical to produce healthy and viable slices. Therefore, prior to dissections, a number of steps were taken to minimise contamination. A lab coat, gloves, hairnet and PPE mask were worn throughout the procedure and all surfaces were wiped with 70% ethanol. Sterile autoclaved paper towels were prepared in advance and laid down in all areas of the bench and laminar hood, which would come into contact with dissection equipment. Dissection tools were sterilized in advance by treating with 0.1% Virkon (VWR International, Leicestershire, UK) for a minimum of 20 minutes before being autoclaved. Tools were also sprayed with 70% ethanol immediately before use. Sylgard® elastomer (Dow Corning MI, USA) was used to line the base of 150mm plastic petri dishes used in the dissection. The Sylgard® and razor blades (Wilkinson Sword, Bucks, UK), used in the slicing procedure, were autoclaved and sprayed with 70% ethanol before use. Using a McIlwain tissue chopper, brains were sliced on a PTFE chopping disk that was washed with detergent and sterilised by immersion in 70% ethanol for at least one hour before use. All equipment and tools were allowed to dry fully before coming

into contact with brain tissue. 70% ethanol was liberally sprayed on gloves throughout the procedure. The Petri dishes that would contain brain tissue were always placed on metal heat sinks kept on ice to ensure that temperatures were kept as low as possible for optimal survival of the tissue. The metal heat sinks were cleaned with detergent, autoclaved and sprayed with 70% ethanol before use.

2.1.3 Dissection of mouse brain

4 male mouse pups (P5 – 6) were humanely killed by decapitation. The head was handled with sterile tissue while angled surgical scissors were used to cut the skin along the midline from the base of the neck to the nose. The skin was peeled back to access the skull. An incision was made at the posterior end of the skull above the cerebellum on the left and right hemispheres. The skull was cut along the midline in the anterior direction and a third incision was made on either side above the olfactory bulbs. The two panels of the skull were pulled back using the tips of the surgical scissors to reveal the brain underneath. A small spatula was used to gently remove the brain and transfer it to a petri dish containing ice-cold dissection media (Gey's balanced salt solution, 5 mg/ml D-glucose (Sigma G8270), 1% antibiotic-antimycotic solution (10,000 units/mL penicillin G, 10 mg/ml streptomycin sulphate and 25 mg/mL amphotericin B, Sigma A5955)). The above procedure was repeated until brains from four pups were removed.

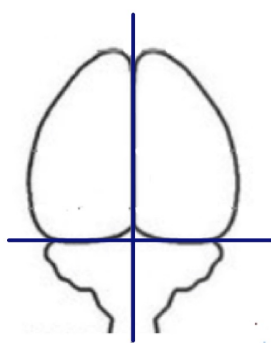


Figure 2.1: Schematic diagram showing the dissection of P5-6 mouse brain. Tissue was cut coronally and midbrains were removed.

Once the brain tissue was in media, it was important that subsequent steps were performed as quickly as possible to prevent warming of the tissue. Using a razor blade (Wilkinson Sword, Bucks, UK) a coronal cut was made at the posterior end of the brain to remove the cerebellum (Figure 2.1). The hemispheres were then separated and transferred using a spatula to a 35 mm petri dish that was filled with ice-cold dissection media and placed on a cold heat

sink. The hemispheres were taken to a cell culture hood and transferred to a Petri dish lined with a Sylgard® containing fresh dissection media and placed on a heat sink. The olfactory bulbs were removed with a razor blade. The midbrain was gently teased apart from the cortex with ultrafine forceps, under a stereomicroscope. When placed face up, the hippocampus could be seen running along the middle of the cortex.

2.1.4 Slicing of the brain and separation of hippocampal slices

A McIlwain Tissue Chopper (Mickle Lab Engineering Co, Surrey, UK) was used to prepare hippocampal slices. The PTFE chopping disk (pre-cooled in dissection media) was placed on the cutting table and ice-cold dissection media was used to moisten the disk. A clean sterile razor blade was attached to the chopper and aligned perpendicular to the disk surface. The sections of cortex were transferred using a spatula and orientated face up so that the longitudinal axis of the hippocampus was parallel to the blade. Excess dissection medium was drained using a fine tip Pasteur pipette. 400µm-thick slices (Figure 2.2) were cut at 50% chopping speed and 50% force (arbitrary units).

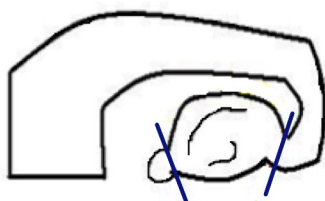


Figure 2.2: Schematic diagram showing the hippocampus attached to cortex tissue of a typical P5-6 mouse brain. The brain was cut into 400µm thick slices and the hippocampus was trimmed at the fimbria and at the end of the dentate gyrus as shown by blue lines.

Ice-cold dissection media was applied immediately after slicing and the tissue was transferred using a spatula back to the dissection dish now containing fresh media. The slices were gently teased apart under a stereomicroscope using ultra fine surgical forceps with care taken to avoid touching the hippocampus. The cortex was dissected from the hippocampus using ultrafine Vannas scissors. Each slice was then trimmed at the fimbria of the hippocampus and at the end of the dentate gyrus. A cut-off sterile plastic Pasteur pipette was used to transfer the slices to a fresh 35mm petri dish containing ice-cold dissection media. On average, 20 – 24 slices were obtained from each P5 – 6 mouse brain. Figure 2.3 shows a hippocampal slice before plating.



Figure 2.3: Brightfield image of a typical P6 hippocampal slice before plating

2.1.5 Plating of hippocampal slices onto membranes

The petri dish containing the slices was placed on a pre-cooled metal heat sink and transferred to a sterile laminar flow hood. As the slices were no longer placed on ice, it was important that the plating procedure was performed as quickly as possible to prevent warming. Typically, the complete plating procedure took 15 minutes.

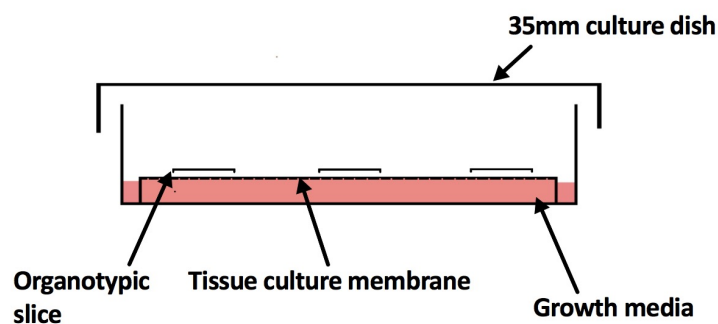


Figure 2.4: Schematic diagram showing slices plated on tissue culture membranes with growth media underneath according to the 'air-liquid' interface technique.

A cut-off Pasteur pipette was used to transfer 6 – 7 slices to each tissue culture membrane insert (Millicell CM, Millipore Corporation, Billerica, MA, USA) placed inside a sterile 35mm petri dish. The membranes allowed the slices to remain in contact with air and receive nutrients *via* diffusion from the medium underneath (Figure 2.4). The slices were gently distributed evenly around the membrane and away from the edge of the insert. The excess dissection media was then drained with a fine tipped Pasteur pipette. 1ml of ice-cold growth media (50% Eagle minimal essential medium with Earle's salts, L-glutamine and bicarbonate (Sigma M4655), 25% Hank's balanced salt solution (Sigma H9394), 25% heat-inactivated horse serum (Sigma H0146), 3mM L-glutamine (Sigma G7513), 5 mg/ml D-glucose (Sigma

G8270), 1% antibiotic-antimycotic solution (Sigma A5955) and 10mM HEPES (VWR 441485H); pH 7.2) was added under each membrane insert so that it covered the base of the petri dish completely but did not flow over the insert. Individual lids were placed on each 35mm dish. The dishes were then placed inside a large glass dish in which they were transferred to a humidified incubator at 37°C with 5%CO₂ in room air.

2.1.6 Maintenance of slices during culture

Slices were cultured for 11 – 14 days before experiments. Media was replaced with pre-warmed (37°C) growth media the day after slices were plated and then every alternate day thereafter until the slices were used for an experiment. During the media change procedure, old media was drained from underneath the membrane using a fine tipped Pasteur pipette and replaced with fresh growth media without disturbing the membrane. Slices would typically flatten over the culture period as is expected during extended culture (more than 7 days)[195].

2.1.7 The *in vitro* traumatic brain injury procedure

The traumatic injury used in this study is a focal contusion injury using a weight drop model (Figure 2.5), in which a free falling small stylus creates a primary focal injury in each slice.

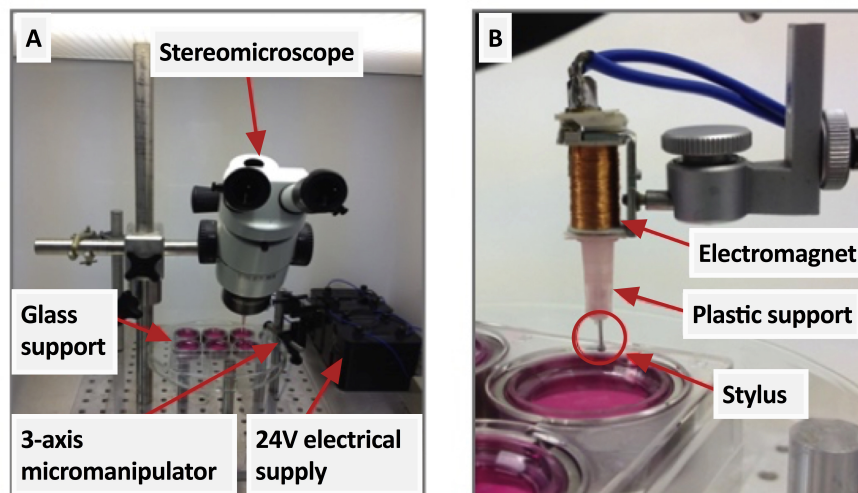


Figure 2.5: *In vitro* TBI device consisting of a 24V electrical supply (4x6V batteries) that delivered an electrical current to an electromagnet. A stylus with known mass was attached to the electromagnet via a plastic support. A 3-axis micromanipulator was used to position the slice directly below the stylus so that the impact would be in the CA1 region of the slice. A cold light source was positioned underneath a glass support and along with a stereomicroscope, enabled accurate positioning of the slice. To inflict the focal injury, the stylus was dropped onto the slice so that it made contact with the surface, and was immediately retracted due to the force of the electromagnet. Photograph courtesy Katie Harris.

The device used to produce the traumatic injury is based on that described by Coburn *et al.*[103] and was built by Katie Harris (PhD thesis 2014, Imperial College London). Focal injury was inflicted on the slice by dropping a round tipped stylus of a known mass from a fixed height onto the CA1 region of a slice. The device consisted of a 24V electrical supply (4x6V batteries) that delivered an electrical current to an electromagnet with the stylus attached to it due to magnetic force and *via* a plastic support. A cold light source was positioned underneath the glass petri dish to allow accurate positioning over the CA1 region. The 3-axis micromanipulator and stereomicroscope were used to adjust the position of the stylus over the slice. By pressing a relay switch, the stylus would momentarily drop from the electromagnet and make contact with the surface of the slice. Impact energy (joules, J) was calculated using the equation:

$$\text{Impact energy (J)} = \text{mass (kg)} \times \text{acceleration (m/s}^2\text{)} \times \text{height (mm)}$$

The stylus measured 1.3mm in diameter, had mass 70 mg and the height from which the stylus was dropped was 5mm. These parameters produced an impact with energy of 3.4μJ.

2.1.8 Preparation of slices for an experiment

On the day of an experiment, custom-made metal rings were placed in 6-well cell culture plates (NUNC, UK) and the wells were filled with 7ml of Experimental Medium (75% Minimum essential media with Earle's salts, L-glutamine and bicarbonate (Sigma M4655), 25% Hank's balanced salt solution (Sigma H9394), 2mM L-glutamine (Sigma G7513), 5mg mL⁻¹ D-glucose (Sigma G8270), 10mM HEPES (VWR 441485H), 1% Antibiotic-antimycotic suspension (Sigma A5955), 4.5μM propidium iodide (PI, Sigma 81845); pH 7.2). The rings were used to support the cell-culture membranes and raise them to an appropriate height to allow imaging under a fluorescence microscope. The plates were incubated for 1 hour at 37°C in a humidified incubator with 5%CO₂. Following this, the culture media of the slices was removed and membranes were transferred using forceps to the 6-well plate with pre-warmed media. The level of media was topped up with a fine tipped Pasteur pipette to prevent formation of bubbles. The plate was then returned to the incubator for one hour to allow PI to equilibrate.

2.1.9 Randomisation of wells and allocation to experimental conditions

Each experiment typically consisted of 12 wells of slices split across two 6-well plates. Slices were randomly assigned to sham and TBI groups before the pre-experiment viability screen. Equal numbers of sham and TBI wells were allocated to each injury quantification experiment. In experiments where the effect of a treatment (drugs and/or noble gases) was studied, TBI injured slices were allocated to either treatment or control injury groups. As analysis of slice injury levels was a purely quantitative process, no blinding took place.

2.1.10 Fluorescence imaging of slices

PI is a membrane impermeable dye that only enters cells with damaged cell membranes, where it binds irreversibly to DNA and RNA. When bound it becomes highly fluorescent with a peak emission in the red region of the visible spectrum. PI is used frequently as an indicator of neuronal integrity and cell viability and is known to be non-toxic to neurons[224].

One hour after the membranes were transferred to experimental media, slices were imaged to conduct a pre-experiment viability screen. Any non-viable slices were excluded from further analysis. A Nikon Eclipse 80i fluorescence microscope equipped with a digital camera and software (Micropublisher 3.3 RTV camera and Qcapture Pro 7, QImaging, Surrey, BC, Canada) was used to capture images of the slices in a RGB 24-bit TIF format. To compensate for the lamp intensity dropping over time, a ‘calibration’ image was obtained using a standard reference slide (Fluor-Ref Texas red, Omega Optical, Brattleboro, VT, USA), with standard exposure time of 11 milliseconds and 10x objective. For the calibration, image neutral density filters (ND4 and ND8) were placed in the fluorescence illumination light path and filter cube 4 was used. The calibration image was used to calculate a relative exposure time for each imaging session using the formula:

$$\text{Relative exposure time} = \frac{\text{Calibration reference value}}{\text{Mean red intensity of calibration image}} \times \text{Standard exposure time}$$

The calibration reference value was 127.12 (determined by taking the mean value of the lamp intensity over 80 hours, the period during which the intensity was the most stable), and the standard exposure time was 2.0s. A detailed discussion of the selection and standardization of

this exposure time is presented in chapter 3. Figure 2.6 shows the red channel intensity of the emission of standard slide when illuminated by the mercury bulb through an illumination/emission filter cube. This indirectly measures the change in intensity of the mercury lamp. The mean red channel intensity is plotted over 120 hours (Figure 2.6A) and over 80 hours (Figure 2.6B).

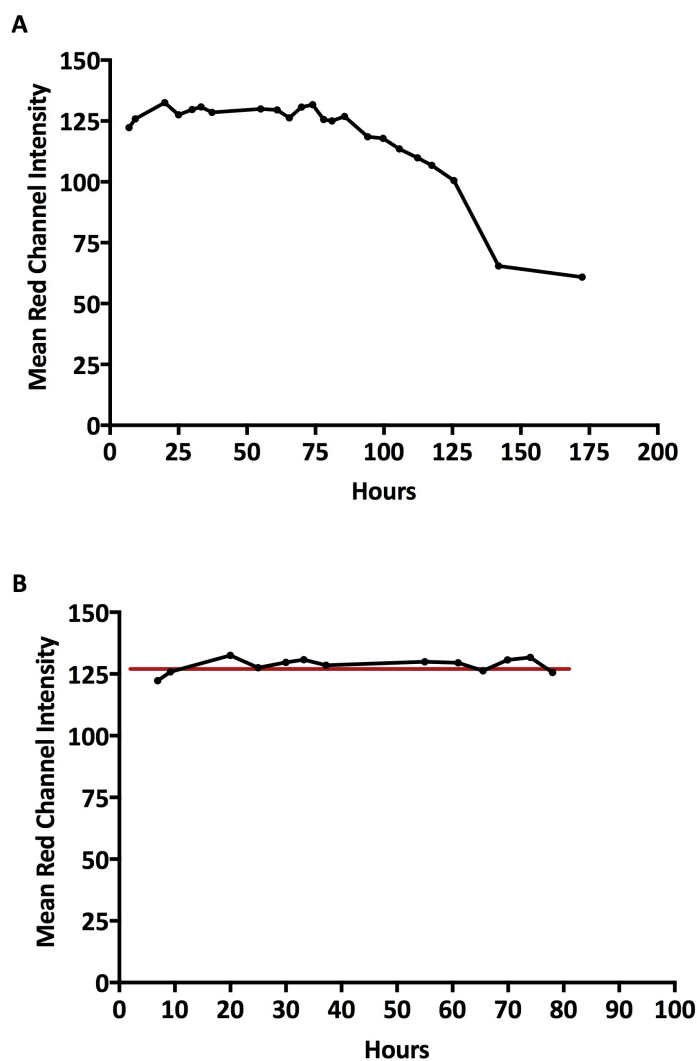


Figure 2.6: Mean change in intensity of a typical high-pressure mercury bulb measured by the red channel intensity of the emission of a standard slide when illuminated by the mercury bulb through an illumination/emission filter cube. (A): mean red channel intensity is plotted over 120 hours and (B): mean red channel intensity is plotted over 80 hours, during which the intensity was the most stable.

After calibration, slices were imaged under a 2x objective using the relative exposure time calculated for that session. A hairdryer held at arms length was used to remove condensation that built up under the lid, which would otherwise attenuate the fluorescence signal. Each slice was imaged individually. Following the T0 viability screen, slices were returned to the 37°C/5% CO₂ humidified incubator to allow their temperature to equilibrate back to 37°C.

2.1.11 Impact Procedure

Before the impact procedure, the cell culture hood (where the device was kept), and all equipment were thoroughly cleaned with 70% ethanol. The stylus was sterilised with 70% ethanol and placed under a UV light for one hour before use. A hairnet and PPE mask was also worn to avoid contamination. The 6-well plate was taken to the hood and the well to be impacted was positioned below the stylus with the light source focussed from underneath the glass petri dish. As membranes may be of a slightly different height, the height of the stylus above the membrane for each well was adjusted separately. The stylus was lowered to a point where it just touched an area on the membrane free from slices. The relay switch was pressed a number of times to ensure that the stylus was firm. This position was then read off the micromanipulator and assigned the '0' position. Each slice was impacted from 5mm above the '0' position for the particular well. The stylus was positioned above the CA1 region using the stereomicroscope. The stylus was then raised to 5mm above the '0' position and the relay switch was pressed to allow the stylus to drop and return to its original position immediately, observed under a microscope to ensure accuracy. The procedure was carried out quickly in order to minimise the chance of contamination, each plate, typically with 20 slices, took between 5 and 10 minutes).

2.1.12 Application of experimental gases following TBI

Following the impact procedure, the 6-well plate was covered with the lid and transferred to a small custom-built hyperbaric pressure chamber (Figure 2.7). The chamber had a volume of 0.925 liters and contained a high-speed fan for rapid gas mixing. It was housed in an incubator set at 37°C and was capable of maintaining pressures (measured on an individual pressure gauge attached) as high as 6atm for several days. The chamber underwent a flushing procedure, with the inlet and outlet valves both open, where the chamber was flushed with a humidified control gas mixture (75%N₂/20%O₂/5%CO₂), bubbled through a sealed plastic container of autoclaved water) for 5 minutes at 5 L/min (measured using a flow meter), which ensured better than 99.99% gas replacement[104]. The outlet valve was then sealed and the appropriate partial pressure of noble gas (see Table 2.1) was added slowly, over 1 minute, before sealing the inlet valve. For all experiments presented in chapter 3, 50% control helium gas was added, unless otherwise stated.

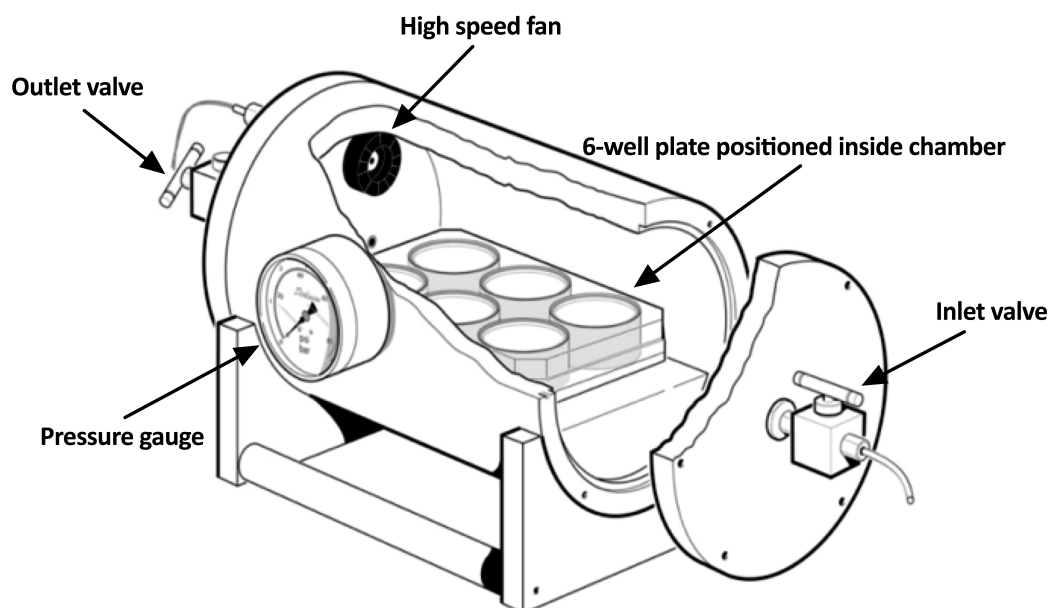


Figure 2.7: Schematic diagram of custom-made hyperbaric chamber with integrated pressure gauge and mixing fan

The partial pressures of oxygen and carbon dioxide were fixed at 0.20atm and 0.05atm, respectively. The high-speed fan was switched on for 5 minutes to ensure rapid mixing of gases. Temperature was maintained at 37°C inside the chamber throughout the experiments. Care was taken to minimise the amount of time for which the incubator was open whilst the flushing and pressurizing procedures took place so as to minimise changes in temperature.

Table 2.1: Experimental gases tested matched against their appropriate controls

Experimental gas	Partial pressure	Control gas	Partial pressure
Xenon	0.25atm	Helium	0.25atm
	0.5atm		0.5atm
Argon	0.25atm	Helium	0.25atm
	0.5atm		0.5atm

When the slices were removed from the chamber at 24, 48 and 72 hours ('t24', 't48' and 't72') post injury for fluorescence imaging (outlined in section 2.1.10), pressure was slowly released (over 1 minute). After imaging, the slices were transferred back to the pressure chamber and underwent the flushing and pressurizing procedures with the appropriate gases. For experiments where 'maximal injury' was measured, slices were submerged in 70% ethanol and left at 4°C overnight. Injury was quantified as described in section 2.1.17. Data presented in chapter 3 describes the refinement of quantification of injury development in

slices and used the steps described above. A schematic diagram showing the timeline of the traumatic injury protocol used is shown in Figure 2.8.

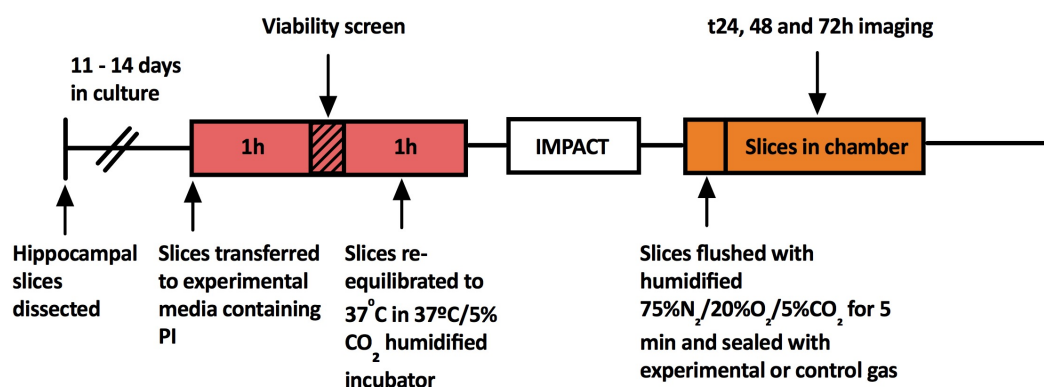


Figure 2.8: Schematic diagram showing timeline of the traumatic injury protocol for *in vitro* TBI in hippocampal slices

Data presented in chapter 4 explores combination treatments with noble gases and other drugs. A modified traumatic injury neuroprotection protocol was used for these experiments and additional steps are described in sections 2.1.13 - 2.1.16.

2.1.13 Combination neuroprotection experiments

Progesterone and meloxicam were evaluated alone and in combination with xenon and argon to test the potential of these noble gases in combination therapy following TBI. In order to increase clinical relevance experimental drugs were applied only after the trauma. This required a solution exchange immediately after the impacting procedure. The following sections describe how experimental drugs were made up, serially diluted to different concentrations and administered to the slices.

2.1.14 Experimental drugs and appropriate vehicles

All reagents were purchased from Sigma-Aldrich (Gillingham, UK) unless otherwise stated. Table 2.2 summarises the various concentrations of progesterone (P0130) and meloxicam sodium salt hydrate (M3935) tested.

Table 2.2: Table showing drugs tested and their concentrations

Drug	Concentrations tested
Progesterone	3μM, 10μM and 25μM
Meloxicam	0.1μM, 1μM, 10μM, 25μM and 50μM

As the drugs were administered to slices using a solution exchange, they were dissolved in experimental media. Due to limited aqueous solubility, this required the use of the vehicle dimethyl sulfoxide, DMSO (D8418).

2.1.15 Formulating concentrations of drug and vehicle solutions

Progesterone (molecular weight: 314.46g/mol) was made up in DMSO as a 250mM stock concentration by addition of 0.078g of progesterone to 1ml of DMSO. The stock solution was vortexed until dissolved and then diluted to the final drug concentrations (Table 2.2) in 100ml of experimental media, which was pre-warmed to 37°C. Stock solutions were stored at 4°C after use. The dilutions ensured that DMSO was present in no higher than a final concentration of 0.01% v/v (i.e., 10µL in 100ml experimental medium) as it was previously established that at this concentration, DMSO did not affect the level of control traumatic injury (Paul Banks Thesis, Imperial College London, 2010).

Meloxicam (molecular weight: 373.38g/mol) was also made up in DMSO as a 500mM stock concentration by addition of 0.086g meloxicam in 0.46ml DMSO. The stock solution was vortexed until dissolved. The stock was serially diluted to stock concentrations of 250mM, 100mM, 10mM and 1mM, which were diluted to final drug concentrations shown in table 2.1, in 100ml of experimental media. Stock solutions were stored at 4°C after use.

For each experiment with these drugs, control slices were treated with media containing DMSO, which was present in control experimental media in the same concentration as was present in experimental media containing the drug. DMSO was present in no higher than a final concentration of 0.01% v/v in meloxicam and progesterone treated slices as well as DMSO vehicle treated slices.

2.1.16 Administering drugs to slices post trauma

The experimental media containing the drug and vehicle was titrated to pH 7.2 and then filtered (0.22µm Stericup filters, Millipore, Watford, UK). A fresh 6-well plate with metal rings was filled with media containing drug and vehicle. The plates were left to equilibrate to 37°C in a humidified incubator with 5%CO₂ for 1 hour before the impact procedure. 50ml Falcon tubes were filled with media containing drug or vehicle to top up the media in the plates after the solution exchange. These tubes were incubated at 37°C. Immediately after

impact, slices were transported to a laminar flow hood. Using forceps, the membranes were carefully transferred to the pre-warmed 6-well plate with experimental media containing drug or vehicle. The level of media was topped up with a fine tipped Pasteur pipette to avoid any air bubbles. Slices were then stored in a hyperbaric chamber as described in section 2.1.12 and imaged over 72 hours as described in section 2.1.10 to quantify injury. A schematic diagram showing the timeline of the traumatic injury and neuroprotection protocol used for these experiments is shown in Figure 2.9.

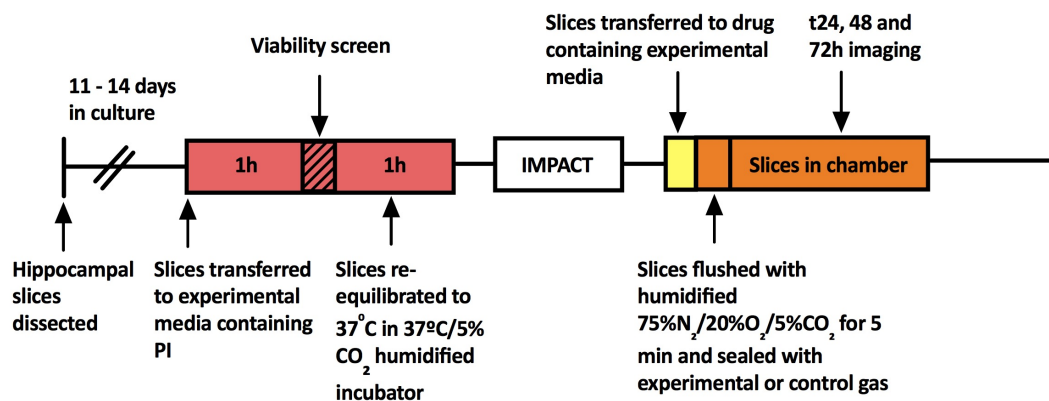


Figure 2.9: Schematic diagram showing timeline of the traumatic injury and neuroprotection protocol for *in vitro* TBI in hippocampal slices

2.1.17 Quantification of traumatic injury

Figure 2.10 shows the key processes in quantifying the development of a traumatic injury using the *in vitro* TBI model. Figure 2.10A and B are examples of PI fluorescent images of TBI and sham slices, represented in greyscale. Slice images were 3 megapixels, with each pixel having 256 intensity levels (0-255). ImageJ software (NIH, Maryland, USA) was used to separate the red, green and blue channels of the image. Using only the red channel, histograms were plotted to show the distribution of pixels over the 256 intensity levels (Figure 2.10C). This process was automated using a custom designed macro in ImageJ and visual basic access (VBA) in Microsoft Office Excel. Figure 2.10D shows histograms of a TBI slice (red line) and sham slice (black line) on a truncated y-axis (i.e., up to 8000 pixels). The large peak in the histogram is due to the background of the image (area not including the slice), containing pixels in intensity levels 0-40 (marked in blue). The more injured a slice, the greater the PI fluorescence, and the greater the number of pixels occupying higher intensity levels. To illustrate, in Figure 2.10D the histogram of the sham slice has fewer pixels in intensity levels 75-255, in contrast to the histogram of the TBI slice. To quantify the

traumatic injury, the number of pixels above a certain intensity threshold (marked in green, Figure 2.10D) was integrated (area shaded in orange, Figure 2.10D). In this way, the degree of injury of slices was quantified and compared. For all experiments, a threshold of 50 was selected. The justification for this choice of threshold is explained in chapter 3.

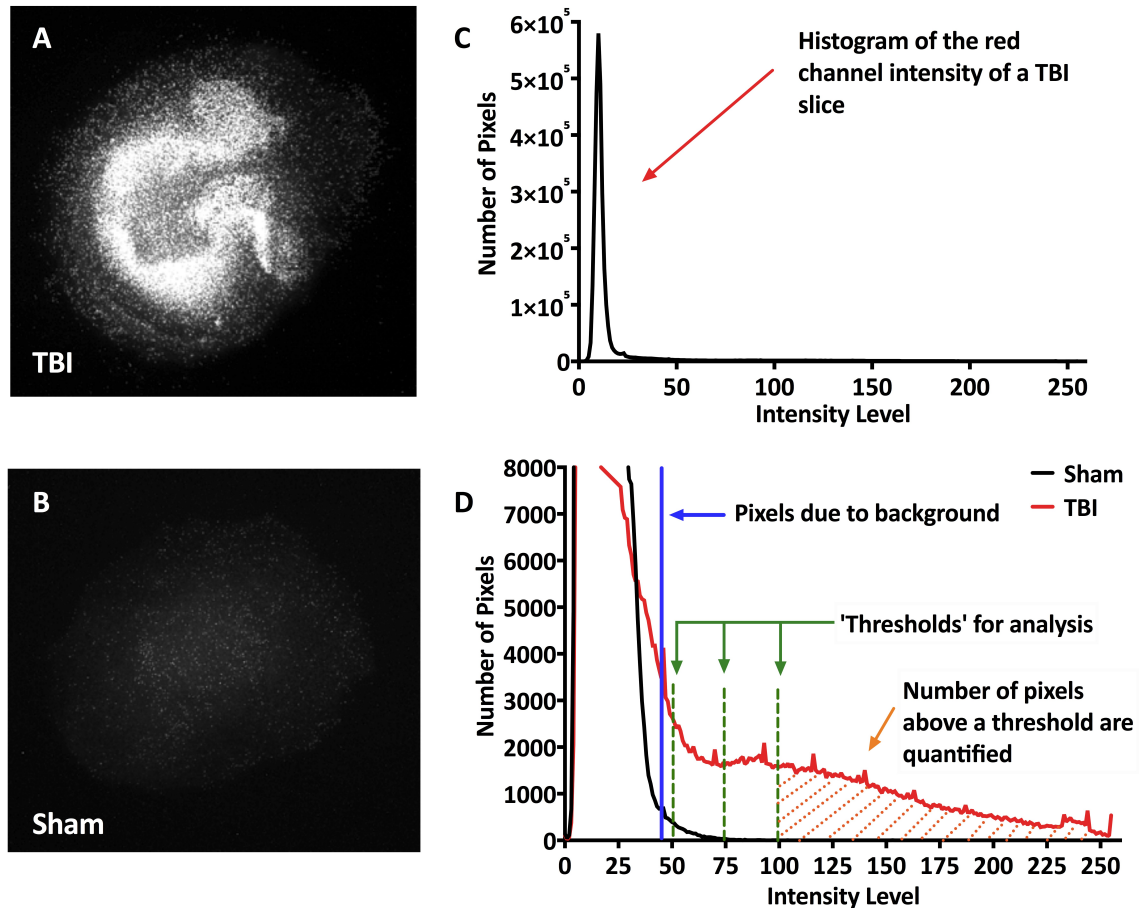


Figure 2.10: A schematic representation of the injury quantification process. Images of propidium iodide fluorescence in a cultured organotypic hippocampal slice at 72 hours (A) after being subjected to traumatic injury, ‘TBI’ and (B) ‘sham’ injury (sham slices undergo the same manipulations as TBI slices except for the injury). (C) Histogram of the red channel intensity of an image of a TBI slice, obtained with ImageJ. (D) Slice histogram profiles of a single TBI (red line) and a single sham (black line) slice at 72 hours on a truncated y-axis (i.e., up to 8000 pixels), showing the area or number of pixels in the histogram occupied by the background (black portion of 2A or 2B) of the image and the ‘thresholds’ – intensity levels above which the number of pixels is quantified for measurement of injury.

As explained in section 2.1.10, slices were screened for viability before an experiment to exclude any slices with compromised viability. This assessment was primarily quantitative – any slice where the distribution of pixels reached 10 or more pixels at intensity levels above a threshold of 70 were excluded. The reasons for this choice are explained in further detail in chapter 3. Additionally, any slice that had a focal lesion (for example, due to the dissection process), cell debris visible in the frame or where the impact was not in the CA1 region at 24

hours, was excluded from analysis. The viability of a typical experiment was approximately 70%.

2.1.18 Data analysis and statistical testing

For injury quantification experiments, each condition (exposure time) was tested at least twice, with additional replicates for exposure times that were more relevant in making the final selection. For neuroprotection experiments, each condition was repeated for a minimum of 3 experiments to obtain as large a sample size as possible for accurate results. Three replicates of a particular condition with a similar result were deemed sufficient to demonstrate reproducibility. Data presented is normalised to the helium control vehicle injury at 72 hours. All graphs were produced using Graphpad Prism 7 software.

Statistical significance was tested using a two-way repeated measures ANOVA with a Tukey's *post hoc* test, implemented using SigmaPlot (Systat Software Inc., California). Factor 1 was the treatment (sham, TBI, TBI + drug) and factor 2 was the time after injury (24 hours, 48 hours and 72 hours). Factor 1 was the repeated factor. Significance was defined as a $p < 0.05$.

2.2 **Modelling traumatic brain injury *in vivo***

2.2.1 The Controlled Cortical Impact (CCI) model of *in vivo* TBI

The Controlled Cortical Impact (CCI) model is one of the most widely used *in vivo* models of blunt TBI. To create the injury, a pneumatic or electromagnetic impact device is used to drive a rigid impactor tip onto the exposed intact Dura of the brain, causing deformation of the underlying cortex[76]. The impact is delivered to the intact Dura through a unilateral craniotomy, in our model between Bregma and lambda on either side of the skull. The damage caused by the impact may develop throughout the ipsilateral hemisphere and extend to the contralateral hemisphere[214].

2.2.2 Subjects and ethical approval

All experiments were carried out after the approval of the Ethical Review Board, Imperial College London (project license: 70/7466) and in accordance with the United Kingdom

Animal Scientific Procedures Act, 1986. Experiments were performed on pathogen-free adult male Sprague-Dawley rats (age: 8 – 10 weeks, weight: 280 – 300g), which were obtained from Harlan, Bicester, UK. A maximum of four animals per cage were housed on a 12-hour light/dark cycle with *ad libitum* access to food and water. Animals were cared for in compliance with the guidelines provided by Imperial College Central Biomedical Services (CBS). All experiments were performed in surgical procedure rooms in Imperial College CBS. All surgeries and procedures involving animals were carried out by me. A total of 50 animals were used and were randomly allocated to the following groups: naïve (n = 9), sham (n = 5) and CCI (n = 30). An animal was allocated to each of naïve, sham and CCI groups from each cage of animals. Additionally, 6 animals were used in the setting-up phase, to test and optimize the anesthetic circuit. Animals typically arrived from the supplier at least one week before surgery to allow for acclimatisation to the CBS environment. They were handled, weighed and monitored daily.

2.2.3 Surgical set up

All surfaces and equipment were cleaned with 70% ethanol. Animals were placed inside an anaesthetic box and were anaesthetized with 4% isoflurane, which was delivered in pure oxygen gas at 2L/min *via* an oxygen concentrator in an anaesthesia circuit with active scavenging using a Cardiff Aldasorber. Buprenorphine (0.03mg/kg) was administered by subcutaneous injection to provide analgesia. Animals were placed in a stereotaxic frame (MyNeuroLab Digital Stereotaxic Frame, Leica, Milton Keynes, UK) and stabilised with ear bars. Lacri-lube ophthalmic ointment (Allergan, Marlow, UK) was applied to the animal's eyes to prevent discomfort and the anaesthetic concentration adjusted as required to maintain surgical anaesthesia. Anaesthesia was maintained through administration of 2.0 – 3.5% isoflurane in 100% oxygen gas at 1.5L/min *via* a nose cone in spontaneously breathing animals for the entire duration of the surgery. Physiological monitoring included respiratory rate, pulse oximetry, heart rate, non-invasive blood pressure cycled at 3 minutes measured via a tail cuff (Kent Coda Monitor, Kent Scientific, UK) and core body temperature which was monitored and maintained at 37°C for the duration of the surgery by means of a rectal probe and feedback-controlled heating pad (CMA 450 Temperature Controller, Harvard Apparatus, Cambridge, UK). Care was taken to ensure that an aseptic environment was maintained with the use of surgical drapes (3M Healthcare, Bracknell, UK) and good aseptic technique.

2.2.4 Surgery

The skin was swabbed with povidone-iodine antiseptic solution (Vetasept, York, UK) and the depth of anaesthesia was confirmed by absence of the pedal reflex in response to a toe pinch. Following a midline scalp incision made under a stereomicroscope (Carl Zeiss, OPMI 1-FC, F-170, Jena, Germany), the temporal fascia muscle was separated from the skull with sterile cotton tipped buds (NuCare, Stewartby, UK) dipped in 0.9% saline (Oxoid, Thermo-Fisher Scientific, Paisley, UK). A high-speed drill (Labset Uni-Drive, Paggen Werkzeugtechnik GmbH, Starnberg, Germany) with a 0.6mm burr bit (Braun Medical, PfiEFFewiesen, Germany) was used to make a 6x7mm craniotomy over the right parietal cortex, following the lambdoid and coronal sutures and along the zygomatic arch (Figure 2.11).

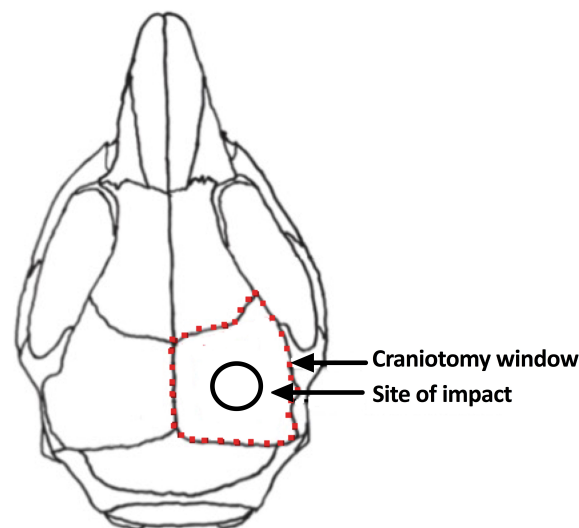


Figure 2.11: Outline of the skull showing craniotomy window (dashed red line) along the lamboid and coronal sutures and zygomatic arch and the site of impact

Without disrupting the Dura mater, the bone flap was lifted medially and rested upright on the sagittal suture. The metal tip of the CCI device (Leica MyNeuroLab, Leica, Illinois, USA) was positioned perpendicular to the brain surface and in contact with the Dura so that the tip just touched the Bregma (Figure 2.12).

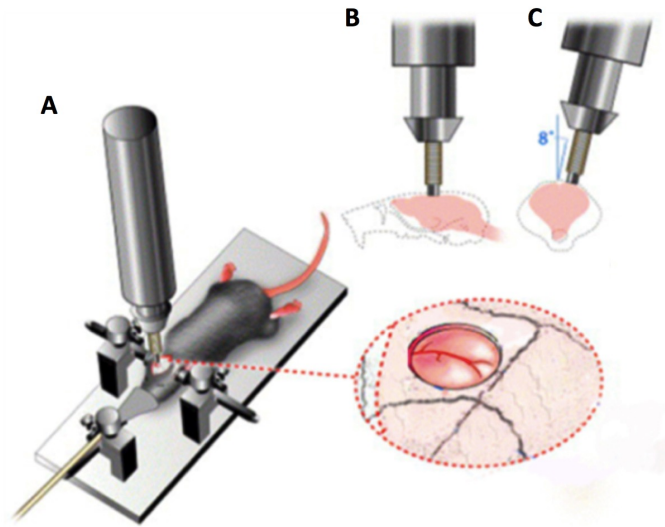


Figure 2.12: (A) Animal placed in frame with actuator overhead for positioning over impact site (shown in inset). The tip was positioned perpendicular to the brain surface. (B) Sagittal view of impact site. (C) Caudal view of impact site.

The tip was then adjusted $-5.8\text{mm} (\pm 0.2\text{mm})$ and $+1.9 (\pm 0.3\text{mm})$ from the Bregma, above the intact Dura and in the centre of the craniotomy window. Error represents standard error of mean anterior-posterior and medio-lateral measurements. Figure 2.13 shows the digital CCI device. The digital display was preset to the desired impact velocity and dwell time. The tip was lowered to the surface of the Dura at the Bregma (confirmed by the contact sensor which indicates the exact point of contact). The tip was zeroed to the surface of the Dura and then retracted. The tip was then lowered by the desired depth. Upon impact, the tip accelerates down to the extended distance, reaching the pre-set velocity by the end of the travel. It remains there for the dwell time and then retracts automatically. One impact using either a 3mm or 5mm diameter flat metallic tip was delivered at a velocity of 4m/s, to deform the Dura to an impact depth of either 1.5mm or 2mm with a dwell time of 400msec. Several combinations of impact parameters were tested to calibrate this model. The final selection of parameters was an impactor tip of 5mm diameter, impact velocity of 4m/s, dwell time of 400msec and depth of 2mm.



Figure 2.13:The electromagnetic digital CCI device mounted on a stereotactic frame. Photograph taken from Leica MyNeurolab CCI device manual.

Following application of the impact, the bone flap was replaced and the area was cleaned thoroughly of blood and any bone residue using paper filter triangles (Sugi Sponge Points, Kettenbach GmbH & Co. KG Eschenburg, Germany) dipped in sterile saline. The flap was sealed with tissue glue (Histoacryl, GP Supplies, London, UK). The skin was sutured using 3/8 circle conventional cutting prime needle and 5-0 coated vicryl polyglactin absorbable sutures (Ethicon, Maidenhead, UK) and cleaned with saline. The animals were removed from the anaesthetic circuit and closely monitored on a heat pad until the return of the righting reflex. Sham animals were subjected to the same anaesthesia, analgesia and set up. However, they received only a skin incision and scraping of the exposed skull cranial suture lines with the same drill tip (turned off) and no craniotomy or CCI injury. The surgery typically lasted 45 – 60 minutes per animal.

2.2.5 Recovery and post-surgical care

Animals were allowed to recover from anaesthesia breathing room air. They were returned to individual pre-warmed recovery cages and monitored for at least 2 hours post-surgery, with access to food and water. All animals (CCI and sham treated) were housed in individual cages following the surgery as described in section 2.2.2. Naïve rats had no anaesthesia or surgery and were housed under similar conditions in individual cages. Animals were carefully monitored every 2 hours after surgery for at least 4 hours, and then daily from 24 hours post-surgery using a modified Morton and Griffiths score[225].

2.2.6 Post-surgery sample collection and processing

Brain tissue and blood samples were obtained after animals were terminally anaesthetized with 5% isoflurane and euthanized by cervical dislocation followed by decapitation. Specific time points of 3 and 24 hours post injury were selected for blood and brain tissue sampling and histology outcomes. A small cohort of animals was additionally sacrificed at 1-hour post impact and 3 hours post impact to observe the development of a primary injury or lesion at the impact site. This was necessary in the early phases of the project to test reproducibility of the impact and surgery.

2.2.7 Preparation of samples for contusion volume measurement

To measure the contusion volume, brain samples were obtained 24 hours post injury. Whole brains were extracted following decapitation and flash frozen on powdered dry ice. Brains were transported on dry ice and stored in a freezer at -80°C until they were cut on a cryostat. On the day of cryosectioning, frozen brains were removed from a freezer at -80°C and allowed to equilibrate to -21°C in a cryostat tissue slicer (Leica CM3050, Leica Microsystems, Milton Keynes, UK) at -21°C for 30 minutes. Brains were then embedded in Optimal Cutting Temperature media (Thermo Fisher Scientific, Paisley, UK). Coronal sections ($10\mu\text{m}$ thick) were cut with a distance of approximately $550\mu\text{m}$ between sections or brain points, across the entire brain. Multiple slices (4 – 6 slices) were cut at each brain point and there were approximately 20 – 22 brain points. Brain sections were collected on Superfrost Plus microscope slides (Thermo Fisher Scientific, Loughborough, Leicestershire, UK) and stored at -20°C until histological processing.

2.2.8 Collection of blood and brain samples for ELISA immunoassays

Following decapitation, blood samples were collected from the head and neck region into EDTA-filled tubes (Vacutainer K2EDTA Tube, BD, Oxford, UK), frozen on and transported on dry ice to a freezer at -80°C .

Immediately following decapitation, the whole brain was extracted from the skull and placed on an autoclaved chilled 1mm interval brain matrix (Zivic Instruments, Pittsburg, USA), placed on wet ice. The hemispheres were divided and sliced into 3mm thick coronal sections, centred on the contusion using a razor blade as previously described[226] for cytokine

immunoassays. This method was selected because it was previously reported to provide adequate tissue with a stronger signal than whole ipsilateral hemispheres[227,228]. Brain tissue for naïve and sham animals were taken from a similar anatomical location at 8mm from the anterior prefrontal cortex. Figure 2.14 shows the region of sampling. Tube tare weights were recorded before the tissue was harvested so that the wet mass of samples could be calculated. Brain samples were placed in 1.5ml Eppendorf tubes and flash-frozen in liquid nitrogen. Tubes containing brain samples were then transferred to a freezer at -80°C for storage.

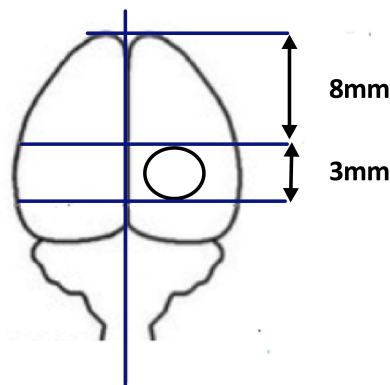


Figure 2.14: Coronal hemisphere slicing for sample collection. Hemispheres were divided before slicing as indicated. 1mm slices were collected and for CCI brains, slice thickness was centered on contusion epicenter and spanned the entire 3mm diameter of the impact. Sham and naïve samples were collected from a similar anatomical region, which started at 8mm from the anterior prefrontal cortex. Measurements were guided by reference to Waxholm Space atlas of the Sprague-Dawley rat brain (Papp et al. 2014[229]).

2.2.9 Cresyl violet histology and calculation of contusion volume

Cresyl violet staining is routinely used to determine the contusion volume post CCI injury[230]. The intensity of cresyl staining indicates cell viability, where injured or dead cells appear pale in contrast to a blue or purple stain.

2.2.10 Determining an appropriate concentration of cresyl violet solution

The concentration of the cresyl violet solution and staining protocol had to be optimized for this study of contusion volume analysis. During optimization tests, different concentrations of cresyl violet solution including 0.4%, 0.6% and 0.8% were tested. Stained ‘test’ slides were then imaged using a digital camera and stereomicroscope to compare the contrast between injured and healthy cells. Following optimization tests, a 0.83% solution of cresyl violet solution was selected because it gave the best contrast between injured cells and healthy cells.

The solution was made up with 0.83% cresyl violet acetate (Acros Organics, Leicestershire, UK) in a mixture of 16.5% ethanol and 82.6% water, stirred on heat for 6 hours and without heat overnight and double filtered (Whatman filter paper grade 413, 150mm, VWR International, Leicestershire, UK) to remove un-dissolved solute. Double filtering was essential to ensure no particles or clumps of powder remained in solution which would cause a residue to form on slices.

2.2.11 Staining and imaging of brain slices using cresyl violet solution

The slides were stained by sequential submersion in 70% ethanol (2 minutes), 0.83% cresyl violet (10 minutes), distilled water, 70% ethanol and 96% ethanol (2 rinses of 1 minute each) and 96% D-Limonene (5 minutes, Atom Scientific, Cheshire, UK). Slides were air dried for 5 minutes before being mounted with Roti-Histokitt mounting media (Carl Roth, Karlsruhe, Germany) and a glass coverslip (Menzel Gläser, Thermo Scientific, Loughborough, Leicestershire, UK). Slides were stored at room temperature until imaged. Cresyl violet stained slides were imaged with a digital camera (Scopetek DCM510; Scopetek Opto-Electric, Hangzhou, China) attached to a stereomicroscope (Wild model M8, Heerbrugg, Switzerland). Imaging parameters were optimized and standardized for all slices.

2.2.12 Calculating the contusion volume from imaged brain slices

The contusion was evident as a difference in intensity of the stain. The area of both hemispheres and the contused brain tissue was measured using FIJI (<http://fiji.sc/Fiji>). 2 – 4 brain slices were used at each brain point to obtain a mean value for each area by tracing around the contralateral and ipsilateral sides of the brain and the contused area. The contusion volume was calculated by multiplying the mean contusion areas at each brain point, A , by the distance between brain sections, d , according to the formula:

$$\text{Contusion Volume} = \frac{d}{2} \times (A_1 + A_n) + d \times (A_2 + A_3 + \dots + A_{n-1})$$

Total injury contusion volume was calculated using the above equation.

2.2.13 Detection of pro-inflammatory cytokines using ELISA

The concentrations of pro-inflammatory cytokines IL-6, TNF and CCL-2 were measured using enzyme-linked immunoassay (ELISA). The expression profile and time course of these inflammatory markers is relevant for understanding the injury development on a molecular level. A 'sandwich' ELISA is a powerful ELISA assay format, highly efficient in sample antigen detection. The analyte to be measured is bound between two primary antibodies – the capture and detection antibody. The following pre-coated sandwich ELISA plate kits from Invitrogen (Renfrew, UK) were used: Rat IL-6 ELISA Kit (KRC0061), Rat TNF- α ELISA Kit (KRC3011) and Rat MCP-1 ELISA Kit (KRC1011). These kits are validated for use in brain and plasma homogenate samples with high inter and intra assay precision.

2.2.14 Homogenisation of brain tissue

All chemicals obtained from Sigma-Aldrich UK, unless stated otherwise. Protocols were obtained from Invitrogen, UK.

Brain samples were thawed at room temperature, 8 times the mass of chilled extraction buffer containing guanidine-HCl (5M, pH 8-9) and Tris-HCl (50mM) was added in 50-100 μ L aliquots with manual homogenization using a micropestle in microcentrifuge tube (Eppendorf 1.5mL). The tubes were then agitated on a plate shaker at 100rpm at room temperature for 3-4 hours. The homogenate was stored at -80 $^{\circ}$ C. Protease inhibitor cocktail (PIC) lyophilized powder (P-2714) was prepared by adding 10mL PBS to the vial to make x10 PIC solution. Dilute chilled PBS-PIC solution was made by adding 50 μ L of x 10 PIC for every 1ml PBS. One hour prior to the ELISA assay, frozen homogenate samples were thawed at room temperature. 10 times the volume of cold PBS-PIC solution was added and mixed thoroughly. The sample was centrifuged at 16,000x g for 20 minutes at 4 $^{\circ}$ C. The resulting supernatant was collected using a Pasteur pipette and stored in fresh micro-centrifuge tubes in a fridge at 4 $^{\circ}$ C. Just prior to addition to ELISA wells, the sample was diluted 1:2 using ELISA standard diluent (Invitrogen, UK) and allowed to equilibrate to room temperature.

2.2.15 Preparation of blood samples for ELISA

To prepare for the ELISA immunoassay, the tubes were thawed at room temperature for at least 2 hours. Tubes were centrifuged (Eppendorf mini centrifuge, Eppendorf, Stevenage,

UK) at 2000xg for 15 minutes at 4⁰C. The resulting supernatant consisted of the plasma and was removed using a Pasteur pipette. Any plasma not required for that particular ELISA session was immediately returned to the freezer at -80⁰C. Immediately prior to performing the immunoassay, the supernatant was diluted 1:2 according to the manufacturer's instructions.

2.2.16 ELISA assay

The ELISA kits were stored at 4⁰C and allowed to equilibrate to room temperature before the assays were conducted. A series of dilutions of cytokine standards of known concentration were prepared in order to generate standard cytokine concentration versus optical density (OD) curves. For every assay run, wells were allocated to high and low range standard controls; zero controls (i.e., standard diluent buffer in the place of sample), and 4 'blank' dry wells for the subtraction of background signal. Samples and standards were run in duplicate each time. The assay was conducted as per manufacturer's instructions. Briefly, 50µL of incubation buffer was added to each well (except blanks). 50µL standards, high and low controls or diluted samples were added to the appropriate wells. 50µL biotin conjugate was added to each well (except blanks). The plate was covered and incubated for 2 hours at room temperature or at 37⁰C (for IL-6 assay) as per manufacturer's instructions. The solutions were aspirated and the plate was washed 4 times with 1X wash buffer. 100µL streptavidin-HRP was added to each well (except blanks). The plate was covered and incubated for 30 minutes at room temperature. Once again, the solutions were aspirated and the plate was washed 4 times with 1X wash buffer. 100µL stabilised chromogen was then added to each well. The plate was then covered and incubated for 30 minutes at room temperature in the dark. Finally, 100µL stop solution was added into each well, causing a colour change from blue to yellow. The absorbance was read on Tecan Sunrise ELISA plate reader with a 450nm filter using Magellan 6 software.

SigmaPlot® 12.5 software and ELISA standard curve macro were used to transform optical density (OD) recordings into values for protein concentration. The standards were used to plot a standard curve using 4 or 5-parameter logistic regression model to predict unknown values. For brain samples, cytokine concentration was normalised to the mean total protein concentration in the homogenates measured using Coomassie (Bradford) Protein Assay Kit (Thermo Scientific 23200). Duplicate OD values with a coefficient of variance (CV) of >10%

were not included in the final data analysis. Further, OD levels below the mean of the zero controls + 2 standard deviations of the zero wells were deemed below the reliable limit of detection for that plate and determined plate sensitivity (equating to $\approx$ 4pg/ml for TNF, 5pg/mL for IL-6 and 12pg/ml for CCL-2), as per kit instructions.

2.2.17 Data analysis and statistics

For the calculation of contusion volume, data presented (contusion volume, mm³) were calculated from the mean of contusion area (mm²) of 2 – 4 slices at each brain section of a number of brains. For cytokine measurements, data presented (graphs) represent mean cytokine concentrations in brain tissue or blood plasma from different experimental groups. Graphs were created and statistical tests were implemented using Graphpad Prism 7.0 software. Error bars represent standard errors of the mean. Statistical tests conducted between groups were: one or two-way ANOVA with multiple comparisons comparing each mean with every other mean with a Tukey's *post hoc* test. Significance was defined as $p < 0.05$.

2.3 **Developing a model of traumatic brain injury using *Drosophila Melanogaster***

2.3.1 Modelling traumatic injury using *Drosophila*

The development of the *Drosophila* model of TBI was inspired by the work of Katzenberger and colleagues[223] and was aimed at modelling a contact or contusion type brain injury. In the assay, flies (loaded in vials) were subjected to strike(s) against a hard surface. This insult causes the brain to deform against the cuticle, resulting in a traumatic injury to the brain in addition to polytrauma to the proboscis and thorax. The following sections describe how the traumatic injury assay was set up; injury and treatment were delivered and quantified.

2.3.2 *Drosophila* breeding cycles and maintenance

Canton Special (Canton-S) flies were used for experiments, as they are a common wild type strain used in the laboratory. Breeding cycles were set up to obtain cohorts of flies between the ages 1 – 4 days ('young flies') and 11 – 14 days ('adult flies') for experiments. Sexual maturity for both male and female *Drosophila* occurs at 7 days of age. Male and female flies

were distinguished from each other under a microscope by the black pigmentation on the posterior abdominal segment of the males, which is absent on females. Carbon dioxide (CO₂) anaesthesia was selected instead of cooling so as to not pre-expose the flies to hypothermia, which was used as a treatment in this model. Flies were anaesthetised with CO₂ on a pad, grouped into equal pairs and were gently moved to breeding bottles, with food inside them, using a paintbrush. Bottles were sealed with a tight fitting cotton plug and left in an incubator at 25⁰C. Eggs hatched within 24 hours and over the course of the next 7 days, larvae went through a cycle of events leading up to pupation. Breeding bottles were then placed at room temperature (21⁰C) and a 12-hour light/dark cycle until adults emerged from the pupa case. During this time, the parents were removed from the breeding bottles by anaesthetising them. The tip of a CO₂ delivery tube was inserted into the breeding bottle. After a minute, flies began to immobilise. The bottle was emptied gently on a CO₂ pad. Care was taken not to disturb the developing pupae, which were attached to the sides of the bottle. Parent flies were removed and re-bred in a separate bottle. At 9 days, flies began to emerge from the pupa case. Using CO₂ anaesthesia, male flies were removed and stored in a separate bottle to prevent further breeding, which would otherwise cause a mix-up of flies with different ages. This process was repeated for 3 days and on the day of an experiment; flies were randomly selected from stock bottles containing flies at ages between 1 – 4 days (for experiments with young flies) and at 11 – 14 days (for experiments with adult flies). Flies were bred on a continuous cycle and breeding bottles were discarded every 10 days to avoid using stale food. Flies were fed on standard food prepared according to the Bloomington protocol[231].

2.3.3 The trauma device

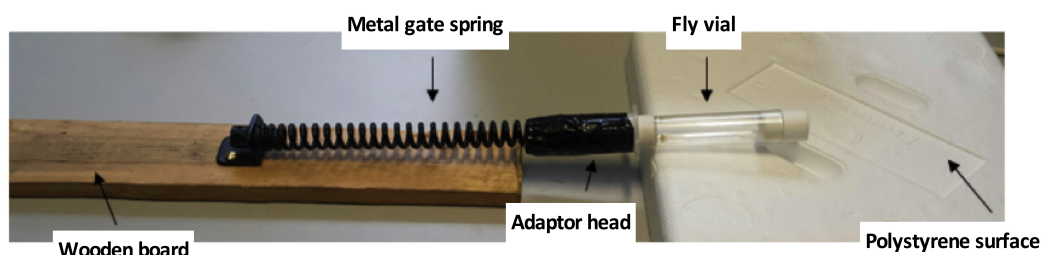


Figure 2.15: Custom made traumatic injury device used to inflict trauma on flies. Flies are loaded into a plastic vial slotted into an adaptor head. The attached spring is pulled back and the vial is allowed to drop and contact a hard polystyrene surface.

Figure 2.15 shows the device used to model traumatic injury. A 21.5cm long metal gate spring (Screwfix, London, UK) was screwed into a wooden board (38cm x 6.5cm). An

adaptor head to slot the fly vials was created out of a cut off 50ml syringe and a small plastic cap, 5cm in length and 2.3cm in diameter. These dimensions ensured a tight fit of the plastic fly vial so that it would not fall out following the impact procedure. The adaptor head was attached to the end of the spring using plastic coiling that was tightly secured around the end of the spring using high strength duct tape. It was important that the spring was held very tightly in place so that movement within the syringe was limited, which would otherwise reduce the force of the impact. The wooden plank was secured to a laboratory bench using large G-clamps to ensure that the device would not move during the procedure. The lid of a polystyrene box was used as a hard landing surface for the vials during the trauma procedure. This material also prevented rebound strikes. The distance from the spring to the foam was fixed as the spring was screwed to the board at the point that enabled the vial to contact the foam as horizontally as possible.

2.3.4 Variables considered

The effect of injury severity, the point in the flies' circadian rhythm at which they received trauma and age were investigated. The impact procedure always took place in a room at 21⁰C. Experiments on young and mature flies were conducted separately. Male flies were used for all experiments.

2.3.5 Preparation of fly groups on the day of trauma

On the day of an experiment, flies from breeding bottles were anaesthetised using CO₂. They were separated and randomly allocated from a number of different breeding bottles into groups of 10. Each group of 10 flies had flies aged either 11-14 days (for experiments with mature flies) or 1-4 days (for experiments with young flies). Each group of 10 flies was loaded into a plastic fly vial of length 9.5cm and diameter 2.3cm. Vials were sealed tightly with a polystyrene plug. Flies were allowed to recover from anaesthesia for two hours, which was deemed sufficient for flies to regain consciousness and movement. It was important that trauma was delivered to freely moving and active flies so that the outcome measured would be as a result of the trauma.

2.3.6 The traumatic injury procedure

Following a two-hour recovery period, the vials were randomly allocated to receive different injury severities determined by the angle at which the vial was pulled back (eg. 90⁰, 110⁰ and 180⁰) and by the number of strikes, (eg. 1, 5, 7 and 10 strikes). Each vial was loaded into the adaptor head and the spring was pulled back at the required angle from which point the vial was allowed to fall free and contact the polystyrene surface. A recovery time of 5 minutes was allowed between strikes for flies that received multiple strikes. Once flies had received the required number of strikes, they were allowed to recover for 5 minutes. This was important to ensure that flies that were temporarily incapacitated would not get trapped in the food once transferred to food containing vials. They were then carefully transferred to food containing vials without using anaesthesia and stored at 21⁰C. Sham flies received the same treatment (i.e., the anaesthesia and transfer to vials) but were not subjected to any strikes.

2.3.7 Treatment with hypothermia

Prophylactic hypothermia has been proposed as an option that combats the early stages of TBI pathology. Testing whether hypothermia provides protection against traumatic injury in flies would validate the assay as a means to study effects and mechanism of hypothermia after traumatic injury. *Drosophila* are poikilotherms that equilibrate with the temperature of the surroundings within seconds. Adult *Drosophila* are known to prefer temperatures in the range of 24⁰C – 26⁰C[232,233]. However, *Drosophila* are able to tolerate large fluctuations of 15⁰C[234]. Based on this, two temperatures were selected for hypothermia treatment: 10⁰C, which is on the lower end of the 15⁰C range, and 4⁰C, which lies outside their adaptable range and is considerably cooler. Following the receipt of a number of strikes, flies that were due to receive hypothermia treatment were not transferred to food containing vials. Instead, they were allowed to recover for 5 minutes from the injury procedure and were then placed in either a cold room at 4⁰C or in a fridge specially controlled at 10⁰C for 30 minutes. The vials were placed on their side in a test tube holder to prevent the tube from coming into direct contact with any chilled surface, which may otherwise freeze flies that were temporarily incapacitated due to the cooling. Flies were then transferred to food containing vials without using anaesthesia and stored in a room at 21⁰C. Sham flies received the CO₂ anaesthesia and transfer as described in section 2.3.5 and the hypothermia treatment but not the injury.

2.3.8 Quantification of outcome

The outcome measured for this assay was survival post trauma. The number of flies alive was counted every 24 hours for a continuous period of 14 - 31 days. Flies were transferred to vials containing fresh food every 7 days to prevent drying out. A complete schematic representation of the traumatic injury protocol is shown in Figure 2.16.

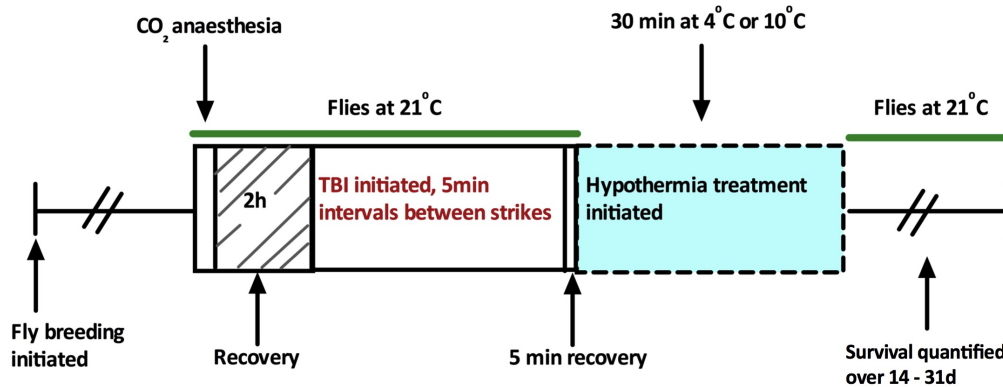


Figure 2.16: Schematic representation of *Drosophila* traumatic injury protocol. Following breeding and maturation to the appropriate age, flies are transferred to empty vials and allowed a recovery period of 2 hours from CO₂ anaesthesia. Vials are then loaded in the device and the spring is pulled back by the appropriate angle (eg. 90°, 120° and 180°). The vial was allowed to fall free and contact the polystyrene surface causing the injury. A recovery period of 5 minutes is allowed between successive strikes (eg. 1, 5, 7 and 10 strikes). Following a final recovery period of 5 minutes, flies are subjected to hypothermia treatment for 30 minutes at 4°C or 10°C. Flies are then returned to an environment at 21°C and survival is quantified over 14 - 31 days.

2.3.9 Data analysis and statistical testing

Data presented in the form of bar graphs are mean survival of flies as a fraction of the total number at a particular time point. Data is normalised to survival of the sham group and errors are SEM. A one-way ANOVA with a Tukey's multiple comparisons *post hoc* test was used to test significance between groups, with significance defined as $p < 0.05$. *Drosophila* survival over a number of days is presented using Kaplan Meier survival curves, which is a non-parametric method of estimating subject survival rates that takes into account the fact that some subjects may have died during a study whereas others may survive beyond the end of the trial period. Statistical significance is assessed using a log rank test, which calculates the chi-square to compare the curves (eg. sham versus trauma). The hazard ratio, defined as the slope of the survival curve, is a measure of how rapidly the subjects die. Hazard ratio values are used to compare different groups. Significance was defined as $p < 0.05$. A more detailed explanation of Kaplan Meier analysis is presented in chapter 6.

3 Chapter 3: Developing an injury quantification methodology in an *in vitro* model of traumatic brain injury

3.1 Overview and purpose of research

One of the advantages of the *in vitro* TBI model is that the analysis of injury can be a purely quantitative process, based on evaluating the magnitude of increase in fluorescence intensity over 72 hours. As explained in chapter 2, section 2.1.10, the quantification of injury depends on the changes in distribution of pixels from digitalized 8-bit images of the slice fluorescence. Injury in organotypic slices was compared between different conditions (eg. sham injury, TBI, TBI + drug) by the integration of pixels above a certain standard or ‘threshold’ intensity level between 0 and 255. In order to reliably quantify injury, any parameters that affect the brightness and sharpness of the images had to be kept constant throughout all experiments. This means that the microscope and software used to take images as well as image acquisition settings had to be consistent throughout. Although image acquisition settings were previously in use for a number of years, an out-dated version of the software, QCapture Pro, was in use. This software had to be replaced when the computer used to capture images had a hard disk failure. Unfortunately, when the new software was used with the same settings, as previously to image the slices, the injury was unable to be quantified as before. This was because the histograms of red channel intensity had a different profile to that which was obtained previously and the same threshold could not be applied to analyse the data. Certain ‘hidden’ image compression settings in the previous version of the software were not recorded, making it impossible to find corresponding settings in the newer software. Although a limitation, this presented the opportunity to understand the injury quantification process better, and to develop a more resilient and adaptable method to quantify injury. The aim was to achieve an injury development pattern as similar as possible to published data[93] obtained previously in our laboratory using the same *in vitro* TBI model. New image acquisition settings, an accompanying method of analysis, threshold values and slice viability criteria were established so that the OHSC model would allow the accurate quantification of traumatic injury development.

This chapter describes the steps taken to establish image acquisition settings, a method of analysis, thresholds and slice viability criteria. The data are presented and discussed. The

neuroprotective efficacy of argon is determined using the new methodology and then compared to published results[93].

3.2 Key terms and concepts in the injury quantification process

As described in chapter 2, section 2.1.17, slice injury was quantified by plotting histograms of red channel intensity of an image and the number of pixels above an intensity threshold are integrated (see Figure 2.10). Table 3.1 summarises key terms and definitions that are used throughout chapter 3.

Table 3.1: Key terms used in injury quantification process

Term	Definition and relevance to injury quantification process
Intensity level	Fluorescent slice images (24 bit, RGB images in .tif format) contained 3 megapixels, with each pixel having 256 intensity levels (0-255) ranging from, black or 0, to a maximum brightness of 255. The more injured a slice, the greater the PI fluorescence, and the greater the number of pixels occupying higher intensity levels.
Histogram	Histogram of the red channel plotted to show the distribution of pixels over the intensity levels.
Background	The large peak in a histogram due to the background of the image (outside the area of slice), which contains pixels in intensity levels 0-40.
Exposure time	The digital camera exposure time (seconds) used to capture the image.
Threshold	Intensity level above which pixels are integrated.
Viability	Viability was assessed quantitatively (see below) and qualitatively before starting an experiment to eliminate slices with compromised health.
Viability criteria	Criteria to reliably identify slices with compromised health, which would go bad (i.e., die even without traumatic injury) during the experiment.
Gain	Image acquisition gain setting that affects the sensitivity of the camera detector (purely electronic camera setting).
Gamma	Camera gamma setting controlling contrast and brightness of acquired image

3.2.1 Variables investigated and their effect on the injury quantification process

The *in vitro* TBI model depended on a number of variables:

1. OHSC – the strain of mice (C57/BL6), their age (P5-6), slice-culture technique (air-liquid interface technique[205]) and culture period pre-experiment (11 – 14 days) were kept constant.
2. Traumatic injury procedure – the protocol including preparation of slices for an experiment, the imaging time-points (pre-experiment viability screen ‘T0’, imaging at 24 hours, 48 hours and 72 hours – ‘T24’, ‘T48’ and ‘T72’), impact device, energy (3.4μJ), and the injury development environment (custom built pressure chambers), were all kept constant.
3. Settings for fluorescent imaging – the settings for fluorescence imaging had to be established, including:
 - a. Calibration of microscope mercury lamp intensity: To quantify how the lamp intensity dropped over time, a calibration image was obtained using a standard reference slide and standard exposure time. To compensate for the change in lamp intensity, so that slice images from different sessions could be compared, a corrected or relative exposure time was determined for each imaging session. A standard exposure time had to be selected (11 milliseconds). Based on this exposure time, a calibration reference value was calculated (127.12). These values were used to calculate the relative exposure time for each imaging session (see chapter 2, section 2.1.10)
 - b. Slice image acquisition settings:
 - a. Gain and colour correction settings: The colour correction of the red, blue and green channels were kept constant at 1.0 each (i.e., no colour correction). The gain was kept constant at 1.0 and the size of the image was kept constant at 2048 x 1536 pixels.
 - b. Gamma correction value: Calibration experiments presented an opportunity to adjust the gamma value, which combined with digital binning settings of the older version of the software, was producing intensity histograms with multiple spikes. Figure 3.1 shows an example of a histogram obtained using the previous version of the software (A) and with the newer version of the software (B). The spikes in the traces seen in

Figure 3.1A occurred at irregular intervals in the histograms and may have resulted in erroneous pixel counts in some images.

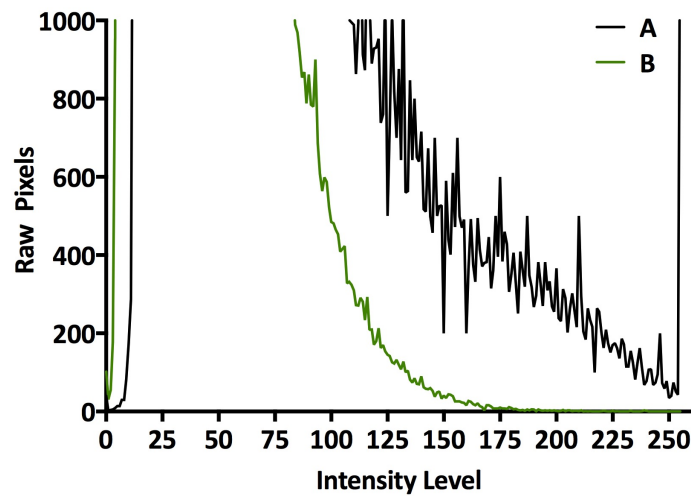


Figure 3.1: Histograms showing the distribution of pixels in a single PI fluorescent TBI slice at 72 hours imaged with previous image acquisition settings and QCapture Pro 5 software (A, black line) which contains an abundance of irregular spikes versus with new image acquisition settings and QCapture Pro 7 software (B, green line).

- c. Exposure time: the exposure time was the most important factor to consider. A longer exposure time results in a brighter image, but also increases the likelihood of saturating the pixels. Figure 3.2 shows the difference between slice images at a low exposure time (1.0s) and a higher exposure time (2.0s). Too low an exposure time would not allow the experimenter to differentiate between PI stained areas of the slice and the background (i.e., in Figure 3.2A, between the slice and the dark portion of the image), while too high an exposure time would cause saturation of the image. Therefore, the aim was to find an appropriate exposure time that would:
 - i. Discriminate between PI stained regions of the slice and the background
 - ii. Not result in saturation of the image at the last time point for imaging (72 hours)
 - iii. Allow the experimenter to distinguish, quantitatively and qualitatively, between sham slices and TBI slices. In Figure 3.2, a short exposure time of 1.0s shows a small difference between histogram profiles of sham and TBI slices (TBI injury is 2.4 times sham injury), while a long exposure time of 2.0s shows a larger difference (TBI injury is 4 times sham injury).

- iv. Show the development of injury between different time points (i.e., there should be a quantifiable difference between slices at 24 hours and at 48 hours and between 48 hours and 72 hours)
- d. Threshold for injury quantification: The threshold selected to detect a difference between sham and TBI slices was dependent on the exposure time. In figure 3.2, a low threshold was more appropriate for a low exposure time as at higher intensity levels, the histogram profiles of slices appear closer together. As the exposure time would be different, a threshold for analysis would need to be determined.
- e. Viability criteria: As the imaging settings are different, new quantitative viability criteria need to be identified.

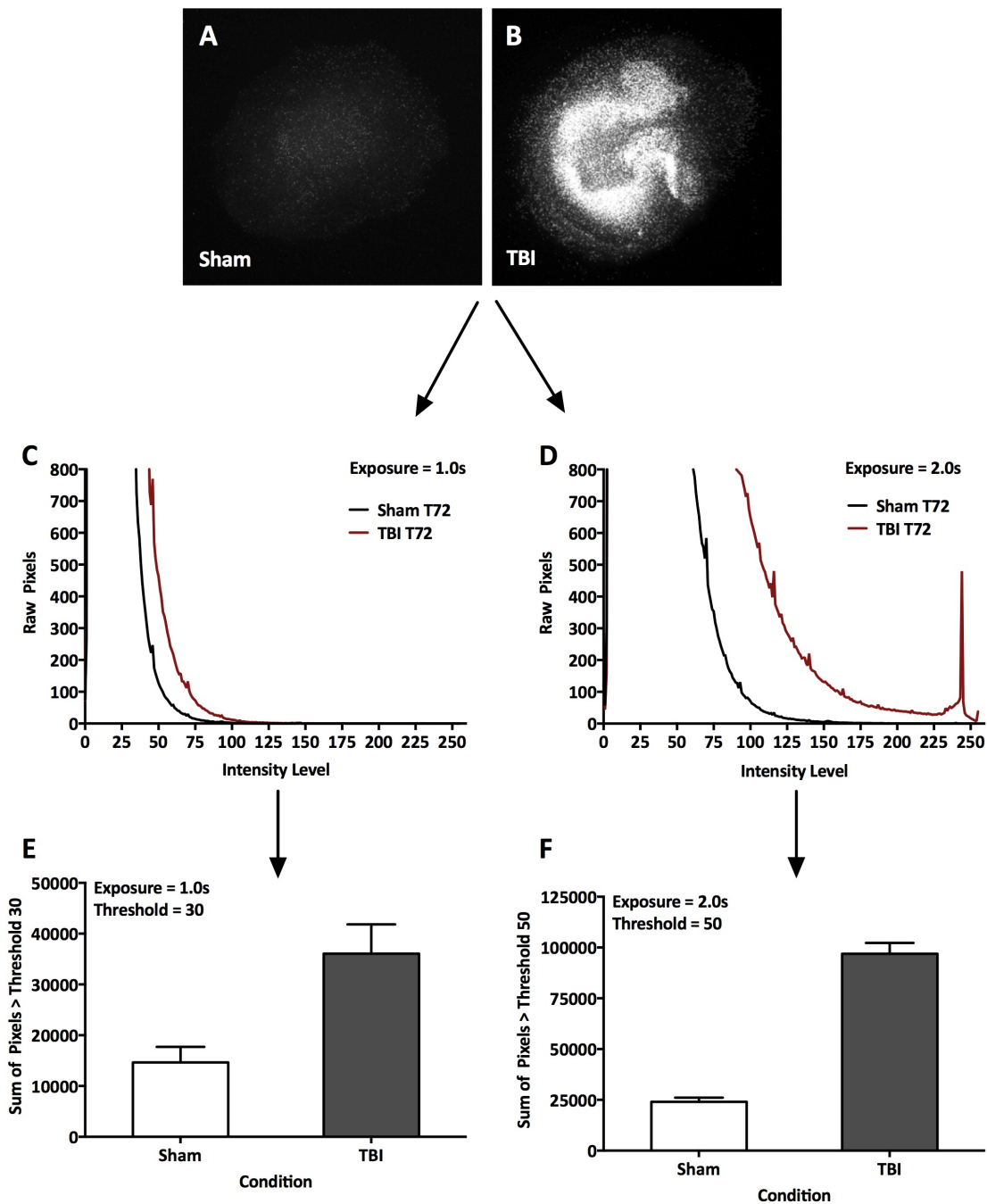


Figure 3.2: Diagram showing the difference between imaging with a long (2.0s) and short (1.0s) exposure times. Representative PI fluorescent image of a cultured organotypic hippocampal slice at 72 hours after (A) sham and (B) TBI injury. (C) Histograms showing the distribution of pixels in sham (black line) and TBI (red line) slices imaged using an exposure time of 1.0s. (D) Histograms showing the distribution of pixels in sham (black line) and TBI (red line) slices imaged using an exposure time of 2.0s. Histograms in (B) and (C) represent a mean value from a number of slices for each condition. (E) Histograms shown in (C) are transformed into bar chart showing injury quantified above a threshold of 30 at 72 hours, where TBI injury is 2.4 x sham injury. (F) Histograms shown in (D) are transformed into bar chart showing injury quantified above a threshold of 50 at 72 hours, where TBI injury is 4 x sham injury.

3.2.2 Design of experiments

The image acquisition settings and threshold for analysis were evaluated simultaneously because they both affect the quantification of injury development. In an ideal circumstance, the same set of slices would be used to assess a range of exposure times, avoiding the effect of variable slice health and allowing a rapid selection to be made. However, if the slices were kept outside the 37°C chamber for a long time (as testing several exposure times on one set would require), they would cool down. Moderate cooling has been shown to be neuroprotective in this model[103]. The average time taken to image a complete set of slices (one 6 well plate) with one exposure time is 30 minutes. In order to compare different exposure times and reduce exposure to hypothermia, the number of exposure times tested for each experiment was limited to two (with the exception of an initial screening experiment). Experiments were designed so that only one variable (exposure time) changed for each experiment. A minimum (1.0s) and maximum (2.5s) exposure time was chosen based on an initial screening experiment. Following this, exposure times above and below the midpoint of the range were compared to a ‘reference’ exposure time (1.5s). Experiments with each exposure time were repeated at least twice, to compare injury development in OHSC prepared in different weeks. The exposure times that were more important and relevant to the final decision were tested several more times. Thresholds for analysis were chosen based on criteria discussed in detail in section 3.3.2. The same thresholds were used to analyse histograms generated using exposure times that were close together (eg. 1.5s and 1.6s). A major challenge was to determine viability criteria to quantitatively exclude unhealthy or injured slices before starting an experiment. Viability criteria depend on the exposure time. At a shorter exposure time, unhealthy slices are less likely to show bright PI staining and therefore more likely to be scored as ‘viable’ when or in fact they are not viable. Conversely, slices imaged with a longer exposure time are more likely to count as ‘not viable’ because all pixels appear brighter. It was not practical to come up with a test for every exposure time tested. Therefore, in each experiment, slices were imaged at a ‘test’ exposure time and the ‘reference’ exposure time of 1.5s. The viability of the slices was assessed by eye using images taken with an exposure time of 1.5s. Slices that had focal, dense or intense PI staining before traumatic injuries were excluded. Once the final exposure time was selected, it was used to design quantitative viability criteria.

3.3 Exposure time and threshold

As discussed above, the exposure time is an important factor in the quantitative analysis of injury development. Too short an exposure time would not allow distinction between sham and TBI slices at each time point or the injury development between time points in the same slices. Too long an exposure time might saturate the image preventing further injury development, which in this model is expected to occur until 72 hours. The following sections describe the process of selection of an appropriate exposure time and threshold.

3.3.1 Pixel distribution and injury quantification

Raw histograms are useful to visually examine the increase in number of pixels at higher intensity levels, indicated by a rightward shift in the traces from time-point to time-point, (eg. from T24 to T48) and to determine thresholds to quantify injury development. There is a large peak in number of pixels at lower intensity levels (0-50), which is attributed to the contribution of the background of the image and is common to all images.

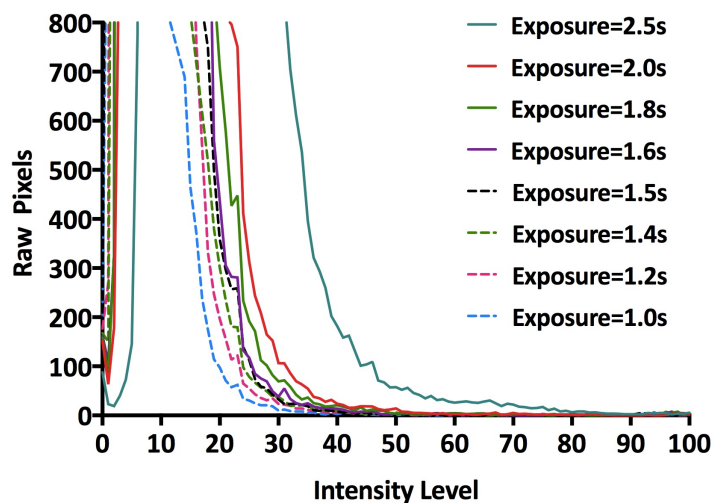


Figure 3.3: Histograms of ‘background’ images (an image of a blank space in a well containing slices) at 72 hours, at exposure times 1.0, 1.2, 1.4, 1.5, 1.6, 1.8, 2.0 and 2.5s. Lines are the mean of n= 5 images per exposure time. All images are of the same area on the same wells (5 wells) but taken at different exposure times.

Figure 3.3 shows the effect of exposure time on the background of an image. It is essential that the exposure time selected does not shift the pixels occupied by the background past the threshold used to analyse the data. This means that the threshold selected must be above the intensity level where the contribution of the background has fallen to zero or a very low count. Increasing the exposure time increases the rightward shift of the peak in the

background histogram (Figure 3.3). The contribution of background pixels with exposures 1.0s to 1.5s falls to zero between intensity levels 30 to 40. This indicates that when using short exposures, a low threshold can be used to analyse the data. When exposures 1.5s to 2.0s are used, the contribution of background pixels falls to zero or becomes very small between intensity levels 40 to 50. Therefore, the threshold has to be increased accordingly.

Next, pixel distribution in sham and TBI slices was compared. The histograms in Figure 3.4 show the distribution of pixels in TBI slices compared to sham slices at 24, 48 and 72 hours, imaged using an exposure time of 1.0s, 1.2s, 1.5s, 1.6s, 1.8s, 2.0s and 2.5s.

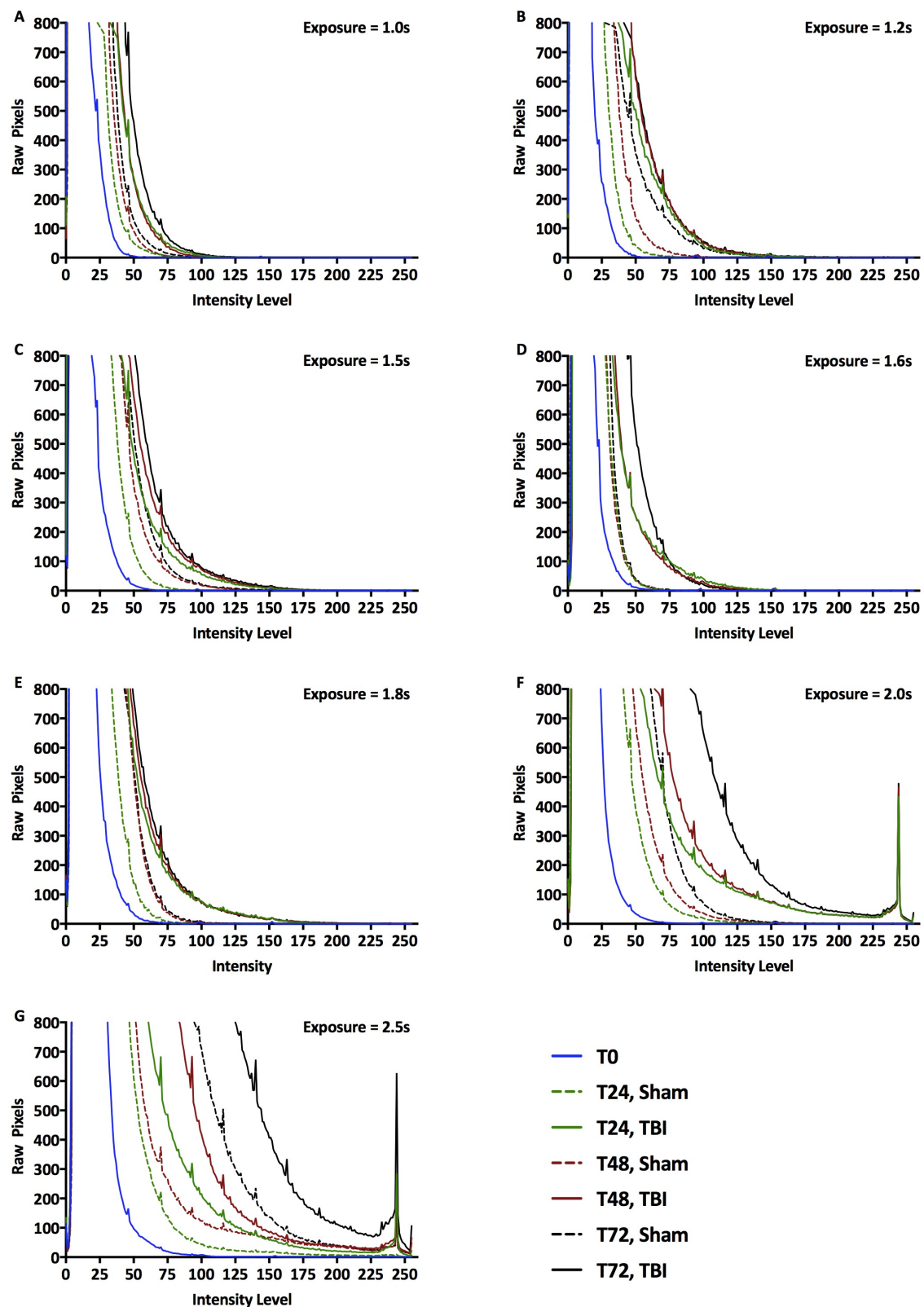


Figure 3.4: Development of a traumatic injury, compared to sham over 72 hours represented by histograms showing the distribution of pixels before injury (T0) and at 24 (T24), 48 (T48) and 72 (T72) hours post injury. Slices were imaged using exposures of (A) 1.0s (Sham: n = 29, TBI: n = 19), (B) 1.2s (Sham: n = 7, TBI: n = 6), (C) 1.5s (Sham: n = 36, TBI: n = 32), (D) 1.6s (Sham: n = 11, TBI: n = 7), (E) 1.8s (Sham: n = 11, TBI: n = 7), (F) 2.0s (Sham: n = 64, TBI: n = 79) and (G) 2.5s (Sham: n = 28, TBI: n = 29). Data presented are means of image histograms from the number of slices indicated (n).

As seen in Figure 3.4A – G, increasing the exposure time increases the extent of the absolute shift in traces between both sham slices and injured slices at each time point. The first point to note is that the intensity levels at which the T0 histograms had zero or a very low pixel count was different at the different exposure times. At an exposure time of 1.0s and 1.2s, T0 histograms have a low pixel count (under 100 pixels) above intensity level 40 (Figure 3.4A and B). At an exposure time of 1.5s, 1.6s and 1.8s, T0 histograms have a low pixel count above intensity levels 50 – 60 (Figure 3.4C, D and E). At an exposure time of 2.0s, T0 histograms have a low pixel count above intensity level 70 (Figure 3.4F) and at 2.5s, T0 histograms have a low pixel count above intensity level 100 (Figure 3.4G). This is relevant because with the appropriate exposure time, there should be a clear shift in the pixel intensity distribution following injury, i.e., at T24, the magnitude of fluorescence intensity should be significantly more than at T0. If the T0 histogram is considered as the basal level of PI fluorescence, an appropriate exposure time should distinguish between sham and TBI slices at each time point. An exposure time when combined with an appropriate threshold, should also distinguish between sham slices at T24, T48 and T72 and between TBI slices at T24, T48 and T72. This is the case at each exposure time tested. However, these differences are magnified at longer exposure times (Figure 3.4F and G: 2.0s and 2.5s), making it easier to quantify differences between conditions.

Furthermore, as the exposure time is increased, the histograms spread further apart with time. Correspondingly, the number of image intensity levels occupied by pixels at each time point increases. For example, in Figure 3.4A, the T0 trace (which has the lowest injury) tails off at intensity level 45 and the T72 TBI trace (which has the highest injury) tails off at intensity level 100. In contrast, in Figure 3.4G, the T0 trace tails off at intensity level 75 and the T72 TBI trace saturates at intensity level 256. Comparing Figure 3.4A with Figure 3.4F shows that doubling the exposure time shifts (to higher intensity levels) the tail-off points for T24, T48 and T72 histograms. With an exposure of 1.0s, T24 to T72 histograms (sham and TBI) tail off between intensity levels 70 to 110, whereas with an exposure of 2.0s, they begin to tail off above intensity level 120. T48 and T72 TBI slice histograms cause some saturation, evident in the large peaks at intensity levels 255 – 256. This occurs because the 256 intensity levels in an 8-bit image are not sufficient to distinguish all of the levels of fluorescence intensity produced using this exposure time, which is a potential drawback of using a long exposure time. When comparing the histograms produced using short exposure times with those produced using long exposure times, it became clear that increasing the exposure time

shifts all pixels to higher values, but also increases the absolute difference between them. This translates into a wider range of intensity levels for pixels to shift into. A caveat of selecting a short exposure time is that pixels that should shift to higher intensity levels on account of being subjected to injury may not as they might be underexposed. This means that at each subsequent time point, the full extent of secondary injury development would not be detected. This would lead to injury development being artificially and unknowingly confined. It is important that compared to uninjured shams there is a clearly distinguishable injury in the untreated TBI slices that develops over time, against which the neuroprotective efficacy of treatments can be measured. Ultimately, as exposure time and threshold mutually affect injury quantification, they were evaluated together to select the best combination.

3.3.2 Analysis of injury development at different thresholds

Examining fluorescence histograms enabled the identification of appropriate thresholds to analyse injury development at each of the exposure times tested. The objective was to determine a standardised threshold such that injury development in slices from different experimental groups (sham, TBI etc.) could be quantified. As exposure time and threshold interdependently affect the quantification of injury development, ultimately the best combination of the two that produced an appropriate injury development was selected. An ‘appropriate’ injury development refers to TBI injury which changes over time, is different to sham and has the same magnitude as previous experiments under the same conditions[93].

Two strategies were considered when evaluating thresholds, illustrated schematically in Figure 3.5. The first approach was to choose the threshold at which TBI and Sham slices first begin to differ in terms of fluorescence emission (Figure 3.5A = threshold 50). The second approach was to choose a threshold that at a certain time point, say 72 hours, gave the greatest difference between a TBI and sham slice in terms of the number of pixels occupied above that threshold. This means that the ratio of number of pixels in a TBI slice to number of pixels in a sham slice is the highest (Figure 3.5B = threshold 75). Options (A) and (B) were tested and the results at different exposure times are shown in Figure 3.6 - Figure 3.9.

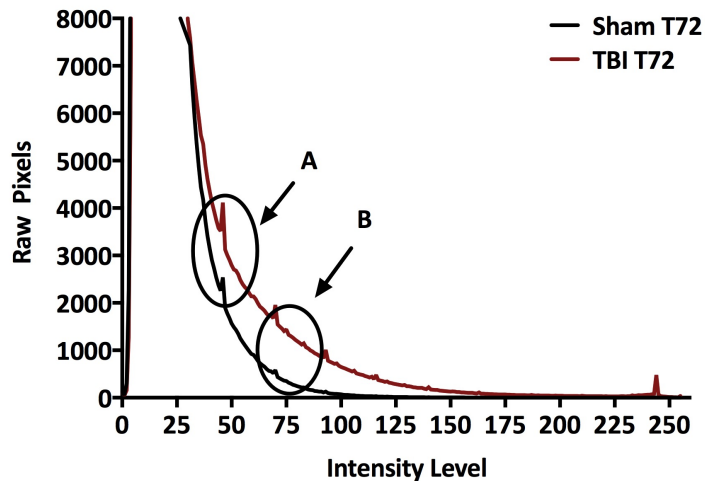


Figure 3.5: Histograms to highlight two options explored when choosing a threshold to quantify injury. Histograms show the distribution of pixels across 256 intensity levels in sham (black line) and TBI (red line) slices at 72 hours post injury (A) Oval outline highlights option 1; the threshold at which TBI and Sham slices first begin to differ in terms of fluorescence emission. (B) Oval outline highlights option 2; the threshold at which the ratio of number of pixels in a TBI slice to number of pixels in a sham slice is the highest. Slices were imaged with an exposure time of 2.0s and data shown are means of image histograms from a number of slices; sham: n= 64 and TBI: n= 79.

While the advantage of choosing the second approach (B) was that a greater difference between sham and injured slices would be detected at every time point, the disadvantage was that increased fluorescence contained in lower intensity levels would not be counted, thereby possibly underestimating injury development. If, in addition to a shift in pixels from lower to higher intensity levels, there was a higher proportion of pixels at each low intensity level, that would still result in an overall increase in fluorescence. However, using the first approach (A), it would be possible to detect this. In addition, choosing the intensity level at which sham and TBI slices first differ would be standardised throughout experiments with different exposure times and would avoid loss of information. The histograms in Figure 3.4 were used to decide the exposure times according to the two options, detailed in Table 3.2 below.

Table 3.2: Table listing thresholds tested according to two approaches considered when deciding an appropriate threshold to analyse injury quantification

Exposure Time (s)	Threshold at which ratio of number of pixels of TBI slice to sham slice is the highest	Threshold at which sham and TBI slices first differ
1.0	50	30
1.2	50	30
1.5	75	50
1.6	75	50
1.8	75	50
2.0	75 and 100	50
2.5	75 and 100	50

As some exposure times produced similar results, for clarity Figure 3.6 - Figure 3.9 show injury development at only 1.0s, 1.5s, 2.0s and 2.5s.

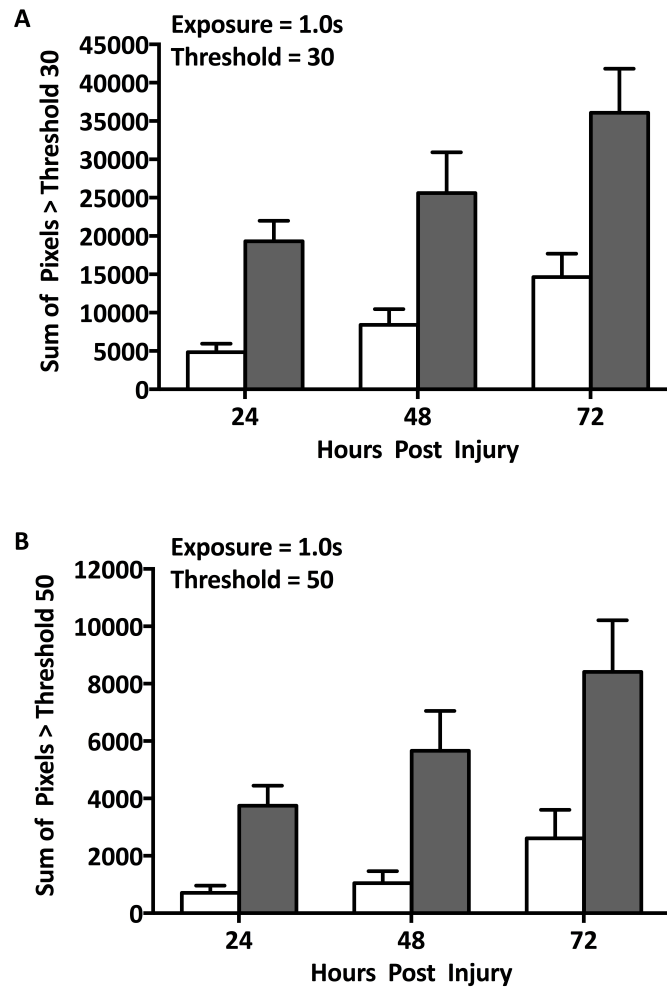


Figure 3.6: Development of traumatic injury (grey bars) compared to sham (white bars) over 72 hours; slices were imaged using an exposure time of 1.0s and analysed by quantifying number of pixels above an intensity level of (A) 30 and (B) 50 (Sham: n = 29, TBI: n = 19 from an approximately 18 animals). Data represent means and error bars are SEM.

When slices imaged at 1.0s were analysed at threshold 30, the maximum number of pixels in injured slices, at 72 hours, reaches a value of $35,000 \pm 5000$ (Figure 3.6A). Increasing the threshold to 50 decreases the range of pixel values, with the maximum in injured slices at 72 hours being 8000 ± 2000 (Figure 3.6B). Correspondingly, at 72 hours post injury, using threshold 30, the TBI injury is 2.5 times the sham injury whereas, using threshold 50, the TBI injury is 3.2 times the sham injury. A similar trend, with the ratio of TBI to sham increasing at higher thresholds, was observed when the slices were imaged using an exposure time of 1.5s and analysed at thresholds 50 and 75 (Figure 3.7A and B).

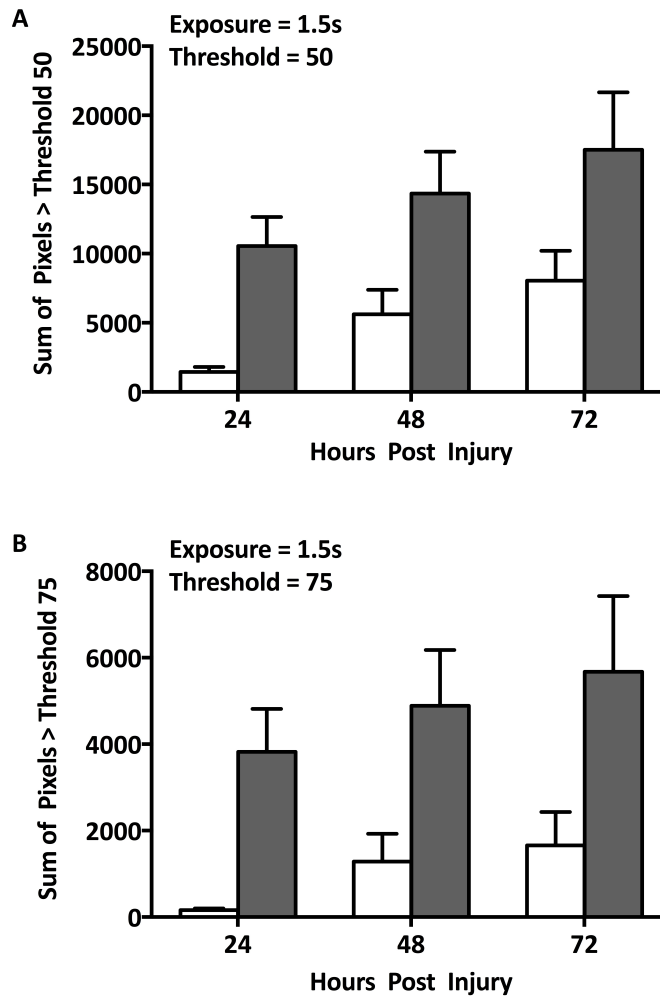


Figure 3.7: Development of a traumatic injury (grey bars) compared to sham (white bars) over 72 hours; slices were imaged using an exposure time of 1.5s and analysed by quantifying number of pixels above an intensity level of (A) 50 and (B) 75 (Sham: n = 36, TBI: n = 32 from approximately 24 animals). Data represent means and error bars are SEM.

When slices imaged at 1.5s were analysed at threshold 50, the maximum number of pixels reaches $18,000 \pm 4000$ (Figure 3.7A), whereas increasing the threshold to 75 decreases the range of maximum pixel values to 6000 ± 1500 (Figure 3.7B). At 72 hours post injury, using threshold 50 the TBI injury is 2.2 times the sham injury, whereas using threshold 75 the TBI injury is 3.4 times the sham injury.

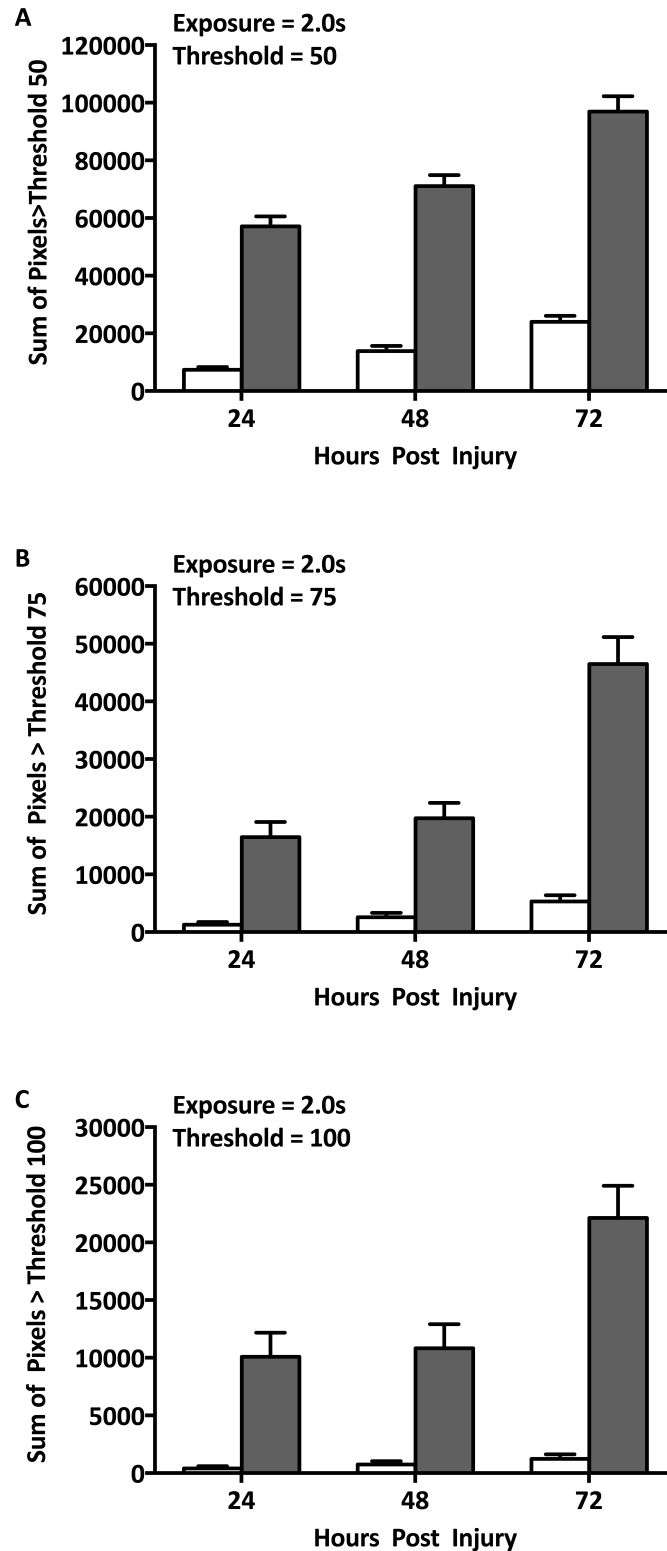


Figure 3.8: Development of a traumatic injury (grey bars) compared to sham (white bars) over 72 hours; slices were imaged using an exposure time of 2.0s and analysed by quantifying number of pixels above an intensity level of (A) 50, (B) 75 and (C) 100 (Sham: n = 64, TBI: n = 79 from approximately 36 animals). Data represent means and error bars are SEM.

The same trend is observed when slices were imaged using higher exposure times i.e., lower maximum pixel values when histograms are analysed at a higher threshold but also a higher

ratio of TBI to sham injury. When slices imaged at 2.0s were analysed at threshold 50 the maximum pixel number in injured slices at 72 hours reaches $95,000 \pm 2000$ (Figure 3.8A). Increasing the threshold to 75 decreased the pixel values to 45000 ± 5000 (Figure 3.8B), and further increasing the threshold to 100 decreased the maximum pixel values to 22000 ± 3000 (Figure 3.8C) at the same time point. Correspondingly, the ratio of pixel numbers in TBI compared to sham slices at each time point also increases. At 72 hours, when analysed at threshold 50, the TBI injury is 4.0 times sham injury, when analysed at threshold 75 TBI is 8.8 times sham injury and at threshold 100 the ratio is 17.8.

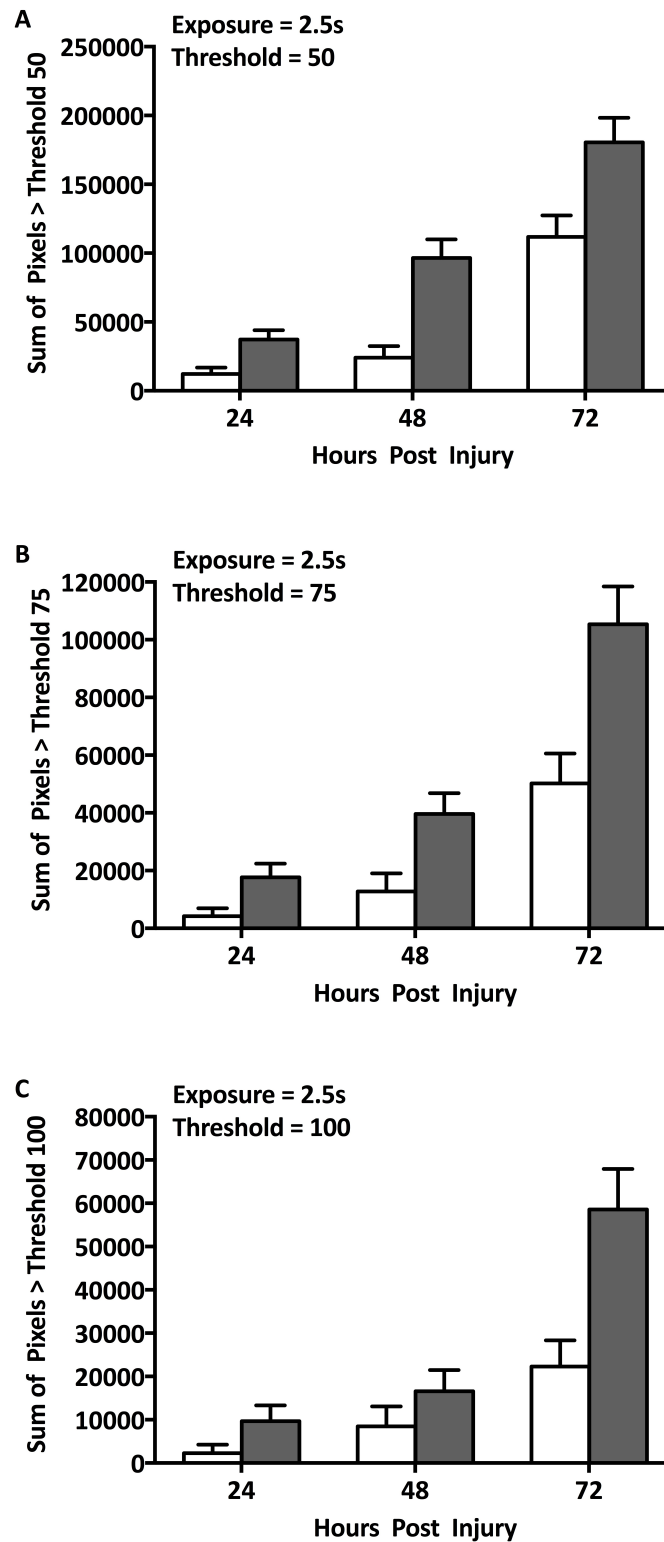


Figure 3.9: Development of a traumatic injury (grey bars) compared to sham (white bars) over 72 hours; slices were imaged using an exposure time of 2.5s and analysed by quantifying number of pixels above an intensity level of (A) 50, (B) 75 and (C) 100 (Sham: n = 28, TBI: n = 29 from approximately 18 animals). Data represent means and error bars are SEM.

Similarly, slices imaged at an exposure time of 2.5s also produced lower maximum pixel values when histograms are analysed at a higher threshold and a higher ratio of TBI to sham

injury. Figure 3.9 shows the slices imaged at 2.5s and analysed at thresholds 50, 75 and 100 (Figure 3.9A, B and C respectively). At threshold 50, maximum pixel number in TBI slices at 72 hours reaches a value of $175,000 \pm 10,000$. Increasing the threshold to 75 decreased the pixel values to $100,000 \pm 10,000$ and further increasing the threshold to 100 decreased the pixel values to $55,000 \pm 10,000$. These absolute pixel values are higher than pixel values for images taken at 2.0s, but follow a similar trend of decreasing as threshold is increased. At 72 hours, when analysed at threshold 50, TBI injury is 1.6 times sham injury, when analysed at threshold 75, TBI injury is 2.0 times sham injury and at threshold 100 the ratio is 2.6.

A key trend observed at all exposure times was that when defining the threshold as the intensity level at which the ratio of number of pixels of TBI slice to sham slice is the highest (option B in Figure 3.5: thresholds 50 for exposure time 1.0s, threshold 75 for exposure time 1.5s and thresholds 75 and 100 for exposure times 2.0s and 2.5s), the range of the number of pixels decreases. Although the difference between injury development for sham and TBI slices was higher at each time point, the standard error when using these thresholds was correspondingly higher in terms of fractional value. This is because when using higher thresholds, the injury was quantified using values that are in the tail end of the histogram. As the absolute number of pixels decreases at higher intensity levels, there is increased variance. Furthermore, using higher thresholds such as 100, for exposure times of 2.0s and 2.5s to analyse injury development, created an unrealistically large difference between sham and TBI slices. For example, for exposure time 2.0s, the ratio of injury in a TBI compared to a sham slice was 17.8 at threshold 100. This is nearly 6 times more than the ratio observed in the model previously (before using the new software (Figure 3.10), where TBI injury was usually 3 times sham injury at 72 hours. While this might at first seem better, it might also allow misinterpretation of the neuroprotective effect of a drug, as it may artificially seem more protective than it really is.

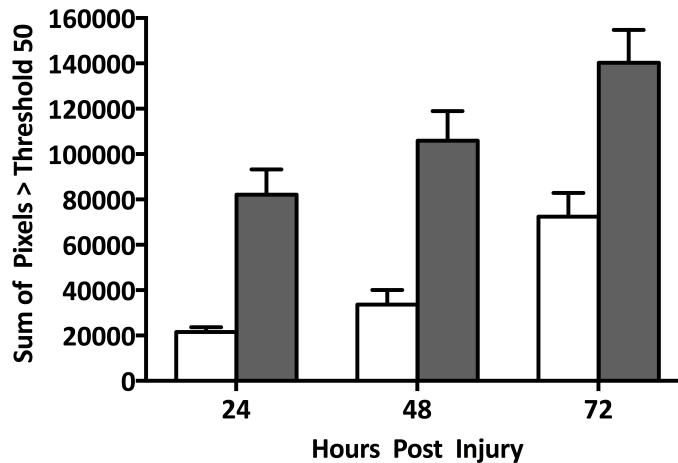


Figure 3.10: Development of a traumatic injury (grey bars) compared to sham (white bars) over 72 hours; slices were imaged using previously established image acquisition settings (exposure time = 1.2s, gamma = 1.29, gain = 1, RGB = 1, software = QCapture Pro 5) and were housed in a humidified incubator at 37°C/5% CO₂ without 0.5atm helium pressure (Sham: n = 39; TBI: n = 29 from approximately 15 animals). Data represent means and error bars are SEM.

To narrow down the choice of thresholds, injury development was compared with data analysed with the previous set up (i.e., before the software was changed, Figure 3.10). Slices were imaged using QCapture Pro 5, an exposure time of 1.2s, gamma value of 1.29, gain and RGB values of 1.0. Here, the absolute pixel values for TBI injury at 72 hours is $125,000 \pm 10,000$ and the point at which the difference between sham and TBI slices first becomes apparent, was used as a threshold (threshold 50). It was high enough to differentiate between TBI and sham slices, but low enough to ensure that quantification was not limited to pixels only in the very tail end of the histogram. Although this data was collected when slices were not stored in the humidified chamber under 0.5atm pressure, previously published work using the same model found no effect of added pressure on sham or TBI injury development[93].

In section 3.2.1, the criteria for choosing an exposure time and threshold were discussed. In addition, it was important that the selection resulted in development of a traumatic injury over time that was most similar to previous data[93] in terms of pixel values and in terms of the ratio between sham and TBI injury at each threshold i.e., a TBI slice at 72 hours should have an injury of approximately 3 times a sham slice at the same time point. Overall, the maximum injury (i.e., TBI injury at 72 hours) should reach a high enough pixel count so that injury development between time points may be differentiated.

Given these criteria, the use of the higher thresholds (at each exposure time) was not suitable. This is because, when histograms are analysed at higher thresholds, the maximum pixel

values (TBI injury at 72 hours) are too low. Also, when some exposure times are used (2.0s and 2.5s), the ratio of TBI to sham injury is too high, which as discussed could lead to misinterpreting the efficacy of a neuroprotective drug. The following combinations were left: exposure time 1.0s and 1.2s analysed at threshold 30, exposure times 1.5s, 1.6s and 1.8s analysed at threshold 50, exposure time 2.0s analysed at threshold 50 and exposure time 2.5s analysed at thresholds 50 and 75. Exposure times below 2.0s were rejected because although the difference between sham and TBI slices was evident, the maximum injury (TBI injury at 72 hours) did not reach a high enough pixel count.

Next, injury development was compared between slices imaged using exposure times 2.0s and 2.5s. The exposure time 2.5s was eliminated because the histogram of 'background' (i.e. no slice) reached a pixel number of 0 only after intensity level 100 (Figure 3.3). This means that when using thresholds 50 and 75 to analyse the data, the background of the image is contributing significantly at every time point. Using a higher threshold to analyse the data increased the variance and lowered the overall pixel numbers, which did not satisfy the other criteria. When analysed at threshold 50, sham slices at 72 hours had an injury that was $62\% \pm 8.6\%$ of TBI injury, implying that this exposure time overly saturated even the sham slices at 72 hours. In contrast, data analysed at a threshold of 50 using exposure time 2.0s occupied a wide enough range of intensity levels and reached high mean pixel numbers (100,000 at 72 hours), sufficient to see a development in injury between time points, as well as being similar to previously published data[93]. Thus the final decision was to use an exposure of 2.0s to image the slices and to quantify injury development by measuring the number of pixels above threshold 50.

3.4 Viability Criteria

Having established a new exposure time and threshold to analyse injury development, quantitative criteria to reliably identify slices whose health was compromised before the experiment (at T0) and which would be ill-representative of traumatic injury development (i.e. they would have PI fluorescence even if uninjured) was defined. For this purpose, sham slices were used from eight experiments, where slices were imaged at an exposure of 2.0s and injury was quantified above threshold 50. Only sham slices were used to evaluate viability criteria because the desired upper 'limit' of pixel values for a healthy sham slice at the end of

an experiment could be determined more easily, as healthy sham slices should develop very little injury over the course of the 72-hour experiment. Generally, throughout the course of an experiment, sham slices had different outcomes:

1. Slices could be healthy and develop a low level of injury through the experiment i.e., normal viable sham slices
2. They may have bright PI staining at T0 (this may be due to focal injury such as CA1/DG damage which may have occurred during the dissection process or during culturing). The high PI staining is accentuated further at T24, T48 and T72. These sham slices may even have pixel numbers equivalent to TBI slices
3. They can appear 'normal' at T0 but go bad at T24, T48 or T72. In this case, they may either become 'maximally injured' at T24 or T48 and then will not develop much further or at T72 they may suddenly appear highly injured

Viability criteria that did not exclude sham slices that would then develop to 50 – 60% of the TBI injury at 72 hours would not be appropriate. The ideal scenario would be to find reliable criteria that would select for only the slices that developed to no more than 30% of the TBI injury at 72 hours (as in outcome 1 listed above). In order to do this, T0 histograms (before injury) for healthy sham slices (those that did not develop injury through to the end of the experiment) were compared with T0 histograms of unhealthy sham slices to find patterns that could be used to develop objective viability criteria. The criteria was then applied to all slices (sham or TBI).

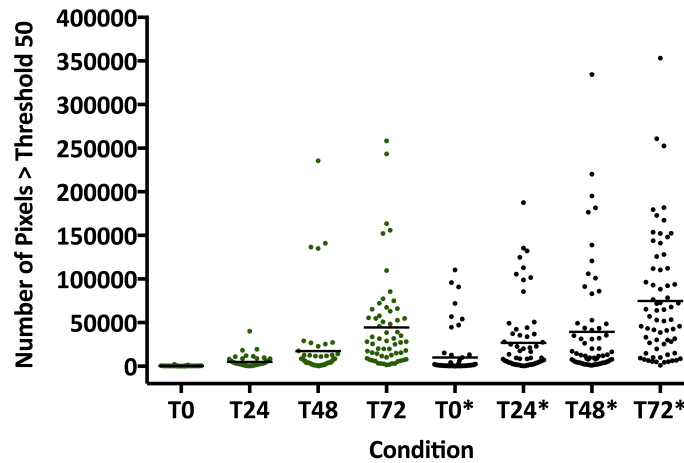


Figure 3.11: Dot plots representing the injury development in individual sham slices (quantified by number of pixels above intensity level 50) before (T0) and at 24, 48 and 72 hours (T24, T48 and T72) after injury. Left-hand 4 columns (Green dots) represent sham slices that were marked viable by eye (VBE) at T0 and right-hand 4 columns with asterisks (black dots) represent sham slices that were marked not viable by eye at T0. Slices were marked not viable if they appeared to have intense, dense or focal PI staining. Some dots are superimposed (curves at the bottom of each group) and straight lines are means. Sham: n = 126.

Figure 3.11 shows the number of pixels above threshold 50 in 126 sham slices before and at 24, 48 and 72 hours after injury. As discussed in section 3.2.2, following T0 imaging, slice viability was assessed by eye, and slices were excluded from analysis if they had intense, dense or focal PI staining. As this was a qualitative procedure, care was taken to ensure that any slice that showed even slightly elevated PI staining was excluded from analysis. Figure 3.11 compares the injury development between slices that were included when assessed by eye (green dots, VBE) and slices that were excluded (black dots, not VBE). A proportion of slices marked viable also develop increased injury and this was especially apparent at 72 hours post injury, where 62% of slices have an injury that is more than half of the mean TBI injury (i.e., more than $50,000 \pm 5000$ pixels) at the same time point. At each time point, there is a clear trend – non-viable slices (black dots) have more variance in injury development, and at T48 and T72, a significant number of non-viable sham slices (black dots) develop injury that is almost comparable to TBI slices at the same time point (the mean TBI injury at 48 hours is $70,000 \pm 4000$ and the mean TBI injury at 72 hours is $100,000 \pm 5000$ pixels above threshold 50). However, when looking at the non-viable slices at T0, the injury development of 86% of slices (black dots) seems comparable to slices marked viable by eye at T0 (very close to 0 pixel values for injury), at a threshold of 50. As extra caution was taken to include only the ‘healthiest’ looking slices as viable for an experiment, some slices in the group marked non viable may have actually been healthy. Not including these slices in experiments would be wasteful.

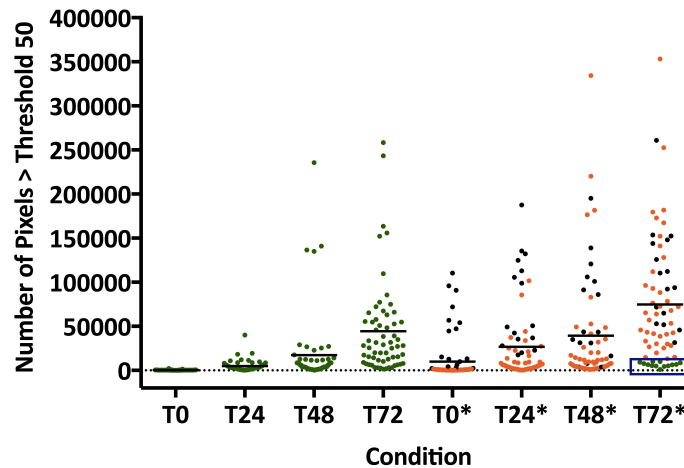


Figure 3.12: Dot plots representing the injury development in individual sham slices (quantified by number of pixels above intensity level 50) of sham slices before (T0) and at 24, 48 and 72 hours (T24, T48 and T72) after injury. Left-hand 4 columns (green dots) represent sham slices that were marked viable by eye at T0 and right-hand 4 columns with asterisks (black dots) represent sham slices that were marked not viable by eye at T0. Orange dots (T0* to T72* columns) represent sham slices that appear to have the same level of injury as slices that were marked viable by eye at T0, but go on to develop high levels of injury. The blue box drawn around green dots at T72* represent healthy slices misidentified as not viable at T0. Slices were marked not viable if they appeared to have intense, dense or focal PI staining. Some dots are superimposed (curves at the bottom of each group) and straight lines are means. Sham: n = 126.

Figure 3.12 shows the injury development quantified by the number of pixels above threshold 50 of the same 126 slices before and at 24, 48 and 72 hours after injury. Injury development is compared between slices that were included when assessed by eye (green dots, VBE) and slices that were excluded (black dots, not VBE). Additionally, non viable slices which had exactly the same level of injury at threshold 50 as slices marked viable by eye at T0 are coloured in orange to ‘track’ the development of injury in these slices. Thus, although initially identified as a subset of non-viable slices, these slices were observed to determine whether they develop injury similar to TBI slices or if they continue to have a low pixel count. Slices that remained healthy (i.e., had injury comparable to VBE slices) at T72 would be the false negatives (slices that were marked ‘not viable’ but are viable) and could be included in the viable group (green dots highlighted with a blue box at T72). Several non-viable slices which had a low level of injury at T0 (58% of the group marked in orange) go on to develop a high injury (above 50,000 pixels) by 72 hours. This indicates two important results:

1. The naked eye is reasonably accurate at determining which slices are unhealthy based on PI staining at T0 and
2. Integrating injury (counting pixels) above a threshold of 50 is not the most appropriate way to distinguish between viable and unhealthy slices for the pre-experimental viability screen.

A higher threshold may be more appropriate because at a threshold of 50, viable and non-viable slices have the same level of injury.

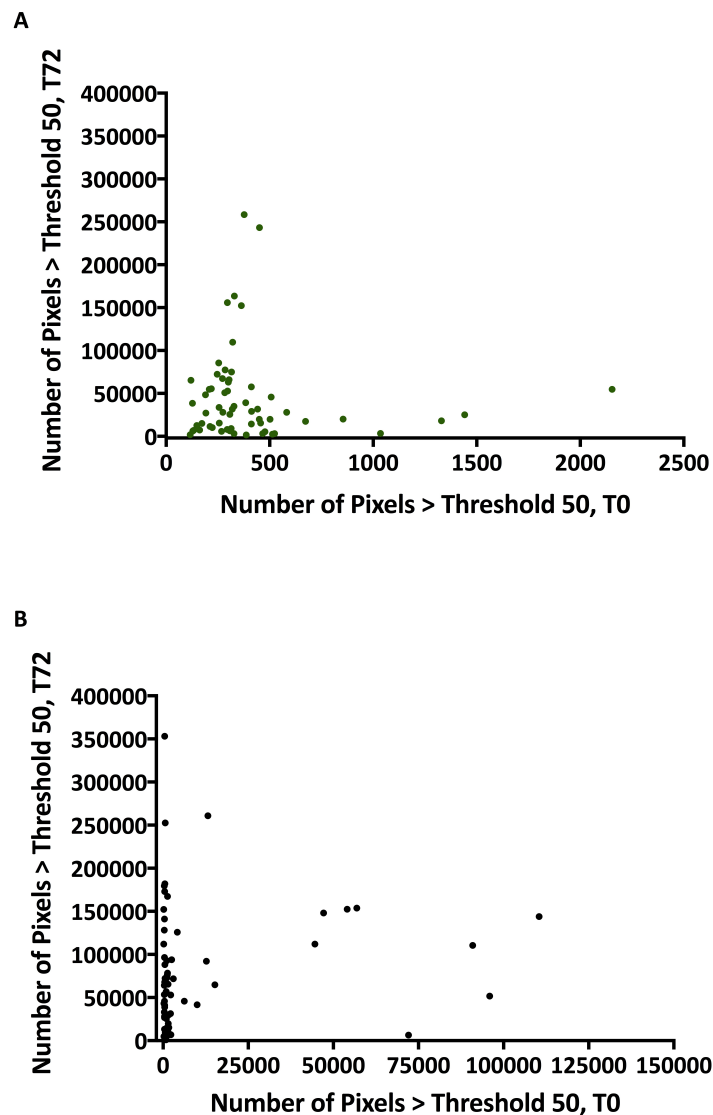


Figure 3.13: Dot plots representing the injury (quantified by number of pixels above intensity level 50) of sham slices before trauma (T0) versus their corresponding injury 72 hours after traumatic injury. (A) Dots represent sham slices that were marked viable by eye (VBE) at T0, $r^2 = 0.0023$ and $n = 61$. (B) Dots represent sham slices that were marked not viable by eye at T0, $r^2 = 0.1852$ and $n = 67$.

Figure 3.13 shows slice injury at time point T0 against the injury at T72 for slices marked viable by eye (A) and not viable by eye (B). For both conditions (slices marked viable or not viable), the correlation between their injury at T0 and T72 was low, represented by r^2 values of 0.0023 and 0.1852 respectively, although greater for non-viable. This indicated that the actual injury level, as measured by the number of pixels above an intensity level at T0, was unlikely to predict whether or not a slice will develop a high level of injury.

One strategy to determine viability criteria was to draw a line at injury level 30,000 at T72 (which represents 30% of the TBI injury at T72) and include any slices from both the 'viable' and 'not viable' group marked by eye that fall under this value into the viable group. However, when tested, this did not exclude slices that, at T0, appeared to have dull global slice injury that did not develop. Therefore, to include the false negatives (slices that were marked 'not viable' but are viable) in the 'viable' group as discussed above, slices from the non-viable group that had a similar level of injury to slices within one standard deviation of the mean of those marked viable at T0 were included in the 'viable' group to determine viability criteria. Although the data shown in Figure 3.11 and Figure 3.12 failed the Shapiro-Wilk test of normality, the standard deviation was still used because the sample size (126 slices) was considered large, obtained over several weeks from a number of animals (in excess of 40 animals) and as the sample size increases, the shape of the sampling distribution approaches a normal distribution based on the principle by Fischer, 1928. Moreover, non-parametric tests would not be as powerful and informative in this situation.

After there were two groups of slices, 'viable' (including slices marked viable by eye and now the false negatives as discussed above) and 'non-viable', their individual histogram traces were examined for patterns. The aim was to distinguish between viable and non-viable slices using non-zero values of the histograms i.e., above intensity levels that represent the major peak in the histogram but below intensity levels where the intensity of staining has dropped to zero values. It was observed that the slices marked viable that do not develop a high level of injury at 72 hours (i.e., the 'viable' slices), produce histograms that begin to approach 0 around intensity levels 60 – 70 at T0. Therefore, healthy slices contained the vast majority of their pixels in low intensity levels. In contrast, it was observed that slices marked not viable (and developed a high level of injury) did not produce histograms that begin to approach 0 at intensity levels 60 – 70 at T0. Instead pixels in these images had shifted to higher intensity levels, and histograms only began to approach 0 at intensity levels above 100. It was observed that the vast majority of slices in the viable group did not have more than 10 pixels in intensity levels above 70. Therefore, an upper limit of acceptable number of pixels at any intensity level above 70 was defined as 10. This means that viable slices should not contain more than 10 pixels at any intensity level above 70. There were, however, some slices in the group marked viable that satisfied the criteria but went on to develop injury; these were the false positive slices (slices marked viable at T0 which develop injury at a later time point).

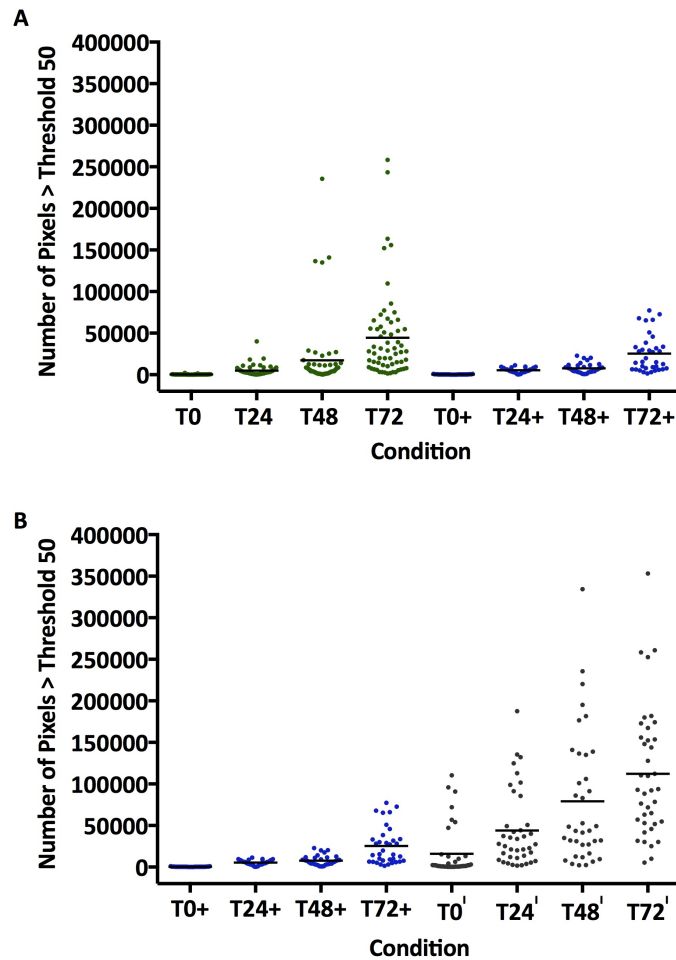


Figure 3.14: Comparison of viability assessed by eye and by using quantitative criteria. (A) Left-hand 4 columns (green dots) represent sham slices that were marked viable by eye at T0 (n = 61) and right-hand 4 columns with + (blue dots) represent sham slices that were marked viable by the criteria (viable slices contain no more than 10 pixels at any intensity level above 70) at T0 (n = 35). **(B)** Left-hand 4 columns with + (blue dots, duplicated from A) represent sham slices that were marked viable by criteria at T0 (n = 35) and right-hand 4 columns with dash (grey dots) represent sham slices that were marked not viable by criteria at T0 (n = 39). Some dots are superimposed (curves at the bottom of each group) and straight lines are means.

Next, injury development in slices marked viable by the criteria was compared with those marked viable by eye (Figure 3.14). At time points T24 and T48, the slices marked 'viable by criteria' do not develop significant injury from T0, indicating that the criteria do indeed predict which slices remain healthy. At T72, the group of slices marked viable by eye has a more diffuse spread and higher injury development than slices marked viable by criteria. Figure 3.14B compares the injury development in slices that were marked viable by the criteria versus those marked not viable by the criteria. At every time point, the variance in injury development is greater in groups of slices marked not viable. Even if the slices had a relatively low injury at T0, those slices go on to develop high injury at subsequent time points. In contrast, slices marked viable remain minimally injured, with little development in

comparison at T72. There will always be a small percentage of slices in an experiment that appear viable at T0 but go on to develop injury at a later point. These outliers develop injury due to an external factor, for example, if the impact procedure takes longer than expected on a particular day and shams are exposed to room air for longer. It is expected that shams will have a small amount of injury through the 72-hour experiment, but provided TBI slices have an injury that is at least 3 times higher than sham injury (from previously established data[93]), the injury level was deemed acceptable.

3.5 Calibrated injury development

Figure 3.15 shows the final development of a control traumatic injury under conditions of 0.5atm helium pressure.

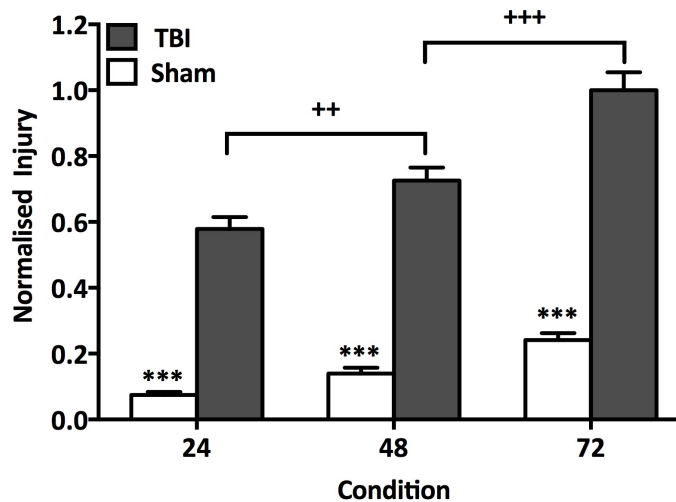


Figure 3.15: Development of a traumatic injury (grey bars) compared to sham (white bars) over 72 hours. Data presented are means, normalised to the injury development of slices with a control traumatic injury at 72 hours. Error bars are SEM. **($p<0.0001$) compared to TBI injury at each time point. ++($p<0.01$) and +++($p<0.0001$) indicates value significantly different from injured slices between time points (brackets drawn). Sham: $n = 150$ and TBI: $n = 117$ from approximately 60 animals.

The development of injury between time points was significantly different. At 24 hours the injury ($57 \pm 3\%$) was significantly different ($p<0.0001$) to the injury at 48 hours ($72 \pm 3\%$), which develops further ($p<0.0001$) at 72 hours ($100 \pm 5\%$). Sham injury remained significantly lower ($p<0.0001$) than TBI injury at each time point. At 72 hours, sham injury was $24 \pm 2\%$ of TBI injury. To assess whether the exposure time and threshold selected caused any saturation of the image, the control injury at 72 hours was compared to the ‘maximal injury’ attainable in the slices.

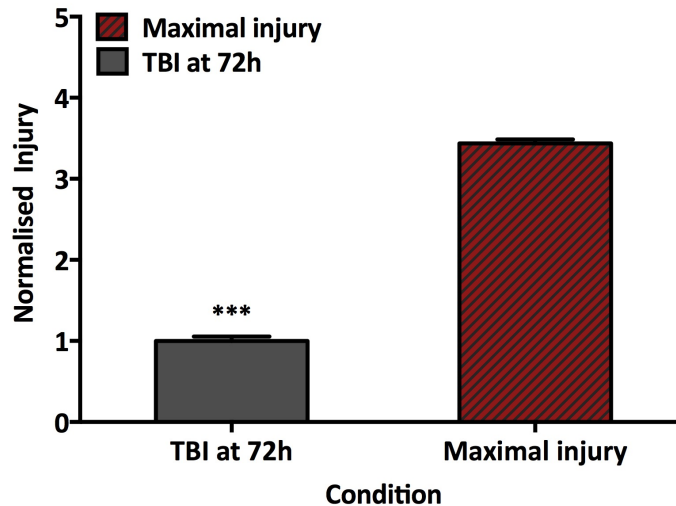


Figure 3.16: A comparison between the control traumatic injury (grey bar) at 72 hours and ‘maximal’ injury (red striped bar) after the slices have been submerged in 70% ethanol for 24 hours at the end of the 72 hour traumatic injury experiment. Data presented are means, normalised to the injury development of TBI slices with a control traumatic injury at 72 hours and error bars are SEM. ***($p < 0.0001$) compared to maximal injury, obtained through an unpaired, two tailed t -test. TBI at 72h: $n = 117$ and maximal injury: $n = 70$.

Figure 3.16 shows that the control injury at 72 hours was $30 \pm 5\%$ of the ‘maximal’ injury, obtained by submerging the slices in 70% ethanol for 24 hours at the end of the 72-hour experiment. This is comparable to the previously established model and indicates that the exposure time chosen does not cause saturation of the slice image, similar to results seen in Harris *et al.*[93].

3.6 Effect of argon on the development of a traumatic injury

The final step was to test the neuroprotective efficacy of a previously tested treatment and compare its level of protection to published data. The noble gas argon was selected for these experiments as it has been tested under the same conditions using this particular *in vitro* TBI model.

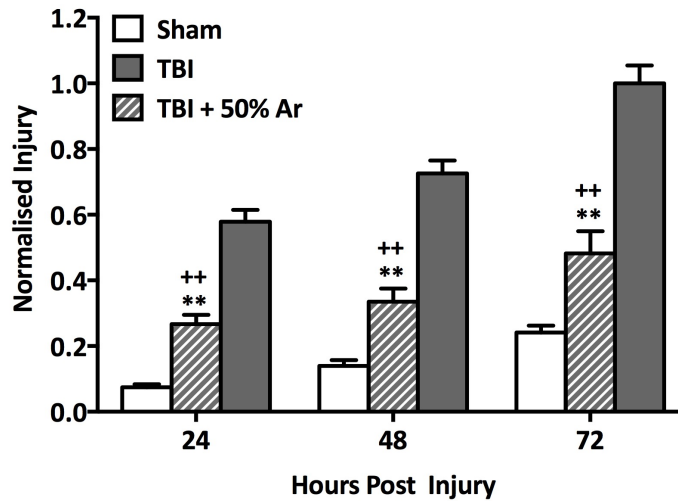


Figure 3.17: Treatment with 0.5atm (50%) argon (grey striped bars) attenuates development of traumatic injury (grey bars) compared to sham (white bars) over 72 hours. Data presented are means, normalised to the injury development of slices with a control traumatic injury with 0.5atm helium at 72 hours. Error bars are SEM. **($p < 0.0001$) and ++($p < 0.001$) compared to TBI injury and sham respectively at each time point. Sham: $n = 150$, TBI: $n = 117$ (from approximately 60 animals) and TBI + 50% Ar: $n = 77$ from approximately 36 animals.

50% argon attenuated the development of injury at each time point (Figure 3.17). At 24 hours, 50% argon was $54 \pm 1\%$ protective. This level of protection remained constant at 48 hours, where argon was $54 \pm 1\%$ protective as well. By 72 hours, argon was $52 \pm 3\%$ protective. The injury in argon treated slices was significantly higher ($p < 0.001$) than sham slices at all time points, thus 50% argon attenuated but did not abolish the development of secondary injury. This is consistent with that published by Harris *et al.*[93], who found that 50% argon was $52 \pm 7\%$ protective at 72 hours post trauma. Loetscher *et al.*[129] found that 50% argon was even more protective, reducing injury to $14 \pm 3\%$ of the control injury at 72 hours however, the injury used here ($3.4\mu\text{J}$) and the laboratory conditions of this data are more similar to Harris *et al.*[93]. Having established that argon was neuroprotective and to a similar degree as found previously, the *in vitro* model was adequately calibrated to study neuroprotection with other treatments, not previously tested.

4 Chapter 4: Combination therapies in an *in vitro* model of TBI

4.1 Overview and purpose of research

The *in vitro* TBI model, calibrated as described in chapter 3, was used to test the neuroprotective efficacy of different drugs and combinations of drugs with noble gases. In order to report a ‘complete’ combination study, each monotherapy was evaluated first and then examined in combination, as opposed to reporting ‘quasi’ combination, which involves the addition of therapies without confirmation of their individual effects in a model[80]. The aims were to:

1. Establish levels of protection with concentrations of xenon and argon (10%, 25% and 50%atm)
2. Test whether progesterone and meloxicam are protective following a traumatic injury *in vitro*, and whether their protective effect (if any) is dose dependent;
3. Determine whether co-administration of these drugs is able to increase efficacy of any sub-maximally effective concentrations of xenon and argon; and
4. Determine whether the combination neuroprotective effect is ‘synergistic’, or ‘additive’ (section 4.2).

4.2 Quantitative analysis of drug combination effects

When two (or more) drugs are given together, their combined effect may be above what is expected from their individual efficacies and so their effect is ‘synergistic’ or super-additive. In contrast, the combined action of some drugs may be consistent with or no more than their individual drug efficacies, in which case their interaction is said to be ‘additive’. Different studies have demonstrated that synergistic and additive effects are not simply a property of the two drugs, but also depend on the doses of each drug in the combination.

There are several methods to assess drug synergy and additivity including the cooperative synergy effect method, response additivity synergy, Loewe additivity synergy and Bliss independence. Briefly, cooperative effect synergy (also known as Gaddum’s non-interaction mechanism or the highest single agent approach) states that a drug combination displays synergism if the combined effect is greater than the effect of both drugs in the combination

acting alone i.e., if $E_{(a+b)} > E_{(a)}$ and $E_{(a+b)} > E_{(b)}$. This method although approved by the US Food and Drug Administration (FDA), does not account for additivity and unless it is already known that one of the drugs in the combination is inert, is not very informative. Response additivity synergy states that the combined additive effect is equal to the sum of the individual effects of each drug and synergy is observed when the observed combination effect exceeds the additive effect i.e., if $E_{(a+b)} > E_{ADD(a+b)}$. This method is not applicable to situations where curvilinear dose response curves are observed[235–237]. Loewe additivity synergy is a graphical method to analyse drug combinations. This method is used to derive pairs of dose ‘a’ of drug A and dose ‘b’ of drug B that add to achieve a constant effect, which is then plotted as an isobologram. This method is often considered the gold standard for assessing drug combinations, but has certain limitations (discussed further in chapter 7, section 7.2.10), which made its use in this project challenging.

This project used the dose-effect data from individual drugs and the effect in question in this study is neuroprotection quantified by the intensity of PI fluorescence following injury. In this chapter, the Bliss Independence model founded by Bliss in 1939[238,237,236] is used to evaluate whether drugs in combination therapy are additive or synergistic. Additionally, the results obtained from the Bliss Independence model are compared with estimates made to construct isobolograms. The Bliss Independence model focuses on treatment effect enhancement and is one of the most frequently used reference models[239,235]. It is based on the principle that drugs elicit their effects independently and the expected combination effect is calculated based on the probability of the independent effects[240]. This is not the direct addition of effects which may be without meaning, if say for example dose A gives a 60% E_{max} effect and dose B gives a 70% E_{max} effect. The dose response data of each individual drug is used to determine the expected combination effect and this is calculated using the complete additivity of probability theory:

$$\textbf{Combination effect}_{ab,0} = x_a + x_b - x_a \cdot x_b$$

where, drug A provides x_a percent protection at dose a and drug B provides x_b percent protection at dose b.

This expected effect (termed the ‘independent’ or ‘additive’ effect) is then statistically compared with the observed drug combination effect to determine whether a departure from additivity, such as synergism or subadditivity has occurred. If the observed combination

effect is higher than the predicted effect, the doses in combination are said to be synergistic, whereas if the observed effect is less than the expected effect, the doses in combination are said to be subadditive. When the observed effect is equal to the predicted effect, this is suggestive of an additive effect in combination. These situations are summarised as:

$$\text{Combination effect}_{ab,O} \leftarrow \begin{cases} > \text{Combination effect}_{ab,P} & \text{Synergy} \\ = \text{Combination effect}_{ab,P} & \text{Additivity} \\ < \text{Combination effect}_{ab,P} & \text{Antagonism} \end{cases}$$

Overall, results of drug combination experiments could provide new insight into the efficacy of combination therapies to treat the highly complex and systemic nature of TBI, for which it is becoming increasingly clear that monotherapy approaches may not be the most effective[75,241,242].

4.3 Adapting the *in vitro* TBI model to administer drugs other than noble gases

In this study, progesterone and meloxicam were applied first, followed by xenon or argon where relevant. The experimental drugs were applied exogenously after trauma was inflicted on slices, to increase clinical relevance. This required a solution exchange immediately after the impacting procedure. Before beginning experiments with drug addition, it was necessary to test whether the solution exchange affected injury development in the slices (Figure 4.1).

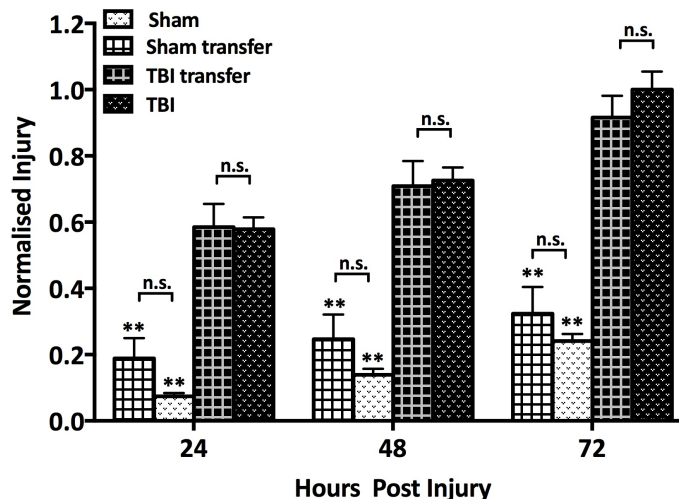


Figure 4.1: The transfer of wells and solution exchange after impact has no effect on development of traumatic injury over 72 hours. Sham injury in slices that were transferred (grey bars with black pattern) to a fresh 6-well plate containing pre-warmed experimental media is not significantly different to sham injury in slices that had no transfer (white bars with black pattern) at each time point. TBI injury in slices that were transferred (black bars with grey pattern) is also not significantly different to TBI injury in slices that had no transfer (black bars with white pattern) at each time point. ******($p < 0.001$) compared to TBI injury at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham: $n = 150$, TBI: $n = 117$ (from approximately 66 animals), sham transfer: $n = 37$ and TBI transfer: $n = 49$ (from approximately 24 animals).

A solution exchange *via* transfer of wells into a fresh 6-well plate with pre-incubated experimental media at 37°C had no effect on the development of injury. There was no significant difference between transfer and no transfer for sham injury at each time point ($p > 0.09$). There was also no significant difference between transfer and no transfer for TBI injury at each time point ($p > 0.9$).

The drugs were administered to slices dissolved in experimental media. Due to their limited aqueous solubility, this required the use of DMSO (see section 2.1.16). Control slices (sham or TBI + no treatment and TBI + noble gas for combination experiments) underwent a solution exchange and were treated with media containing DMSO.

4.4 Neuroprotective effect of xenon

The protective effect of xenon was studied over a range of different concentrations to determine the most appropriate concentrations for combination experiments. Xenon was administered as added pressure in addition to a mixture of 20%O₂/75%N₂/5%CO₂. Figure 4.2 to Figure 4.4 show the effect of 50%, 25% and 10% xenon on the development of injury. The

control slices in each case received the same pressure of helium (which is not known to have any pharmacological effect) to control for any effect of added pressure.

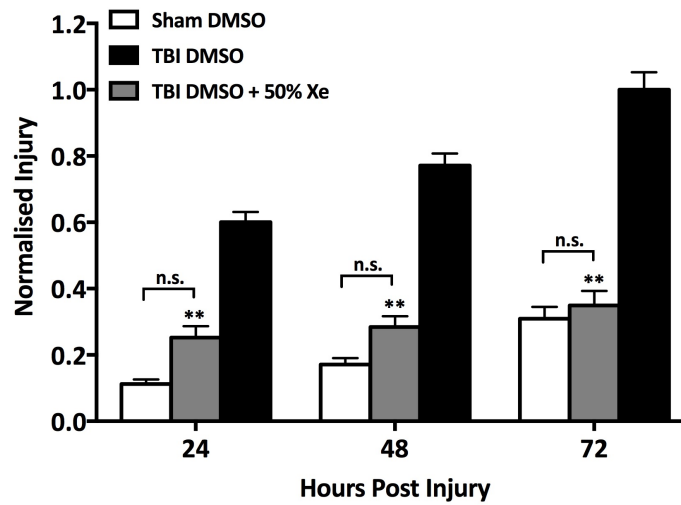


Figure 4.2: The effect of 50%atm xenon on the development of traumatic injury. At 24, 48 and 72 hours post injury, 50% xenon treated slices (grey bars) are not significantly different to uninjured sham slices (white bars). Sham and TBI slices were exposed to 50% helium to control for any effect of pressure. **($p < 0.001$) compared to TBI injury (black bars) at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 120$, TBI DMSO: $n = 102$ (from approximately 60 animals), TBI + 50% xenon: $n = 29$ (from approximately 30 animals).

Treatment with 50% xenon reduced the injury to 0.42 ± 0.03 at 24 hours, 0.36 ± 0.03 at 48 hours and 0.34 ± 0.04 at 72 hours, of the control injury at each time point (Figure 4.2). This was not significantly different ($p > 0.2$) to uninjured sham slices at all 3 time points.

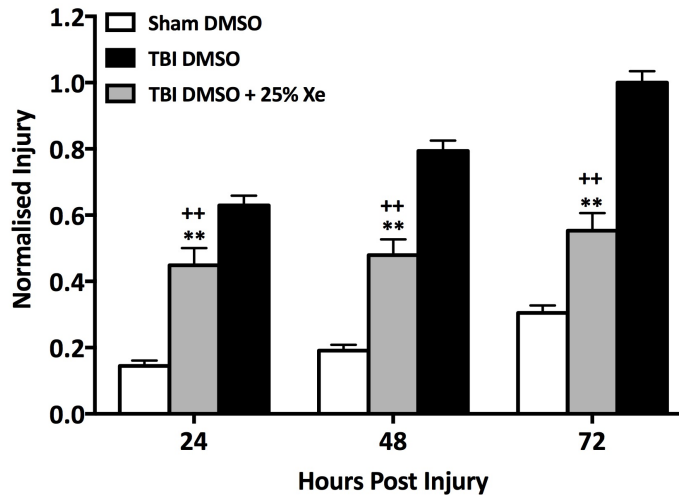


Figure 4.3: The effect of 25%atm xenon on the development of traumatic injury. At 24, 48 and 72 hours post injury, 25% xenon treated slices (light grey bars) had reduced levels of injury. Sham (white bars) and TBI (black bars) slices were exposed to 25% helium to control for any effect of pressure. Bars represent mean injury and error bars are SEM. **($p < 0.001$) and ++($p < 0.001$) compared to TBI injury and sham, respectively at each time point. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 95$, TBI DMSO: $n = 97$ (from approximately 60 animals), TBI + 25% xenon: $n = 31$ (from approximately 24 animals).

Treatment with 25% xenon reduced the injury to 0.70 ± 0.05 of untreated TBI at 24 hours, 0.60 ± 0.04 of untreated TBI at 48 hours and 0.55 ± 0.05 of untreated TBI at 72 hours (Figure 4.3), however this reduction was not down to the level of sham slices ($p < 0.01$). Therefore, treatment with 25% xenon attenuated the development of injury unlike treatment with 50% xenon, which abolished it. Next, the effect of an even lower concentration of xenon on injury development was tested.

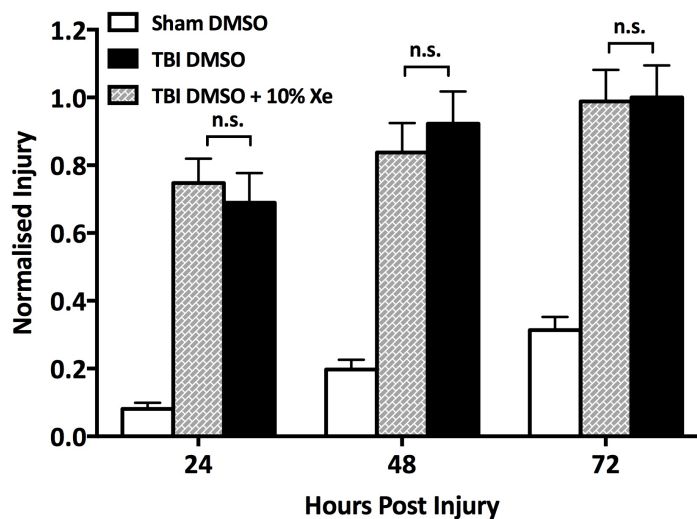


Figure 4.4: The effect of 10%atm xenon on the development of traumatic injury. At 24, 48 and 72 hours post injury, 10% xenon treated slices (grey patterned bars) were not significantly different to TBI injury (black bars). Sham (white bars) and TBI slices were exposed to 10% helium to control for any effect of pressure. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: n = 14, TBI DMSO: n = 22 and TBI + 10% xenon: n = 28 (from approximately 12 animals).

Figure 4.4 shows that treatment with 10% xenon was not protective at any time point ($p=0.99$).

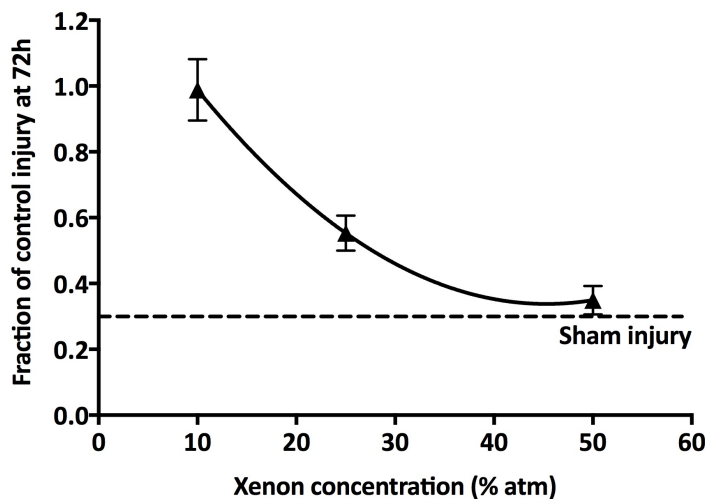


Figure 4.5: Concentration response relationship for neuroprotection by xenon at 72 hours following traumatic injury. 10% xenon was not protective (0.98 ± 0.09 of control injury). 25% and 50% xenon were protective, reducing the injury to 0.55 ± 0.05 and 0.34 ± 0.04 of the control injury, respectively. The dotted line indicates the level of injury of uninjured sham slices, which represents the baseline or maximum protection. A second order polynomial (quadratic) curve was fitted through the points. Error bars are SEM. TBI + 50% xenon: n = 29, TBI + 25% xenon: n = 31 and TBI + 10% xenon: n = 28.

The data in Figure 4.2 - Figure 4.4 are summarised in Figure 4.5, which shows the fraction of control injury at 72 hours post trauma when slices are treated with 10%, 25% and 50% xenon. The level of sham injury (dotted line) is 0.30 ± 0.03 of the TBI injury at 72 hours.

10% xenon exhibited no protective effect (0.98 ± 0.09 of control injury). 25% xenon attenuated injury development to 0.55 ± 0.05 of the control injury ($p < 0.001$) while 50% xenon abolished injury to 0.34 ± 0.04 of the control injury respectively ($p < 0.001$). Thus, xenon exhibited a concentration dependent effect across the range of concentrations tested.

4.5 Neuroprotective effect of argon

Similarly, the protective effect of 50%, 25% and 10%atm argon was investigated (Figure 4.6 - Figure 4.8).

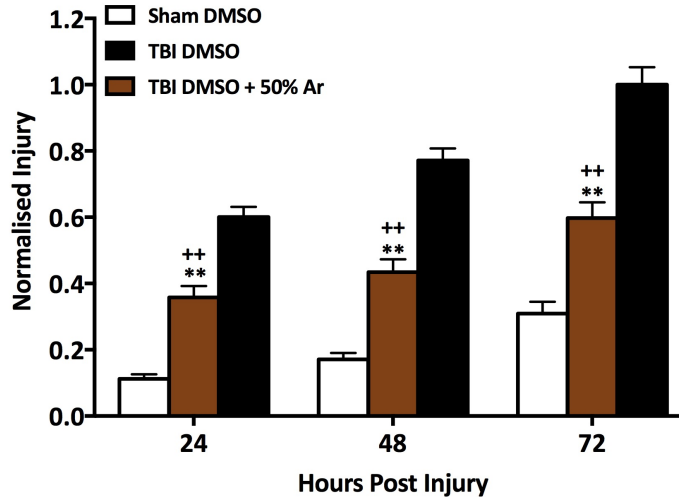


Figure 4.6: The effect of 50% atm argon on the development of traumatic injury. At 24, 48 and 72 hours post injury, 50% argon treated slices (brown bars) had reduced levels of injury. Sham (white bars) and TBI (black bars) slices were exposed to 50% helium to control for any effect of pressure. **($p < 0.001$) and ++($p < 0.001$) compared to TBI injury and sham slices, respectively at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 120$, TBI DMSO: $n = 102$: TBI DMSO (from approximately 60 animals) and TBI + 50% argon: $n = 62$ (from approximately 36 animals).

Figure 4.6 shows that treatment with 50% argon reduced the development of injury at each time point to 0.58 ± 0.03 at 24 hours, 0.56 ± 0.03 at 48 hours and 0.59 ± 0.05 at 72 hours, compared to control injury at each time point, however this reduction was not down to the level of sham injury ($p < 0.001$). The injury in 50% argon treated slices did not develop significantly between 24 and 48 hours ($p = 0.08$) but does between 48 and 72 hours ($p < 0.001$). Therefore, 50% argon attenuated, but does not abolish, the development of injury. Next, a lower concentration of argon was tested (Figure 4.7).

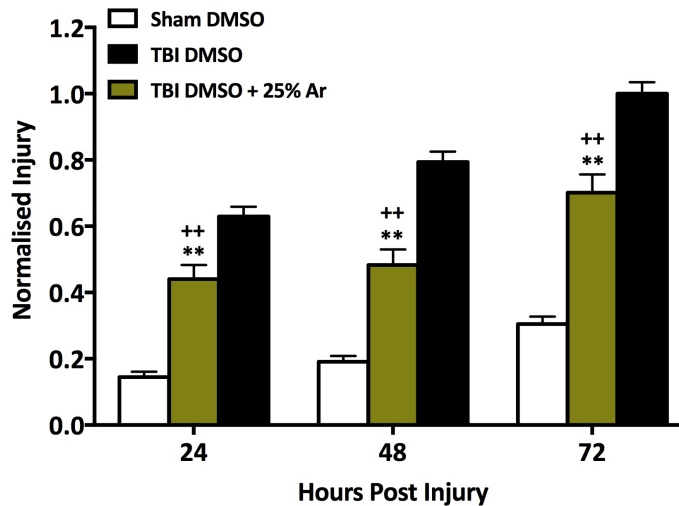


Figure 4.7: The effect of 25%atm argon on the development of traumatic injury. At 24, 48 and 72 hours post injury, 25% argon treated slices (green bars) had reduced levels of injury. Sham (white bars) and TBI (black bars) slices were exposed to 25% helium to control for any effect of pressure. **($p<0.001$) and ++($p<0.001$) compared to TBI injury and sham, respectively at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 95$, TBI DMSO: $n = 97$ (from approximately 60 animals) and TBI + 25% argon: $n = 61$ (from approximately 48 animals).

Treatment with 25% argon reduced the development of injury at each time point to 0.69 ± 0.04 at 24 hours, 0.60 ± 0.05 at 48 hours and 0.70 ± 0.05 at 72 hours of the control injury at each time point. However this reduction was not down to the level of sham injury ($p<0.001$) at any time point. The injury in 25% argon treated slices did not develop between 24 and 48 hours ($p=0.32$) but developed between 48 and 72 hours ($p<0.001$). Treatment with 25% argon also attenuated the development of injury but to a lesser extent than 50% argon.

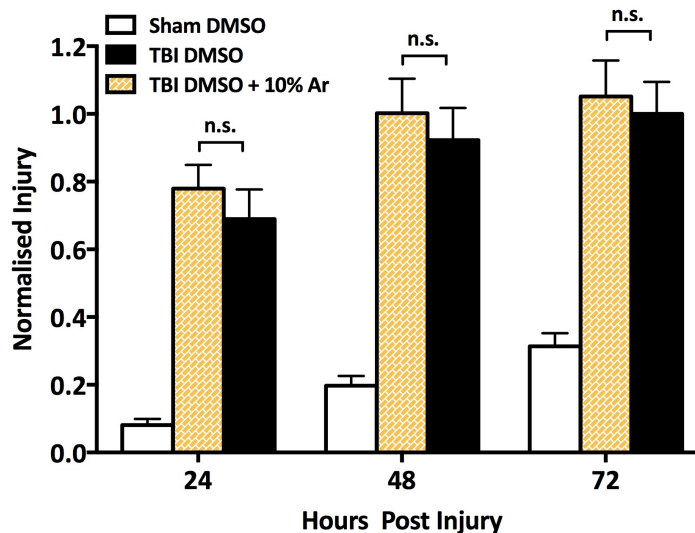


Figure 4.8: The effect of 10%atm argon on the development of traumatic injury. At 24, 48 and 72 hours post injury, 10% argon treated slices (orange patterned bars) were not significantly different to TBI injury (black bars). Sham (white bars) and TBI slices were exposed to 10% helium to control for any effect of pressure. Bars represent mean injury from a number of slices and error bars are standard errors. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: n = 14, TBI DMSO: n = 22 and TBI + 10% argon: n = 31 (from approximately 12 animals).

Finally, the effect of 10% argon on injury development was tested (Figure 4.8), which was not protective at any time point ($p=0.33$).

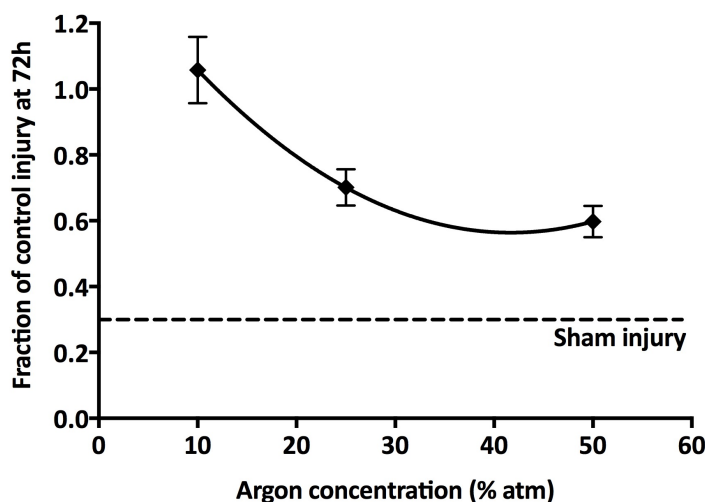


Figure 4.9: Concentration response relationship for neuroprotection by argon at 72 hours following traumatic injury. 10% argon (1.05 ± 0.10 of control injury) was not protective, but 25% and 50% argon were protective, reducing the injury to 0.70 ± 0.05 and 0.59 ± 0.04 of the control injury respectively. The dotted line indicates the level of injury of uninjured sham slices, which represents the baseline or maximum protection. A second order polynomial (quadratic) curve was fitted through the points. Error bars are standard errors and TBI + 50% argon: n = 62, TBI + 25% argon: n = 61 and TBI + 10% argon: n = 31.

The data in Figure 4.6 - Figure 4.8 are summarised in Figure 4.9, which shows the fraction of control injury at 72 hours post trauma when slices are treated with 10%, 25% and 50% argon. The level of sham injury, 0.30 ± 0.03 of the TBI injury at 72 hours, is shown by a dotted line.

10% argon (1.05 ± 0.1 of control injury) exhibited no protective effect but 25% and 50% argon were protective, reducing injury to 0.70 ± 0.05 and 0.59 ± 0.04 of the control injury at 72 hours respectively ($p < 0.001$). Thus, argon exhibited a concentration dependent effect across the range of concentrations tested.

4.6 Neuroprotective effect of progesterone

The effect of progesterone was tested over a range of concentrations to determine whether it is protective following injury in this model and whether its effects are concentration dependent. The concentrations used (3 μ M, 10 μ M and 25 μ M) were based on *in vitro* experiments in an OGD model, where a 25% reduction in cell death was observed following treatment with 10 μ M progesterone[141]. As 25% and 50% xenon and argon would be used in combination experiments with progesterone, the effect of progesterone alone was investigated under 25% and 50% helium in order to control for any possible effect that pressure alone might have on the efficacy of the drug (eg. due to an alteration of its binding properties).

4.6.1 Neuroprotective effect of progesterone at 50%atm partial pressure of helium

The effect of progesterone alone on sham slices was first tested. 3 μ M, 10 μ M and 25 μ M progesterone had no effect on uninjured sham slices which remained as healthy as untreated sham slices over the 72-hour time period (Figure 4.10).

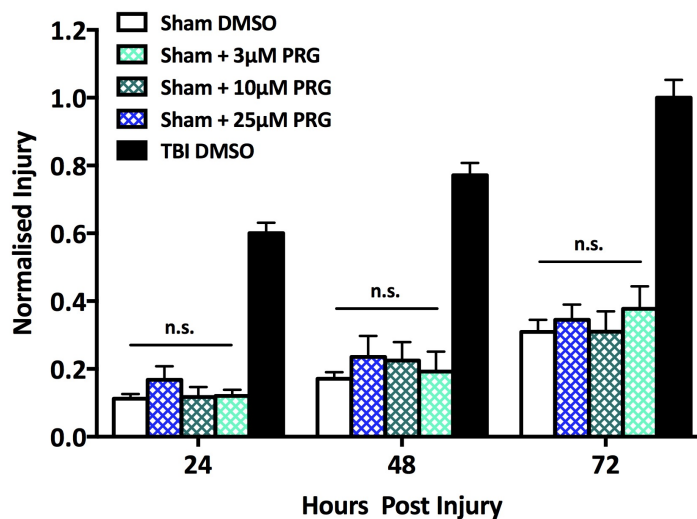


Figure 4.10: The effect of 3µM, 10µM and 25µM progesterone (PRG) on uninjured sham slices. Treatment with 25µM (blue hatched bars), 10µM (teal hatched bars) and 3µM (cyan hatched bars) progesterone had no effect on sham injury (white bars) at any time point. Bars represent mean injury from a number of slices and error bars are SEM. The data have been normalised to the TBI injury (black bars) at 72 hours. Sham DMSO: n = 120, TBI DMSO: n = 102 (from approximately 60 animals), sham + 25µM progesterone: n = 10, sham + 10µM progesterone: n = 16 and sham + 3µM progesterone: n = 5 (from approximately 6 animals).

As it was established that progesterone had no effect on sham injury, the different concentrations were then tested following TBI injury (Figure 4.11). Progesterone was neuroprotective at all three of the concentrations tested and that reduction in injury was dependent on the concentration of progesterone.

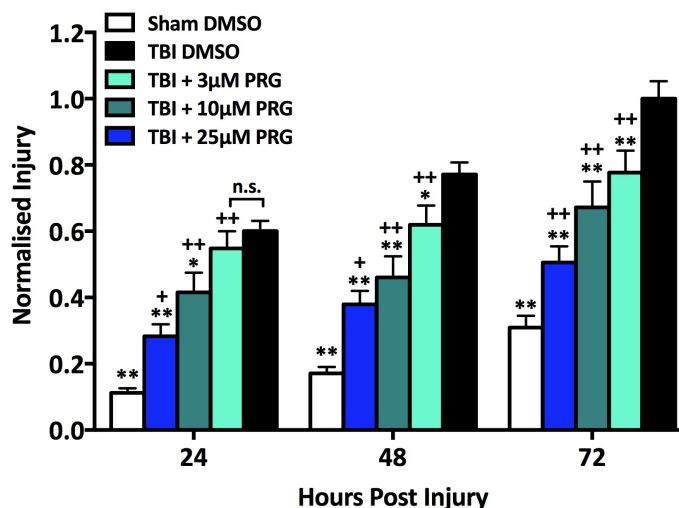


Figure 4.11: The effect of 3µM, 10µM and 25µM progesterone (PRG) on the development of traumatic injury at 50% helium pressure. At 24 hours, 3µM progesterone (cyan bars) was not protective, but at 48 and 72 hours it decreased the level of injury. Treatment with 25µM (blue bars) and 10µM (teal bars) progesterone reduced, but did not abolish injury at all three time points. * (p<0.05) and ** (p<0.001) compared to TBI (black bars). + (p<0.05) and ++ (p<0.001) compared to sham (white bars) injury at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: n = 120, TBI DMSO: n = 102 (from approximately 60 animals), TBI + 25µM progesterone: n = 45 (from approximately 30 animals), TBI + 10µM progesterone: n = 33 (from approximately 30 animals) and TBI + 3µM progesterone: n = 62 (from approximately 36 animals).

3 μ M progesterone is not protective at 24 hours ($p=0.65$), but is protective at 48 hours ($p=0.03$) and at 72 hours ($p<0.001$). 10 μ M progesterone is protective at 24 hours ($p=0.02$) and this effect is sustained at 48 hours and 72 hours ($p<0.001$). Similarly, 25 μ M progesterone is also protective at all three time points ($p<0.001$). In each case, the comparison and significance is made to the control TBI injury at the appropriate time point. None of the concentrations abolish injury to the level of the sham ($p>0.05$).

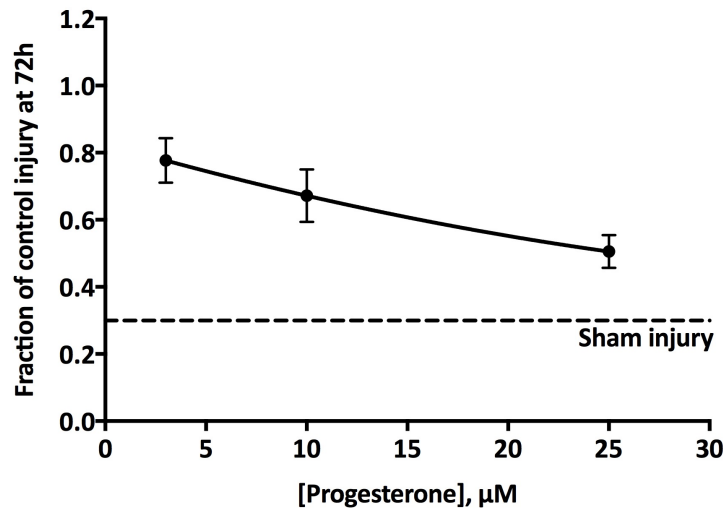


Figure 4.12: Concentration response relationship for progesterone (administered with 50% helium pressure) at 72 hours following traumatic injury. 3 μ M, 10 μ M and 25 μ M progesterone were protective, reducing injury to 0.77 ± 0.06 , 0.67 ± 0.07 and 0.50 ± 0.04 of the control injury respectively. The dotted line indicates the level of injury of uninjured sham slices, which represents the baseline or maximum protection. A second order polynomial (quadratic) curve was fitted through the points. Error bars are SEM. TBI + 25 μ M progesterone: $n = 45$, TBI + 10 μ M: $n = n = 33$ and TBI + 3 μ M progesterone: $n = 62$.

Figure 4.12 shows the fraction of control injury at 72 hours post trauma when slices are treated with 3 μ M, 10 μ M and 25 μ M progesterone. The level of sham injury (dotted line) was 0.30 ± 0.03 of the TBI injury at 72 hours. Progesterone exhibited a protective effect at a concentration of 3 μ M, 10 μ M and 25 μ M, reducing the injury to 0.77 ± 0.06 , 0.67 ± 0.07 and 0.50 ± 0.04 of the control injury respectively, at 72 hours ($p<0.001$). Progesterone exhibited a concentration dependent protective effect across the concentrations tested under 50% helium pressure.

4.6.2 Neuroprotective effect of progesterone at 25% atm partial pressure of helium

Figure 4.13 shows the effect of progesterone under 25% helium pressure.

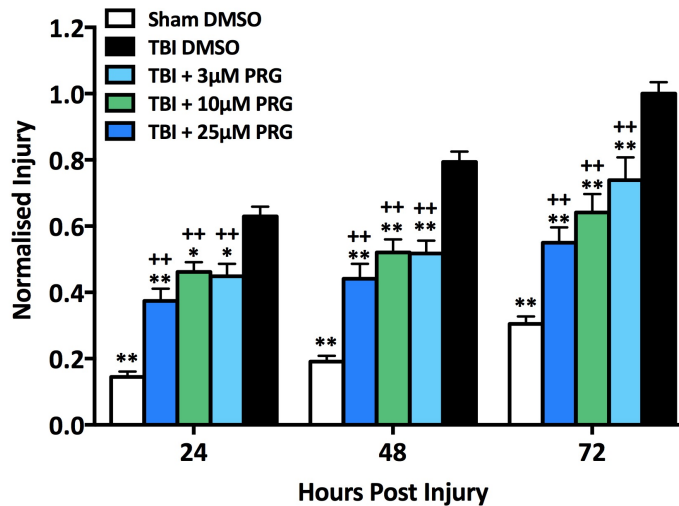


Figure 4.13: The effect of 3µM, 10µM and 25µM progesterone (PRG) on the development of traumatic injury at 25% helium pressure. At 24 hours, 48 hours and 72 hours post injury, treatment with 25µM (blue bars), 10µM (green bars) and 3µM (light blue bars) progesterone decreased but did not abolish injury. *($p < 0.05$) and **($p < 0.001$) compared to TBI (black bars). ++($p < 0.001$) compared to sham (white bars) injury at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 95$, TBI DMSO: $n = 97$ (from approximately 60 animals), TBI + 25µM progesterone: $n = 23$ (from approximately 30 animals), TBI + 10µM progesterone: $n = 40$ (from approximately 30 animals) and TBI + 3µM progesterone: $n = 26$ (from approximately 36 animals).

When applied with 25% helium pressure, 3µM progesterone was protective at 24 hours ($p = 0.04$), 48 hours ($p < 0.001$) and 72 hours ($p < 0.001$). 10µM progesterone was also protective at 24 hours ($p = 0.02$) and this effect was sustained at 48 hours and 72 hours ($p < 0.001$). Similarly, 25µM progesterone was protective at all three time points ($p < 0.001$). In each case, the comparison and significance is made to the control TBI injury at the appropriate time point. None of the concentrations abolished injury to the level of the sham ($p > 0.05$), similar to the effects at 50% helium pressure. As protection obtained under 25% and 50% helium pressure was similar, this indicates that there was no effect of applied pressure on progesterone's efficacy.

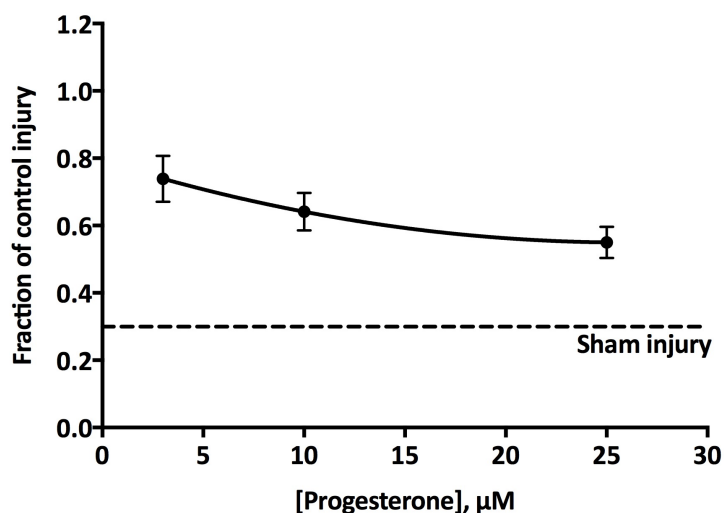


Figure 4.14: Concentration response relationship for progesterone (administered with 25% helium pressure) at 72 hours following traumatic injury. Progesterone exhibited a protective effect at a concentration of 3 μM , 10 μM and 25 μM , reducing the injury to 0.73 ± 0.06 , 0.64 ± 0.05 and 0.55 ± 0.04 of the control injury respectively. The dotted line indicates the level of injury of uninjured sham slices, which represents the baseline or maximum protection. A second order polynomial (quadratic) curve was fitted through the points. Error bars are SEM. TBI + 25 μM progesterone: $n = 23$, TBI + 10 μM progesterone: $n = 40$ and TBI + 3 μM progesterone: $n = 26$.

Figure 4.14 shows the fraction of control injury at 72 hours post trauma when slices are treated with 3 μM , 10 μM and 25 μM progesterone under 25% helium pressure. The level of sham injury (dotted line) was 0.30 ± 0.03 of the TBI injury at 72 hours. Progesterone exhibited a protective effect at a concentration of 3 μM , 10 μM and 25 μM , reducing the injury to 0.73 ± 0.06 , 0.64 ± 0.05 and 0.55 ± 0.04 of the control injury respectively ($p < 0.001$ compared to control). Thus, progesterone exhibited a concentration dependent protective effect across the range of concentrations tested at 25% helium pressure.

4.7 Combination treatments with noble gases and progesterone

Having tested the effect of progesterone, xenon and argon on injury development, the next step was to determine whether co-administration of progesterone and the noble gases xenon and argon is more protective than sub-maximal concentrations of noble gases administered alone and whether the combined protective effect is greater than the individual efficacies of the drugs. In order to determine if the interaction was synergistic or additive, different doses of progesterone, xenon and argon were tested together. For argon, the doses selected were 25% and 50%atm as argon at these concentrations reduced injury to 0.70 ± 0.05 and 0.59 ± 0.05 , but neither dose abolished the injury and were thus both sub-maximally effective. To compare the combined protective effect with that produced by progesterone and xenon, 25%

xenon was selected, which individually reduced injury to 0.55 ± 0.05 of the control injury at 72 hours. Although 50% xenon was maximally effective, reducing injury to 0.34 ± 0.04 of the control, experiments were carried out with progesterone and 50% xenon for completeness. Progesterone at concentrations of $3\mu\text{M}$, $10\mu\text{M}$ and $25\mu\text{M}$ were tested in combination with the gases. The results below are presented as the effect of progesterone in combination with 25% argon, 50% argon, 25% xenon and 50% xenon. The combined effect is shown compared to the effect of the gas alone, progesterone alone and the control sham and TBI injury. In each case, the injury (quoted in brackets) is calculated as a proportion of the control TBI injury at each time point. The comparison and significance is made to the particular condition (eg. TBI + Ar) at the appropriate time point.

4.7.1 Neuroprotection with co-administration of progesterone and 25% argon

25% argon was administered in combination with $3\mu\text{M}$, $10\mu\text{M}$ and $25\mu\text{M}$ progesterone. Figure 4.15 shows the effect of co-administering $3\mu\text{M}$ progesterone with 25% argon.

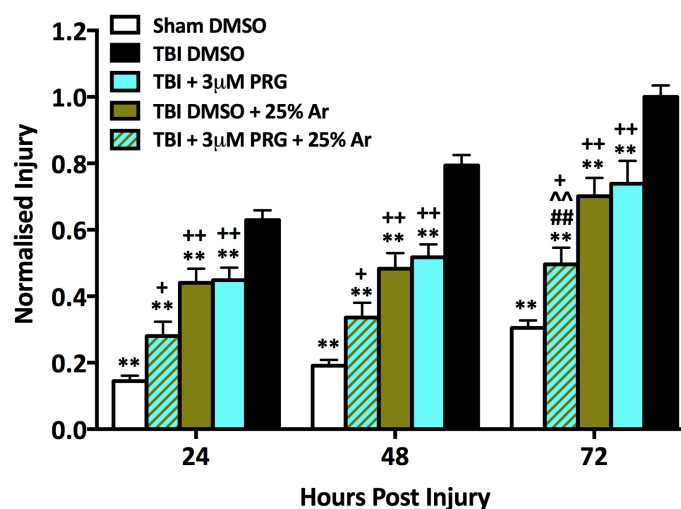


Figure 4.15: Neuroprotection with co-administration of $3\mu\text{M}$ progesterone (PRG) and 25% argon following a traumatic injury. At 24 hours, 48 hours and 72 hours post injury, treatment with a combination of $3\mu\text{M}$ progesterone and 25% argon (light blue striped bars) reduced injury development. ##($p < 0.001$) and ^^($p < 0.001$) compared to TBI + 25% Ar (green bars) and TBI + $3\mu\text{M}$ progesterone (light blue bars) respectively, at each time point. **($p < 0.001$) and ++($p < 0.001$) compared to control TBI (black bars) and sham (white bars) respectively, at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 95$, TBI DMSO: $n = 97$ (from approximately 60 animals), TBI + $3\mu\text{M}$ progesterone: $n = 26$ (from approximately 36 animals), TBI DMSO + 25% Ar: $n = 61$ (from approximately 48 animals) and TBI + $3\mu\text{M}$ progesterone + 25% Ar: $n = 50$ (from approximately 24 animals).

At 24 hours, the combination (0.45 ± 0.04) was not more protective than argon alone ($p > 0.05$). By 48 hours, the injury following treatment with the combination had not developed (0.42 ± 0.04) but was still not significantly different to argon acting alone

($p=0.06$). However, by 72 hours, the combination attenuated injury (0.49 ± 0.05) to a significantly lower level than progesterone ($p=0.006$) and argon acting alone ($p=0.003$). Although the combination of $3\mu\text{M}$ progesterone and 25% argon reduced injury at every time point, this reduction was not down to the level of sham injury ($p>0.05$ compared to sham at each time point). This suggests that progesterone in combination with 25% argon was able to attenuate the development of injury more effectively than either drug acting alone at 72 hours. Thus, the efficacy of 25% argon is increased when co-administered with a low dose of progesterone.

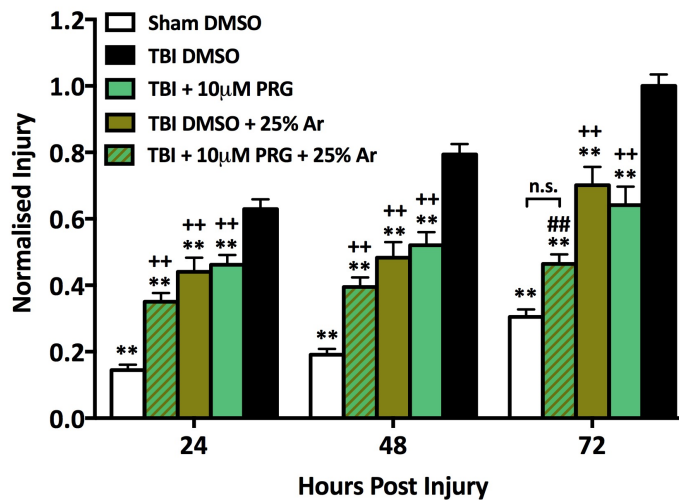


Figure 4.16: Neuroprotection with co-administration of $10\mu\text{M}$ progesterone (PRG) and 25% argon following a traumatic injury. At 24 hours, 48 hours and 72 hours post injury, treatment with a combination of $10\mu\text{M}$ progesterone and 25% argon (light green striped bars) reduced injury development. ##($p<0.001$) compared to TBI + 25% Ar (green bars) at each time point. **($p<0.001$) and ++($p<0.001$) compared to control TBI (black bars) and sham respectively, at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 95$, TBI DMSO: $n = 97$ (from approximately 60 animals), TBI + $10\mu\text{M}$ progesterone: $n = 40$ (from approximately 30 animals), TBI DMSO + 25% Ar: $n = 61$ (from approximately 48 animals) and TBI + $10\mu\text{M}$ progesterone + 25% Ar: $n = 27$ (from approximately 24 animals).

Next, a higher dose of progesterone, $10\mu\text{M}$ was tested in combination with 25% argon (Figure 4.16). At 24 hours, the combination (0.56 ± 0.02) was not significantly more protective than argon or progesterone alone ($p>0.5$). By 48 hours, the injury following treatment with the combination was similar (0.49 ± 0.02) but not significantly different to argon or progesterone acting alone ($p>0.5$). However, at 72 hours, protection achieved by the combination (0.46 ± 0.02) was higher than the protective effect of 25% argon alone ($p<0.001$). There was no significant difference between the sham injury and the combination effect ($p=1.0$) at 72 hours, which is indicative of a higher protective effect compared to a combination of $3\mu\text{M}$ progesterone and 25% argon.

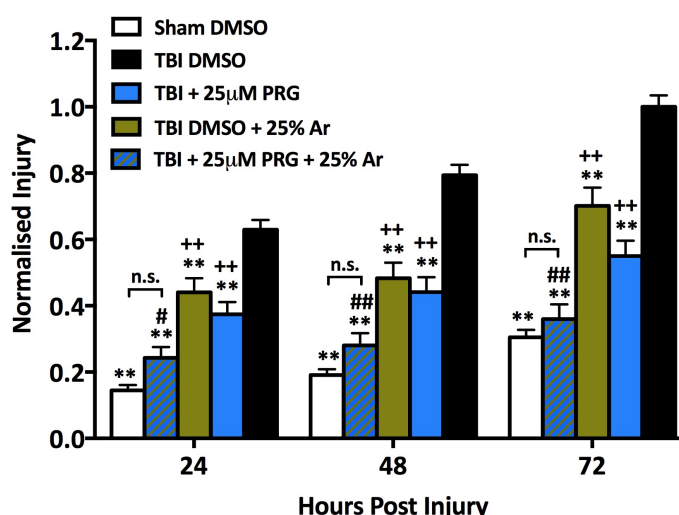


Figure 4.17: Neuroprotection with co-administration of 25µM progesterone (PRG) and 25% argon following a traumatic injury. At 24 hours, 48 hours and 72 hours post injury, treatment with a combination of 25µM progesterone and 25% argon (blue striped bars) abolished injury to the level of the sham (white bars). ##($p<0.001$) compared to TBI + 25% Ar (green bars) at each time point. **($p<0.001$) and ++($p<0.001$) compared to control TBI (black bars) and sham respectively, at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 95$, TBI DMSO: $n = 97$ (from approximately 60 animals), TBI + 25µM progesterone: $n = 23$ (from approximately 30 animals), TBI DMSO + 25% Ar: $n = 61$ (from approximately 48 animals) and TBI + 25µM progesterone + 25% Ar: $n = 33$ (from approximately 18 animals).

Treatment with a combination of 25µM progesterone and 25% argon abolished the development of injury at all 3 time points (Figure 4.17). At 24 hours, the combination (0.38 ± 0.03) was more protective than argon (0.69 ± 0.04) alone ($p=0.01$). By 48 hours, the injury following treatment with the combination had not developed significantly (0.35 ± 0.04) and was significantly lower than injury following treatment with 25% argon (0.60 ± 0.05) acting alone ($p=0.007$). Similarly at 72 hours, injury following treatment with combination had not developed any further (0.36 ± 0.04) and was significantly lower than the protective effect of 25% argon (0.30 ± 0.05) alone ($p<0.001$). There was no significant difference between the sham injury and the combination effect ($p=1.0$) at any time point. Thus, while treatment with 25% argon alone attenuates the development of injury, in combination with 25µM progesterone, it is able to abolish the development of injury.

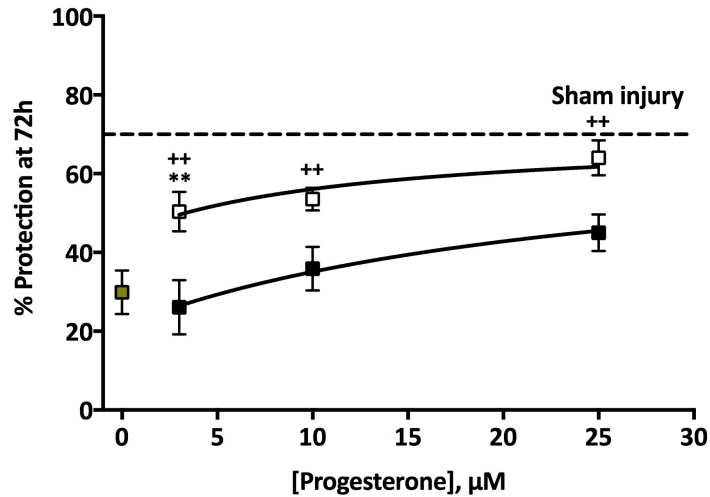


Figure 4.18: Percentage protection exhibited by progesterone (filled squares), 25% argon (green square) and progesterone + 25% Ar (open squares) at 72 hours following traumatic injury. **($p < 0.001$) compared to TBI + progesterone at the corresponding concentration. ++($p < 0.001$) compared to TBI + 25% argon. The dotted line indicates the level of injury of uninjured sham slices, which represents the maximum attainable protective effect at 72 hours. A 3-parameter agonist-response curve was fitted through the points. Error bars are SEM. TBI + 25µM progesterone: $n = 23$, TBI + 10µM progesterone: $n = 40$, TBI + 3µM progesterone: $n = 26$, TBI + 25% Ar: $n = 61$, TBI + 25µM progesterone + 25% Ar: $n = 33$, TBI + 10µM progesterone + 25% Ar: $n = 27$ and TBI + 3µM progesterone + 25% Ar: $n = 50$.

The data in Figure 4.15 - Figure 4.17 summarised in Figure 4.18, which shows the percentage protection at 72 hours post trauma when slices are treated with progesterone alone, 25% argon alone and progesterone administered in combination with 25% argon. Sham slices, which represent the maximum attainable level of protection at 72 hours ($69 \pm 4\%$) is shown by a dotted line. Progesterone's neuroprotective effect increased with concentration, from $22 \pm 7\%$ at 3µM to $33 \pm 8\%$ at 10µM and $49 \pm 5\%$ at 25µM. Neuroprotection by progesterone increased when it was co-administered with 25% argon, to $50 \pm 5\%$ at 3µM, $54 \pm 3\%$ at 10µM and $64 \pm 4\%$ at 25µM. At all 3 concentrations - 3µM ($p = 0.003$), 10µM and 25µM ($p < 0.001$) of progesterone, the combined protective effect was significantly higher than 25% argon alone, which exhibited $29 \pm 5\%$ protection. However, the combined protection of argon and progesterone at 72 hours was significantly different to protection with progesterone alone at 3µM ($p < 0.001$), but did not reach significance at 10µM ($p = 0.08$) and 25µM ($p = 0.09$). It may be concluded that the combined effect of 25% argon and lower doses of progesterone reduced injury more effectively than 25% argon at 72 hours, while the combination with 25µM progesterone abolished injury at 72 hours. To quantify the interaction between 25% argon and progesterone, the observed combination effect was compared to the predicted combination effect, which was calculated as described in section 4.2. At 3µM ($48 \pm 7\%$), 10µM ($55 \pm 5\%$) and 25µM ($61 \pm 4\%$) progesterone, the predicted combined protective effect (quoted in brackets) was equal within error to the observed effects. This suggests that 25%

argon in combination with 3 μ M, 10 μ M and 25 μ M progesterone has an additive protective effect at 72 hours.

4.7.2 Neuroprotection with co-administration of progesterone and 50% argon

Having established that there is an additional protective effect at 72 hours with co-administration of 25% argon with all of the concentrations of progesterone tested and that it was possible to abolish injury development at 72 hours by co-administration of 25 μ M progesterone and 25% argon, the next step was to test whether injury can be abolished with a higher dose of argon but lower doses of progesterone in combination and whether this is possible at earlier time points. 3 μ M, 10 μ M and 25 μ M progesterone were tested with 50% argon. Figure 4.19 shows the effect of co-administering 3 μ M progesterone and 50% argon.

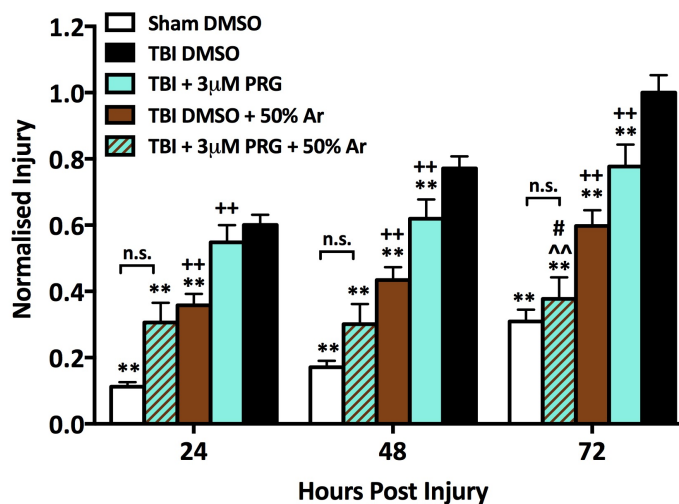


Figure 4.19: Neuroprotection with co-administration of 3 μ M progesterone (PRG) and 50% argon following a traumatic injury. At 24 hours, 48 hours and 72 hours post injury, treatment with a combination of 3 μ M progesterone and 50% argon (light blue striped bars) reduced injury development. #($p < 0.05$) and ^^($p < 0.001$) compared to TBI + 50% Ar (brown bars) and TBI + 3 μ M progesterone (light blue bars) respectively, at each time point. **($p < 0.001$) and ++($p < 0.001$) compared to control TBI (black bars) and sham (white bars) respectively, at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 120$, TBI DMSO: $n = 102$ (from approximately 60 animals), TBI + 3 μ M progesterone: $n = 62$ (from approximately 36 animals), TBI DMSO + 50% Ar: $n = 62$ (from approximately 36 animals) and TBI + 3 μ M progesterone + 50% Ar: $n = 25$ (from approximately 12 animals).

At 24 hours, the combination (0.51 ± 0.06) was not significantly more protective than argon alone ($p > 0.5$). By 48 hours, the injury following treatment with the combination had not developed any further (0.38 ± 0.06), but was not significantly different to argon ($p = 0.43$) alone. However, by 72 hours, protection obtained by the combination (0.37 ± 0.06) was higher than the protective effect of 50% argon (0.59 ± 0.05) alone ($p = 0.04$) and of 3 μ M progesterone alone ($p < 0.001$). There was no significant difference between the sham injury

and the combination effect ($p=0.25$) at any time point. At 72 hours, it may be concluded that the combination abolished injury. Therefore, even the lowest dose of progesterone tested, in combination with 50% argon, was effective at abolishing injury at the 72-hour time point. This indicates an increased potency for the combined effect of progesterone and 50% argon as opposed to argon acting alone.

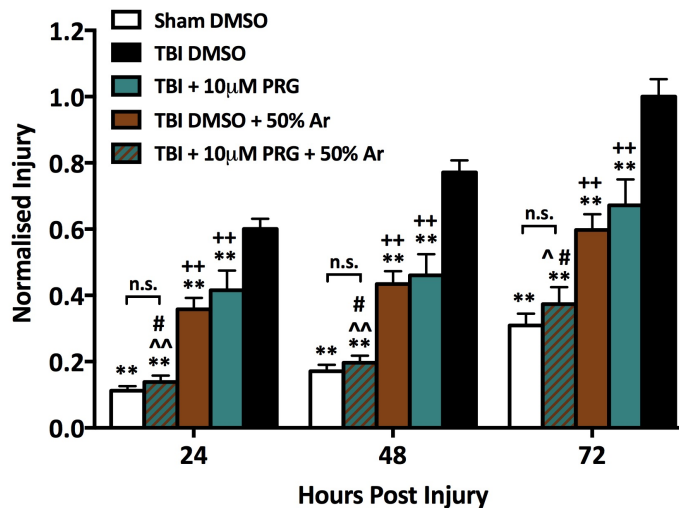


Figure 4.20: Neuroprotection with co-administration of 10µM progesterone (PRG) and 50% argon following a traumatic injury. At 24 hours, 48 hours and 72 hours post injury, treatment with a combination of 10µM progesterone and 50% argon (teal striped bars) abolished injury to the level of the sham (white bars). #($p<0.05$) compared to TBI + 50% Ar (brown bars) at each time point. ^($p<0.05$) and ^^($p<0.001$) compared to TBI + 10µM progesterone (teal bars) at each time point. **($p<0.001$) and ++($p<0.001$) compared to control TBI (black bars) and sham respectively, at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 120$, TBI DMSO: $n = 102$ (from approximately 60 animals), TBI + 10µM progesterone: $n = 33$ (from approximately 30 animals), TBI DMSO + 50% Ar: $n = 62$ (from approximately 36 animals) and TBI + 10µM progesterone + 50% Ar: $n = 25$ (from approximately 12 animals)

Treatment with a combination of 10µM progesterone and 50% argon abolished the development of injury at all 3 time points (Figure 4.20). At 24 hours, the combination (0.23 ± 0.02) was more protective than argon (0.58 ± 0.03) alone ($p=0.02$). This effect was consistent at 48 hours – slices treated with the combination had not developed further injury (0.25 ± 0.02) and protection was significantly higher than argon (0.56 ± 0.03) alone ($p=0.009$). By 72 hours, the protective effect of the combination (0.37 ± 0.05) was still higher than the protective effect of 50% argon (0.59 ± 0.05) alone ($p=0.01$). Additionally, the combined protective effect was also higher than 10µM progesterone acting alone at 24 hours ($p=0.007$), 48 hours ($p=0.003$) and 72 hours ($p=0.01$). There was no significant difference between the sham injury and the combination effect ($p=0.9$) at any time point, indicating that the combination, unlike either monotherapy, was able to abolish injury. Thus, increasing the

concentration of progesterone 3-fold in combination with 50% argon achieved substantially higher protection than the attenuation of injury seen with 50% argon alone.

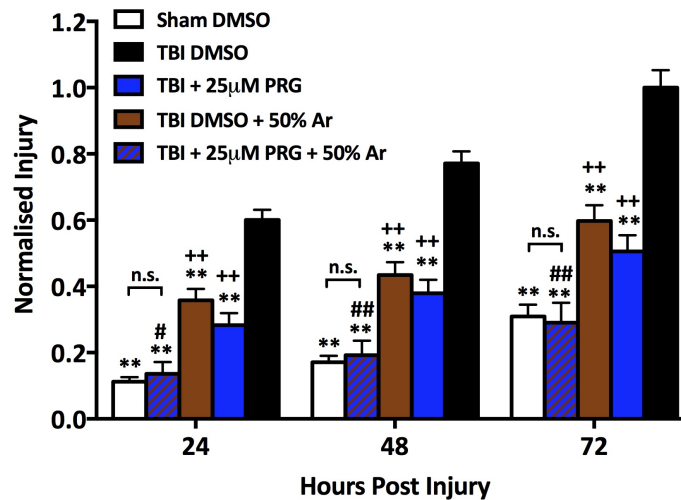


Figure 4.21: Neuroprotection with co-administration of 25µM progesterone (PRG) and 50% argon following a traumatic injury. At 24 hours, 48 hours and 72 hours post injury, treatment with a combination of 25µM progesterone and 50% argon (blue striped bars) abolished injury development to the level of sham injury (white bars). #($p < 0.05$) and ##($p < 0.001$) compared to TBI + 50% Ar (brown bars) at each time point. **($p < 0.001$) and ++($p < 0.001$) compared to control TBI (black bars) and sham respectively, at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 120$, TBI DMSO: $n = 102$ (from approximately 60 animals), TBI + 25µM progesterone: $n = 45$ (from approximately 30 animals), TBI DMSO + 50% Ar: $n = 62$ (from approximately 36 animals) and TBI + 25µM progesterone + 50% Ar: $n = 25$ (from approximately 12 animals).

To further characterise the increased protective effect with progesterone and 50% argon, a higher dose of progesterone in combination with argon was tested. Treatment with a combination of 25µM progesterone and 50% argon, unlike either of the monotherapies, abolished the development of injury at all 3 time points (Figure 4.21). Similar to a combination of 10µM progesterone and 50% argon, co-administering 25µM progesterone with 50% argon increased its efficacy from attenuating to abolishing injury development at all time points. At 24 hours, the combination (0.22 ± 0.04) was more protective than argon (0.58 ± 0.03) alone ($p = 0.03$). By 48 hours, the injury following treatment with the combination had not developed significantly (0.24 ± 0.04) and was still higher than argon (0.56 ± 0.03) acting alone ($p = 0.01$). Similarly at 72 hours, the increased protective effect by the combination (0.29 ± 0.06) was sustained compared to the protective effect of 50% argon (0.59 ± 0.05) alone ($p < 0.001$). There was no significant difference between the sham injury and the combination effect ($p = 1.0$) at any time point, indicating that the combination, 50% argon acting alone, was able to abolish injury.

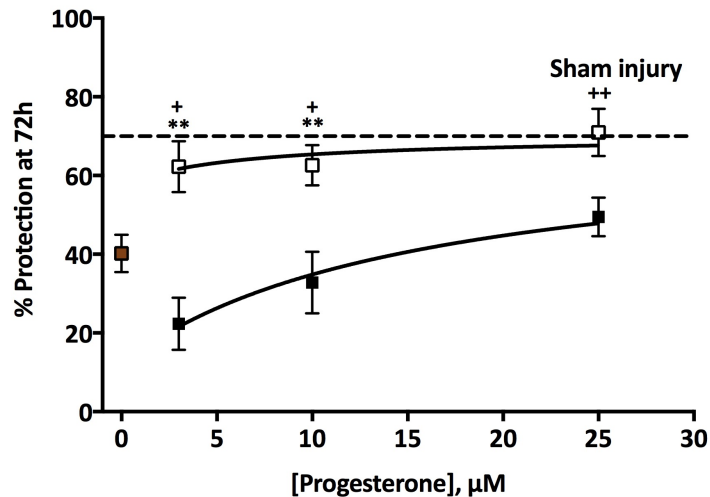


Figure 4.22: Percentage protection exhibited by progesterone (filled squares), 50% argon (brown square) and progesterone + 50% Ar (open squares) at 72 hours following traumatic injury. **($p<0.001$) compared to TBI + progesterone at the corresponding concentration. +($p<0.05$) and ++($p<0.001$) compared to TBI + 50% argon alone. The dotted line indicates the level of injury of uninjured sham slices, which represents the maximum attainable protective effect at 72 hours. A 3-parameter agonist-response curve was fitted through the points. Error bars are SEM. TBI + 25µM progesterone: $n = 45$, TBI + 10µM progesterone: $n = 33$, TBI + 3µM progesterone: $n = 62$, TBI + 50% Ar: $n = 62$, TBI + 25µM progesterone + 50% Ar: $n = 25$, TBI + 10µM progesterone + 50% Ar: $n = 33$ and TBI + 3µM progesterone + 50% Ar: $n = 25$.

The data in Figure 4.19 to Figure 4.21 are summarised in Figure 4.22, which shows the percentage protection at 72 hours post trauma when slices were treated with progesterone alone, 50% argon alone and progesterone administered in combination with 50% argon. Progesterone's neuroprotective effect increased with concentration, from $22 \pm 7\%$ at 3µM to $33 \pm 8\%$ at 10µM and $49 \pm 5\%$ at 25µM. 50% argon alone exhibited $40 \pm 5\%$ protection. Neuroprotection by progesterone was increased when it was co-administered with 50% argon, to $63 \pm 6\%$ at a dose of 3µM, $63 \pm 5\%$ at a dose of 10µM and $71 \pm 6\%$ at a dose of 25µM. The combined protection of argon and progesterone at 72 hours is significantly different to protection with progesterone acting alone at 3µM and 10µM doses ($p<0.001$), but did not reach significance ($p=0.06$) at 25µM. Similarly, adding 3µM ($p=0.04$), 10µM ($p=0.01$) and 25µM progesterone ($p<0.001$) to 50% argon was more effective at reducing injury than 50% argon alone at 72 hours. Thus, combining 50% argon with all concentrations of progesterone abolished injury at 72 hours and this was significantly different to the both the effect of 50% argon acting alone as well as to progesterone acting alone at lower concentrations. Whereas 25µM progesterone was required in combination with 25% argon to abolish injury, with 50% argon even the lowest dose tested - 3µM progesterone, abolished injury. To quantify the interaction between 50% argon and progesterone, the observed combination effect was compared to the predicted combination effect, as described previously. At 3µM progesterone, the predicted combined protective effect ($54 \pm 6\%$) was

less than the observed effect ($63 \pm 6\%$). Furthermore, in contrast to the parallel upwards shift in percentage protection seen at $10\mu\text{M}$ and $25\mu\text{M}$ progesterone concentrations in Figure 4.22, the shift at and $3\mu\text{M}$ progesterone is more than expected. This is indicative of a more than additive, i.e., synergistic effect by 50% argon and $3\mu\text{M}$ progesterone. At $10\mu\text{M}$ ($60 \pm 7\%$) and $25\mu\text{M}$ ($70 \pm 4\%$) progesterone, the predicted combined protective effect (quoted in brackets) was equal within error to the observed effects, indicative of additivity.

4.7.3 Neuroprotection with co-administration of progesterone and 25% xenon

Having established that the addition of different concentrations of progesterone is able to increase efficacy of argon, 25% xenon was tested in combination with $3\mu\text{M}$, $10\mu\text{M}$ and $25\mu\text{M}$ progesterone.

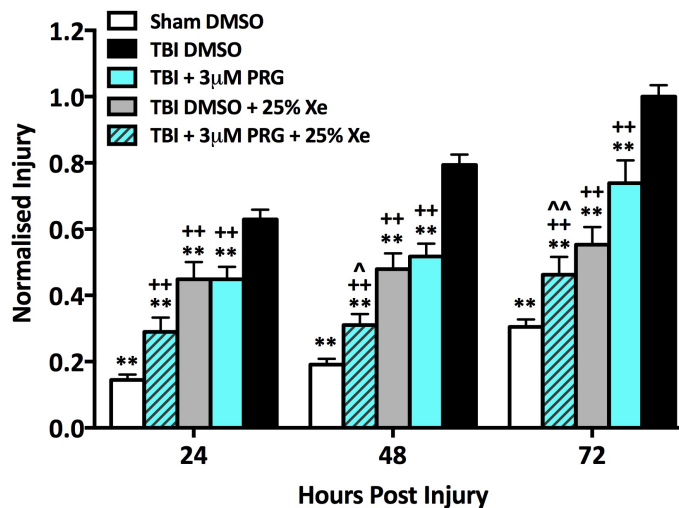


Figure 4.23: Neuroprotection with co-administration of $3\mu\text{M}$ progesterone (PRG) and 25% xenon following a traumatic injury. At 24, 48 and 72 hours post injury, treatment with a combination of $3\mu\text{M}$ progesterone and 25% xenon (blue and grey striped bars) reduces injury development. ^^($p < 0.001$) compared to TBI + $3\mu\text{M}$ progesterone (light blue bars) at each time point. **($p < 0.001$) and ++($p < 0.001$) compared to control TBI (black bars) and sham respectively, at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 95$, TBI DMSO: $n = 97$ (from approximately 60 animals), TBI + $3\mu\text{M}$ progesterone: $n = 26$ (from approximately 36 animals), TBI DMSO + 25% Xe: $n = 31$ (from approximately 24 animals) and TBI + $3\mu\text{M}$ progesterone + 25% Xe: $n = 31$ (from approximately 12 animals).

Treatment with a combination of $3\mu\text{M}$ progesterone and 25% xenon reduced but did not abolish the development of injury at all 3 time points (Figure 4.23). At 24 hours, the combination (0.45 ± 0.04) was not more protective than xenon ($p = 0.11$) alone. By 48 hours, the injury following treatment with the combination had not developed (0.39 ± 0.03) and was significantly different to progesterone alone ($p = 0.02$). Although the combination appeared more protective than xenon alone, this effect did not reach significance ($p = 0.08$). By 72 hours, injury following treatment with the combination (0.46 ± 0.05) had developed.

However, although the combination was more protective than progesterone alone ($p<0.001$), it was not significantly different to that of 25% xenon alone ($p=0.64$) and nor did it abolish injury to the level of the sham at ($p=0.02$). Thus, at no time point did co-administration of 3 μ M progesterone increase efficacy of 25% xenon.

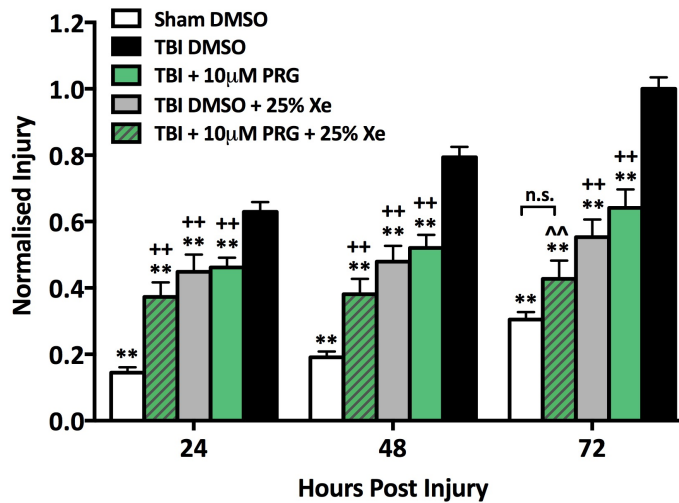


Figure 4.24: Neuroprotection with co-administration of 10 μ M progesterone (PRG) and 25% xenon following a traumatic injury. At 24, 48 and 72 hours post injury, treatment with a combination of 10 μ M progesterone and 25% xenon (green and grey striped bars) reduced injury development. ^^($p<0.001$) compared to TBI + 10 μ M progesterone (light green bars) at each time point. **($p<0.001$) and ++($p<0.001$) compared to control TBI (black bars) and sham (white bars) respectively, at each time point. Bars represent mean injury and error bars are standard errors. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 95$, TBI DMSO: $n = 97$ (from approximately 60 animals), TBI + 10 μ M progesterone: $n = 40$ (from approximately 30 animals), TBI DMSO + 25% Xe: $n = 31$ (from approximately 24 animals) and TBI + 10 μ M progesterone + 25% Xe: $n = 35$ (from approximately 12 animals).

The dose of progesterone was increased to 10 μ M in combination with 25% xenon to determine whether a higher dose is able to increase xenon's efficacy (Figure 4.24). Treatment with a combination of 10 μ M progesterone and 25% xenon reduced but did not abolish the development of injury at all 3 time points. At 24 hours, the combination (0.59 ± 0.04) was neither more protective than xenon ($p=0.78$) nor progesterone ($p=0.60$) alone. By 48 hours, the injury following treatment with the combination had not developed (0.48 ± 0.05) but was neither significantly different to xenon ($p=0.56$) nor progesterone ($p=0.15$) acting alone. By 72 hours, injury following treatment with the combination (0.43 ± 0.05) was higher than the protective effect of progesterone alone ($p=0.005$), but not significantly different to that of xenon alone ($p=0.31$). However, it can be concluded that the combination abolished injury to the level of the sham at 72 hours ($p=0.13$). Thus, similar to the combination 3 μ M progesterone and 25% xenon, the addition of 10 μ M progesterone was unable to increase efficacy of 25% xenon at any time point.

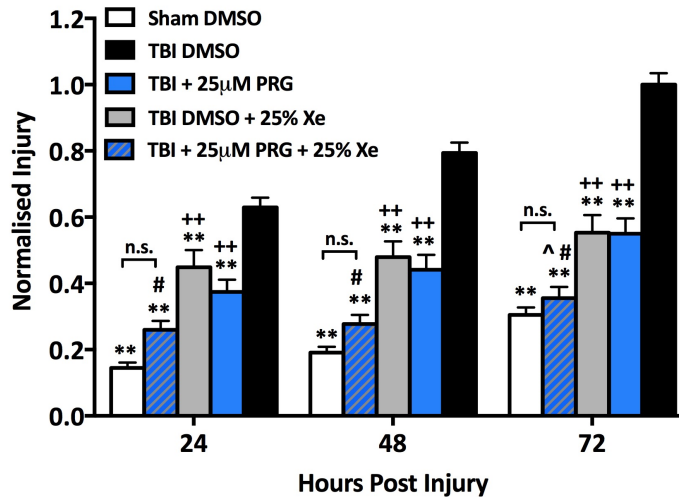


Figure 4.25: Neuroprotection with co-administration of 25µM progesterone (PRG) and 25% xenon following a traumatic injury. At 24, 48 and 72 hours post injury, treatment with a combination of 25µM progesterone and 25% xenon (blue and grey striped bars) abolished injury development to the level of sham slices (white bars). # ($p < 0.05$) compared to TBI + 25% Xe (grey bars) at each time point. ^ ($p < 0.05$) compared to TBI + 25µM progesterone (blue bars) at each time point. ** ($p < 0.001$) and ++ ($p < 0.001$) compared to control TBI (black bars) and sham respectively, at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 95$, TBI DMSO: $n = 97$ (from approximately 60 animals), TBI + 25µM progesterone: $n = 23$ (from approximately 60 animals), TBI DMSO + 25% Xe: $n = 31$ (from approximately 24 animals) and TBI + 25µM progesterone + 25% Xe: $n = 32$ (from approximately 12 animals).

Finally, the dose of progesterone was further increased in combination with 25% xenon. Treatment with a combination of 25µM progesterone and 25% xenon abolished the development of injury at all 3 time points (Figure 4.25). At 24 hours, the combination (0.40 ± 0.03) was more protective than xenon alone ($p = 0.02$). Although it appeared more protective than the effect of progesterone alone, this was not significant ($p = 0.44$). By 48 hours, the injury following treatment with the combination had not developed significantly (0.34 ± 0.03) and was significantly different to xenon acting alone ($p = 0.01$) but not to progesterone alone ($p = 0.11$). However, by 72 hours protection achieved by the combination (0.36 ± 0.03) was higher than the protective effect of 25% xenon ($p = 0.01$) and 25µM progesterone ($p = 0.03$) alone. There was no significant difference between the sham injury and the combination effect ($p = 0.34$) at any time point, indicating that the combination, unlike either monotherapy, was able to abolish injury. Thus, by increasing the concentration of progesterone to 25µM in combination with 25% xenon, it is possible to abolish injury at all time points. This shows that the combination effect is concentration dependent and that a sufficient increase in concentration of progesterone is able to increase efficacy of 25% xenon to the level of the sham.

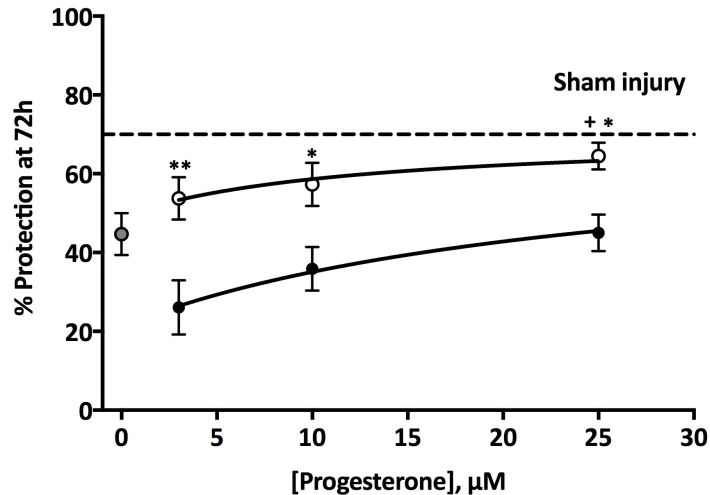


Figure 4.26: Percentage protection exhibited by progesterone (filled circles), 25% xenon (grey circle) and progesterone + 25% Xe (open circles) at 72 hours following traumatic injury. *($p < 0.05$) and **($p < 0.001$) compared to TBI + progesterone at the corresponding concentration. +($p < 0.05$) compared to TBI + 25% xenon. The dotted line indicates the level of injury of uninjured sham slices, which represents the maximum attainable protective effect at 72 hours. A 3-parameter agonist-response curve was fitted through the points. Error bars are SEM. TBI + 25µM progesterone: $n = 23$, TBI + 10µM progesterone: $n = 40$, TBI + 3µM progesterone: $n = 26$, TBI DMSO + 25% Xe: $n = 31$, TBI + 25µM progesterone + 25% Xe: $n = 32$, TBI + 10µM progesterone + 25% Xe: $n = 35$ and TBI + 3µM progesterone + 25% Xe: $n = 31$.

The data in Figure 4.23 - Figure 4.25 are summarised in Figure 4.26, which shows the percentage protection at 72 hours post trauma when slices are treated with progesterone alone and progesterone administered in combination with 25% xenon. Sham slices (dotted line) represent the maximum attainable level of protection at 72 hours ($69 \pm 4\%$). Progesterone's neuroprotective effect increased with concentration, from $26 \pm 7\%$ at 3µM to $36 \pm 6\%$ at 10µM and $45 \pm 5\%$ at 25µM. When co-administered with progesterone, the protective effect of 25% xenon increased from $44 \pm 5\%$ (acting alone) to $54 \pm 5\%$ at 3µM progesterone, $57 \pm 6\%$ at 10µM progesterone and $65 \pm 3\%$ at 25µM progesterone. The combined protective effect at 72 hours was significantly higher than protection with progesterone acting alone at 3µM ($p < 0.001$), 10µM ($p = 0.005$) and at 25µM ($p = 0.03$). Further, although co-administration of 3µM and 10µM progesterone did not increase protection achieved with 25% xenon, co-administration of 25µM progesterone increased the efficacy of 25% xenon ($p = 0.01$) to abolishing injury at 72 hours. Protection attained with the combination also approaches the maximum level of protection at 25µM progesterone, whereas in contrast, the protection offered by progesterone acting alone begins to peak at $45 \pm 5\%$ with 25µM. To quantify the interaction between 25% xenon and progesterone, the observed combination effect was compared to the predicted combination effect, as described previously. At 3µM ($59 \pm 6\%$), 10µM ($65 \pm 5\%$) and 25µM ($70 \pm 4\%$) progesterone, the predicted combined protective effect (quoted in brackets) was equal within error to the observed effects. Thus, 25% xenon in

combination with 3 μ M, 10 μ M and 25 μ M progesterone has an additive protective effect at 72 hours post trauma.

4.7.4 Neuroprotection with co-administration of progesterone and 50% xenon

For completeness, a set of experiments with 50% xenon and the 3 concentrations of progesterone were conducted. It was expected that the combination would abolish injury, consistent with the maximal effect of 50% xenon. Figure 4.27 shows the effect of administering 50% xenon in combination with 3 μ M progesterone.

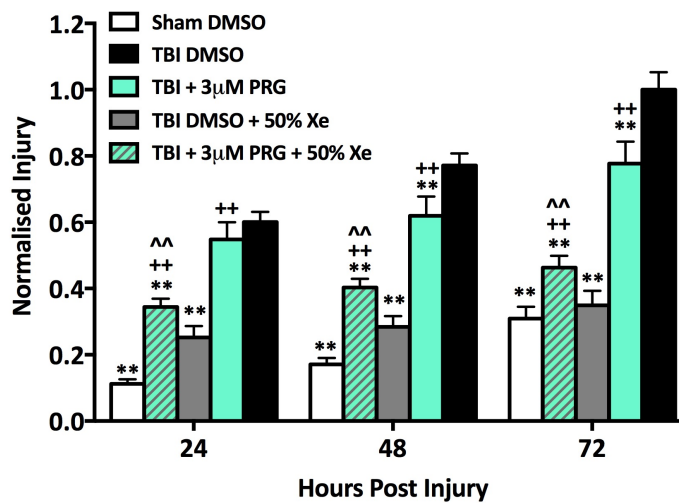


Figure 4.27: Neuroprotection with co-administration of 3 μ M progesterone (PRG) and 50% xenon following a traumatic injury. At 24, 48 and 72 hours post injury, treatment with a combination of 3 μ M progesterone and 50% xenon (cyan and grey striped bars) is more effective at reducing injury than treatment with 3 μ M progesterone alone. ^^($p < 0.001$) compared to TBI + 3 μ M progesterone (cyan bars) at each time point. *($p < 0.001$) and ++($p < 0.001$) compared to control TBI (black bars) and sham (white bars) respectively, at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 120$, TBI DMSO: $n = 102$ (from approximately 60 animals), TBI + 3 μ M progesterone: $n = 62$ (from approximately 36 animals), TBI DMSO + 50% Xe: $n = 29$ (from approximately 30 animals) and TBI + 3 μ M progesterone + 50% Xe: $n = 46$ (from approximately 12 animals).

3 μ M progesterone and 50% xenon did not abolish injury to the level of the sham slices at any time point ($p < 0.001$). At 24 hours, the combination (0.56 ± 0.03) was not more protective than xenon alone ($p = 0.79$) but was more protective than progesterone alone ($p = 0.02$). By 48 hours, the injury following treatment with the combination had not developed (0.51 ± 0.03) and was still not significantly different to xenon ($p = 0.59$) but was higher than progesterone ($p = 0.01$) acting alone. Similarly, by 72 hours protection obtained by the combination (0.46 ± 0.05) was not higher than the protective effect of 50% xenon ($p = 0.63$) but was higher than progesterone ($p < 0.001$) acting alone. Thus, at all 3 time points the combination achieved a

higher degree of protection than progesterone alone but not more than as xenon, although this trend was not significant ($p=0.58$).

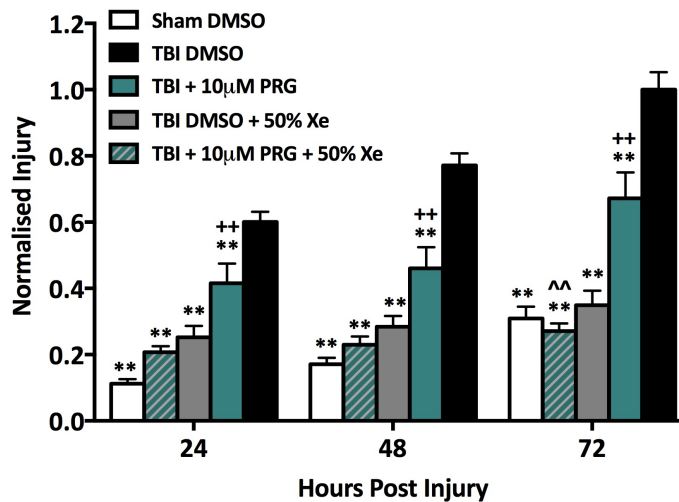


Figure 4.28: Neuroprotection with co-administration of 10µM progesterone (PRG) and 50% xenon following a traumatic injury. At 24, 48 and 72 hours post injury, treatment with a combination of 10µM progesterone and 50% xenon (teal and grey striped bars) abolished the development of injury to the level of sham injury (white bars), similar to xenon treated slices (grey bars). ^^($p<0.001$) compared to TBI + 10µM progesterone (teal bars) at each time point. ** ($p<0.001$) and ++ ($p<0.001$) compared to control TBI (black bars) and sham respectively, at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 120$, TBI DMSO: $n = 102$ (from approximately 60 animals), TBI + 10µM progesterone: $n = 33$ (from approximately 30 animals), TBI DMSO + 50% Xe: $n = 29$ (from approximately 12 animals) and TBI + 10µM progesterone + 50% Xe: $n = 17$ (from approximately 6 animals).

A similar trend was seen when testing 10µM progesterone and 50% xenon in combination (Figure 4.28). The development of injury at all time points was abolished and not significantly different to sham injury ($p=0.98$). At 24 hours, the combination (0.30 ± 0.02) was not more protective than xenon alone ($p=0.99$) or progesterone alone ($p=0.21$). By 48 hours, the injury following treatment with the combination had not developed (0.29 ± 0.02) and was still not significantly different to xenon ($p=0.98$) or progesterone ($p=0.13$) acting alone. Similarly, by 72 hours protection obtained by the combination (0.27 ± 0.05) was not higher than the protective effect of 50% xenon ($p=0.93$) but was higher than progesterone ($p<0.001$) acting alone. The lack of additional protection with 50% xenon and progesterone may be due to the highly potent effect of 50% xenon. In order to confirm this, the combination 25µM progesterone and 50% xenon was tested.

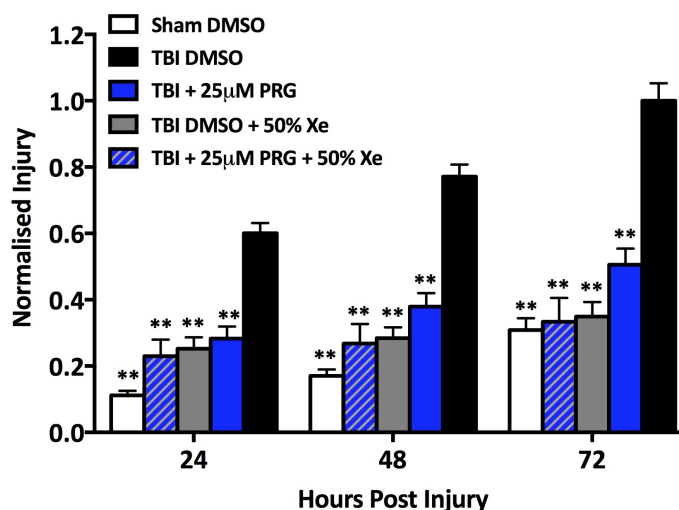


Figure 4.29: Neuroprotection with co-administration of 25µM progesterone (PRG) and 50% xenon following a traumatic injury. At 24, 48 and 72 hours post injury, treatment with a combination of 25µM progesterone and 50% xenon (blue and grey striped bars) abolished injury development to the level of sham injury (white bars), similar to xenon treated slices (grey bars). **($p < 0.001$) compared to TBI (black bars) injury at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 120$, TBI DMSO: $n = 102$ (from approximately 60 animals), TBI + 25µM progesterone: $n = 45$ (from approximately 30 animals), TBI DMSO + 50% Xe: $n = 29$ (from approximately 12 animals) and TBI + 25µM progesterone + 50% Xe: $n = 17$ (from approximately 6 animals).

As expected, treatment with a combination of 25µM progesterone and 50% xenon (Figure 4.29) abolished the development of injury at all 3 time points to the level of the sham ($p = 0.81$). At 24 hours, 48 hours and 72 hours, injury after treatment with the combination (0.38 ± 0.05 , 0.33 ± 0.05 and 0.33 ± 0.07 respectively) was also not significantly higher than progesterone acting alone ($p = 0.97$, $p = 0.74$ and $p = 0.33$ respectively at each time point). As expected, it could be concluded that 50% xenon alone was too potent to observe any further protective effect in combination with 25µM progesterone.

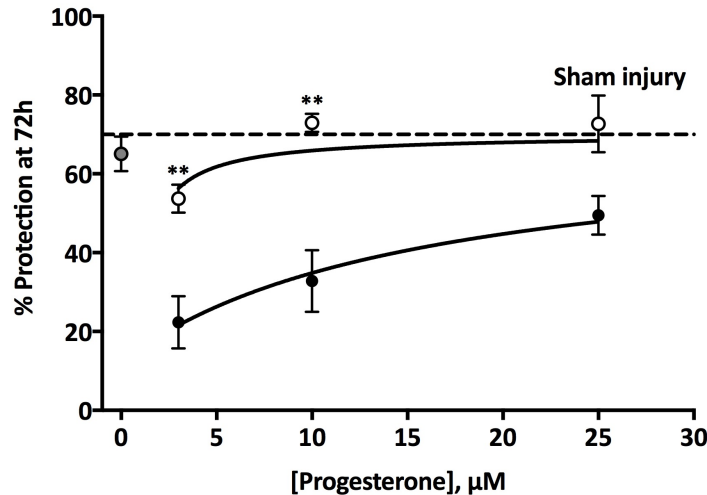


Figure 4.30: Percentage protection exhibited by progesterone (filled circles), 50% xenon (grey circle) and progesterone + 50% Xe (open circles) at 72 hours following traumatic injury. **($p < 0.001$) compared to TBI + progesterone at the corresponding concentration. The dotted line indicates the level of injury of uninjured sham slices, which represents the maximum attainable protective effect at 72 hours. A 3-parameter agonist-response curve was fitted through the points. Error bars are SEM. TBI + 25μM progesterone: $n = 45$, TBI + 10μM progesterone: $n = 33$, TBI + 3μM progesterone: $n = 62$, TBI DMSO + 50% Xe: $n = 29$, TBI + 25μM progesterone + 50% Xe: $n = 17$, TBI + 10μM progesterone + 50% Xe: $n = 17$ and TBI + 3μM progesterone + 50% Xe: $n = 46$.

The data in Figure 4.27 - Figure 4.29 are summarised in Figure 4.30, which shows the percentage protection at 72 hours post trauma when slices are treated with progesterone alone and progesterone administered in combination with 50% xenon. Sham slices, which represent the maximum attainable level of protection at 72 hours ($69 \pm 4\%$) is shown by a dotted line. Progesterone's neuroprotective effect increased with concentration, exhibiting $22 \pm 7\%$ protection at 3μM, $33 \pm 8\%$ protection at 10μM and $49 \pm 5\%$ protection at a dose of 25μM. At 72 hours, 50% xenon was $65 \pm 4\%$ protective when administered alone. Neuroprotection by progesterone was increased when it was co-administered with 50% xenon, to $54 \pm 4\%$ at a dose of 3μM, $72 \pm 2\%$ at a dose of 10μM and $67 \pm 7\%$ at a dose of 25μM. The combined protection of xenon and progesterone at 72 hours was significantly different to protection with progesterone acting alone at 3 and 10μM doses ($p < 0.001$), but did not reach significance ($p = 0.33$) at 25μM. Thus, the combined effect of 50% xenon and progesterone achieves higher levels of protection and that this is significantly different to the effect of progesterone acting alone at lower concentrations. The combination effect approached the maximum level of protection at 10μM progesterone, whereas in contrast, the protection offered by progesterone acting alone begins to peak at $49 \pm 5\%$ with 25μM. Moreover, as expected, the protection with the combination was also not significantly different to 50% xenon acting alone. To quantify the interaction between 50% xenon and progesterone, the observed combination effect was compared to the predicted combination effect, as described previously. In the case

of 50% xenon and different concentrations of progesterone, the predicted combination effect values calculated were higher than the maximum possible protection attainable (i.e., taking into account the sham injury, indicated by the dotted line in Figure 4.30). In these situations, the predicted combination effect was considered maximal, as the calculated values are less meaningful. At 3 μ M progesterone, the observed effect was less than maximal, which is indicative of a less than additive effect. However, at 10 μ M and 25 μ M progesterone, the predicted combined protective effect (maximum protection) was equal to the observed effects. Therefore, while 50% xenon may have a less than additive effect in combination with 3 μ M progesterone, it acts additively in combination with 10 μ M and 25 μ M progesterone at 72 hours after trauma.

4.8 Neuroprotective effect of meloxicam

After observing that the efficacy of sub-maximal noble gas treatments may be increased when they are co-administered with progesterone, the effect of meloxicam was tested. 0.1 μ M, 1 μ M, 10 μ M, 25 μ M and 50 μ M meloxicam were tested to determine whether meloxicam is protective following injury and whether its effects are concentration dependent. In an OGD model of injury in OHSC, Llorente *et al.* observed a 50% reduction in injury following treatment with 50 μ M meloxicam[192]. First, the effect of meloxicam on sham slices was first tested to ensure that the drug has no toxic effect on the uninjured slices (Figure 4.31).

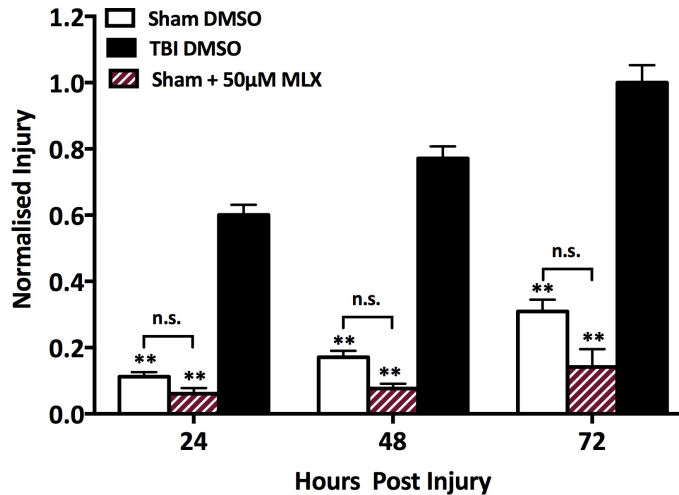


Figure 4.31: Treatment with 50µM meloxicam (MLX, maroon hatched bars) had no effect on sham injury (white bars) at 24, 48 and 72 hours post injury. **($p < 0.001$) compared to TBI (black bars) injury at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 120$, TBI DMSO: $n = 102$ (from approximately 60 animals), sham + 50µM meloxicam: $n = 5$ (from approximately 5 animals).

At the highest concentration tested (50µM), meloxicam had no effect on sham injury. Sham slices treated with meloxicam remained as healthy as untreated sham slices over the 72-hour time period. Thus, the different concentrations of meloxicam could then be tested following TBI injury.

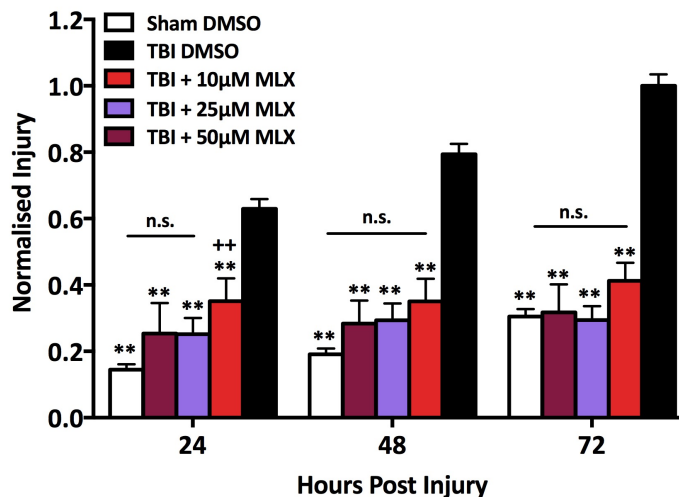


Figure 4.32: The effect of 10µM, 25µM and 50µM meloxicam (MLX) on traumatic injury development at 25% helium pressure. At 24, 48 and 72 hours post injury, treatment with 10µM (red bars), 25µM (purple bars) and 50µM (maroon bars) meloxicam abolished the development of injury, compared to sham (white bars). **($p < 0.001$) compared to TBI (black bars) injury and ++($p < 0.001$) compared to sham injury (white bars) at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 95$, TBI DMSO: $n = 97$ (from approximately 60 animals), TBI + 10µM meloxicam: $n = 12$ (from approximately 12 animals), TBI + 25µM meloxicam: $n = 13$ (from approximately 6 animals) and TBI + 50µM meloxicam: $n = 11$ (from approximately 12 animals).

10 μ M, 25 μ M and 50 μ M meloxicam were extremely neuroprotective in this TBI model (Figure 4.32). Treatment with 50 μ M abolished the development of injury at 24 (0.40 ± 0.09), 48 (0.35 ± 0.07) and 72 (0.31 ± 0.08) hours to the level of the sham ($p=0.58$). 25 μ M meloxicam had a similar effect and abolished the development of injury at 24 (0.40 ± 0.05), 48 (0.36 ± 0.05) and 72 (0.29 ± 0.04) hours. Although treatment with 10 μ M meloxicam did not abolish injury at 24 hours (0.56 ± 0.07 , $p=0.02$ compared to sham), by 48 (0.44 ± 0.07) and 72 (0.41 ± 0.05) it did ($p=0.10$ and $p=0.35$ respectively). Thus it was concluded that 10 μ M, 25 μ M and 50 μ M meloxicam are highly potent and concentrations 10 times lower were tested.

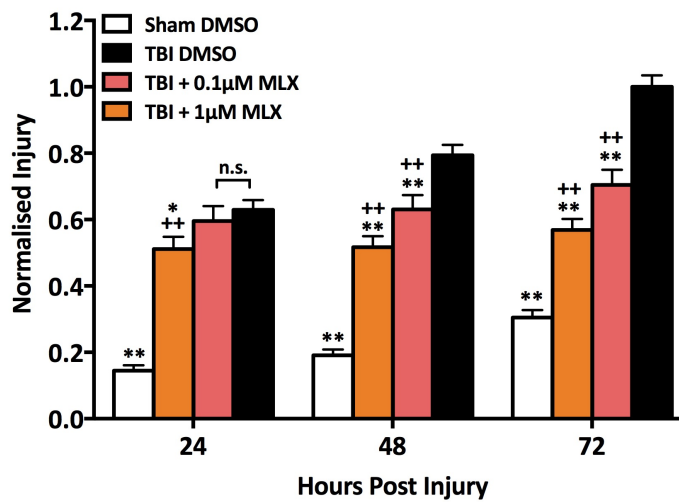


Figure 4.33: The effect of 0.1 μ M and 1 μ M meloxicam (MLX) on traumatic injury development at 25% helium pressure. At 24, 48 and 72 hours post injury, treatment with 0.1 μ M (pink bars) and 1 μ M (orange bars) reduced but did not abolish injury to the sham (white bars). **($p<0.001$) and ++($p<0.001$) compared to TBI (black bars) and sham respectively, at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 95$, TBI DMSO: $n = 97$ (from approximately 60 animals), TBI + 0.1 μ M meloxicam: $n = 57$ (from approximately 36 animals) and TBI + 1 μ M meloxicam: $n = 77$ (from approximately 48 animals).

Neither 0.1 μ M nor 1 μ M meloxicam abolished the injury to the level of sham injury (Figure 4.33). 0.1 μ M meloxicam was not protective at 24 hours (0.95 ± 0.04 , $p=0.75$ compared to sham) but exhibited a protective effect at 48 (0.79 ± 0.04) and 72 (0.70 ± 0.04) hours ($p<0.001$). Treatment with 1 μ M meloxicam was protective at 24 hours (0.82 ± 0.04 , $p=0.01$), 48 hours (0.64 ± 0.03) and 72 hours (0.56 ± 0.03) post injury ($p<0.001$).

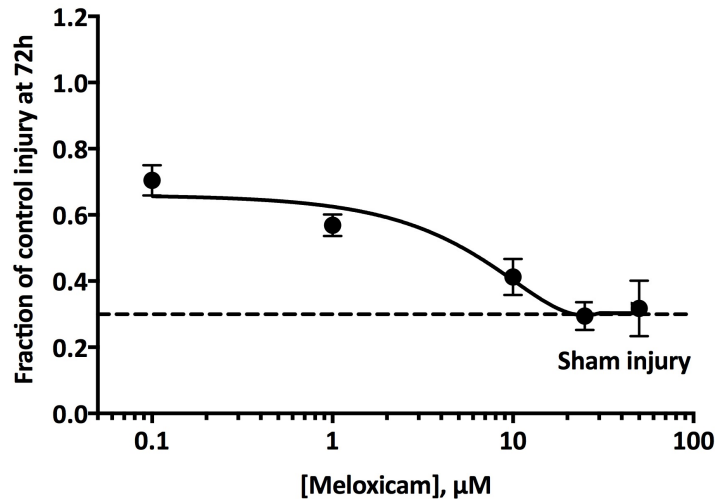


Figure 4.34: Concentration response relationship for neuroprotection by meloxicam (administered with 25% helium pressure) at 72 hours following traumatic injury. Meloxicam abolished injury at concentrations of 25 μM (0.29 ± 0.04) and 50 μM (0.31 ± 0.08) and exhibited a protective effect at concentrations of 0.1 μM , 1 μM and 10 μM , reducing the injury to 0.70 ± 0.04 , 0.56 ± 0.03 and 0.41 ± 0.05 of the control injury respectively. The dotted line indicates the level of injury of uninjured sham slices, below which it would not be possible to attain a protective effect. Error bars are SEM. TBI + 0.1 μM meloxicam: $n = 57$, TBI + 1 μM meloxicam: $n = 77$, TBI + 10 μM meloxicam: $n = 12$, TBI + 25 μM meloxicam: $n = 13$ and TBI + 50 μM meloxicam: $n = 11$.

The data in Figure 4.32 - Figure 4.33 are summarised in Figure 4.34, which shows the fraction of control injury at 72 hours post trauma when slices are treated with 0.1 μM , 1 μM , 10 μM , 25 μM and 50 μM meloxicam. Sham injury (dotted line) is 0.30 ± 0.03 of the TBI injury at 72 hours. Meloxicam abolished injury at concentrations of 25 μM (0.29 ± 0.04) and 50 μM (0.31 ± 0.08) and exhibited a protective effect at concentrations of 0.1 μM , 1 μM and 10 μM , reducing the injury to 0.70 ± 0.04 , 0.56 ± 0.03 and 0.41 ± 0.05 of the control injury respectively ($p < 0.001$ compared to control). Thus, meloxicam exhibits a concentration response relationship across the range of concentrations tested at 25% helium pressure. Meloxicam's protective effect approached a plateau at a concentration of 10 μM and abolished injury development completely at higher doses.

4.9 Combination treatments with meloxicam and noble gases

After establishing the effect of meloxicam on injury development, the next step was to determine whether co-administration of meloxicam and the noble gases is more protective than the monotherapies. Similar to combination experiments with progesterone, in order to study synergistic and additive properties of the combination, different doses of meloxicam, xenon and argon were tested together. As meloxicam appeared to be highly potent at

concentrations above 10 μ M, 0.1 μ M and 1 μ M doses, which reduced injury to 0.70 ± 0.04 and 0.56 ± 0.03 of the control respectively at 72 hours, were tested. For the same reason, 25% xenon and argon were used for combination experiments. The aim was to determine whether combining low doses of meloxicam with 25% noble gases would be more protective than noble gases and indeed low doses of meloxicam, alone. The results below are presented as the effect of meloxicam in combination with 25% argon, followed by 25% xenon. The combined effect is measured against the effects of the gas alone, meloxicam alone and the control sham and TBI injury. In each case, the injury (quoted in brackets) is calculated as a proportion of the control TBI injury at each time point. The comparison and significance is made to the particular condition (eg. TBI + Ar) at the appropriate time point.

4.9.1 Neuroprotection with co-administration of meloxicam and argon

The first combination tested was 0.1 μ M meloxicam and 25% argon.

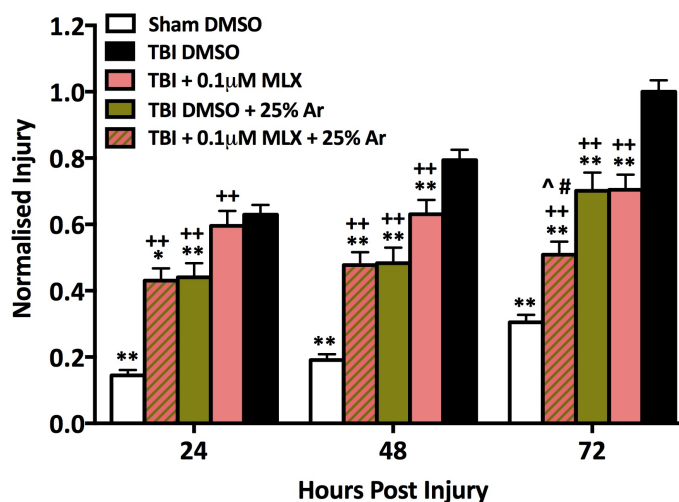


Figure 4.35: Neuroprotection with co-administration of 0.1 μ M meloxicam (MLX) and 25% argon following a traumatic injury. At 24, 48 and 72 hours post injury, treatment with a combination of 0.1 μ M meloxicam and 25% argon (pink and green striped bars) reduced injury development. #($p < 0.05$) compared to TBI DMSO + 25% Ar (green bars) at each time point. ^($p < 0.05$) compared to TBI + 0.1 μ M meloxicam (pink bars) at each time point. **($p < 0.001$) and +($p < 0.001$) compared to control TBI (black bars) and sham (white bars) respectively, at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 95$, TBI DMSO: $n = 97$ (from approximately 60 animals), TBI + 0.1 μ M meloxicam: $n = 57$ (from approximately 36 animals), TBI DMSO + 25% Ar: $n = 61$ (from approximately 48 animals) and TBI + 0.1 μ M meloxicam + 25% Ar: $n = 39$ (from approximately 18 animals).

Treatment with a combination of 0.1 μ M meloxicam and 25% argon reduced injury at all 3 time points (Figure 4.35). At 24 hours (0.69 ± 0.04) and 48 hours (0.59 ± 0.04), the combination was not more protective than 25% argon alone ($p = 1.0$). However, by 72 hours, injury following treatment with the combination (0.51 ± 0.04) had still not developed. The

protective effect of the combination was now higher than the protective effect of both meloxicam ($p=0.01$) and argon ($p=0.01$) alone. In addition, the combination did not abolish injury to the level of the sham ($p<0.001$).

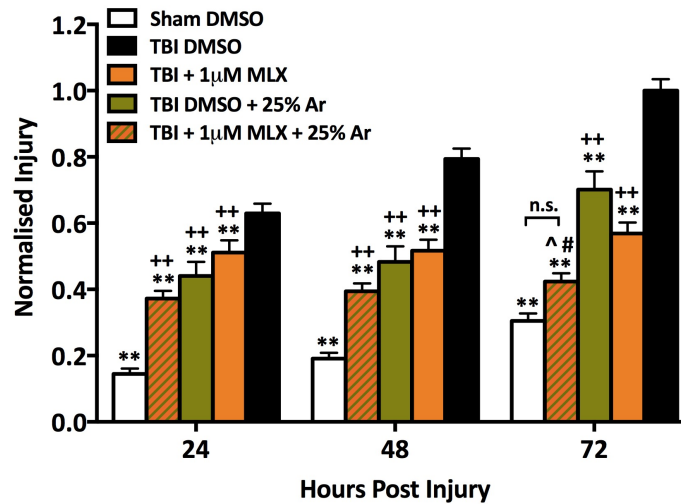


Figure 4.36: Neuroprotection with co-administration of 1µM meloxicam (MLX) and 25% argon following a traumatic injury. At 24, 48 and 72 hours post injury, treatment with a combination of 1µM meloxicam and 25% argon (orange and green striped bars) reduced injury development. #($p<0.05$) compared to TBI DMSO + 25% Ar (green bars) at each time point. ^($p<0.05$) compared to TBI + 1µM meloxicam (orange bars) at each time point. **($p<0.001$) and ++($p<0.001$) compared to control TBI (black bars) and to sham (white bars) respectively, at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 95$, TBI DMSO: $n = 97$ (from approximately 60 animals), TBI + 1µM meloxicam: $n = 77$ (from approximately 48 animals), TBI DMSO + 25% Ar: $n = 61$ (from approximately 48 animals) and TBI + 1µM meloxicam + 25% Ar: $n = 60$ (from approximately 18 animals).

Following the protective effect seen with 0.1µM meloxicam and 25% argon, the protective effect with 1µM and 25% argon was tested (Figure 4.36). 1µM meloxicam and 25% argon reduced injury at 24 and 48 hours but abolished the development of injury at 72 hours compared to the sham. At 24 hours (0.59 ± 0.02) and 48 hours (0.49 ± 0.02), the combination was not more protective than argon alone ($p>0.4$). However, by 72 hours, injury following treatment with the combination (0.42 ± 0.02) had still not developed. The protective effect of the combination was now higher than the protective effect of both meloxicam ($p=0.02$) and argon ($p=0.04$) alone. In addition, unlike treatment with 0.1µM and 25% argon, treatment with 1µM and 25% argon abolished injury to level of the sham ($p=0.07$).

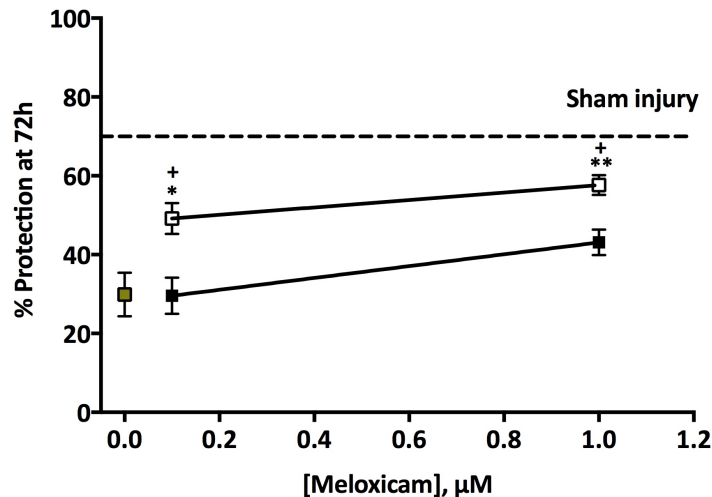


Figure 4.37: Percentage protection exhibited by meloxicam (filled squares), 25% argon (green square) and meloxicam + 25% Ar (open squares) at 72 hours following traumatic injury. *($p < 0.05$) and **($p < 0.001$) compared to TBI + meloxicam at the corresponding concentration. +($p < 0.05$) compared to TBI DMSO + 25% Ar. The dotted line indicates the level of injury of uninjured sham slices, which represents the maximum attainable protective effect at 72 hours. A line was fitted through the points. Error bars are SEM. TBI + 0.1µM meloxicam: $n = 57$, TBI + 1µM meloxicam: $n = 77$, TBI DMSO + 25% Ar: $n = 61$, TBI + 0.1µM meloxicam + 25% Ar: $n = 39$ and TBI + 1µM meloxicam + 25% Ar: $n = 33$.

The data in Figure 4.35 - Figure 4.36 are summarised in Figure 4.37, which shows the percentage protection at 72 hours post trauma when slices are treated with meloxicam alone, 25% argon alone and meloxicam administered in combination with 25% argon. Sham slices, which represent the maximum attainable level of protection at 72 hours ($69 \pm 4\%$) is shown by a dotted line. Meloxicam's neuroprotective effect increased with concentration, exhibiting $30 \pm 5\%$ protection at 0.1µM and $43 \pm 3\%$ protection at 1µM doses. 25% argon was $29 \pm 5\%$ protective when administered alone. When co-administered with 25% argon, the protective effect of meloxicam increased to $49 \pm 4\%$ at 0.1µM and $58 \pm 2\%$ at 1µM meloxicam. Therefore, the protective effect of meloxicam in combination with 25% argon increases with increasing concentrations of meloxicam. The combined protective effect at 72 hours was significantly different to protection with 25% argon acting alone at 0.1µM meloxicam ($p = 0.01$) and 1µM ($p = 0.04$). Thus, co-administration of low doses of meloxicam increased efficacy of 25% argon by 20% at 0.1µM meloxicam and by 30% to abolish injury at 1µM meloxicam. Further, the combined protective effect is also significantly different to meloxicam acting alone at 0.1µM ($p = 0.01$) and 1µM ($p = 0.002$) doses. Protection attained with the combination also begins to approach the maximum level of protection at a concentration of 1µM meloxicam, whereas in contrast, the protection offered by meloxicam acting alone approaches the maximum level of protection at a dose of 10µM. Therefore, the addition of 25% argon appears to make a dose that is 10 times lower as effective. Moreover,

to quantify the interaction between 25% argon and meloxicam, the observed combination effect was compared to the predicted combination effect, as described previously. At 0.1 μ M (51 $\pm$ 4%) and 1 μ M (60 $\pm$ 3%) meloxicam, the predicted combined protective effect (quoted in brackets) was equal within error to the observed effects. This suggests that 25% argon in combination with 0.1 μ M and 1 μ M meloxicam has an additive protective effect at 72 hours.

4.9.2 Neuroprotection with co-administration of meloxicam and xenon

Having observed that the combined protection with meloxicam and argon was higher than the protection obtained with either monotherapies at two concentrations of meloxicam, the next step was to observe whether there is a similar interaction between meloxicam and xenon.

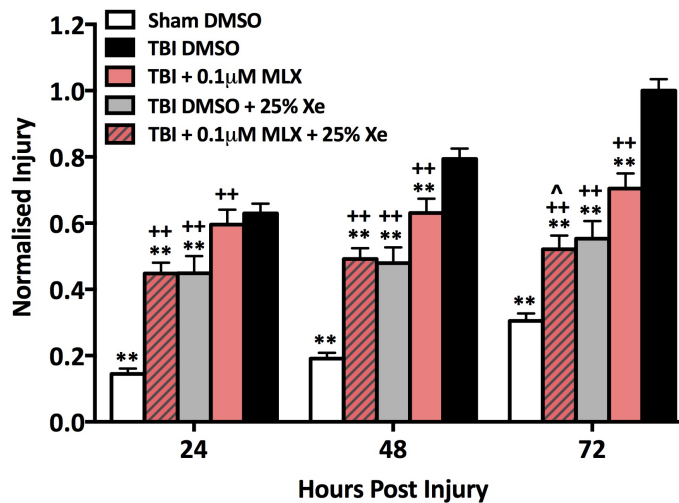


Figure 4.38: Neuroprotection with co-administration of 0.1 μ M meloxicam (MLX) and 25% xenon following a traumatic injury. At 24, 48 and 72 hours post injury, treatment with a combination of 0.1 μ M meloxicam and 25% xenon (pink and grey striped bars) reduced injury development. [^]($p < 0.05$) compared to TBI + 0.1 μ M meloxicam (pink bars) at each time point. ^{**}($p < 0.001$) and ⁺⁺($p < 0.001$) compared to control TBI (black bars) and sham (white bars) respectively, at each time point. Bars represent mean injury and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 95$, TBI DMSO: $n = 97$ (from approximately 60 animals), TBI + 0.1 μ M meloxicam: $n = 57$ (from approximately 36 animals), TBI DMSO + 25% Xe: $n = 31$ (from approximately 24 animals) and TBI + 0.1 μ M meloxicam + 25% Xe: $n = 30$ (from approximately 18 animals).

Figure 4.38 shows that treatment with a combination of 0.1 μ M meloxicam and 25% xenon reduced but did not abolish injury at all three time points ($p < 0.001$ compared to sham). At 24 hours (0.70 ± 0.03) and 48 hours (0.62 ± 0.03), the combination was not more protective than 25% xenon ($p = 1.0$) or meloxicam ($p > 0.11$) alone. However, by 72 hours, the protective effect of the combination (0.52 ± 0.04) was now higher than the protective effect of meloxicam ($p = 0.02$) but not xenon ($p = 0.99$) alone.

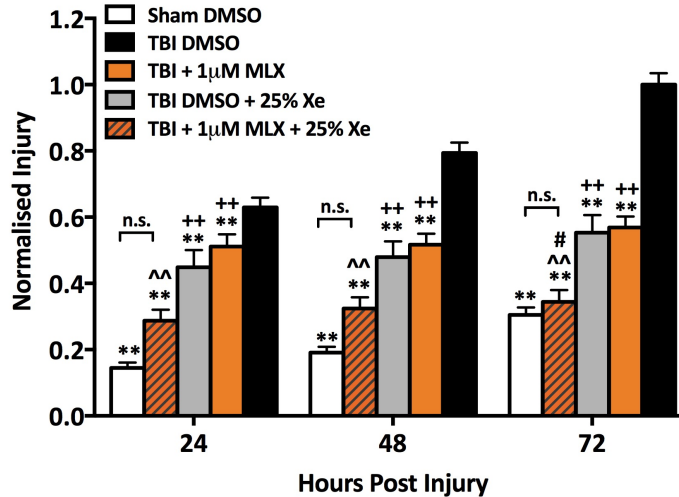


Figure 4.39: Neuroprotection with co-administration of 1µM meloxicam (MLX) and 25% xenon following a traumatic injury. At 24, 48 and 72 hours post injury, treatment with a combination of 1µM meloxicam and 25% xenon (orange and grey striped bars) abolished injury development to sham levels (white bars). #($p < 0.05$) compared to TBI DMSO + 25% Xe (grey bars) at each time point. ^^($p < 0.001$) compared to TBI + 0.1µM meloxicam (orange bars) at each time point. **($p < 0.001$) and ++($p < 0.001$) compared to control TBI (black bars) and sham respectively, at each time point. Bars represent mean injury from a number of slices and error bars are SEM. The data have been normalised to the TBI injury at 72 hours. Sham DMSO: $n = 95$, TBI DMSO: $n = 97$ (from approximately 60 animals), TBI + 1µM meloxicam: $n = 77$ (from approximately 48 animals), TBI DMSO + 25% Xe: $n = 31$ (from approximately 24 animals) and TBI + 1µM meloxicam + 25% Xe: $n = 33$ (from approximately 18 animals).

Next, the effect of 1µM meloxicam in combination with 25% xenon was tested (Figure 4.39), which abolished the level of injury at all three time points ($p = 0.2$, compared to sham). At 24 hours, the combination (0.45 ± 0.03) was more protective than meloxicam ($p < 0.001$) alone but not more protective than xenon alone ($p = 0.10$). By 48 hours, the injury following treatment with the combination had not developed (0.40 ± 0.03) and there was still no difference in protective effect between the combination and xenon ($p = 0.13$), but the combination was more protective than meloxicam ($p = 0.005$) alone. However, by 72 hours the injury following treatment with the combination had still not developed (0.34 ± 0.04) and the protective effect of the combination was now higher than the protective effect of both meloxicam ($p < 0.001$) and xenon ($p = 0.01$) alone.

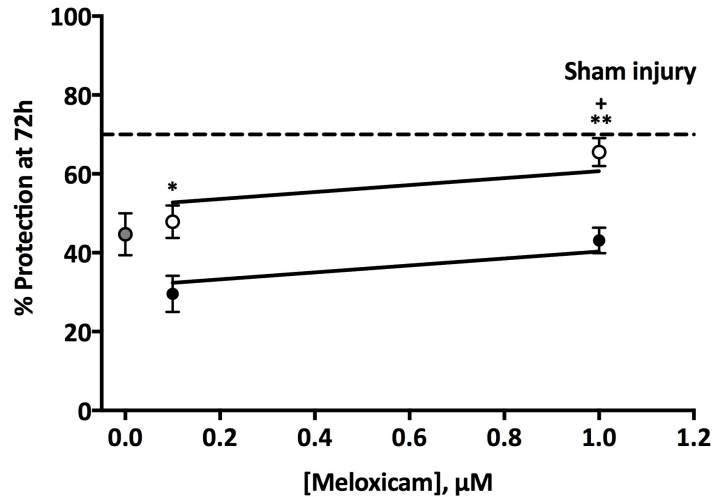


Figure 4.40: Percentage protection exhibited by meloxicam (filled circles), 25% xenon (grey circle) and meloxicam + 25% Xe (open squares) at 72 hours following traumatic injury. *($p < 0.05$) and **($p < 0.001$) compared to TBI + meloxicam at the corresponding concentration. +($p < 0.05$) compared to TBI DMSO + 25% Xe. The dotted line indicates the level of injury of uninjured sham slices, which represents the maximum attainable protective effect at 72 hours. A line was fitted through the points. Error bars are SEM. TBI + 0.1 μM meloxicam: $n = 57$, TBI + 1 μM meloxicam: $n = 77$, TBI + 0.1 μM meloxicam + 25% Xe: $n = 30$ and TBI + 1 μM meloxicam + 25% Xe: $n = 33$.

The data in Figure 4.38 - Figure 4.39 are summarised in Figure 4.40, which shows the percentage protection at 72 hours post trauma when slices are treated with meloxicam alone, 25% xenon alone and meloxicam administered in combination with 25% xenon. Sham slices (dotted line) represent the maximum attainable level of protection at 72 hours ($69 \pm 4\%$). Meloxicam's neuroprotective effect increased with concentration, exhibiting $30 \pm 5\%$ protection at 0.1 μM and $43 \pm 3\%$ protection at 1 μM . When co-administered with meloxicam, the protective effect of 25% xenon increased to $48 \pm 4\%$ at a dose of 0.1 μM meloxicam and $66 \pm 4\%$ at a dose of 1 μM meloxicam. The combined protective effect at 72 hours was significantly different to protection with meloxicam acting alone at 0.1 μM ($p = 0.02$) and 1 μM ($p < 0.001$) concentrations. By increasing the concentration of meloxicam in combination therapy with 25% xenon, it was possible to abolish injury at 72 hours, whereas 25% xenon alone provides only $44 \pm 5\%$ protection. The combination makes 25% xenon 30% more effective than the monotherapy. Further, similar to the combination effect with argon, protection attained with 25% xenon and meloxicam reached the maximum level of protection at 1 μM meloxicam, whereas in contrast, the protection offered by meloxicam acting alone approaches the maximum level of protection only at 10 μM . Therefore, the addition of 25% xenon appears to make a dose that is 10 times lower as effective. To quantify the interaction between 25% xenon and meloxicam, the observed combination effect was compared to the predicted combination effect, as described previously. At 0.1 μM meloxicam, the observed

effect was less than the predicted effect ($61 \pm 4\%$), which is indicative of a less than additive effect. However, at $1\mu\text{M}$ meloxicam, the predicted combined protective effect ($69 \pm 3\%$) was equal to the observed effects. Therefore, while 25% xenon may have a less than additive effect in combination with $0.1\mu\text{M}$ meloxicam, it acts additively in combination with $1\mu\text{M}$ meloxicam at 72 hours post trauma.

4.10 Isobolographic analysis of combination experiments

For the purpose of comparison to the Bliss independence method, isobolograms were constructed for each combination therapy tested (Figure 4.41) using the Loewe additivity approach described in section 4.2. Data were extrapolated from each concentration response curve to construct the isobolograms.

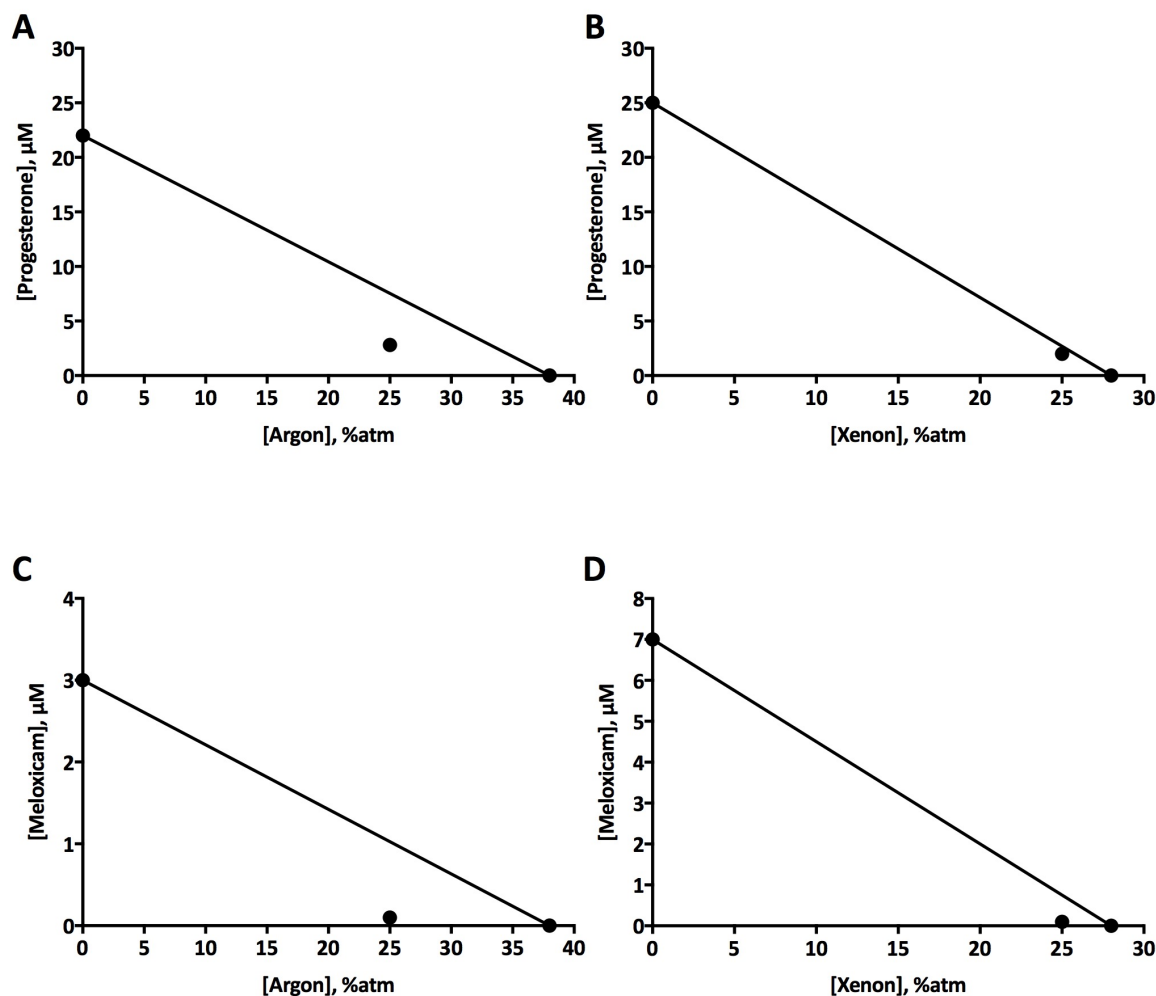


Figure 4.41: Isobolograms for the combinations (A) progesterone and argon, (B) progesterone and xenon, (C) meloxicam and argon and (D) meloxicam and xenon, the isoboles (lines of additivity) are constructed at the EC_{45} concentrations for combinations involving argon and at the EC_{50} concentrations for combinations involving xenon. The isoboles connect the pair of concentrations which have the same (specified) effect when acting as monotherapies. Where the combination effect (lone dot in each isobologram) is situated below the isobole, that combination is said to be synergistic while the presence of the combination effect above the isobole indicates sub-additivity.

The isobolograms shown in Figure 4.41 suggest that (A) progesterone and argon act synergistically, (B) progesterone and xenon are additive, (C) meloxicam and argon are synergistic and (D) meloxicam and xenon are additive though with a tendency towards synergy. While these isobolograms provide a holistic overview of the interaction between drugs in combination, it is not possible to conclude whether each dose combination tested is additive or synergistic. For example, Figure 4.41A shows that progesterone and argon act synergistically, however when the Bliss independence method is additionally considered, not all combinations involving progesterone and argon act synergistically – in fact, only one combination (3μM progesterone + 50% argon) is proposed to act synergistically while the rest are proposed to act in an additive manner. Similarly, Figure 4.41C shows that argon and

meloxicam act synergistically. However, when Bliss independence values are considered, all combinations of argon and meloxicam are proposed to act in an additive manner, though the estimates are extremely close. The results of the Bliss independence method are in agreement with the isobolograms for progesterone and xenon (Figure 4.41B) and for progesterone and meloxicam (Figure 4.41D) with regard to the additive effects seen however, in each situation there is one combination (3 μ M progesterone + 50% xenon and 0.1 μ M meloxicam + 25% xenon) that acts in a sub-additive manner. Thus, the isobolograms presented above are useful in providing an overview of interactions between the drug combinations – for example, that argon is proposed to act synergistically with both progesterone and meloxicam warrants further investigation. However, for a more detailed breakdown of which particular combinations of concentration ratios are synergistic or additive, the Bliss independence method is a more informative approach.

4.11 Summary of combination experiments

Table 4.1 shows a summary of the combination experiments alongside conclusions from both the Bliss independence and Loewe additivity method of assessing drug interaction.

Table 4.1: Table showing summary of combination experiments; Bliss predicted effect shown in brackets

Combination therapy		Effect (%) protection)	Drug interaction	
Noble gas (atm)	Drug (μ M)		Loewe additivity	Bliss independence
25% argon	3 μ M progesterone	50 $\pm$ 5%	Synergistic	Additive (48 $\pm$ 7%)
25% argon	10 μ M progesterone	54 $\pm$ 3%		Additive (55 $\pm$ 5%)
25% argon	25 μ M progesterone	64 $\pm$ 4%		Additive (61 $\pm$ 4%)
50% argon	3 μ M progesterone	63 $\pm$ 6%		Synergistic (54 $\pm$ 6%)
50% argon	10 μ M progesterone	63 $\pm$ 5%		Additive (60 $\pm$ 7%)
50% argon	25 μ M progesterone	71 $\pm$ 6%		Additive (70 $\pm$ 4%)
25% xenon	3 μ M progesterone	54 $\pm$ 5%	Additive	Additive (59 $\pm$ 6%)
25% xenon	10 μ M progesterone	57 $\pm$ 6%		Additive (65 $\pm$ 5%)
25% xenon	25 μ M progesterone	65 $\pm$ 3%		Additive (70 $\pm$ 4%)
50% xenon	3 μ M progesterone	54 $\pm$ 4%		Sub-additive (abolish)
50% xenon	10 μ M progesterone	72 $\pm$ 2%		Additive (abolish)
50% xenon	25 μ M progesterone	67 $\pm$ 7%		Additive (abolish)
25% argon	0.1 μ M meloxicam	49 $\pm$ 4%	Synergistic	Additive (51 $\pm$ 4%)
25% argon	1 μ M meloxicam	58 $\pm$ 2%		Additive (60 $\pm$ 3%)
25% xenon	0.1 μ M meloxicam	48 $\pm$ 4%	Additive	Sub-additive (61 $\pm$ 4%)
25% xenon	1 μ M meloxicam	66 $\pm$ 4%		Additive (69 $\pm$ 3%)

5 Chapter 5: Development of an *in vivo* model of traumatic brain injury to quantify contusion volume and profile cytokine expression

5.1 Overview and purpose of research

This chapter describes experiments conducted to set up and calibrate an *in vivo* model of TBI using the controlled cortical impact (CCI) injury. The CCI model is recognised for its ability to reproduce many pathological elements representative of clinical blunt-traumatic brain injury, including contusion, cortical tissue loss, acute subdural haematoma, axonal injury, concussion, blood brain barrier disruption, hemorrhage and even coma[76,78,211]. The contusion volume at 24 hours after injury was the outcome chosen to calibrate the CCI model, in terms of the parameters of tip diameter, velocity, dwell time and penetration depth that are known to influence lesion size and injury development. In parallel with measurements of contusion volume, the expression of the inflammatory cytokines TNF, IL-6 and the chemokine CCL-2 were quantified in blood and brain tissue at different time points post trauma using ELISA assays.

In the results presented, I performed all the surgeries, collected all the blood and brain tissue samples, performed all of the histology and contusion volume measurements, and some of the ELISA assays. I am grateful to Dr. Sarah Ciechanowicz who kindly contributed her ELISA data to cytokine expression results displayed in Figure 5.7 to Figure 5.12.

5.2 Considerations when setting up the model

The device used to produce a CCI injury consists of an impactor tip attached to a shaft that is accelerated by either a pneumatic piston or an electromechanical actuator. For this study, the latter was used and was mounted onto the arm of a stereotaxic manipulator. The device enabled fine adjustment of dwell time, depth and velocity. Moreover, an important consideration when setting up an *in vivo* study involving neurosurgery is the choice of anaesthesia. The goal of the current study was to calibrate a model that would then be

available for use by other members of the laboratory to test neuroprotective treatments including noble gases and combination treatments. It was desired that the model is adaptable to study neurological changes in the acute period post TBI as well as over a longer term. In addition, the option to administer treatment gases at different time points including early time points post trauma was desired. The volatile anaesthetic isoflurane was chosen because it has a short half-life and recovery (including the return of the righting reflex) occurred within minutes after CCI in the current study and in other studies[76]. Sham animals were subjected to a skin incision and light scratching of the skull under an equivalent duration of anaesthesia compared to CCI injured animals to control for any impact of anaesthesia on the outcomes measured. Further, a flat tip was chosen over a rounded tip for this study because flat tip impactors are known to cause more extensive cortical haemorrhage and neuronal loss[243]. Another important consideration was the location and size of the craniotomy as this may cause different effects[244]. In this study, a 6x7mm craniotomy was made over the right parietal cortex, following the lambdoid and coronal sutures and along the zygomatic arch. This enabled the injury to be centred at AP -5.8mm and ML +1.9 from the Bregma. The craniotomy location and size and injury coordinates are similar to CCI studies using rats[215,244–246].

As the model was being set up for the first time in our laboratory, it was important to monitor survival and pay particular attention to animal welfare. The survival rate at 24 hours post trauma in the animals I performed CCI surgeries on was 93%, 2 out of 30 animals died following the CCI impact. All sham animals survived. Figure 5.1 shows the percentage weight change at 24 hours post trauma as an indicator of animal health in sham animals and CCI animals with two different sets of impact parameters. The parameters used with the 5mm tip (red dots) were the highest injury parameters used in this study.

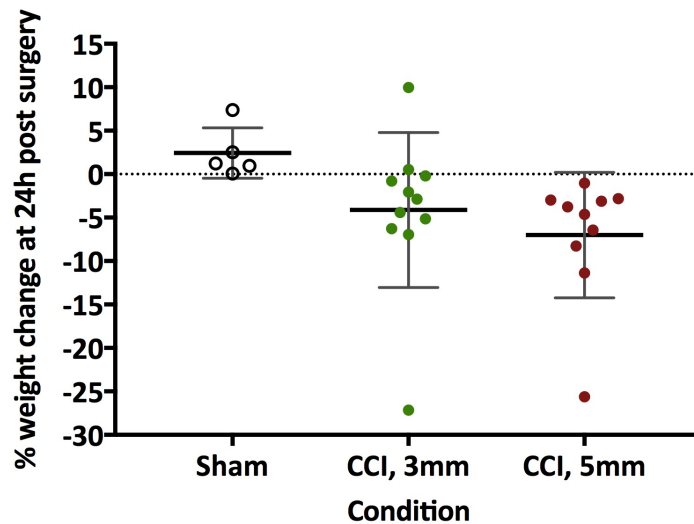


Figure 5.1: Percentage weight change at 24 hours after sham (anaesthesia, skin incision but no craniotomy or trauma) and CCI surgeries with impact parameters: velocity = 4m/s, dwell time = 400msec, tip diameter = 3mm, penetration depth = 1.5mm (green dots, ‘CCI, 3mm’) and velocity = 4m/s, dwell time = 400msec, tip diameter = 5mm, penetration depth = 2mm (red dots, ‘CCI, 5mm’). Dots represent percentage weight change at 24 hours from ‘n’ animals and error bars are standard deviations. Dashed line indicates the level of no weight change. Sham: n = 5, CCI 3mm: n = 11 and CCI 5mm: n = 10.

Sham animals did not lose weight 24 hours after trauma. One animal had a small increase (7%) in body weight. Some CCI animals lost a small percentage of their body weight after trauma. On average, no more than 6% weight was lost in the 3mm impact parameters group (green dots) with the exception of one animal, which lost 27% of its body weight. In the 5mm parameters CCI group (red dots), no more than 11% weight was lost with also the exception of one animal that lost 25% of its body weight. The percentage body weight loss was within the limits of the Morton and Griffiths scoring system[225] used to monitor animal welfare in this study.

5.3 Histology and contusion volume

Nissl stains are a category of basophilic stains used to visualise cell bodies in brightfield microscopy. A Nissl stain is basic and good for staining acidic molecules like DNA and RNA. Nissl stains specifically label RNA within cells, providing contrast to ribosomes and rough endoplasmic reticulum. Cresyl violet is one of the most commonly used Nissl stains and stains both neurons and glia. The redistribution of stained structures in injured or regenerating neurons allow Nissl staining to reveal the physiological state of neurons and glial cells[247]. The intensity of cresyl violet staining indicates cell viability, where injured

or dead cells appear pale in contrast to a blue or purple stain in healthy cells. Figure 5.2 shows an example of how the contusion area was obtained to calculate the contusion volume.

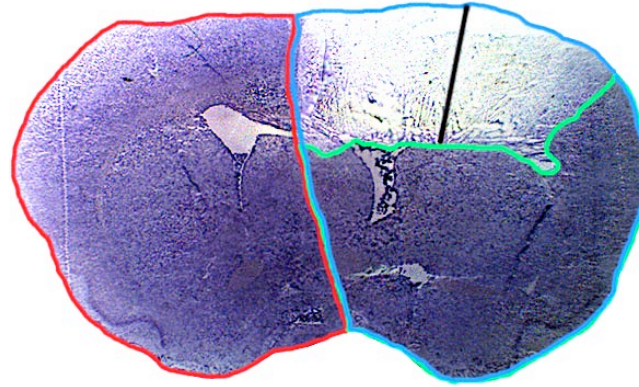


Figure 5.2: Typical cresyl violet stained slice from a CCI injury. The area of the contralateral hemisphere (red outline), ipsilateral hemisphere (blue outline) and area of the healthy tissue in the ipsilateral hemisphere (green outline) were traced to obtain the contusion areas used to calculate contusion volume. The depth of the lesion was measured at 24 hours post injury by drawing a line from the surface of the brain in the centre of the contusion to the upper outline of the healthy tissue (black line). CCI injury parameters: velocity = 4m/s, dwell time = 400msec, tip diameter = 5mm, penetration depth = 2mm. The contusion area appears white against the healthy cresyl stained tissue.

Slices were imaged as described in chapter 2, section 2.2.11. The area of the contralateral (red outline) and ipsilateral (blue outline) hemispheres was traced using FIJI software. Next, the area of the healthy tissue in the ipsilateral hemisphere (green outline) of the slice was traced. The contusion area is obtained by subtracting the area of the healthy tissue from the area of the hemisphere. The contusion volume was calculated by multiplying the contusion areas at each brain point, A , by the distance between brain sections, d , according to the formula:

$$\text{Contusion Volume} = \frac{d}{2} \times (A_1 + A_n) + d \times (A_2 + A_3 + \dots + A_{n-1})$$

Figure 5.3 shows coronal brain slices taken from a CCI animal 24 hours after injury with the CCI parameters: tip diameter = 3mm, velocity = 4m/s, dwell time = 400msec and penetration depth = 1.5mm.

A



B

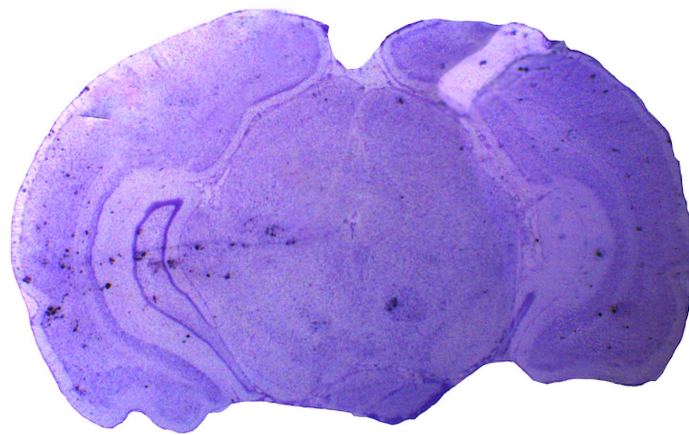


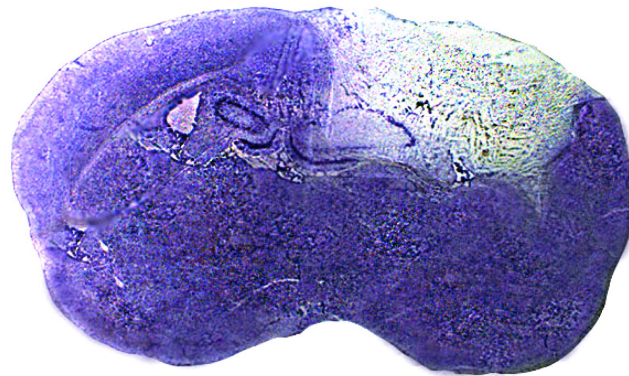
Figure 5.3: Typical cresyl violet stained slices at (A) +4.7mm from bregma (site of impact) and (B) +7.6mm from bregma from a CCI injury with parameters: velocity = 4m/s, dwell time = 400msec, tip diameter = 3mm, penetration depth = 1.5mm. The contusion area in the upper right appears light purple in contrast to the dark cresyl violet stained healthy tissue.

The slices above show the damaged tissue at the site of impact (+4.67mm from bregma) and towards the end of the impact site (+7.6mm from bregma). In the slices shown above, a contusion was not evident at the centre of the site of impact, 24 hours after injury however the tissue appeared torn towards the end of the impact site. A very small contusion was evident in the majority of CCI brains at these parameters and this was calculated to be 4.7 ± 2.3 mm (from 3 brains), however, this damage does not extend to other areas of the cortex, as would be expected with a ‘moderate’ level of CCI injury.

Next, the tip diameter was increased to 5mm, which is used more frequently for CCI injury in rats[244]. Figure 5.4 shows coronal slices at the site of impact (Figure 5.4A) and towards the

end (Figure 5.4B) of a contusion obtained with CCI injury parameters: tip diameter = 5mm, velocity = 4m/s, dwell time = 400msec, penetration depth = 2mm.

A



B

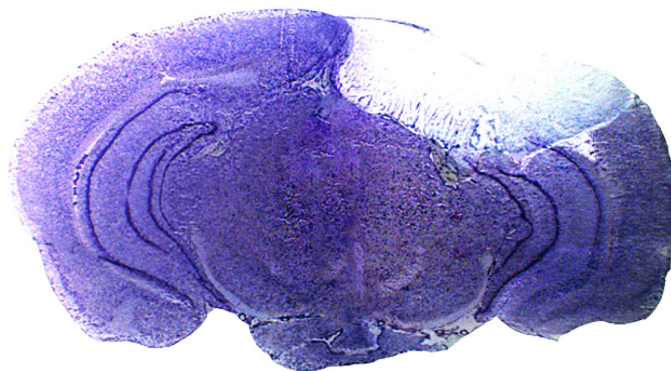


Figure 5.4: Typical cresyl violet stained slices at (A) +5.25mm from bregma and (B) +8.72mm from bregma, from a CCI injury with parameters: velocity = 4m/s, dwell time = 400msec, tip diameter = 5mm, penetration depth = 2mm. The contusion area appears white against the healthy cresyl stained tissue.

Using a tip diameter of 5mm produced a clear and recognisable contusion, significantly larger than the contusion seen when a 3mm tip was used. Next, the other parameters were adjusted to find a combination of parameters that produced a moderate injury. Increasing the velocity to 6m/s resulted in too severe an injury that caused death of the animal immediately after impact. However, adjusting the impact depth resulted in variation in the contusion volume. Figure 5.5 compares the contusion volume obtained with two different penetration depths: 1.5mm (white bars) and 2mm (grey bars), when all other parameters were kept constant, i.e., velocity = 4m/s, dwell time = 400msec and tip diameter = 5mm.

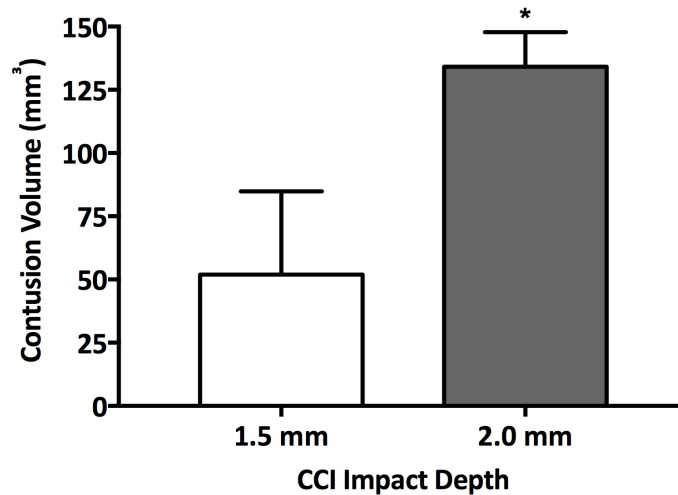


Figure 5.5: Variation in contusion volume at 24 hours after injury, as a function of CCI impactor penetration depth. Contusion volume was calculated as described in Methods section 2.2.9. Impact parameters were: velocity = 4m/s, dwell time = 400msec, tip diameter = 5mm, penetration depth = 1.5mm (white bar) and 2mm (grey bar). Bars represent mean values and error bars are SEM. Injury at 2mm depth: n = 9 and injury at 1.5mm depth: n = 2. *(p<0.05) compared to injury with 1.5mm depth, obtained from the results of a two-tailed unpaired *t*-test.

Using a penetration depth of 1.5mm produced a contusion volume of $52 \pm 33\text{mm}^3$, whereas increasing the penetration depth to 2mm more than doubled the contusion volume to $134 \pm 14\text{mm}^3$. In this model, all parameters must be adjusted relative to each other – there is no one combination of parameters that will give a ‘severe’, ‘moderate’ or ‘mild’ injury. After selecting values for velocity, tip size and dwell time, studies have often only varied impact depth as a measure of severity of injury as it has shown to correlate with physiological and behavioural outcomes[248]. A tip diameter of 3mm did not produce a large contusion and when a higher velocity and lower dwell time (6m/s and 200msec) in combination with a 5mm tip were tested, too severe an injury was produced resulting in death of the animal immediately after injury.

Next, to test whether the injury had developed by 24 hours, the depth of the lesion was measured at 24 hours post injury by drawing a line from the surface of the impact site to the bottom of the contusion as shown in Figure 5.2. If the lesion were deeper than 1.5mm or 2mm (the impact depth at the time of injury) from the surface, it would indicate development of injury.

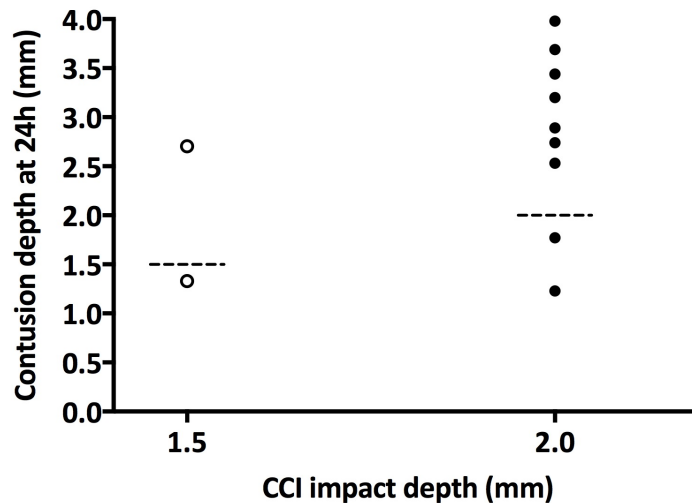


Figure 5.6: Variation in contusion impact depth at 24 hours from the surface of the brain as a function CCI impactor penetration depth at the time of injury. Contusion depth was calculated by measuring depth of contusion at the impact site from images of cresyl violet stained brain slices. Impact parameters were: velocity = 4m/s, dwell time = 400msec, tip diameter = 5mm, penetration depth = 1.5mm (open circles) and 2mm (filled circles). Dots represent contusion depth from ‘n’ animals. Dashed lines indicate level of CCI impactor penetration depth at the time of injury. Injury at 2mm depth: n= 9 and injury at 1.5mm depth: n = 2.

Figure 5.6 shows the penetration depths (1.5mm and 2mm) versus the contusion depth at 24 hours. The final parameters (velocity = 4m/s, dwell time = 400msec, tip diameter = 5mm, penetration depth = 2mm) produced an injury in which the contusion is clearly evident and develops because in 7 out of 9 brains, the depth into the cortex is greater than impact depth of injury (2mm) from the surface.

5.4 Cytokine profiling after CCI injury

5.4.1 ELISA assay format

A ‘sandwich’ ELISA is the most powerful ELISA assay format and is highly efficient in sample antigen detection. The analyte to be measured is bound between two primary antibodies – the capture and detection antibody. This format has several advantages. It is highly specific and therefore the sample does not have to be purified before analysis, which works well for blood plasma and brain samples that are likely to be contaminated. Additionally, the format allows signal amplification that provides increased sensitivity (up to 2 to 5 times more sensitive than direct or indirect ELISA) for detecting and quantifying small amounts of antigens. A disadvantage is the potential for cross-reactivity of the secondary antibody, and therefore secondary antibodies that have been cross-adsorbed to remove

antibodies with an affinity for the capture antibody are generally used. Overall, the sandwich ELISA format is a sensitive and robust test.

5.4.2 TNF expression after CCI injury

The concentration of the inflammatory cytokine TNF was measured in brain and blood plasma at different times after injury in animals from both ‘lower injury’ and ‘higher injury’ parameter groups using an ELISA assay. Brain and plasma samples were measured at 3 hours and 24 hours after injury in the groups with ‘lower injury’ parameters (velocity = 4m/s, dwell time = 400msec, tip diameter = 3mm, penetration depth = 1.5mm) and at 24 hours in the plasma of animals injured with the ‘higher injury’ parameters (velocity = 4m/s, dwell time = 400msec, tip diameter = 5mm, penetration depth = 2mm). TNF levels after injury was compared to those of sham (anaesthesia, skin incision but no craniotomy or trauma) and naïve groups. Figure 5.7 shows the concentrations of TNF in plasma samples.

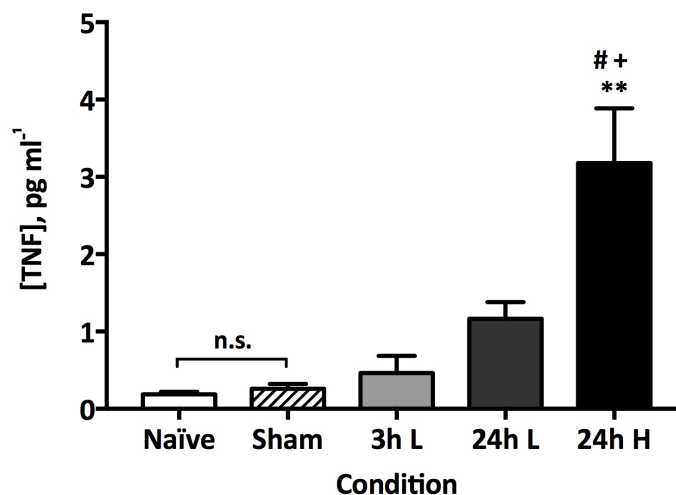


Figure 5.7: Concentration of TNF following CCI injury in plasma of naïve (n = 5), sham at 24 hours (n = 4), CCI at 3 hours (n = 2), CCI at 24 hours (n = 6) with injury parameters: tip diameter = 3mm, velocity = 4m/s, penetration depth = 1.5mm, dwell time = 400msec, and CCI at 24 hours (n = 6) with injury parameters: tip diameter = 5mm, velocity = 4m/s, penetration depth = 2mm, dwell time = 400msec, labelled ‘24h’. **($p < 0.01$), #($p < 0.05$) and +($p < 0.05$) compared to sham, CCI injury at 3 hours and CCI injury with lower parameters at 24 hours respectively. Bars represent mean concentration from ‘n’ animals and error bars are SEM.**

TNF concentrations in the plasma of animals subjected to CCI with ‘lower injury’ parameters showed no increase at 3 hours and 24 hours compared to sham and the difference between concentrations at 3 hours ($0.46 \pm 0.22 \text{ pgml}^{-1}$) and 24 hours ($1.16 \pm 0.21 \text{ pgml}^{-1}$) hours did not reach significance. Although there was a trend towards increase in TNF at both time points compared to sham, this did not reach significance. However, the situation was different with animals subjected to higher injury parameters, where the concentration of TNF in the plasma

at 24 hours ($3.18 \pm 0.70 \text{ pgml}^{-1}$) was significantly different ($p=0.001$) to sham ($0.26 \pm 0.06 \text{ pgml}^{-1}$). It was also significantly different ($p=0.01$) to the 24-hour ‘lower injury’ parameter CCI group ($1.16 \pm 0.21 \text{ pgml}^{-1}$). Figure 5.8 shows the concentration of TNF in brain samples.

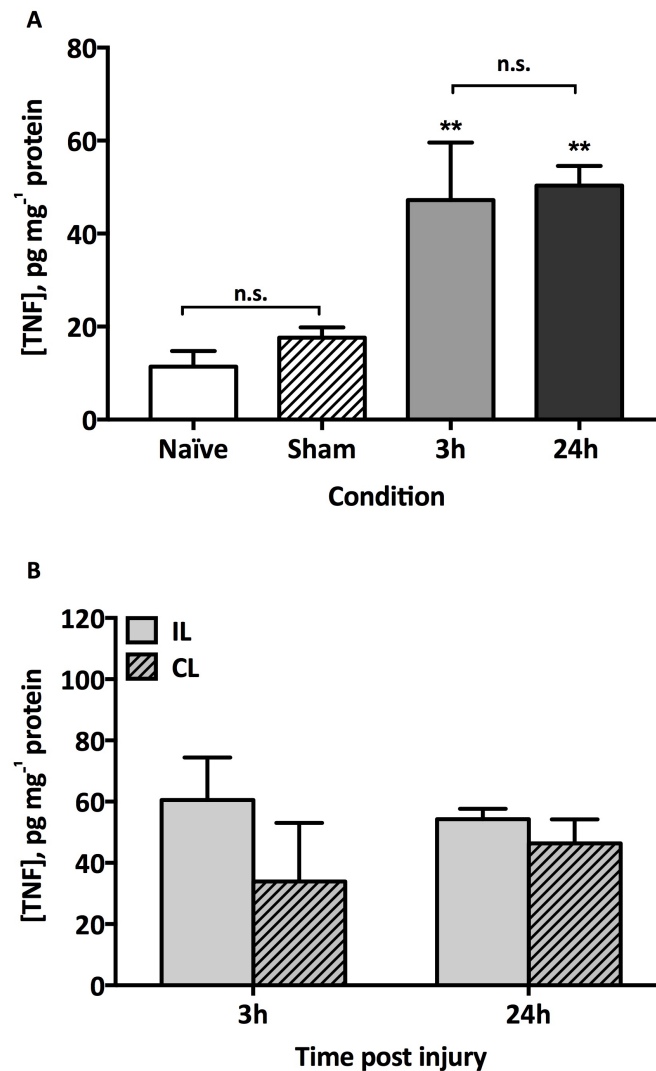


Figure 5.8: Concentration of TNF following CCI injury in (A) brain tissue of naïve ($n = 4$), sham at 24 hours ($n = 3$), CCI at 3 hours ($n = 2$), CCI at 24 hours ($n = 4$) with injury parameters: tip diameter = 3mm, velocity = 4m/s, penetration depth = 1.5mm, dwell time = 400msec, and (B) separate brain hemispheres, the difference in concentration between ipsilateral (IL) and contralateral (CL) hemispheres did not reach significance ($p>0.05$). $ (p<0.001)$ compared to sham. Bars represent mean concentration from ‘n’ animals and error bars are SEM.**

TNF concentration was significantly elevated in brains at both 3 hours ($47.2 \pm 12.4 \text{ pgmg}^{-1}$, $p<0.001$) and 24 hours ($45.5 \pm 12.4 \text{ pgmg}^{-1}$, $p<0.007$) after CCI injury in ‘lower injury’ group compared to sham ($17.0 \pm 3.7 \text{ pgmg}^{-1}$). There was no difference between brain TNF concentration at 3 hours and 24 hours in the CCI injury groups (Figure 5.8A). When levels of TNF in ipsilateral (injured) and contralateral hemispheres were compared there was a slightly higher concentration in ipsilateral hemisphere at 3 hours after injury, but this was not

significant while at 24 hours after injury the levels were almost identical in each hemisphere. (Figure 5.8B).

Taken together, these results suggest that TNF is a marker of inflammation detectable in the brain and plasma following CCI injury and that the concentration of TNF in the plasma at 24 hours correlates positively with injury severity.

5.4.3 IL-6 expression after CCI injury

As described above, the concentration of IL-6 was measured using an ELISA assay at 3 and 24 hours in the brain and plasma of animals injured with the ‘lower injury’ parameters and at 24 hours in the plasma of animals injured with the ‘higher injury’ parameters. Concentrations of IL-6 in plasma after injury were compared to those of sham and naïve groups (Figure 5.9).

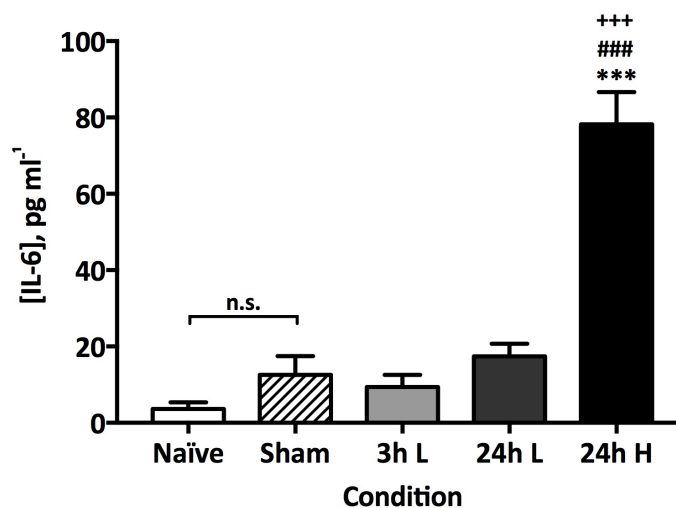


Figure 5.9: Concentration of IL-6 following CCI injury in plasma of naïve (n = 5), sham at 24 hours (n = 5), CCI at 3 hours (n = 2), CCI at 24 hours (n = 5) with injury parameters: tip diameter = 3mm, velocity = 4m/s, penetration depth = 1.5mm, dwell time = 400msec; n = 2) and CCI at 24 hours (n = 8) with injury parameters: tip diameter = 5mm, velocity = 4m/s, penetration depth = 2mm, dwell time = 400msec, labelled ‘24h***’. ***($p < 0.0001$), ###($p < 0.0001$) and +++($p < 0.0001$) compared to sham, CCI injury at 3 hours and CCI injury with lower parameters at 24 hours respectively. Bars represent mean concentration from ‘n’ animals and error bars are SEM.

No difference in IL-6 levels between sham and CCI groups was detected in the plasma of animals subjected to lower injury parameters at 3 hours and 24 hours. The mean level of IL-6 at 24 hours ($17.4 \pm 3.3 \text{ pg ml}^{-1}$) was numerically higher than that at 3 hours ($9.4 \pm 3.2 \text{ pg ml}^{-1}$), although this difference was not significant. However, the level of IL-6 in the plasma of animals subjected to higher injury parameters ($78.18 \pm 8.49 \text{ pg ml}^{-1}$) was significantly higher to sham ($p < 0.0001$) and the 24-hour ($p < 0.0001$) lower parameter CCI groups. Figure 5.10 shows IL-6 concentrations measured in brain tissue.

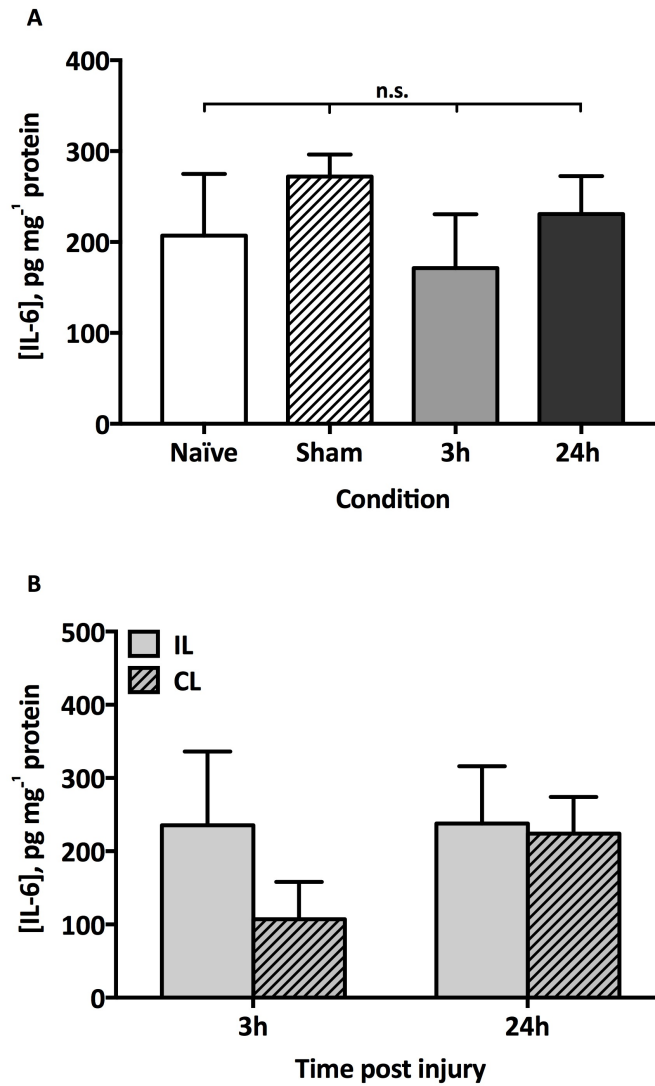


Figure 5.10: Concentration of IL-6 following CCI injury in (A) brain tissue of naïve ($n = 6$), sham at 24 hours ($n = 3$), CCI at 3 hours ($n = 2$), CCI at 24 hours ($n = 4$) with injury parameters: tip diameter = 3mm, velocity = 4m/s, penetration depth = 1.5mm, dwell time = 400msec, and (B) separate brain hemispheres, difference in concentration between ipsilateral (IL) and contralateral (CL) hemispheres did not reach significance ($p > 0.05$). Bars represent mean concentration from 'n' animals and error bars are SEM.

There was no difference in IL-6 concentration in brain between sham and CCI injured animals at either time point (Figure 5.10A). When IL-6 levels were compared between ipsilateral and contralateral hemispheres, at 3 hours again there was a trend for ipsilateral levels of IL-6 to be higher ($235 \pm 100 \text{ pgmg}^{-1}$) than contralateral levels ($107 \pm 51 \text{ pgmg}^{-1}$) but this did not reach significance (Figure 5.10B).

These results suggest that IL-6 is a marker of inflammation in the plasma following CCI injury and that its concentration at 24 hours correlates positively with injury severity.

5.4.4 CCL-2 expression after CCI injury

As described above, the concentration of the chemokine CCL-2 was quantified using an ELISA assay at 3 hours and 24 hours in the brain and plasma of animals injured with the ‘lower injury’ parameters and at 24 hours in the plasma of animals injured with the ‘higher injury’ parameters. CCL-2 levels were compared to those of sham and naïve animals. Figure 5.11 shows the concentrations of CCL-2 in the plasma.

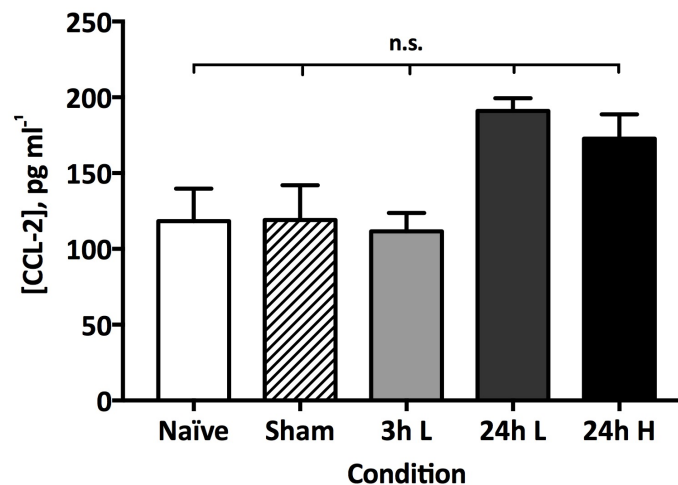


Figure 5.11: Concentration of CCL-2 following CCI injury in plasma of naïve ($n = 6$), sham at 24 hours ($n = 5$), CCI at 3 hours ($n = 2$), CCI at 24 hours ($n = 5$) with injury parameters: tip diameter = 3mm, velocity = 4m/s, penetration depth = 1.5mm, dwell time = 400msec; $n = 2$) and CCI at 24 hours ($n = 8$) with injury parameters: tip diameter = 5mm, velocity = 4m/s, penetration depth = 2mm, dwell time = 400msec, labelled ‘24h**’. Bars represent mean concentration from ‘ n ’ animals and error bars are SEM.

CCL-2 concentrations in the plasma of animals subjected to ‘lower injury’ parameters increased from $112 \pm 12.2 \text{ pgml}^{-1}$ at 3 hours to $191 \pm 8.5 \text{ pgml}^{-1}$ at 24 hours, although the difference did not reach significance. There no difference between the level of CCL-2 at 24 hours in the plasma of animals subjected to ‘higher injury’ parameters ($172.7 \pm 16.2 \text{ pgml}^{-1}$) and those subjected to ‘lower injury’ at the same time point. Moreover, the difference between all three CCI groups and the sham group ($119 \pm 23 \text{ pgml}^{-1}$) did not reach significance, although there is a trend towards an increase in CCL-2 concentration for both CCI groups at 24 hours compared to sham. Figure 5.12 shows the concentration of CCL-2 measured in the brain.

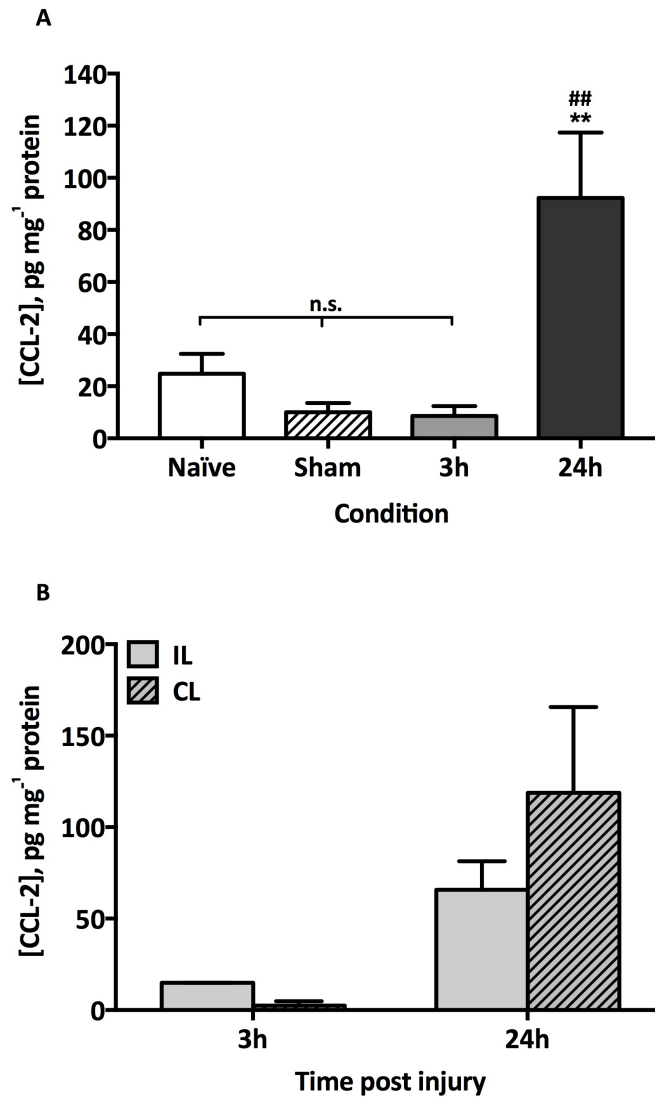


Figure 5.12: Concentration of CCL-2 following CCI injury in (A) brain tissue of naïve (n = 5), sham at 24 hours (n = 3), CCI at 3 hours (n = 2), CCI at 24 hours (n = 5) with injury parameters: tip diameter = 3mm, velocity = 4m/s, penetration depth = 1.5mm, dwell time = 400msec, and (B) separate brain hemispheres, difference in concentration between ipsilateral (IL) and contralateral (CL) hemispheres did not reach significance ($p > 0.05$). **($p < 0.001$) and ##($p < 0.001$) compared to sham and CCI injury at 3 hours, respectively. Bars represent mean concentration from 'n' animals and error bars are SEM.

Figure 5.12A shows that brain levels of CCL-2 were significantly ($p = 0.0003$) increased at 24 hours post-CCI ($71 \pm 23 \text{ pgmg}^{-1}$) compared to sham ($10 \pm 4 \text{ pgmg}^{-1}$). However, no difference between ipsilateral (injured) and contralateral hemisphere was observed (Figure 5.12B).

These results suggest that CCL-2 may be an early marker of inflammation detectable in the brain following CCI injury.

5.4.5 Evaluation of sensitivity of ELISA assay format

In the data shown (TNF assay), some cytokine concentrations were near the lower limits of detection of the ELISA plates. The low readings may have caused the higher variance seen in some of the graphs because results were interpreted from the lower end of the standard curve. The low readings were interpreted with caution. A standard curve was run each time an assay was conducted and this was used to interpret results. A low and a high control were also run each time. Although values that were below the reliable detection limit of the plate were not used, in the future, I would use an ultrasensitive ELISA kit that matches the range of results in brain and plasma samples obtained from this model more closely (for example, Rat TNF Ultra-sensitive ELISA, Invitrogen KRC3014). Another alternative would be to use multiplex immunoassay magnetic beads (for example, Luminex Multiplex Assay, Invitrogen), which produces results that are comparable to ELISA. Although a drawback of Luminex technology is that it requires a special instrument that quantifies spectral signal, it offers significant advantages over the ELISA assay. The Luminex assay can be custom designed to measure multiple cytokines simultaneously hence significantly cutting the time required to obtain results, has lower cross reactivity and uses very small volumes of sample.

6 Chapter 6: Establishing a *Drosophila* model of traumatic injury

6.1 Overview and purpose of research

This chapter describes experiments conducted to set up a HTS assay for TBI, using *Drosophila Melanogaster*. The injury model is based on the work of Katzenberger and colleagues, who introduced the concept of using *Drosophila* to model TBI in 2014[223]. These findings were used to set up a model of polytrauma including a contact type TBI. The model was used to test the effect of treatment with hypothermia, or cooling, on survival. A number of factors were investigated when developing the model, such as injury severity, which is a function of the number of impacts and the force of each impact, the age of the flies, and the circadian time at which flies received the trauma. Following experiments in which the effect of trauma on survival was tested, the effect of hypothermia treatment at 4⁰C and 10⁰C on *Drosophila* survival was determined.

These experiments were done in collaboration with Dr. Giorgio Gilestro's (Department of Life Sciences, Imperial College London) laboratory and I am grateful to Dr. Gilestro and his team for their advice on fly biology and the project. I would particularly like to acknowledge Anne Petzold for teaching me the fly breeding technique and for her general advice on working with *Drosophila*. I would also like to acknowledge Alessandro Prete, who assisted with data collection.

6.2 Kaplan Meier survival analysis

Drosophila survival is presented using Kaplan Meier (K-M) survival curves, which are a non-parametric method of estimating subject survival rates that takes into account the fact that some subjects may have died during a study whereas others may survive beyond the end of the trial period. Established by Edward Kaplan and Paul Meier in 1958[249], Kaplan Meier analysis uses a mathematical formula to estimate the proportion of subjects alive at any point during the trial, based on information from those who have died, and is plotted over time as a 'staircase' curve, a diagram of which is shown in Figure 6.1.

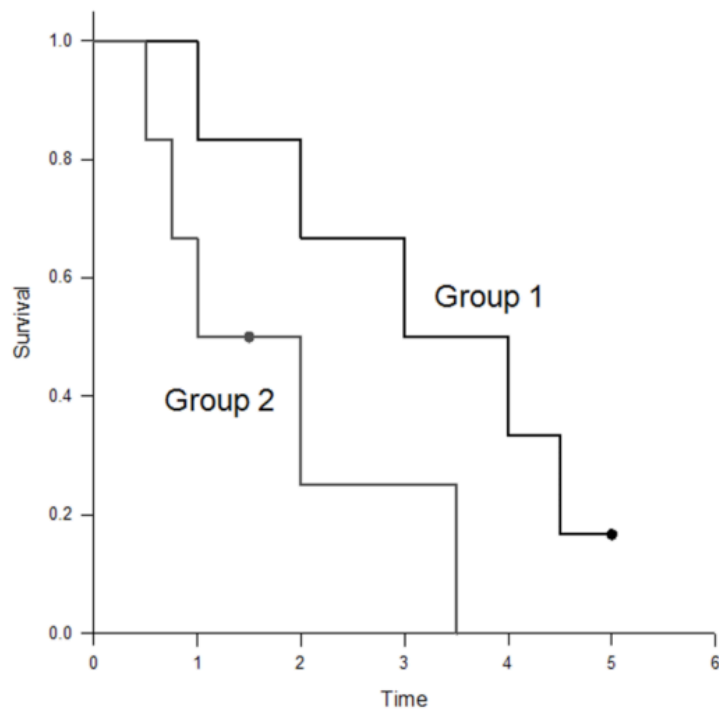


Figure 6.1: Schematic diagram of Kaplan Meier curve (taken from Rich *et al.*, 2010[250])

The curve shows a series of horizontal steps known as ‘intervals’, which represent an event of interest (or end-point). An interval is terminated when an event of interest occurs. The lengths of horizontal lines represent the survival duration for that interval. Vertical distances between horizontal lines indicate the change in cumulative probability as the curve advances (shown on y-axis, Figure 6.1). Censored subjects are subjects for whom total survival time cannot be accurately determined because the subject has left the study or survives after the study has ended. Censoring removes the subject from the individuals still at risk, i.e., from the denominator. Censored subjects are indicated as upticks on curves and have no impact on intervals. K-M curves provide a simple means to visualise differences between different curves. However, the difference must also be quantified. Due to the stepwise and non-continuous nature of the curves, calculating a point survival from the curve is difficult and often misrepresentative[250]. This means that simply extracting the survival data at a series of time points from the curves for comparison is not sufficient. The entire curve of mortality must be considered. There is one important assumption made when using K-M curves that applies to the current study. It is assumed that at any time, subjects who were censored have the same survival probability as those who continue to be monitored. In the case of *Drosophila* experiments and indeed even in clinical trials, this is challenging to test.

The survival curves shown have been evaluated using the log rank test and hazard ratios. The log rank test is used to assess statistical significance between groups. It calculates the chi-square for each event time for each group. The summed results for each group are then added to obtain the final chi-square used to compare the full curve of each group. In this way, the entire survival curves over the time period tested are compared with each other. The hypothesis tested is that there is no difference in survival time between groups studied at all time points in the study. A significance level of $p < 0.05$ is considered statistically significant. The hazard ratio calculates a relative event rate in the groups. The hazard is defined as the slope of the survival curve, and measures how rapidly subjects are dying. The calculation of the hazard ratios is based on the log-rank test and assumes that the ratios are consistent over time. Therefore any differences are due to random sampling[251].

In terms of limitations of K-M analysis, there are a few factors to consider. The log rank test is purely a significance test – it does not indicate the size of the difference between conditions. Thus, in the current study, hazard ratios were used. Moreover, K-M analysis only enables one factor at a time to be studied and so cannot be used for multivariate analysis eg. to assess the effect of age and circadian rhythm on fly survival. This may be done using the more complex Cox proportional hazards method[250].

In summary, the chi-square result from the log-rank test indicates whether two curves are statistically different. The hazard ratio of two curves indicates the increased rate of having an event in one curve versus the other. In the results shown below, *Drosophila* are entered into the study before they receive trauma and the event of interest is death.

6.3 Injury severity

In our *Drosophila* model of trauma, flies were loaded into vials that were individually attached to the end of a spring (see chapter 2, Figure 2.15). Injury severity was assessed as a function of (a) the number of strikes the flies receive and (b) the angle at which the spring is deflected (measured from the base of the spring to the flat board to which the spring was attached) before being released and causing the vial containing flies to strike the hard surface. The angle of the vial is a measure of the tension in the spring and therefore the force of the impact. As opposed to a high mortality in the first 24 – 48 hours after trauma, a more delayed

mortality and thus a moderate injury severity was desired for this model because we wanted to study the development of injury by assessing survival post trauma for at least 14 – 31 days. Following a number of successive strikes, flies were temporarily incapacitated and lay in groups at the bottom of the vial. Flies re-gained locomotor activity within the 2-hour recovery period. The time of incapacitation was a function of injury severity.

First, the effect of injury severity on acute mortality was tested. As there was no difference between survival at 24 and 48 hours, both when varying the number of hits and the angle of spring deflection, it was concluded that mortality due to the primary injury (number of strikes) was complete within 24 hours and further mortality (i.e., on subsequent days) was due to the progression of injury. Figure 6.2 shows the effect of number of strikes on *Drosophila* survival at 24 hours post trauma.

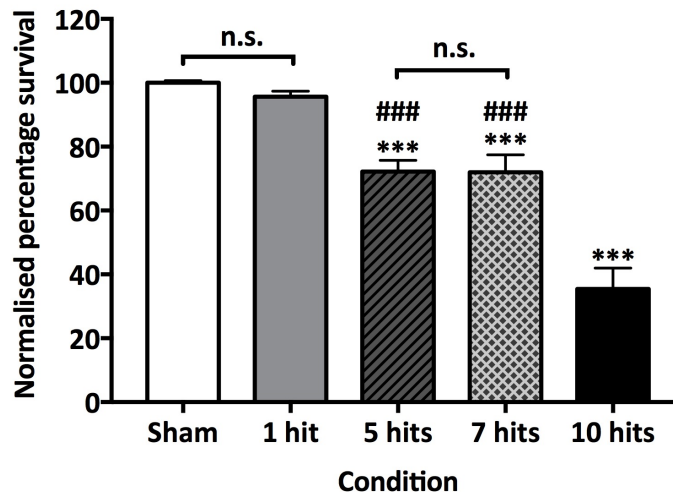


Figure 6.2: The effect of number of 90° hits on *Drosophila* survival at 24 hours post trauma following 1 hit (grey bar), 5 hits (grey bar with stripes), 7 hits (grey bar with pattern) and 10 hits (black bar) normalised to sham survival (white bar). Data represent means and error bars are SEM. ***($p < 0.0001$) and ###($p < 0.0001$) compared to sham and 10 hit groups respectively. Sham: $n = 250$, 1 hit: $n = 290$, 5 hits: $n = 300$, 7 hits: $n = 90$ and 10 hits: $n = 40$

Figure 6.2 shows the effect of 1, 5, 7 and 10 hits delivered with vial held at 90° before releasing spring, on survival at 24 hours post injury. 1 hit ($95 \pm 2\%$) did not significantly reduce survival ($p = 0.7$) compared to the sham group ($100 \pm 1\%$). Increasing the number of hits to 5 and 7 reduced survival significantly ($p < 0.0001$) to $72 \pm 3\%$ and $71 \pm 5\%$ respectively compared to sham survival. The reduction in survival due to 5 and 7 hits was not as severe as reduction in survival with 10 hits ($p < 0.0001$). 10 hits significantly reduced survival 24 hours post injury to $35 \pm 7\%$ compared to sham ($p < 0.0001$).

Figure 6.3 shows the effect of 90° and 110° hits on fly survival at 24 hours post trauma. 5 hits at either 90° or 110° were administered with 5-minute intervals between strikes.

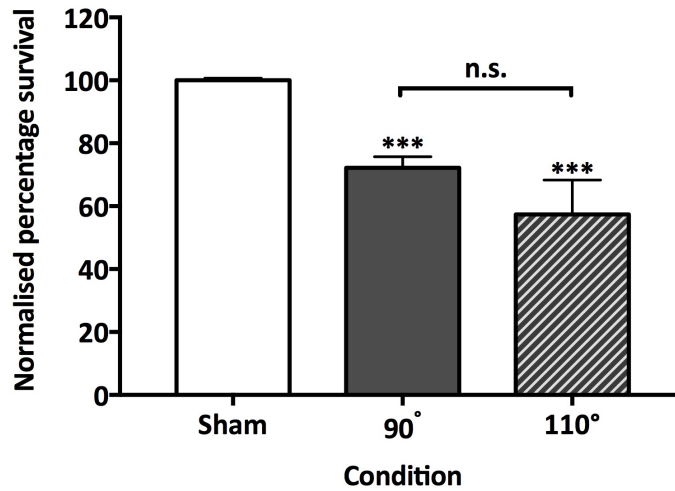


Figure 6.3: The effect of angle of hits on *Drosophila* survival at 24 hours post trauma following 5 hits at 90° (grey bar) and 5 hits at 110° (grey bar with stripes) normalised to sham survival (white bar). Bars represent means and error bars are SEM. ***($p < 0.0001$) compared to sham. Sham: $n = 250$, 5 hits at 90°: $n = 300$ and 5 hits at 110°: $n = 60$.

Both 90° hits and 110° hits caused a significant ($p < 0.0001$) reduction in survival at 24 hours post trauma compared to the sham group. However, the difference in survival between the two trauma groups was not significantly different ($72 \pm 3\%$ and $57 \pm 10\%$ respectively). Moreover, retracting the spring back to 180° produced too severe an insult and caused death of all flies within 4 hours of receiving trauma. This indicates that modest changes in the angle of injury were insufficient to cause changes to the severity of injury. However, exceeding a threshold of in terms of number of hits and angle of deflection does impact acute survival.

Next, the effect of both the number of hits and the angle of injury on *Drosophila* survival over a longer term was assessed. The K-M curves in Figure 6.4A and B show the percentage survival of flies following 1, 5, 7 and 10 hits for 14 days and following 1 and 5 hits for 31 days post trauma respectively. There is an overlap of flies for 1 hit and 5 hits group in Figure 6.4A and B i.e., the same 1 hit and 5 hits groups were studied for 31 days.

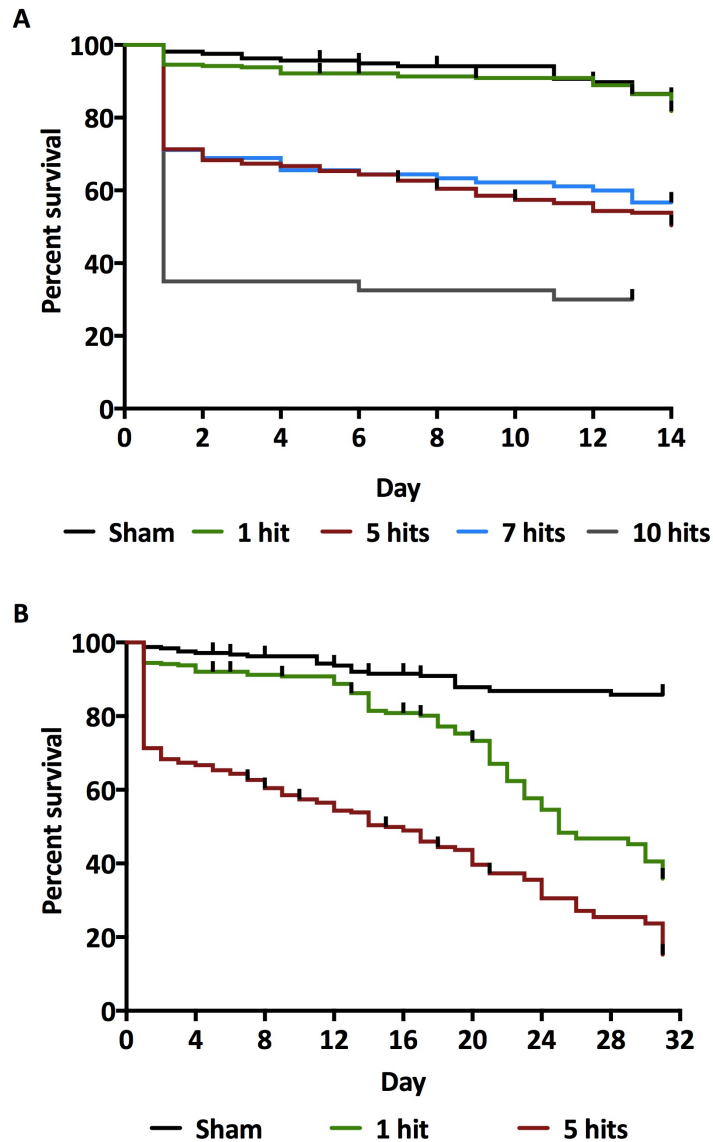


Figure 6.4: The effect of number of 90° hits on *Drosophila* survival shown using Kaplan Meier survival curves. (A) Percentage survival after 1 hit (green line), 5 hits (red line), 7 hits (blue line) and 10 hits (grey line) versus sham (black line) over 14 days post trauma. Log-rank tests indicated no significant difference between sham and 1 hit group over 14 days, but curves for 5, 7 and 10 hits were significantly different ($p < 0.0001$) compared to sham. (B) Percentage survival post 1 hit (green line) and 5 hits (red line) versus sham (black line) over 31 days post trauma. Log-rank tests indicated a significant difference ($p < 0.001$) between sham compared to 1 and 5 hit groups and between 1 and 5 hits groups. Upticks represent points at which data was censored. Sham: $n = 250$, 1 hit: $n = 290$, 5 hits: $n = 300$, 7 hits: $n = 90$ and 10 hits: $n = 40$.

There was no significant difference in survival over 14 days between 1 hit and sham groups, but the survival over 14 days in 5, 7 and 10 hits groups was significantly reduced ($p < 0.0001$) compared to sham. However, when assessed over 31 days (Figure 6.4B), there was a significant difference ($p < 0.0001$) between survival curves with 1 hit compared to sham and between groups that received 1 and 5 hits. By 31 days, percentage survival of flies who received 5 hits was $15 \pm 4\%$, compared with those that received 1 hit which was $36 \pm 6\%$ and

to the sham group of which $85 \pm 3\%$ were likely to survive. Therefore, over time, the group that have received even the mildest insult had a significantly lower survival compared to sham. This indicates that the traumatic insult impairs normal physiological functioning to an extent that increases mortality over time. Moreover, the hazard ratio of 5:1 hit groups was 2.91 (95% confidence intervals, CI = 2.24 to 3.79), indicating that the 5 hits group had a steeper mortality curve than the 1 hit group. Flies who received 5 hits were nearly 3 times more likely to die than those that received 1 hit. This indicates that the mortality over a given time interval due to injury varies proportionally with the number of hits flies receive.

The K-M curves in Figure 6.5 show the percentage survival of flies that receive 5 hits at 90° or at 110° compared to the sham group over 31 days post trauma.

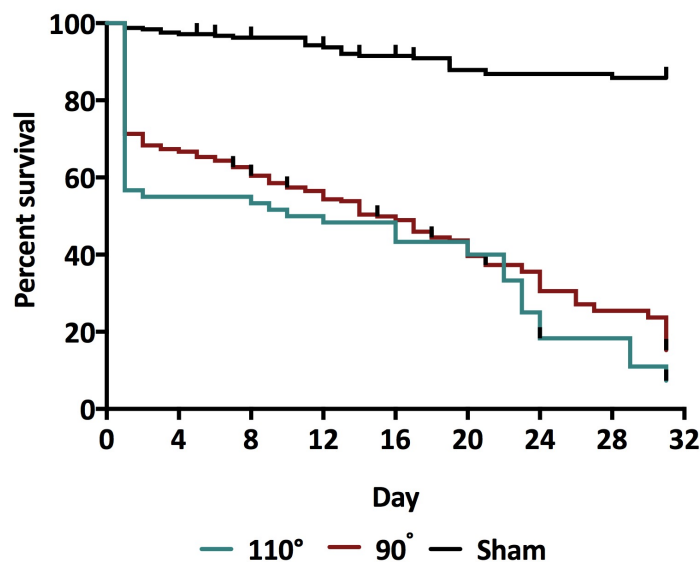


Figure 6.5: The effect of angle of hits on *Drosophila* survival post trauma. Kaplan Meier survival curves showing percentage survival post 5 hits at 90° (red line) and 5 hits at 110° (teal line) versus sham (black line) over 31 days post trauma. Log-rank tests indicated a significant difference ($p < 0.0001$) between percentage survival with 90° and 110° hits compared to sham, but no significant difference between 90° and 110° hits. Upticks represent points at which data was censored. Sham: $n = 250$, 5 hits at 90° : $n = 300$ and 5 hits at 110° : $n = 60$.

Similar to the survival at 24 hours, there was no significant difference ($p = 0.22$) in percentage survival between flies that received 90° hits and those that received 110° hits over 31 days, however, survival in both groups was significantly different ($p < 0.0001$) to survival in the sham group over 31 days. As discussed in section 6.2, when K-M analysis is used, whole curves must be compared rather than extrapolating the survival at specific points from the curves. The hazard ratio of $110^\circ:90^\circ$ groups was 1.18 (95% CI = 0.85 to 1.63), indicating no difference in the rate of mortality between the groups over 31 days. At the end of the study and 31 days after trauma, percentage survival for the 110° hits group had dropped to $7 \pm 4\%$

which was not significantly different to survival in the compared to the 90⁰ hits group, for which survival was $15 \pm 4\%$.

Following these experiments, an injury of 1 hit was chosen as a ‘mild’ injury based on delayed mortality (Figure 6.4) whereas an injury of 5 hits was selected for a ‘moderate’ injury, based on early and long term mortality. 10 hits were deemed too severe a primary injury to observe over a longer experimental time period, as this reduced survival to $35 \pm 7\%$ within 24 hours post injury. An angle of 90⁰ was chosen on pragmatic grounds because it was easier to standardise.

6.4 Effect of age on fly survival post trauma

To determine whether like in humans or rodents, age associated processes increase sensitivity to trauma in *Drosophila*, the effect of age at the time of trauma on long-term survival was tested. Young (age 1 – 3 days old) and mature (11 – 14 days old) flies were subjected to a mild insult of 1 hit and a moderate insult of 5 hits. These ages were selected because the sexual maturity for *Drosophila* is 7 days[252]. Their survival post trauma was compared to sham flies from the same age groups over 21 days and is shown in Figure 6.6.

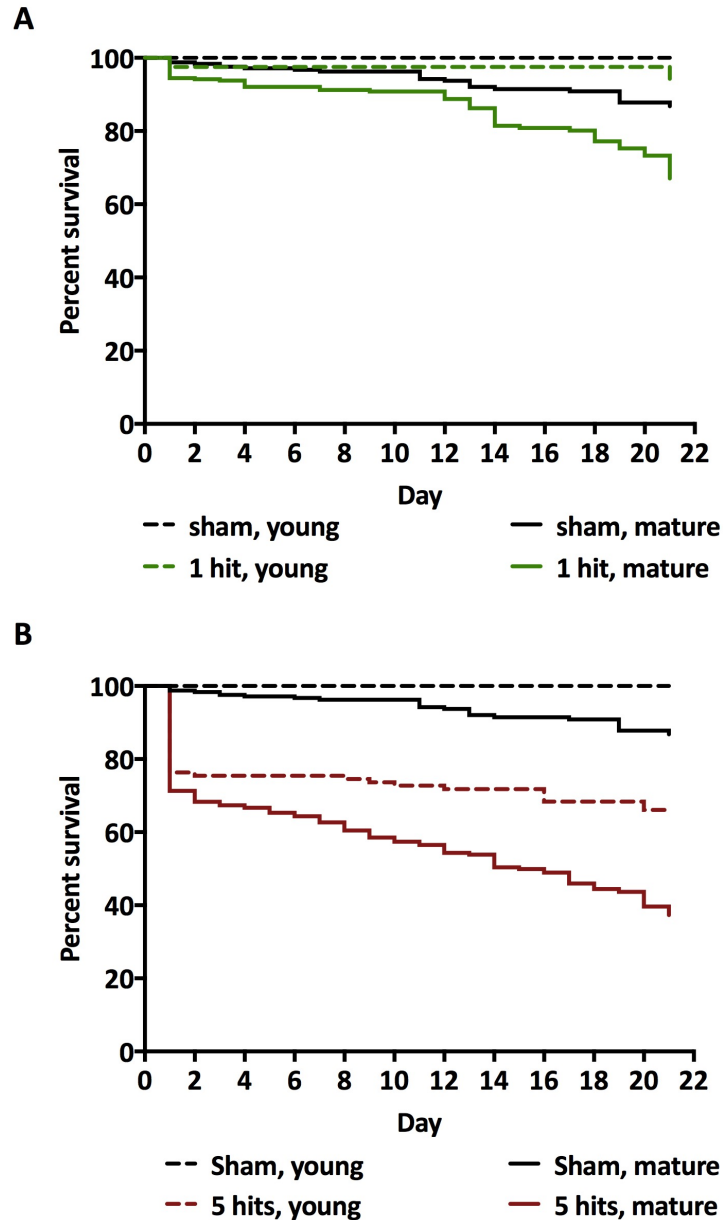


Figure 6.6: The effect of age of *Drosophila* on sensitivity to trauma shown using Kaplan Meier survival curves. (A) Percentage survival after 1 hit at 90° in young flies (green dashed line) compared to mature flies (green solid line) after trauma versus young sham flies (black dashed line) and mature sham flies (black solid line). (B) Percentage survival after 5 hits at 90° in young (red dashed line) flies compared to mature (red solid line) after trauma versus young sham flies (black dashed line) and mature sham flies (black solid line). Log-rank tests indicated a significant difference ($p < 0.0001$) in survival between young and mature flies that had both 1 and 5 hits. Upticks represent points at which data was censored. Sham: $n = 250$, young, 1 hit: $n = 80$, young, 5 hits: $n = 120$, mature, 1 hit: $n = 290$, mature, 5 hits: $n = 300$, young sham: $n = 50$ and mature sham: $n = 250$.

There was no significant difference in survival between young flies that received 1 hit ($93 \pm 3\%$) and the sham group, which had 100% survival until the end of the experiment at 21 days (Figure 6.6A). However, the survival over 21 days in young flies that received 5 hits ($66 \pm 5\%$ survival at the end of 21 days) was significantly different ($p < 0.0001$) compared to sham flies (Figure 6.6B). On the contrary, for mature flies, survival was significantly different

($p < 0.0001$) compared to sham for groups that received both 1 and 5 hits ($67 \pm 4\%$ and $37 \pm 3\%$ survival at the end of 21 days respectively). The survival curves of young and mature flies that received 1 hit is compared in Figure 6.6A. There was a significant difference ($p < 0.0001$) in survival between the two groups, and the hazard ratio was 2.45 (95% CI = 1.57 to 3.79), indicating that mature flies that received 1 hit died 2.5 times as fast as young flies. Similarly, there was a significant difference ($p < 0.0001$) in survival between mature and young flies that received 5 hits, shown in Figure 6.6B. The hazard ratio was 7.17 (95% CI = 5.38 to 9.55), indicating that the rate of mortality in mature flies that received 5 hits was 7 times faster than young flies that received the same injury. Thus, age at the time of injury increases sensitivity to trauma in *Drosophila*.

6.5 Variation in sensitivity to trauma when *Drosophila* are injured at different times in their circadian cycle

In terms of locomotor behaviour, *Drosophila* are known to show two peaks of activity – one in the morning and one in the evening, under constant environmental conditions[253]. To test whether activity level affects sensitivity to trauma, the effect of injuring *Drosophila* at different times in their 24-hour circadian cycle was measured. Figure 6.7 shows the percentage survival following 5 hits at 90° in mature flies during the ‘morning’ (between 1 and 2 hours from the start of subjective day, Figure 6.7A), ‘afternoon’ (between 4 and 6 hours from the start of subjective day, Figure 6.7B) and ‘evening’ (more than 8 hours after the start of subjective day, Figure 6.7C).

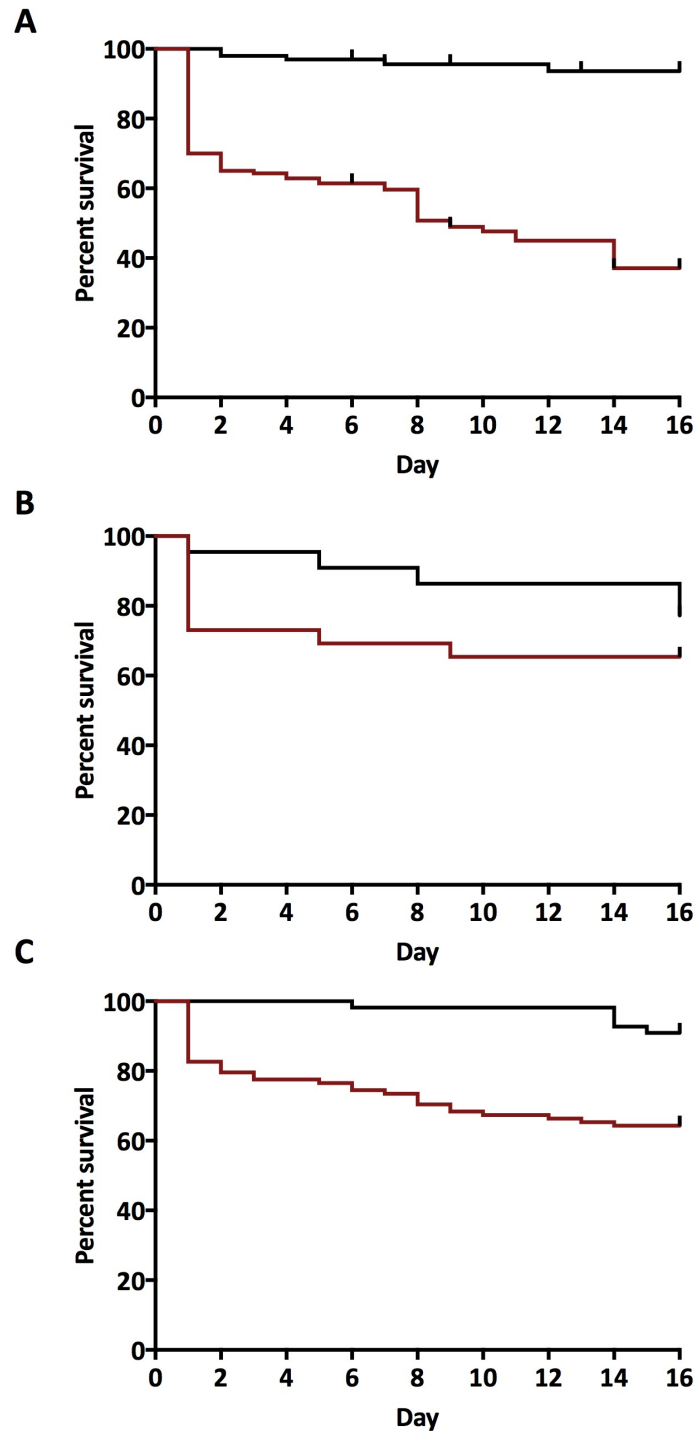


Figure 6.7: Variation in *Drosophila* sensitivity to trauma when flies are injured at different circadian times shown using Kaplan Meier survival curves. (A) Percentage survival after 5 hits at 90° (red line) versus sham (black line) when flies are injured in the ‘morning’ (1 – 2 hours after start of subjective day); 5 hits: $n = 140$ and sham: $n = 100$. (B) Percentage survival after 5 hits at 90° when flies are injured in the ‘afternoon’ (4 – 6 hours after start of subjective day); 5 hits: $n = 30$ and sham: $n = 20$. (C) Percentage survival after 5 hits at 90° when flies are injured in the ‘evening’ (more than 8 hours after start of subjective day); 5 hits: $n = 130$ and sham: $n = 60$. Log-rank tests indicated a significant difference in survival between groups injured in the morning compared with in the afternoon ($p < 0.05$) and the evening ($p < 0.001$). Percentage survival in injured flies was also significantly different ($p < 0.001$) to the sham group at all three phases of the day measured. Upticks represent points at which data was censored.

Percentage survival in injured flies was significantly different ($p < 0.001$) to the sham group at all three phases of the day measured over the course of the experiment. By the end of the study at 16 days, the survival of flies injured in the morning was $37 \pm 5\%$ compared to the sham group, which had $93 \pm 3\%$ survival. In the afternoon group, survival was $65 \pm 9\%$ compared to the sham group in which survival was $77 \pm 9\%$. Flies injured in the evening had $64 \pm 5\%$ survival at the end of the study compared to the sham group, which had $91 \pm 4\%$ survival. There was a significant difference ($p < 0.05$) found in mortality between groups that were injured during the morning and the afternoon as well as during the morning and the evening ($p < 0.01$). No significant difference in mortality was found between flies injured in the afternoon and the evening. Flies that were injured in the morning showed the steepest mortality curve, as indicated by the hazard ratio of 5 hits: sham, of 12.87 (95% CI = 8.33 to 19.88, Figure 6.7A) in the morning, compared with the afternoon (1.70, 95% CI = 0.59 to 4.86, Figure 6.7B) and the evening (4.69, 95% CI = 2.49 to 8.83, Figure 6.7C). Thus, *Drosophila* may be more sensitive to trauma in the early phase of their circadian cycle. There is also a trend to increased sensitivity in the evening compared to afternoon.

6.6 Treatment with hypothermia

Finally, to assess whether the model has the potential to be used as a high-throughput assay to test neuroprotective therapies, the effect of hypothermia was tested on survival. For a model of injury to be ultimately useful in research, it should allow the effects of neuroprotective treatments to be studied and quantified. There is an increasing interest in the use of prophylactic hypothermia as a neuroprotectant in various experimental models of neurological injury. Hypothermia is also simple to administer to *Drosophila* in a controlled environment and so was the treatment of choice. As flies were more sensitive to trauma in the early part of the subjective day, the effect of hypothermia on flies subjected to trauma in the ‘morning’ was investigated. Figure 6.8 shows the effect of treatment with cooling at 4°C on fly survival.

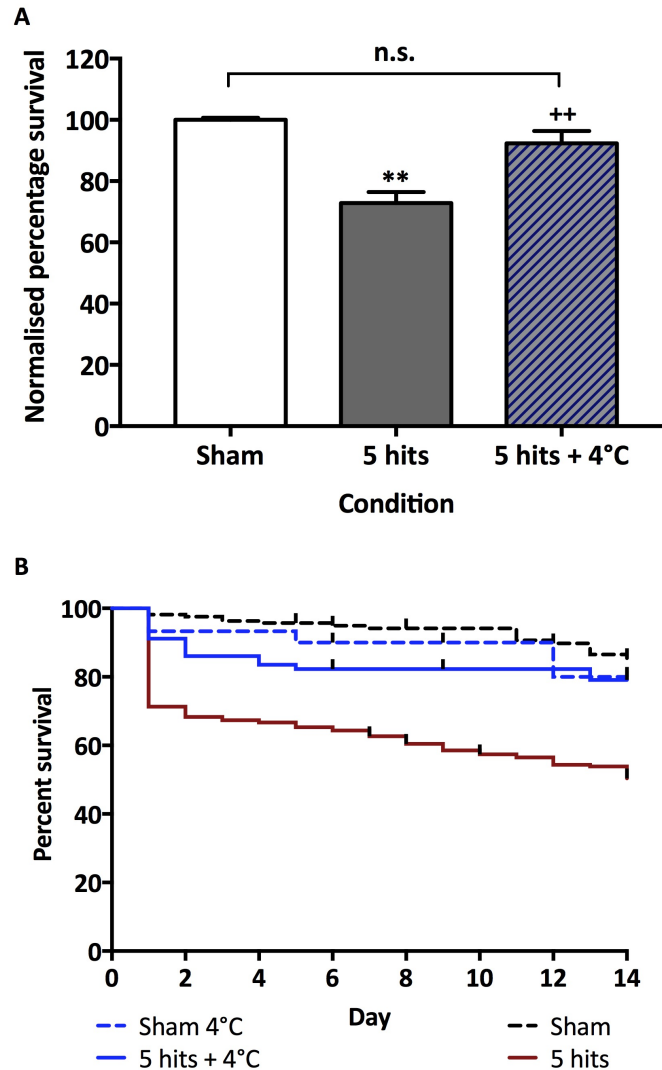


Figure 6.8: Cooling at 4°C for 30 minutes post trauma increases survival at 24 hours and over 14 days following treatment. (A) Survival following 5 hits at 90° (grey bar) and 5 hits at 90° + cooling at 4°C for 30 minutes (grey striped bar) normalised to sham injury (white bar) at 24 hours post trauma. Data represent means and error bars are SEM. **($p<0.001$) and ++($p<0.001$) compared to sham and injury with 5 hits at 90° respectively. (B) Kaplan Meier survival curves showing percentage survival with 5 hits at 90° + cooling at 4°C for 30 minutes (blue line), 5 hits at 90° (red line), sham at 4°C (blue dashed line) and sham (black dashed line). Log-rank tests indicated a significant difference ($p<0.0001$) between the 5 hits group and 5 hits + 4°C group. Upticks represent points at which data was censored. 5 hits at 90° + 4°C cooling: $n = 80$, 5 hits at 90°: $n = 300$, sham at 4°C: $n = 30$ and sham: $n = 250$.

30 minutes of cooling at 4°C decreased the effect of the primary injury (5 hits at 90°) on fly survival at 24 hours, compared to control injury, as indicated by a significant increase ($92 \pm 4\%$ with hypothermia treatment versus $73 \pm 4\%$ without hypothermia treatment) in normalised survival ($p<0.001$) in Figure 6.8A. The injured group treated with hypothermia ($92 \pm 4\%$) was also not significantly different ($p=0.38$) to the uninjured sham group ($100.0 \pm 0.7\%$). However the survival at 24 hours in 5 hits control injury group without hypothermia ($73 \pm 4\%$) was significantly lower than sham ($p<0.0001$). The K-M curve (Figure 6.8B) shows the percentage survival of sham, hypothermia treated and control injury flies over 14

days. Similar to survival at 1-day post trauma, the survival curves of the control injury and hypothermia treated group were significantly different ($p < 0.0001$). The hazard ratio of control injury: hypothermia treated flies was 2.55 (95% CI = 1.73 to 3.73), indicating that the mortality rate in untreated flies that received 5 hits was 2.6 times more than the mortality rate in flies that were subjected to the same injury but treated with hypothermia. There was no difference ($p = 0.56$) found between the survival of sham flies and hypothermia treated injured flies over the study period. By the end of the study, at 14 days post trauma, the survival in the group that received 5 hits followed by 30 minutes of cooling at 4°C was $79 \pm 5\%$, which was not significantly different to survival in the sham group ($85 \pm 3\%$) but was significantly higher ($p < 0.0001$) than survival in the control injury group ($50 \pm 3\%$).

Following the effect seen with hypothermia treatment at 4°C , the effect of cooling at 10°C on survival post injury was tested. Figure 6.9 shows the effect of treatment with 10°C cooling for 30 minutes post trauma.

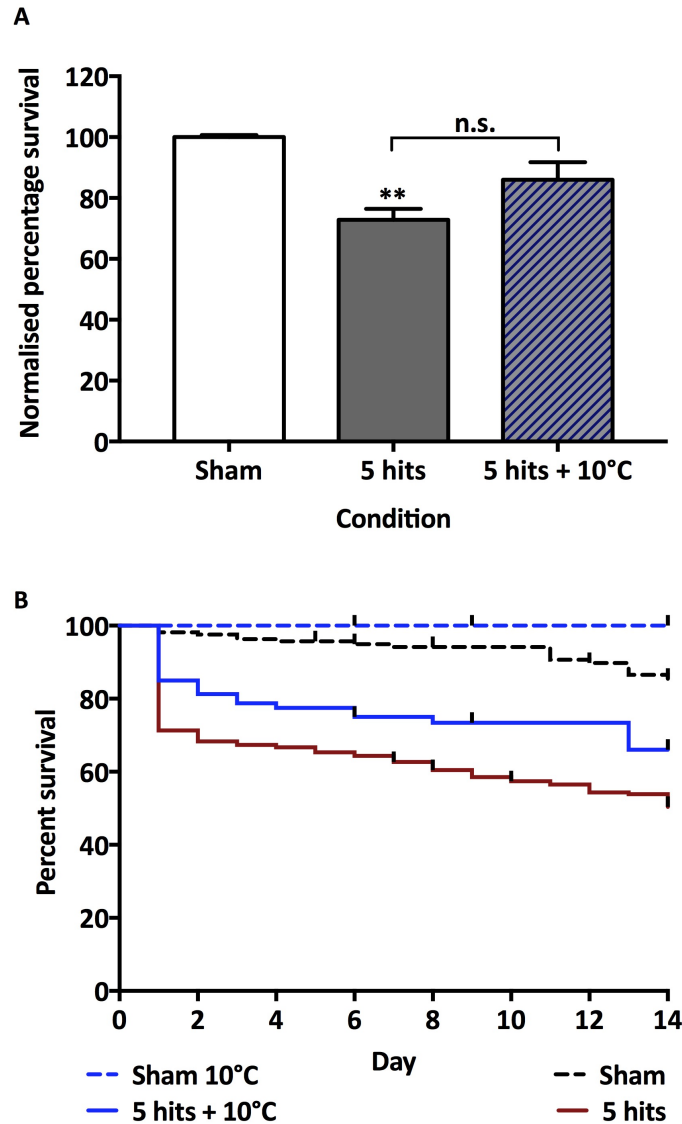


Figure 6.9: Treatment with hypothermia at 10°C post trauma does not improve survival at 24 hours but improves survival over 14 days following treatment. (A) Survival following 5 hits at 90° (grey bar) and 5 hits at 90° followed by cooling at 10°C for 30 minutes (grey and blue striped bar) normalised to sham injury (white bar) at 24 hours post trauma. Data represent means and error bars are SEM. **($p < 0.001$) compared to injury with 5 hits at 90°. (B) Kaplan Meier survival curves showing percentage survival with 5 hits at 90° followed by cooling at 10°C for 30 minutes (blue line), 5 hits at 90° (red line), sham at 10°C (blue dashed line) and sham (black dashed line). Log-rank tests indicated a significant difference ($p < 0.05$) between the 5 hits group and 5 hits + 10°C group. Upticks represent points at which data was censored. 5 hits at 90° + 10°C cooling: $n = 80$, 5 hits at 90°: $n = 300$, sham at 10°C: $n = 30$ and sham: $n = 250$.

At 24 hours post trauma, the survival in the group that had 5 hits followed by 30 minutes of cooling at 10°C was higher ($86 \pm 6\%$) than the control injury group ($72 \pm 4\%$) although the difference was not significant ($p = 0.07$). However, survival in 10°C treated injured flies was significantly higher ($p = 0.02$) than control injury flies over the course of the 14-day study. At 14 days post trauma, the survival in the group that received 5 hits and 30 minutes of cooling at 10°C was $66 \pm 6\%$ whereas the survival in the control injury group was $50 \pm 3\%$. The

hazard ratio of control injury flies to 10⁰C treated flies was 1.59 (95% CI = 1.09 to 2.33) indicating that the rate of mortality of control injury flies was nearly 1.6 times higher than hypothermia treated flies over the 14-day experiment. This suggests a subtle improvement in survival due to 10⁰C cooling although not to the extent of the sham as seen with 4⁰C cooling. Thus, the protective effect of hypothermia treatment in *Drosophila* is increased at lower temperatures.

7 Chapter 7: Discussion

The overall theme of this thesis is a study of models of TBI and drugs and treatments both individually and in combination to treat the condition. The results presented describe the refinement of injury quantification methodology in an *in vitro* model of TBI, followed by a study of noble gases, drugs and combination therapies to treat TBI. In addition, experiments involving the setting up and calibration of an *in vivo* controlled cortical model of TBI are described, including histological outcomes and cytokine expression profiling. Finally, to explore the potential of HTS models to investigate TBI, a model of polytrauma was established using the fruit fly *Drosophila melanogaster* and the effect of hypothermia treatment following injury was investigated. In this chapter the suitability of the various models/outcomes measured to investigate human TBI is discussed, results presented are compared with previously published results and the significance of results presented with regard to future of TBI research is discussed.

7.1 Modelling TBI *in vitro* using organotypic hippocampal slices and the weight drop device

In this thesis, TBI was modelled *in vitro* using OHSC from neonatal C57/BL6 mice and a weight drop device that creates a focal contusion type injury. In the following sections, injury to the hippocampus, and organotypic slices are evaluated as representations of the *in vivo* brain. Following this, advantages and limitations of the *in vitro* traumatic injury procedure and the injury quantification methodology developed in chapter 3 are discussed. Finally, the suitability of organotypic slices and the weight drop model to study combination drug treatments for TBI are discussed.

7.1.1 The hippocampus as the brain region for this *in vitro* study

The hippocampus was chosen as the brain region for investigation in this model because it is one of the most vulnerable regions to damage following a brain injury[254]. Hippocampal atrophy, and particularly CA1 damage, has been documented in both animal and human studies of TBI[255,256]. In a study by Kotapka and colleagues[254], hippocampal damage was noted in 84% of individuals who suffered from non-penetrating head injuries whilst

bilateral neuronal loss in the hippocampus was noted in a further 50% of these individuals. Hippocampal atrophy following TBI may be attributed to the spread of secondary injury, while direct impact to the hippocampus depends on the severity of the insult.

One of the reasons for the sensitivity of the hippocampus to damage after TBI may be due to the high density of receptors thought to play a role in secondary injury development. The hippocampus is a region of high concentration of NMDA receptors. For example, in a study[257] that used C57/BL6 mice, it was previously found that the GluR ϵ 1/NR2 and GluR ϵ 2/NR2B subunits of the NMDA receptor had the highest concentration in the hippocampus, while the GluR ζ 1/NR1 was also highly concentrated in the hippocampus. Within the hippocampus, the same study showed that the highest levels of these subunits were detected in the CA1 followed by the CA3 pyramidal cell layers. Further, GABA_A subunits are heterogeneously distributed in the different hippocampal subfields. A study by Hortnagl and colleagues found that α 1, α 2, α 4, α 5 and β subunits were abundantly expressed in various subfields of the hippocampus as well as the DG[258]. It was subsequently found that TBI induces transient down-regulation in the expression of these subunits[259], increasing excitation. Moreover, basal COX-2 expression is abundant in neurons of the hippocampus – pyramidal and granule layers and is regulated by normal glutamatergic synaptic activity. Neuronal induction occurs subsequently after a TBI and other brain injuries[183]. Strauss and colleagues characterised the temporal expression of COX-2 following TBI in the hippocampus. They found that COX-2 was upregulated as early as 2 hours after injury and progressed from the CA1 region to the CA2 and CA3 regions. A high level of expression persisted for at least 3 days post trauma[186]. Finally, in terms of inflammatory cytokine levels in the hippocampus, various studies examining the anti-inflammatory potential of drugs have noted a hippocampus-specific downregulation of cytokines like TNF and IL-1 β following treatment with anti-inflammatory drugs after TBI[35]. Therefore, the hippocampal area is a good model for excitotoxicity and the inflammatory response, which may also explain its sensitivity to injury. This makes the hippocampus is an important brain region to study when examining the potential of pharmacological compounds in treating TBI.

7.1.2 Organotypic slices as representations of the *in vivo* brain

In vitro cell culture models are important tools in basic science because they enable the study of cellular processes in an isolated system, facilitate the screening of therapeutic molecules and allow precise connections to be made between neuropathological outcomes and molecular changes. In addition, *in vitro* models allow more information to be obtained from each animal, thus reducing the total number of animals required compared to *in vivo* experiments. Organotypic slices represent a major step towards a more realistic simulation of what occurs *in vivo*, compared to isolated cells, as they allow several morphological and synaptic features of the brain to be maintained. Table 7.1 summarises other tissue surrogates that are used in the study of *in vitro* TBI.

Table 7.1: Table summarising alternative tissue surrogates in the study of *in vitro* TBI[194,195,207]

Method	Description	Advantages	Limitations
Primary dissociated cultures	-Mechanically and enzymatically dissociated astrocytes, microglia or neurons from brain tissue	-Effect of injury on a single cell type may be examined or between types when cells are cultured together in defined ratios	-Host age affects cellular phenotype and injury response -Most cultures are performed on monolayers, yet neural cells behave differently when cultured in constructs
Acute slices	Explants of CNS tissue, freshly isolated and prepared in thin slices (<475µm) of the hippocampus, cerebellum, frontal cortex etc.	-No restrictions on the age of the harvested tissue -Anatomical structure, neuronal circuitry, and heterogeneous cell populations found <i>in vivo</i> are preserved. -Extracellular matrix is intact with its <i>in vivo</i> composition and bioactivity which may be critical in dictating the injury response	-Experiments must be completed within 8 hours before the tissue begins to deteriorate -Whether isolation modifies injury response remains an open question -Response may be altered by dilution of secondary injury mediators into the bathing medium
Immortalized cell lines	PC2/PC12 – differentiate into neuronal like cells with processes BV-2 – microglia	-Easy to procure, consistent across laboratories, well characterized, and easy to scale up for large studies	-Entails disruption of normal intracellular signaling cascades and apoptotic machinery -Immortalized cells can suffer from disruption of contact dependency and poor adhesion – problem for inflicting injuries

As an *in vitro* model, organotypic slices have been termed “closest to an *in vivo* situation”[204]. Compared to primary single-cell cultures, the complex three-dimensional architecture is partly preserved in organotypic slices and partly disconnected pathways can also be re-established. Whilst acute slices have been known to preserve the anatomical structure and neural circuitry of an *in vivo* brain[195], a limitation of acute slices is that experiments need to be performed on the day of slicing, which brings into consideration the effect of the tissue explantation and slicing procedure. In contrast, not only do slice cultures maintain the cytoarchitecture of the brain, longer culture periods (in this study 11 – 14 days) for organotypic slices enable them to return to a stable, quiescent state before an experiment. Moreover, while acute slices only remain viable for experiments for 24 hours, organotypic slices may be used for longer experiments.

Moreover, the mix of cell types and particularly the presence of populations of glial cells in OHSC is often not found in simpler *in vitro* models and is relevant to the complex neuropathology of TBI[260]. Astrocytes represent the major non-neuronal cells in the brain, but microglia also comprise approximately 15% of the brain cell population[206]. The axons of oligodendrocytes become myelinated over the first 14 days of the cell culture period in organotypic slices. All of these cell types are present in organotypic slices, enabling their interaction to be studied.

One point to consider is the significance of age and maturity of the slices in culture. The question of studying the developmental situation versus studying a mature adult situation following TBI is complex for two reasons. Firstly, although it has been shown by confocal microscopy that organotypic slices cultured for 1, 2 or 3 weeks were developmentally identical (neuronal structure, spine density, total neuronal number) to brain slices dissected at postnatal day 14, 17 and 21[261], it remains to be investigated whether organotypic slices cultured for more than 2 or 3 months can represent a mature adult neuron. However, the same logic applies to how closely a 2-month old rat or even a 1-year-old mouse compares with an adult human. Secondly, OHSC are only suitable to study the short term effects of a drug post injury because the slices die within a few days of intense or prolonged injury[262]. When evaluating the ability of OHSC to represent the *in vivo* situation following a TBI, one must also evaluate how closely *in vivo* animal models represent the human situation. However, taken together, organotypic slices are an innovative and robust *in vitro* system and the many advantages of this model support its use in studying neuroprotection in TBI.

7.1.3 Advantages and limitations of the *in vitro* traumatic injury procedure

Table 7.2 summarises various techniques used to model TBI in OHSC.

Table 7.2: Table summarising various *in vitro* TBI injury models

Injury method	Description	Advantages	Limitations
Transection Morrison <i>et al.</i> , [195]	Axotomy using a plastic stylet, rotating scribe or blades	-Can be scaled to perform high throughput therapeutic screening -Excitotoxicity and glial activation are observed	-Injury parameters are not prescribed -Clinically relevant for a small percentage of brain injuries.
Compression Adamchik <i>et al.</i> , 2000[207] Adembri <i>et al.</i> , 2004[263] Sieg <i>et al.</i> , 1999 [207,264]	Weight drop method or rolling a stainless steel cylinder over the slice	-Injury severity controlled by adjusting force, depth, shape of weight and duration -Generates a region of primary and secondary injury	-Complete characterization of biomechanics is not possible because it is difficult to measure applied force and tissue deformation
Hydrostatic pressure Adembri <i>et al.</i> , 2004 [263]	Application of transient or static pressure to cultures induces injury	-Accurately reproduces the time course of pressure changes during closed-head TBI or during FPI <i>in vivo</i>	-Pressure must be increased above levels that occur during TBI to achieve a cellular response -Brain deformation is small difficult to verify
Stretch injury Morrison <i>et al.</i> , 2000[194] Morrison <i>et al.</i> , 2011[195]	Stretching membrane upon which slices are cultured	-Precise control of injury parameters -Re-creates axonal injury by injuring cell processes without confounding effects of cell body deformation	-Injury is not homogenous nor well characterised

Among these methods, compression or focal injury[207,264] is the only method that enables the study of secondary injury in slices, attributed to the process of cortical spreading depression (CSD) observed following weight drop injury in slices[265]. CSD is defined as a depolarisation wave in cerebral grey matter that propagates across the brain at a (slow) velocity of 2 – 5 mm/min and is induced by electrical or mechanical stimulation[266]. It is characterised by a depression of evoked and spontaneous EEG activity, which is caused by failure of brain ion homeostasis and efflux of excitatory neurotransmitters[267]. Because a direct injury to the hippocampus depends on severity of insult and whether penetrating or not, it is important that the *in vitro* model allows for the study of secondary injury. For this reason, injury and the effect of treatments was measured at different times over a 72-hour

period. That secondary injury does develop in the model used for this study was shown by Harris *et al.* [93] who found that injury with an impact of 3.4μJ produced a primary injury (measured as $14 \pm 1\%$ of total injury at 72 hour, at 30 minutes post trauma) that developed over 72 hours. Additionally, in the current study with the same impact energy, injury also developed significantly between 24 and 48 hours and between 48 and 72 hours (Figure 3.15). The injury at 24 hours was $57 \pm 3\%$ and at 48 hours it was $72 \pm 3\%$ of the control injury at 72 hours, indicating that there was the development of secondary injury following the initial impact. These values are similar to those of Harris *et al.* who found that the injury at 24 hours was approximately 60% and at 48 hours, 80% of the control injury at 72 hours[93].

The outcome used to quantify injury was cell death indicated by PI fluorescence. PI is one of the most frequently used dyes to study the viability of cells in living slices. An advantage of live cell staining is that the slices can be imaged repeatedly using a fluorescence microscope, which enabled the development of injury at each time point to be quantified in the same slices. Moreover, the use of PI fluorescence is an established technique[93,104,209,263,268] and PI uptake been shown to correlate with cell death[268]. PI enters cells with compromised membranes, binds DNA and RNA and becomes fluorescent, which is detected by a fluorescence microscope. As hippocampi from animals of the same age were used, the slices were all of a similar size. The standardisation of the experimental and fluorescence imaging protocol (e.g. all slices were exposed to PI for the same amount of time, standardisation of image acquisition settings discussed in chapter 3, enabled robust comparisons to be made between treated and untreated injured slices using PI fluorescence.

A limitation of PI is that like other live cell stains (Hoescht, DAPI, ethidium bromide, SYTOX dyes), it is not specific for a particular cell type. In terms of cell death following TBI, there is evidence to suggest that both apoptotic and necrotic mechanisms play a role and occur as a spectrum rather than discreet events. Necrosis occurs on a timescale of minutes and so is harder to target due to a narrow therapeutic window. Clinical and preclinical studies suggest that a second wave of delayed cell death occurs by apoptotic (programmed) cell death[38]. However, both apoptotic and necrotic neurons have been found within contused tissue in the acute traumatic period as well as regions distal from the impact site in the days and weeks following TBI[269]. As this is a model of secondary injury following TBI, both types of cell death would be represented. For the purpose of the current study, the therapeutic properties of drugs were examined from a more holistic viewpoint of whether they are

neuroprotective or not following TBI. The more protective a drug and the earlier this protection is apparent post trauma, the more likely it is that cell death would be arrested.

7.1.4 Advantages and limitations of the injury quantification methodology

The development of an injury quantification methodology presented in chapter 3 was an opportunity to refine the model. A more adaptable and robust method of injury quantification was established, however not without limitations. The biggest challenge was ensuring that the health of the cultures did not affect the decision to eliminate a particular exposure time from consideration, if for example in a particular week a significant proportion of slices were unhealthy. Organotypic slices are highly sensitive to their environment and factors like pH, temperature and culture media. Although a consistent effort was made to ensure that the environment is as sterile as possible, there were a proportion of slices (typically 20 – 30%) lost to poor viability in every experiment. Thus, viability in each set was assessed relative to one another. Slices were imaged at a reference exposure (1.5s) and a ‘test’ exposure for every experiment. Viability for the sets was assessed at the same exposure time (1.5s) and a comparison between experiments was only made if the viability for the sets was similar. This ensured that an exposure time was not incorrectly eliminated due to a high viability failure in that particular week causing an overestimation of injury development. Experiments in which the viability was less than 60% were excluded from analysis to ensure that the effect of poor viability on injury development is minimized.

While this was an extra factor to consider, it also ensured that the viability criteria developed (section 3.4) took into account slices from a number of different experiments, making the final criteria more reliable. This would otherwise not have been possible if only a few experiments were examined, given that slices were initially allotted to ‘viable’ and ‘not viable’ groups based on the absence of PI staining observed by eye. The viability criteria were more stringent as a result of this. Thus, evaluating slices from experiments conducted over several months enabled a quantitative and objective criteria to be developed.

Further, this process presented the opportunity to refine certain aspects of injury quantification in the model. Because the model involves an objective quantification of fluorescence, it is important that any sources of variability in the microscope and settings should be eliminated. This process presented the opportunity to study the effects of optical

parameters on the intensity of the fluorescent image. Experiments were done to determine maximum fluorescence obtainable from a slice in order to ensure that the selection of image acquisition settings did not result in a saturated image. Finally, precise experiments were conducted to decide the calibration intensity reference value used to control for changes in microscope lamp intensity between imaging sessions. Previously, this value was simply an arbitrary number. Image acquisition settings such as exposure time, gain and gamma were adjusted to determine their effect on image intensity levels and arrive at a final calibration reference value which would be used to determine the relative exposure time for imaging each session.

An alternative approach and indeed to validate the methodology developed and extend this work further would involve examining other markers of cell death and measure the change in fluorescence intensity from the site of impact. For example, drawing a mask around the region of primary injury surrounded by further concentric circles extending over the area of the slice would enable the change in fluorescence from time point to time point to be measured relative to the site of impact. The lactate dehydrogenase (LDH) assay is another common measure of cell integrity and similar to PI, is based on changes in cellular membrane permeability upon injury. The MTT assay, which measures the metabolic conversion of yellow MTT dye by active mitochondria to insoluble blue precipitates offers yet another measure of viability. Results obtained using PI in this thesis may be cross-validated with other markers. Additionally, the same process could be repeated with markers that are specific to neurons (NeuN), glial cells (GFAP) and microglia (Iba-1) to isolate the proportion of neurons from glial cells that are affected by injury. Other markers include fluoro-jade (selects for neurons undergoing degeneration and stains cell bodies, dendrites and axon fibres), CD11b (marker of microglia/macrophage recruitment), CD45 (expressed on nucleated hematopoietic cells and CD68 (marker of active phagocytosis).

7.1.5 Rationale for the use of the *in vitro* TBI model to study combination treatments

For proof of principle studies, *in vitro* models offer a realistic screening platform to test combination therapies. The *in vitro* weight drop model is able to reproduce tissue distortion of blunt TBI and activates secondary injury pathways (section 7.1.3). Moreover, the hippocampus contains many of the major targets for the drugs that were studied in

combination treatments (section 7.1.1). An advantage of organotypic slices over *in vivo* models is that the precise control which can be exerted over the extracellular environment[195]. Controlling the concentration of the compounds in the media is particularly important for combination studies because the effects of the combination on cell death could be isolated. Moreover, due to repeated access to the tissue, the effects of treatment at multiple time points could be monitored as opposed to an *in vivo* situation where analysis of the damaged tissue can only be carried out at a single time point after trauma. Thus, the model offered a low-cost and robust platform to screen combination therapies in this study. In another OHSC model based on PI fluorescence, it was recently shown that combination treatment with 17 β -estradiol and memantine (non-competitive NMDA antagonist) was more protective than monotherapies, following stretch TBI injury[270]. Moreover, combination treatment with xenon and hypothermia was shown to be neuroprotective in a weight drop model using OHSC and PI fluorescence[103]. In addition, in another OHSC model of ischemic injury based on PI fluorescence it was shown that combination therapy with MK-801 (NMDA antagonist) and α -phenyl-*tert*-butyl-nitron (free radical scavenger) enhances protection against neuronal damage[271]. These studies support the use of OHSC models of TBI to study combination treatments. The present study will add to the body of evidence for the use of *in vitro* models of TBI to study combination treatments.

7.2 Neuroprotection with noble gases, drugs and combination treatments in an *in vitro* model of TBI

The following sections refer to results presented in chapter 4, aimed at studying neuroprotection with drugs and noble gases individually and in combination. In the present study, all drugs were administered only after TBI injury to increase the clinical relevance of the study. Progesterone and meloxicam were administered *via* a solution exchange that did not cause any additional damage (Figure 4.1) and nor were the drugs toxic to sham slices (Figure 4.10 and Figure 4.31).

7.2.1 Xenon is neuroprotective following TBI *in vitro*

In the present study, treatment with 50% xenon abolished the development of injury at 24, 48 and 72 hours, to the level of sham injury at each time point (Figure 4.2). This is similar to the

results found by Harris *et al*[93]., who showed the same. In the experiments presented here, 25% xenon attenuated, but did not abolish, the development of injury each time point (Figure 4.3). However, treatment with 10% xenon was not protective at any time point (Figure 4.4). To examine the concentration response relationship of xenon, the degree of protection at the 72-hour time point was taken as the reference for comparison (Figure 4.5). As a percentage of control injury, the protective effect of xenon increased with concentration to $45 \pm 5\%$ at 25% xenon and $66 \pm 4\%$ at 50% xenon. It was not possible to get more protection than the level of injury in the sham slices, which represents $29 \pm 4\%$ of control injury at 72 hours. This trend is similar to the concentration response relationship observed by Harris *et al*. [93], where the protective effect of xenon increased from $40 \pm 8\%$ protection at a concentration of 30% to abolishing injury to the level of the sham, at 50% xenon. Few studies have examined the concentration response relationship of xenon following TBI. However, the concentration response relationship in the present study is also similar to that seen in a mouse model of neuronal-glial culture, where xenon (10 – 75%atm) showed a concentration dependent protective effect on injury induced *via* application of NMDA ($IC_{50} = 19 \pm 6\%$) and glutamate ($IC_{50} = 28 \pm 8\%$) [107]. So far, there has been only one study that assessed xenon's neuroprotective efficacy in TBI *in vivo*. Xenon was equally effective at concentrations of 30%, 50% and 70% at reducing contusion volume (by approximately 19%) 24 hours post TBI [108]. This is a promising finding for xenon's translatability to the clinic. On one hand, a 19% reduction in contusion volume is a small improvement and there is certainly the potential to enhance his effect *via* combination therapies with xenon. On the other hand, that 30%, 50% and 70% xenon were equally effective, there is the potential to combine even lower doses of xenon (15 – 20%) and determine how far down in concentration it is possible to go and still achieve neuroprotection. There is also the additional possibility to combine a percentage of argon with 30% xenon and another drug in a 'cocktail' therapy.

7.2.2 Argon is neuroprotective following TBI *in vitro*

In this study, treatment with 50% argon attenuated the development of injury at 24, 48 and 72 hours (Figure 4.6). Treatment with 25% argon also attenuated, although to a lesser extent, the development of injury at each time point (Figure 4.7). However 10% argon was not protective (Figure 4.8). Thus, the neuroprotective effect of argon also displays concentration response dependence (Figure 4.9). As a percentage of control injury, the protective effect of argon increased with concentration to $29 \pm 4\%$ at 25% argon and to $41 \pm 4\%$ at 50% argon.

The protective effect of 25% argon is more potent in the current study than in the study by Harris *et al.*, where at a concentration of 30%, argon was devoid of any protective effect[93]. However, Harris *et al.* observed a similar reduction to $40 \pm 10\%$ of the control injury using 50% argon[93]. The discrepancy at 25% argon may be attributed to sample size, which was over 10 times higher in the present study. Under slightly different conditions (normobaric pressure, higher impact force of 5.26 μ J and a higher threshold above which data was analysed) in a study by Loetscher *et al.*, 25% argon was twice as effective, providing $63 \pm 4\%$ protection at 72 hours. They also observed a higher protective effect with 50% argon, which was $86 \pm 3\%$ effective at 72 hours[129]. Given that there are opposing studies with regards to argon's protective effects at lower concentrations in TBI *in vitro*, the findings of the present study provide more evidence for argon's neuroprotective effect at lower concentrations.

Argon's concentration dependent effect has been demonstrated *in vivo* by Brucken *et al.* who observed that animals treated with 70% argon had significantly less neurological impairment than animals treated with 40% argon in a model of cardiac arrest[125]. In a model of *in vivo* excitotoxic injury, 25% argon reduced the volume of neuronal death although the reduction did not reach significance compared to the untreated control injury. However, in the same study, at 50 hours post trauma, 37.5% and 50% argon significantly reduced neuronal damage with the reduction reaching a peak at 50% argon[272]. In Figure 4.9 we can see that argon approaches its peak level of protection at the 50% concentration *in vitro*. Injury is more complex *in vivo* and NMDA induced excitotoxicity does not directly compare to a clinical setting. Thus, more *in vivo* studies of TBI are required to ascertain whether the neuroprotection with lower doses of argon are translatable *in vivo* as so far, most studies have focussed on concentrations above 50% argon and have used other models of brain injury[125,130].

7.2.3 Progesterone is neuroprotective following TBI *in vitro*

At 50% helium pressure (Figure 4.11), 3 μ M progesterone was not protective at 24 hours, but was protective at 48 and 72 hours. In contrast, 10 μ M and 25 μ M progesterone were protective at all time points. Neuroprotection with progesterone applied at 25% helium pressure was similar, however all 3 concentrations of progesterone were protective at the 3 time points (Figure 4.13). At both partial pressures of helium, progesterone's protective effect was

concentration dependent at 72 hours and levels off at a concentration of 25 μ M, displaying similar concentration dependence (Figure 4.12 and Figure 4.14). At 50% helium pressure, 3 μ M progesterone was $23 \pm 6\%$ protective, 10 μ M progesterone was $33 \pm 7\%$ protective and 25 μ M progesterone was $50 \pm 4\%$ at 72 hours after injury. Similarly, the protective effect of progesterone at 25% helium pressure increased with concentration from $27 \pm 6\%$ at 3 μ M to $36 \pm 5\%$ at 10 μ M and $45 \pm 4\%$ at 25 μ M progesterone, 72 hours after injury. Therefore, there was no effect of added pressure on progesterone's efficacy.

Surprisingly, there have been no studies that have assessed progesterone's efficacy in *in vitro* models of TBI so far. The concentrations of progesterone studied in this thesis were based on those used in an *in vitro* study of OGD ischaemic injury in OHSC[141], where 10 μ M progesterone was 25% protective and 1 μ M progesterone was 10% protective at 24 hours following OGD. It is difficult to directly compare an *in vitro* ischemic injury model with a TBI model because the injury from TBI is less severe due to the focal nature of the injury in this model and because cell death is assessed at 24 as opposed to 72 hours in TBI. However, it is not unexpected that a similar dose of progesterone, when applied to a TBI model, is more protective.

Progesterone has been tested extensively *in vivo*; in over 50 models of TBI, ischemia and intracerebral haemorrhage[70]. The dose response effect of progesterone following TBI (CCI model) has been studied by Goss *et al.* who found that 8 and 16mg/kg progesterone were protective (assessed by performance on Morris water maze), however 32mg/kg was not protective, with the most potent protective effect seen at 16mg/kg. This study showed that low and moderate doses of progesterone are optimal for facilitating recovery but that progesterone loses its protective effect at higher concentrations[273]. Following this study, the vast majority of animal studies with progesterone have used only a single dose – 16mg/kg for rats and 4 or 8mg/kg for mice[59,144,150,160,168,170,171,173,274,275]. The concentrations used in this *in vitro* study correspond with the doses used *in vivo*. When calculated, it was found that 25 μ M progesterone equates to the 8mg/kg dose used for mice and 3 μ M progesterone equates to a 1mg/kg dose used in human clinical trials for TBI (see section 1.4.3.4). However, factors such as bioavailability, route of administration and pharmacokinetics need to be considered which makes a direct comparison between the *in vitro* and *in vivo* situation challenging. As such, a time dependent dose response curve such as the one constructed in the present study is preferable to directly comparing and equating a

dose. No other dose response studies have been conducted *in vivo*; more dose response studies evaluating a variety of outcomes is needed to conclude whether the protective effect of progesterone is lost at higher concentrations. This loss of effect was not observed in the present *in vitro* study, however, doses higher than 25 μ M were not tested so although the protective effect levels off at 25 μ M, we cannot conclude whether higher doses will be less protective. Overall however, progesterone has shown neuroprotective effects *via* a number of mechanisms (see Figure 1.7) which have been translated into positive outcomes in behavioural and functional measures[144,160,168–174].

Progesterone showed promise in Phase II clinical trials but showed no additional benefit compared to placebo in Phase III clinical trials (trials described in section 1.4.3.4). One of the key reasons cited for failure of Phase III testing was the lack of any dose/dosing schedule optimisation prior to testing[179]. Extremely high doses of progesterone were used in the trial; the reasons for this are put down to insufficient pre-clinical testing of the dose response effect[180]. Thus, before embarking upon combination studies with progesterone *in vitro*, it was important to establish dose dependence in this study. Further, in order to test the hypothesis that progesterone may be an effective adjunct therapy with noble gases, it was important to test that the administration of progesterone at different pressures did not interfere with its protective effect. The positive effect of progesterone seen in rodent models demonstrates its ability as a neuroprotective therapy, but its failure in clinical trials points towards the need to adopt a different strategy. Evidence for progesterone's dose dependent protective effect *in vitro* (section 4.6) forms the basis for testing combination therapies following TBI *in vitro*.

7.2.4 Neuroprotection with co-administration of progesterone and argon

After establishing progesterone's concentration dependent effect, the next step was to determine whether co-administration of progesterone and argon is more protective than argon alone and whether the combined protective effect is greater than the individual efficacies of the drugs. In all combination experiments, the expected protection was calculated using the Bliss Independence method based on the complete additivity of probability theory and compared with the actual protection measured. In order to determine if progesterone is able to increase efficacy of argon's sub-maximal protective effect, 3 μ M, 10 μ M and 25 μ M progesterone was tested with 25% and 50%atm argon. At these doses, argon provided $29 \pm$

4% and $41 \pm 5\%$ protection at 72 hours respectively, but neither dose abolished the injury. At 24 and 48 hours, co-administration with $3\mu\text{M}$ and $10\mu\text{M}$ progesterone did not significantly increase efficacy of 25% argon (Figure 4.15 and Figure 4.16). In contrast, the addition of $25\mu\text{M}$ progesterone significantly increased protection with 25% argon at the earlier time points (Figure 4.17). However, by 72 hours, all three concentrations of progesterone significantly increased the protection attained with 25% argon, to $50 \pm 5\%$ at $3\mu\text{M}$, $54 \pm 3\%$ at $10\mu\text{M}$ and $64 \pm 4\%$ at $25\mu\text{M}$ progesterone (Figure 4.18). Protection achieved with a combination of $25\mu\text{M}$ progesterone and 25% argon was also not significantly different to the maximum attainable protection (indicated by sham injury level), which suggests that this combination abolishes injury at 72 hours. Thus, the combined protective effect of progesterone and 25% argon is positively correlated with the concentration of progesterone in the combination. The almost parallel upward shift in dose response curves of progesterone acting alone and in combination with 25% argon suggests an additive effect of the combination (Figure 4.18). The combined protective effects at each concentration of progesterone were also equal (within error) to the expected combined effect calculated using the Bliss independence approach assuming additivity, which suggests that 25% argon in combination with $3\mu\text{M}$, $10\mu\text{M}$ and $25\mu\text{M}$ progesterone has an additive protective effect at 72 hours. These results are significant because by increasing doses of progesterone, the protective effect of 25% argon can be increased from $29 \pm 4\%$ (acting alone) to abolishing injury when co-administered with $25\mu\text{M}$ progesterone.

Next, 50% argon was tested in combination with progesterone to determine if co-administration with doses of progesterone increases protection by argon (from $41 \pm 5\%$) either at an earlier time point or at a lower dose of progesterone. The addition of $3\mu\text{M}$ progesterone does not significantly increase protection by argon at early time points (Figure 4.19), however, the addition of $10\mu\text{M}$ and $25\mu\text{M}$ progesterone abolishes injury at 24 and 48 hours and this effect is significantly different to protection by 50% argon acting alone (Figure 4.20 and Figure 4.21). Protection with $10\mu\text{M}$ progesterone and 50% argon is also significantly different to protection with progesterone alone at early time points. At 72 hours, the combined effect of 50% argon and progesterone abolished injury at all concentrations of progesterone and this was significantly different to the effect of argon and lower concentrations of progesterone acting alone (Figure 4.22). Thus, while with 25% argon the highest concentration of progesterone tested ($25\mu\text{M}$) was required to abolish injury at 72 hours, with 50% argon, co-administration of even the lowest concentration tested ($3\mu\text{M}$)

abolished injury. Moreover, the combined protective effect obtained with 3 μ M progesterone and 50% argon was higher than the expected effect assuming additivity, which indicates a synergistic protective effect at this concentration ratio. The combinations with 10 μ M and 25 μ M progesterone produced an additive combination effect, as protection was consistent with predicted values calculated using the Bliss independence approach. However, when isobolograms were constructed using the Loewe additivity method, an overall synergistic mode of action was suggested between argon and progesterone. Thus, through testing additional concentration ratios of argon and progesterone, additional synergistic combinations such as 3 μ M progesterone and 50% argon may become apparent. As synergistic and additive effects depend on the doses of each drug in the combination[276–279], testing several dose ratios is useful in the development of an optimisation strategy. My finding that the combined protective effect of 3 μ M progesterone and 50% argon is synergistic whereas other combinations are additive is important and suggests this particular combination merits further investigation.

7.2.5 Neuroprotection with co-administration of progesterone and xenon

After determining that different doses of progesterone increase the efficacy of argon, the next step was to test whether a similar effect is seen with co-administration of progesterone and 25% xenon, which is sub-maximally effective, providing $44 \pm 5\%$ protection at 72 hours when administered alone. When administered with 3 μ M and 10 μ M progesterone (Figure 4.23 and Figure 4.24), the combination appeared more protective than xenon alone but this effect did not reach significance at any time point. However, when the dose of progesterone is increased to 25 μ M, the protective effect of the combination was significantly greater than the protective effect of xenon alone at all three time points (Figure 4.25) and abolished injury to sham levels. At 72 hours, the protective effect of the combination was significantly greater than the protective effect of progesterone acting alone at all three time points (Figure 4.26). Based on expected values for the combined effect calculated using Bliss independence, 25% xenon and progesterone in combination have an additive effect. Therefore, the increase in xenon's protective effect when co-administered with progesterone is concentration dependent upon progesterone and increasing the dose of progesterone sufficiently is able to abolish injury to the level of the sham.

For completeness, the combination protective effect was also tested with 50% xenon (Figure 4.27 - Figure 4.30). 50% xenon abolished injury development, providing $65 \pm 5\%$ protection that is not significantly different to the sham at 72 hours and thus the maximum attainable protection. As expected, the addition of progesterone did not increase the protective effect of 50% xenon. At concentrations of $10\mu\text{M}$ and $25\mu\text{M}$ progesterone, the combination of progesterone and 50% xenon reduced injury down to the level of the sham, which is consistent with the expected combination effect calculated using Bliss independence. Isobologram analysis also revealed an additive interaction between xenon and progesterone. However, although an additive effect was expected from the combination of $3\mu\text{M}$ progesterone and 50% xenon, the observed effect was sub-additive ($54 \pm 4\%$ versus abolishing injury as expected). This shows that dose optimisation is important in combination neuroprotection experiments because ranges of dose ratios are usually synergistic, additive and antagonistic. Thus although the isobologram may provide an indicative overview of the interactions between drugs in a combination, the Bliss independence method provides information on which specific concentration ratios are additive, synergistic or sub-additive.

7.2.6 Meloxicam is neuroprotective *in vitro*

Meloxicam was shown to be a potent neuroprotectant in the current study. $50\mu\text{M}$ and $25\mu\text{M}$ meloxicam abolished injury development at 24, 48 and 72 hours. $10\mu\text{M}$ abolished injury at 48 and 72 hours, but not at 24 hours (Figure 4.32). Meloxicam was protective even when concentrations 10 times lower were tested. $1\mu\text{M}$ meloxicam was protective at all 3 time points whereas although $0.1\mu\text{M}$ meloxicam was not protective at 24 hours, it was protective at 48 and 72 hours (Figure 4.33). Meloxicam's protective effect was concentration dependent and reached a maximum at $25\mu\text{M}$. To examine the concentration response relationship of meloxicam, the degree of protection at the 72-hour time point was taken as the reference for comparison (Figure 4.34). At 72 hours, meloxicam abolished injury at concentrations of $25\mu\text{M}$ and $50\mu\text{M}$, providing $71 \pm 4\%$ and $69 \pm 8\%$ protection respectively. These values are not different to the level of sham injury ($29 \pm 4\%$ of the control injury at 72 hours) and thus the maximum attainable protection. At concentrations of $0.1\mu\text{M}$, $1\mu\text{M}$ and $10\mu\text{M}$, meloxicam was $30 \pm 4\%$, $44 \pm 3\%$ and $60 \pm 5\%$ protective, respectively. These protective effects were seen under conditions of 25% helium pressure.

As far as I am aware, meloxicam has not been tested in *in vitro* models of TBI and there have been few studies that tested meloxicam in ischaemic injury models *in vitro*. In an OGD model of ischemic injury in OHSC, Llorente *et al.* found that 50 μ M decreased injury by approximately 50% and that this effect was attributed to modification in the expression of NMDA and AMPA receptor subunits, leading to a decrease in excitotoxicity[192]. The reason for meloxicam's greater protective effect in TBI may be attributed to the fact that TBI injury in the slice model is of a focal nature and less diffuse compared to OGD. In a model of hypoxia re-oxygenation injury in rat brain slices of the whole cortex, meloxicam was effective at reducing tissue damage over a range of concentrations[280]. The IC₅₀ in that study was estimated to be $9.9 \pm 1.3\mu$ M, which is similar to my results, albeit a different injury model. Other differences to note are that the slices in that study were from a different brain region and meloxicam was present in the media before injury. This IC₅₀ concentration also corresponds to the range of plasma concentrations of meloxicam (10^{-6} to 10^{-4} M) in humans[280]. The findings of my study are in agreement with these results from *in vitro* studies of ischaemic injury.

Meloxicam's neuroprotective effect has also recently been shown in two *in vivo* studies of blunt TBI. In a rat weight drop model of TBI, Hakan *et al.* reported a decrease in lipid peroxidation and oedema at 48 hours, restored glutathione and preserved BBB integrity at 24 and 48 hours with 2mg/kg IP meloxicam. They also reported an improvement in neurological examination scores[185]. In another study of closed head injury in mice, 2mg/kg IP meloxicam reduced inflammation but did not alter oedema or functional outcome[193]. The conflicting results of the two studies may be attributed to different time point evaluation (48 hours in Hakan *et al.* versus 6 and 24 hours in Girgis *et al.*), dosing regimen (meloxicam was administered 30 minutes after trauma Hakan *et al.* versus 10 minutes after trauma in Girgis *et al.*), difference in species and injury models used. In terms of how the *in vitro* concentrations used compare to the *in vivo* circumstance, when calculated, it was found that 5 μ M meloxicam equates to the 2mg/kg dose used in mice, which is within the range of concentrations used in the present study. However, as discussed in section 7.2.3, caution must be exerted when comparing an isolated tissue system to an *in vivo* situation. More *in vivo* studies are required to clarify whether meloxicam is neuroprotective after TBI in whole animals. The concentration response relationship of meloxicam's protective effect has also not been assessed to date *in vivo* and my findings *in vitro* indicate that there is merit in assessing concentration dependence of meloxicam *in vivo*. Should meloxicam be taken to further stages

of pre-clinical and indeed clinical testing, this is relevant to avoid a similar situation to that seen with progesterone, where inadequate pre-clinical evaluation of the drug dose response effect led to inappropriate dosing in Phase III trials and was one of the reasons why progesterone ultimately failed Phase III trials[180].

7.2.7 Neuroprotection with co-administration of meloxicam and argon

After establishing the neuroprotective effect of meloxicam, the aim was to determine whether combining low doses of meloxicam with 25% argon would be more protective than argon alone. At 24 and 48 hours, combining 0.1 μ M or 1 μ M meloxicam with 25% argon did not significantly reduce injury compared to 25% argon alone (Figure 4.35 and Figure 4.36). However, by 72 hours, both combinations significantly reduced injury compared to 25% argon alone as well as the individual doses of meloxicam acting alone. At 72 hours after injury, the protective effect of 0.1 μ M meloxicam and 25% argon combined was $49 \pm 4\%$ and of 1 μ M meloxicam and 25% argon combined was $58 \pm 2\%$ (Figure 4.37). The protective effect of 25% argon was doubled from $29 \pm 5\%$ when administered alone to $58 \pm 2\%$ when administered with 1 μ M meloxicam, thus co-administration of low doses of meloxicam increase the efficacy of 25% argon, which provides little protection when administered alone. At both concentrations of meloxicam, the protection provided by the combination with argon is equal to the expected protection assuming additivity, consistent with an additive effect. Additionally, the isobologram in Figure 4.41C indicates an overall synergistic mode of action between argon and meloxicam. Although this is not revealed in the individual concentration ratios evaluated using the Bliss independence approach, testing additional concentration ratios of argon and meloxicam may reveal synergistic effects.

7.2.8 Neuroprotection with co-administration of xenon and meloxicam

Following evidence that low concentrations of meloxicam can increase efficacy of 25% argon, the same effect was tested with 25% xenon. Co-administration of 0.1 μ M meloxicam and 25% xenon reduced injury at all three time points, although not significantly different to the protection by 25% xenon at any time point (Figure 4.38). Increasing the concentration of meloxicam to 1 μ M in the combination abolished injury to the level of the sham at all time points and this was significantly different to the effect of 25% xenon acting alone at 72 hours (Figure 4.39). Thus, it is possible to abolish injury completely by using 1 μ M meloxicam with 25% xenon, which alone is $44 \pm 5\%$ protective at 72 hours. Moreover, when co-administered

with 25% xenon, 0.1 μ M meloxicam was $48 \pm 4\%$ protective while 1 μ M meloxicam was $66 \pm 4\%$ protective at 72 hours, which was significantly higher than protection with meloxicam acting alone at each dose (Figure 4.40). Thus, while meloxicam acting alone is able to abolish injury at 10 μ M, the addition of 25% xenon makes a dose that is 10 times lower as effective. However, although the protective effect of meloxicam in combination with 25% xenon appears to vary with the concentration of meloxicam, unlike argon, the relationship between the two drugs is not additive at both concentrations of meloxicam when evaluated using the Bliss independence approach. While the protection obtained with 1 μ M and 25% xenon coincides with the expected protection assuming additivity, the protective obtained with 0.1 μ M meloxicam and 25% xenon is less than additive by $21 \pm 5\%$. In other words, at a concentration ratio of 0.1 μ M meloxicam and 25% xenon, the expected level of protection was $61 \pm 4\%$, which would have taken the level of protection by the combination to almost as good as the uninjured sham. The isobologram in Figure 4.41D indicates an overall additive interaction between xenon and meloxicam. This further highlights the need for optimisation of dose ratios in combination studies, as concentration ratios of the same drugs in combination can be synergistic, additive and antagonistic.

7.2.9 Mechanisms of combination therapies

Drugs in combination therapy would be most beneficial to clinical TBI if they act on complementary rather than single targets[75,80,81] – this was a key recommendation from a major US convention on combination therapies to treat TBI held in 2008[81]. In this section, potential overlaps between mechanisms of action of the combinations tested that are likely to be seen in hippocampal slices will be discussed.

Amongst the drugs and noble gases studied, progesterone has the widest range of mechanisms of action and in this *in vitro* study, progesterone is likely to act at a number of targets in the hippocampus. The multifunctional nature of progesterone is a particularly attractive feature because it means that more pathways can be targeted. The progesterone receptors (PR α and PR β) are particularly concentrated in the hippocampus of the developing and adult brain[152,281]. Modulation of these receptors after TBI has been suggested to mediate inflammation, ion and water homeostasis and myelin repair[153]. Moreover, progesterone can also be metabolised to metabolites like allopregnanolone, which is a neurosteroid shown to act *via* binding to sites within the hydrophobic domain of GABA_A

receptors, resulting in the potentiation of GABA-induced chloride conductance[143]. This was seen in an OGD model in OHSC, where it was first shown that progesterone metabolises to allopregnanolone following which, inhibition of GABA_A receptors with picrotoxin (non-competitive GABA_A antagonist) prevented protection seen with progesterone and allopregnanolone[141]. This mechanism of action is also relevant clinically, because administration of progesterone increases downstream allopregnanolone levels by several fold[70]. Finally, progesterone has also been reported to inhibit pro-apoptotic enzymes like cytochrome c, activated caspase 3 and upregulation of anti-apoptotic Bcl2 and ERK protein, which helps maintain cell integrity and promote survival[151,160].

To further enhance the protective effect of progesterone, it is recommended that it may be co-administered with agents that dampen excitotoxicity[81]. Xenon is known to dampen excitotoxicity *via* inhibition of the NMDA receptor[93] and *via* activation of TREK-1 channels[94], thus complimenting the actions of progesterone. The mechanisms of argon neuroprotection are unclear (chapter 1, section 1.4.2.3) however, argon shares 3 of its proposed mechanisms (see Figure 1.5) with progesterone. These include the potentiation of the GABA_A receptor[126], anti-apoptotic effects *via* increased postsurvival protein synthesis of Bcl-xL[127] and mediation of the ERK1/2 pathway[128]. Besides sharing mechanisms of action, because the combination of progesterone and each of the noble gases was additive, it is likely that a number of mechanisms of progesterone described above act with those of xenon and argon. However, progesterone is a pleiotropic drug and additional mechanisms of action have been discussed in chapter 1, section 1.4.3.3. In an *in vivo* situation, if progesterone were co-administered with xenon or argon, the combination would be likely to act at additional targets (see Figure 1.7), which may not be specific to the hippocampus. In fact, perhaps synergistic effects of noble gases and progesterone may be seen in brain regions other than the hippocampus altogether.

Meloxicam's protective effects following a TBI stem from its NSAID COX-2 selective inhibitory actions. Under normal physiological conditions, low steady state levels of COX-2 are found in the hippocampus and COX-2 appears to be regulated by normal glutamatergic synaptic activity[282,283]. Studies have shown that toxic concentrations of glutamate and NMDA induced COX-2 overexpression in both neurons and glia[184,186,284]. Pathological COX-2 induction has been associated with delayed cell death which has been correlated with functional deficits following brain injuries[186,284,285]. In the hippocampus specifically, a

‘temporal wave’ of COX-2 mRNA has been observed that starts in the CA1-2 neurons at 2 to 6 hours after trauma and appears in the CA3 and DG by 6 to 24 hours post injury in the rat[186]. Thus, there is a wealth of pre-clinical data which suggests that COX-2 inhibitors and particularly, COX-2 selective inhibitors like meloxicam, may be used to treat neurological conditions, most notably acute conditions like TBI and stroke[183,186,286,287]. In addition, COX enzymes have also been shown to modulate glutamatergic transmission, thus interfering with glutamate induced cell death. In a model of ischaemic brain injury, selective COX-2 inhibition has been shown to reduce cortical brain damage caused by injection of NMDA and hippocampal oxidative damage caused by injection of kainate[190,191]. Thus, there may be an overlap of anti-excitatory effects between xenon and meloxicam when co-administered. Moreover, perhaps meloxicam’s anti-inflammatory action through COX2 inhibition complements xenon’s anti-excitatory actions. A similar logic applies to argon, in which case argon’s anti-apoptotic or inhibitory actions *via* the GABA_A receptor may complement meloxicam’s anti-inflammatory effects.

7.2.10 Evaluation of methods to assess drug interaction in combination experiments

The Bliss independence method[238] was adapted to analyse drug combination data. In this method, the observed combination response is compared with the predicted combination response (see chapter 4, section 4.2), obtained with the assumption that there is no effect from drug-drug interactions. The Bliss independence model focuses on treatment effect enhancement and is one of the most frequently used reference models[239]. It assumes a stochastic process in which two drugs elicit their effects independently and the expected combination effect is calculated based on the probability of the independent effects[240]. As explained previously, this is not the direct addition of effects which may be without meaning, if say for example dose A gives a 60% $E_{\max}$ effect and dose B gives a 70% $E_{\max}$ effect. In contrast, Loewe additivity uses a different approach as it provides the view of the additive dose combinations that give a specific effect level (eg., IC_{50}) and this becomes the basis for detecting synergism or sub-additivity using an isobologram[288]. It is a non-mechanistic, graphical approach and is focussed on dose reduction – i.e., to determine whether the same effect level be achieved by lower doses of the individual drugs[276]. In practice, it has been found that Loewe additivity based models only work well when monotherapy dose response curves are well characterised through parametric models.

When dose response curves are either not available or non-standard (eg. not based on the Hill equation or a four-parameter logistic nonlinear regression model) it is impossible to accurately generate an isobologram using Loewe additivity. In terms of data presented in this thesis, it was not possible to obtain a dose response curve for any monotherapy that went from 0 to 100% protection. This is because the maximum attainable protection is 70%, as it is not possible to get more protection than the level of sham injury (which is 30% of the total injury at 72 hours, based on data presented). The classical isobologram based on Loewe additivity does not account for situations like this. For this reason, Bliss independence was used as the main method to assess synergy and additivity and results are then compared with isobolograms which were made from estimates of EC_{50}/EC_{45} values from the dose response curves. For argon, the EC_{45} value was used because at even the highest concentration tested, argon was not 50% protective.

Thus, even though Loewe additivity is the more widely preferred quantitative assessment of drug interaction[276,289,290], the Bliss independence based model provides a viable alternative. One drawback of this model is that drug combinations often involve drugs with complex mechanisms of action, as is the case with progesterone for example, which has pleiotropic effects. Thus some studies have suggested that assessment of synergism and additivity should not depend on mechanisms of action[236]. Another drawback of this model is that it can incorrectly claim synergy when two identical drugs are combined, however, this situation is not encountered in the present study. It is also more accurate than simply adding the protection levels achieved by the monotherapies, as it uses the complete additivity of probability theory[239]. In the current study, where expected protection was calculated to exceed 70% protection (as was the case in a few instances) the expected protection was simply considered maximal.

An alternative approach would be to use curved isoboles, a method developed by Grabovsky and Tallarida[291] when the isobole of additivity is not necessarily linear. This situation is encountered when the maximum effects of two agonists is different – for example, in the current study, 50% argon offers $40 \pm 5\%$ protection whereas 50 μ M meloxicam offers $70 \pm 8\%$ protection. This approach may work better with data presented in this thesis as it intrinsically assumes neither drug may offer 100% protection as a monotherapy. However, the other challenge when constructing the isobologram is that because it relies on dose reduction, the numbers need to work out such that a particular combination would give

exactly 50% protection if EC₅₀ concentrations of the two drugs as monotherapies are used. This was a challenge in the current study and estimates were made from dose response curves. Perhaps testing more combinations in the future would allow for the generation of more standard dose response curves, which would enable the construction of more accurate curved isobolograms.

In summary, with relevance to the results presented in this thesis, the isobolograms presented are useful in providing an overview of interactions between the drug combinations. However, for a more detailed breakdown of which particular combinations of concentration ratios are synergistic or additive, the Bliss independence method is a more informative approach.

7.2.11 Clinical importance of findings and future work

It has been established both in this thesis and other work[93] that 50% xenon treatment is maximally effective but 50% argon is not as effective *in vitro*. Additionally, argon has been shown to be not as effective as xenon in *in vivo* models of cardiac arrest induced neurological damage and stroke[131,132]. It has been demonstrated in this thesis that combining argon or sub-maximally effective concentrations of xenon with other drugs improves efficacy of the sub-maximal noble gas treatment. At some concentrations in combination, co-administration of the adjuncts with xenon or argon is more effective than the progesterone and meloxicam acting alone as well. This represents an important proof of concept step towards translating noble gas treatment for TBI into the clinic as it presents the opportunity to reduce cost and increase efficacy. In a study by Dingley *et al.*, which aimed at developing a specialised closed circuit delivery system, a 50% mixture of xenon was administered at a cost of \$148.5/hour (at \$30/litre) totalling \$800 (approximately £650) for a 6-hour period[292]. Whereas this may be expensive to sustain in the clinic, the results of this thesis indicate that maximal protection may be achieved *in vitro*, even with lower concentrations of xenon or argon by combining them with adjuncts, thus decreasing costs.

A key feature of noble gases is their lack of chemical reactivity with other drugs, which makes them ideal candidates for combination therapies as it minimises the potential for off target effects. Moreover, the therapeutic efficacy of monotherapies has consistently fallen below expectations in clinical trials for TBI[72,81,180,269]. Arguing that xenon or argon may be administered in combination therapy with other drugs, especially drugs like

progesterone for which there is a wealth of pre-clinical and even early clinical evidence of success, may accelerate their progression to clinical trials more easily than arguing for their use as a single agent, especially when there are economic constraints associated with xenon's widespread use.

This is the first study to investigate combination therapies with xenon, argon and pharmacological agents for TBI. In terms of study design, three important steps were taken to increase clinical relevance. Firstly, as some pharmacological agents may interact or have off-target effects in combination, the dose-duration response efficacy must be optimised in combination doses[75]. This concept was followed in the current study besides trying to determine the lowest concentrations of drugs in combination that would still provide more protection than either monotherapy, which is preferred to selecting the best effective dose of each drug as monotherapies and then combining them[293]. Secondly, more than one therapeutic dose for each compound was tested, both singularly and in combination and thirdly, more than one time point was tested. So far, only one study each has used more than one combination and has assessed more than one time-point in combination therapy for TBI[241]. Consistent with recommendations from a review by Kline *et al.*[241], this was important to ensure that a potential therapy was not 'prematurely discarded' based on a single combination not being effective or a missed temporal effect and thus points once again towards the importance of dose optimisation in combination studies.

The positive results seen with combinations of progesterone/meloxicam and noble gases also raises the question of whether other anti-inflammatory drugs, steroids or even other pleiotropic drugs may be a viable adjunct to noble gases for TBI treatment. For example, vitamin D hormone is a neuroprotective secosteroid hormone shown to be neuroprotective in several injury models[294] through multiple signalling pathways. In an *in vivo* model of focal cortical contusion, combination therapy with progesterone and vitamin D produced better functional outcomes than progesterone alone[274]. Following this, a phase I/II clinical TBI trial showed that patients who received the combination had a significantly higher recovery rate than progesterone alone[295], whilst a similar situation was seen in a model of ischemic stroke[293]. By combining different agents with noble gases it may be possible to isolate additive and synergistic drug targets, whilst increasing our understanding of their mechanism of action.

Future work on combination therapies using the *in vitro* TBI model may involve a study of the mechanism by which the drugs and noble gases tested in this thesis exert their effects. Organotypic slices enable imaging transgenic and transfected cultures using high resolution confocal and time-lapse microscopy[206]. These techniques are particularly relevant to assess morphological plasticity of cells following treatment with neuroprotective drugs or combinations of drugs. Capturing this information at the cellular and sub-cellular levels is not as feasible with whole animals, and this may be crucial to understanding mechanisms of injury prevention and repair following treatment. Further, it would be interesting to determine how injury progresses *in vitro* with the immediate treatment of a noble gas for a specified amount of time followed by the delayed administration of an anti-inflammatory drug or vice versa. Will the additive or synergetic neuroprotective effect be sustained despite withdrawal of noble gas treatment or will protection be no greater than that caused by the anti-inflammatory drug alone? If protection is sustained, this may suggest that the pathways activated by the gas treatment are still active due to complementary actions by anti-inflammatory drugs. This is relevant because combination treatments may be able to increase the therapeutic window for either monotherapy.

7.3 Development of an *in vivo* model of TBI to quantify contusion volume and profile cytokine expression

The next aim was to set up and calibrate an *in vivo* rat model of CCI injury for the first time in our laboratory. Considerable work went into identifying, organising and setting up a facility to perform surgeries, procuring equipment and consumables, testing different anaesthetic circuits and calibrating the CCI device. Considerations when setting up the model were discussed in chapter 5 (see section 5.2). In the following sections, contusion volume and cytokine profiling results will be discussed.

7.3.1 Contusion volume as a histological endpoint

The contusion volume at 24 hours was chosen to calibrate the model because it is a convenient measure of injury that has been shown to correlate with behavioural impairments. The parameters of tip diameter, velocity, dwell time and penetration depth are known to influence lesion size and injury development[244].

With a 3mm tip, varying the penetration depth, velocity and dwell time produced an injury with a very small contusion: $4.7 \pm 2.3\text{mm}$ (Figure 5.3). However, studies that examine CCI injury in rats more frequently use a tip diameter of 5mm[296–301]. A 5mm tip with velocity = 4m/s, dwell time = 400msec and an impact depth of either 1.5 or 2mm produced a larger contusion (Figure 5.4). After selecting values for velocity, tip size and dwell time, studies have often only varied impact depth as a measure of severity of injury[248] as it has shown to correlate with elevated intracranial pressure, decreased blood and cerebral perfusion pressures[302], histological alterations[248] and functional deficits such as performance on the Morris water maze[303]. For example, Stover and colleagues used a 5mm bolt with a velocity of 7m/s and dwell time of 300msec with either a 1mm penetration depth or a 1.5mm depth to produce a more severe cortical contusion[304]. Dixon, who first described the CCI injury model, has suggested that beyond a velocity of 3m/s, penetration depth is a greater determinant of injury severity[215], whilst the tip material and dwell time are less well characterised. In the present study, using a penetration depth of 1.5mm produced a contusion volume of $52 \pm 33\text{mm}^3$, whereas increasing the penetration depth to 2mm more than doubled the contusion volume to $134 \pm 14\text{mm}^3$ (Figure 5.5), therefore, contusion volume varies with impact depth. Increasing the velocity to 6m/s and reducing the dwell time to 250msec resulted in an injury that caused the death of the animal immediately after the impact. Thus, the injury parameters velocity = 4m/s, dwell time = 400msec, tip diameter = 5mm and impact depth = 1.5mm represent a ‘moderate injury and the parameters velocity = 4m/s, dwell time = 400msec, tip diameter = 5mm and impact depth = 2mm were taken to represent a ‘moderate to severe’ injury in the current model. The depth of injury also depends on the zero-point used when setting the desired depth on the impactor[215]. In the present study, the tip was zeroed to the dura surface, although there is a lack of standardisation with some groups also zeroing the tip to the skull (before craniotomy). In general, to produce a moderately severe injury in rats, a tip diameter of 5 – 6mm, velocity of 4m/s, penetration depth of 1 – 3mm from the dural surface with a dwell time of 50 – 250msec is recommended[244]. There was no data in the literature with exactly the same parameters and a corresponding value for contusion volume at 24 hours. However, the parameters used in this study were similar to those used by the following groups: Ling[300], Dekosky[21], Watson[227], Turtzo[305] and Heck[297], who define the injury produced with these parameters as “moderate to severe”. Unfortunately these groups did not publish values for contusion volume 24 hours post injury. For a point of reference, another group that used a 2.5mm tip diameter, 6m/s velocity and 2mm penetration depth obtained a contusion volume of 60mm^3 , 24 hours after CCI[226]. One major drawback

of the CCI model is that there is no standardisation across labs with respect to which combinations of parameters constitute a mild, moderate or severe injury[215]. Therefore pilot testing, such as the experiments done in the present study, is important to determine the parameters required to produce the desired pathology.

7.3.2 Cytokine Profiling after injury

The inflammatory cascade following TBI is regulated by both pro- and anti-inflammatory cytokines[34]. TNF, IL-6 and CCL-2 are three cytokines released in response to TBI and their expression profile may provide information on the extent and type of tissue damage following CCI injury. The cytokines were detected in the brain and blood plasma at different time points using ELISA assays (chapter 5, Figure 5.7 - Figure 5.12). Table 7.3 refers to concentrations of cytokines in the brain and blood from other studies that evaluated CCI injury in rats.

Table 7.3: Table showing inflammatory cytokine protein concentrations post CCI in rat models of TBI. Brain parenchymal levels refer to ipsilateral hemispheres following injury

Cytokine	Tissue/fluid	Level	Time (h)	Reference
TNF	Brain	55 pg/mg	4	Dalgard <i>et al.</i> , 2012
	Brain	10 pg/mg	24	Dalgard <i>et al.</i> , 2012
	Brain	50 pg/mg	24	Chen <i>et al.</i> , 2008
	Plasma	No change		Harting <i>et al.</i> , 2008
IL-6	Brain	250 pg/mg	24	Lee <i>et al.</i> , 2012
	Brain	280 pg/mg	24	Chen <i>et al.</i> , 2008
	Plasma	No change		Harting <i>et al.</i> , 2008
CCL-2	Brain	500 pg/mg	24	Dalgard <i>et al.</i> , 2012

7.3.2.1 Expression of TNF in the brain and blood plasma following injury

TNF is the most extensively characterised cytokine in neurological diseases, including TBI[306,307]. Produced as a membrane bound 26kDa precursor molecule, TNF is cleaved to a 17kDa active cytokine by a conversion enzyme and is present in low levels in the healthy brain. As a consequence, its precise function in physiological conditions remains poorly characterised. However, it plays a pivotal role in initiating and regulating the cytokine cascade during an inflammatory response[34]. A pro-inflammatory cytokine, TNF is released

rapidly after injury, which suggests that activated microglia or neuronal cells as opposed to infiltrated leukocytes produce it[308]. Elevated levels of TNF in brain homogenates have been observed within a few hours after TBI in different models of focal trauma[34,40,218,227,228], however the results are less clear with blood plasma sampling[41]. In human brain injury, TNF has mainly been detected in the CSF[309]. The increased production of TNF has conventionally been associated with neurologic deficits in rodents following experimental TBI[310,311].

The data presented in chapter 5, section 5.4.2 substantiate the idea that TNF is an early marker of inflammation detectable in the brain and plasma following CCI injury. With lower injury parameters (velocity = 4m/s, dwell time = 400msec, tip diameter = 3mm, penetration depth = 1.5mm), TNF concentration was elevated at 3 hours ($0.46 \pm 0.22\text{pgml}^{-1}$) and 24 hours ($1.16 \pm 0.21\text{pgml}^{-1}$) compared to sham ($0.26 \pm 0.06\text{pgml}^{-1}$). When higher injury parameters (velocity = 4m/s, dwell time = 400msec, tip diameter = 5mm, penetration depth = 2mm) were used, the concentration of TNF in the plasma at 24 hours ($3.18 \pm 0.70\text{pgml}^{-1}$) was significantly increased compared to sham ($0.26 \pm 0.06\text{pgml}^{-1}$) and to lower injury 24-hour ($1.16 \pm 0.21\text{pgml}^{-1}$) groups. Thus, the concentration of TNF in the plasma correlates positively with injury severity (Figure 5.7). Surprisingly, there have been few studies in the literature that have examined TNF levels in the plasma following experimental TBI injury even though detection in the plasma (alongside the CSF) is potentially the most clinically relevant method of assessing and monitoring cytokine levels. Harting and colleagues who studied TNF expression post CCI injury in rats saw no change in plasma TNF levels following a ‘moderately severe’ CCI injury[312]. While the TNF concentration obtained with lower injury parameters in my study are consistent with this, there was a significant increase in TNF levels when higher injury parameters were used. In a study of human head injury, 36% of patients had a TNF concentration above 2pgml^{-1} within 24 hours of injury in CSF[313] and in a study of CCI injury, Stover and colleagues found that TNF levels in the CSF of rats subjected to injury peaked at approximately 3pgml^{-1} 24 hours after trauma[314]. Although not a direct comparison, these results are similar to the values obtained in the present study with higher injury parameters, which is promising and indicates that future studies are warranted to confirm the positive correlation of TNF in the plasma with injury severity.

In the brain (Figure 5.8), TNF concentration was significantly elevated at both 3 hours ($47.2 \pm 12.4\text{pgmg}^{-1}$) and 24 hours ($45.5 \pm 12.4\text{pgmg}^{-1}$) after CCI injury in lower injury groups

compared to sham ($17.0 \pm 3.7 \text{ pgmg}^{-1}$). In rat models of CCI, TNF can be detected in as early as 1 hour and studies have suggested that levels peak between 4 and 8 hours post injury[227] or at 24 hours post injury[228]. The values obtained for TNF in the brain at 3 hours and at 24 hours in the present study closely correlate with those obtained by Chen *et al.*, 2008 and Dalgard *et al.*, 2012 (see Table 7.3). These studies used the same CCI model of injury and similar injury parameters. While the TNF concentration decreased 3-fold at 12 hours and up to 24 hours post injury in the study by Dalgard *et al.*, 2012, the same trend was not observed in the present study, where TNF levels remained elevated at 24 hours. Conversely, in the study by Chen *et al.*, 2008, the TNF concentration appeared to increase up to 24 hours at which point it peaked (see Table 7.3). Thus, further studies may be required to confirm the true peak levels for TNF. Further, contralateral levels of TNF at 3 and 24 hours were higher than in the study by Dalgard *et al.*, 2012, which could indicate that the injury secondary injury development of penumbra from the site of impact may be wider than in other models.

The exact function of TNF in secondary injury post TBI remains controversial. For example, in an animal study by Kim *et al.*, recombinant TNF was injected into the brain and induced BBB breakdown, cerebral inflammation and intracranial leukocyte recruitment[315]. From another study that examined the ability of compounds to inhibit TNF transcription and bioactivity following closed head injury in rodents it was concluded that TNF is implicated in neurotoxicity and worsens outcome within 24 hours post trauma[316]. The results from knockout studies have been particularly controversial. Some studies have shown that mice lacking TNF or TNF receptors suffered BBB damage and neurological deficits after injury[317]. In another study, deletion of TNF expression in knockout mice resulted in mild behavioural deficits compared to controls in the acute phase post injury, however, while wild type mice recovered from injury by 2 – 3 weeks post trauma, TNF knockouts displayed persistent motor deficits in the long term[318]. Therefore, it appears that while earlier studies suggested a detrimental role for TNF following TBI[319], more recent studies suggest otherwise[320]. Taken together, the literature suggests that while TNF may ameliorate injury and deficits in the acute phase post trauma, endogenous TNF may be neurotropic and encourage repair in the long term.

7.3.2.2 Expression of IL-6 in the brain and blood plasma following injury

IL-6 is a multifunctional factor that is important in host defence[321]. IL-6 belongs to the neuropoietin family of cytokines[322] and its release is triggered by the elevations in TNF

and IL-1. IL-6 is expressed by astrocytes, microglia and neurons and has also been implicated in TBI, although not exclusively expressed in the CNS. In TBI patients, high concentrations of IL-6 have been detected in the CSF[307] and in the plasma[323]. In various experimental models of TBI, IL-6 has been detected in brain tissue but less so in the plasma[34,40,41,218,324–326].

Figure 5.9 suggests that IL-6 may be an early marker of inflammation in the plasma following CCI injury and that its concentration correlates positively with injury severity. With lower injury parameters, IL-6 concentration showed a numerical increase at 3 hours ($9.4 \pm 3.2 \text{ pgml}^{-1}$) and a further increase at 24 hours ($17.4 \pm 3.3 \text{ pgml}^{-1}$). Moreover, no significant difference in IL-6 levels between sham and CCI groups was detected in the plasma of animals subjected to lower injury parameters at either of these time points. When higher injury parameters were used, the concentration of IL-6 in the plasma at 24 hours ($78.2 \pm 8.5 \text{ pgml}^{-1}$) was significantly increased compared to sham ($12.5 \pm 4.9 \text{ pgml}^{-1}$) and to lower injury 24-hour groups. Similar to TNF, there have been few studies that have examined IL-6 levels in the plasma following CCI injury in rats. Harting and colleagues report no change in IL-6 levels in the plasma following a ‘moderately severe’ CCI injury[312]. While the IL-6 concentration obtained with lower injury parameters in my study is consistent with this, there was a significant and dramatic increase (4 – fold) in IL-6 levels when higher injury parameters were used. Plasma IL-6 levels were over 100 pgml^{-1} in a study that examined TBI injured patients at 24 hours post injury[323]. Although the levels cannot be compared with that of a rodent study, the increase in IL-6 at 24 hours warrants future investigation of IL-6 levels in the plasma.

In the brain however, the results obtained in this study were less clear. The increase in IL-6 concentration CCI injured animals (lower injury parameters) was not different compared to sham and at either time point (Figure 5.10) although levels numerically increased from 3 to 24 hours. However, the values in the ipsilateral hemisphere at 24 hours ($238 \pm 78 \text{ pgmg}^{-1}$) correspond to ipsilateral values of IL-6 obtained at 24 hours post injury by Lee *et al.*, 2012 and Chen *et al.*, 2008 (see Table 7.3)[226,228]. Similar to TNF, contralateral levels of IL-6 ($107 \pm 51 \text{ pgmg}^{-1}$) were higher than published studies using the CCI model[226,228], again indicating a more widespread injury. Across a range of models expression of IL-6 is reported to peak within 2 and 8 hours[34,324–326], but only Chen *et al.*, 2008 have examined peak

value concentrations in the CCI model specifically where they found IL-6 to peak at 24 hours.

The functions of IL-6 may be more clearly understood than those of TNF however evidence for a dual role, neurotropic and deleterious, is abundant. For example, elevated IL-6 in the plasma of TBI patients has been correlated with increased concentrations of acute phase proteins including C-reactive protein, fibrinogen and α 1-antitrypsin, and with severe BBB damage[327,328]. It was concluded from this study that IL-6 may be synthesised in the brain and crosses the damaged BBB to influence the functions of other organs. Another study demonstrated a correlation between elevated IL-6 levels and raised ICP as well as poor clinical outcomes[329]. However, IL-6 and its receptor are constitutively expressed in the healthy brain, indicating that IL-6 may be involved in maintaining normal physiological functions of the CNS. From studies that used Glial fibrillary acidic protein (GFAP) IL-6 transgenic mice, it was concluded that elevated IL-6 production after cortical freeze lesion injury is neuroprotective. IL-6 deficient mice showed increased oxidative stress, decreased cell survival and slower wound healing[330]. The anti-inflammatory effects of IL-6 are further supported by its abilities to induce nerve growth factor synthesis, inhibit TNF synthesis, promote neuronal differentiation and survival and dampen NMDA mediated toxicity effects[328].

7.3.2.3 *Expression of CCL-2 in the brain and blood plasma following injury*

Finally, CCL-2 is produced by astrocytes and to a lesser extent, fully reactive microglia, following traumatic injury[331]. CCL-2 is a member of the C-C chemokine family, known to attract monocytes both *in vitro* and *in vivo*. The expression of CCL-2 and its receptor CCR2 is elevated in multiple cell types and is known to drive the acute immune response to trauma[332,333]. Studies suggest that CCR2 co-localises with activated microglia following excitotoxic injury[334], neurons and astrocytes[332].

In the present study, CCL-2 concentrations in the plasma of animals subjected to lower injury parameters numerically increased from 3 hours ($112 \pm 12 \text{ pgml}^{-1}$) to 24 hours ($191 \pm 9 \text{ pgml}^{-1}$), although the difference did not reach significance (Figure 5.11). Moreover, increasing the severity of injury did not produce a corresponding increase in CCL-2 expression as there no difference between the level of CCL-2 at 24 hours in the plasma of animals subjected to higher injury parameters ($173 \pm 16 \text{ pgml}^{-1}$) and those subjected to lower injury at the same

time point. Although there was a trend towards an increase in CCL-2 expression following injury compared to sham, the difference between CCI groups (at higher and lower injury parameters) and sham ($119 \pm 23 \text{pgml}^{-1}$) did not reach significance. From these results, it may be concluded that CCL-2 may not be suitable as a plasma biomarker following blunt TBI.

In the brain (Figure 5.12), CCL-2 levels were significantly increased at 24 hours post-CCI ($71 \pm 23 \text{pgmg}^{-1}$) compared to sham ($10 \pm 4 \text{pgmg}^{-1}$). However, no difference between ipsilateral (injured) and contralateral hemisphere was observed. These values are lower than those published by Dalgard *et al.*, 2012 who observed an increase in CCL-2 concentration at 4 hours up to 12 hours where the value peaks, following which at 24 hours, the level was 500pgmg^{-1} (see Table 7.3). There have been very few studies looking at CCL-2 concentrations post TBI although CCL-2 expression in the plasma of TBI patients has been observed to increase 20% post injury[335]. CCL-2 overexpression correlates with macrophage infiltration and deficit[228]. Studies additionally suggest that CCL-2 contributes to brain lesion development following excitotoxic injury[334] and stroke[336]. As CCL-2 is a powerful chemo-attractant, further studies with CCL-2 expression and testing at earlier time points could elucidate beneficial effects for its inhibition after TBI.

7.3.2.4 *The potential of TNF, IL-6 and CCL-2 as biomarkers of human TBI*

One of the big picture goals of TBI research is to develop biomarkers, which are sensitive early measures of outcome that can provide information sooner than the currently available neurological scores assessed only 6 months after TBI. Based on their functions described in sections 7.3.2.1 - 7.3.2.3, the expression profile of TNF, IL-6 and CCL-2 can potentially provide information about the extent of tissue damage at different time points post trauma. When considering whether testing the concentrations of these cytokines post trauma may be translatable into the clinic, a number of factors need to be considered.

Firstly, the majority of animal studies have focussed on evaluating cytokine levels in brain tissue as opposed to CSF or blood samples. While this is useful to characterise the inflammatory response at the cellular level and test the effect of neuroprotective drugs on different inflammatory cascades, measurement of brain cytokine levels as an early biomarker is challenging after human TBI. Microdialysis measurements in patients can provide ongoing measurements of cytokine levels in the brain parenchyma, but is currently an expensive

and invasive technique[337]. Moreover, cytokine concentrations vary depending on the tissue or fluid they are measured in and on the time post injury at which they are measured. The results from my experiments support the findings[228] that TNF and IL-6 are early markers of inflammation, high levels of which can be detected for up to at least 24 hours post trauma in the brain. They also conflict with another published study in which there was no change to the plasma levels of TNF and IL-6 after injury[312], which is promising because it warrants further investigations into the potential of cytokines as the more clinically relevant plasma biomarkers. Due to a compromised BBB, it is possible to measure CNS generated cytokines in the peripheral blood. In fact, elevated levels TNF and IL-6 have been linked with increased ICP and decreased cerebral perfusion pressure[338], although these studies are small.

Further, the data from rodent models can be different from human data due to the nature of the injury. For example, in human TBI, patients may present with polytrauma, which may also cause a systemic inflammatory response. Depending on the extent and nature, this secondary response may mask the inflammatory response of the brain. Further characterising the temporal profile of plasma cytokines following *in vivo* TBI could be informative. Complimenting these readings with CSF measurements could provide a CNS specific measurement as changes in CSF concentrations do not appear to be affected by peripheral responses[339]. Perhaps animal models of polytrauma are required to simulate a more realistic scenario. Additionally, compared to rodents, the human brain is gyrencephalic, has a larger mass and a different ratio of white to grey matter. Future studies could involve linking up-regulation of cytokine markers and the complete expression and temporal profiles within the CSF, blood and brain. Notwithstanding, cytokine measurements in *in vivo* models of TBI should be assessed more frequently because they provide insight into mechanisms of neuroprotective agents. In the CCI model in particular, standardisation of injury severity would be particularly useful to compare different studies.

7.4 Developing a *Drosophila Melanogaster* model of traumatic injury

The final aim was to establish a high throughput model for polytrauma including TBI, using *Drosophila Melanogaster*. The following sections discuss the potential of *Drosophila* and the spring deflection trauma device to model TBI *in vivo*, followed by the effects of injury

severity, age of the flies, circadian time at which flies received the trauma and hypothermia treatment on survival following trauma. Finally, a discussion of ideas to extend and refine the *Drosophila* model is presented.

7.4.1 *Drosophila* as a model organism for the study of TBI

Drosophila was the first complex organism to have its genome sequenced[340]. The subsequent discoveries of homologies between the human and fly genome have highlighted its potential as a model to study human biology and disease process. The fly is often stated to be at the forefront of biology, where discoveries are elucidated first in the fly and then translated to mammalian systems. The basic biology of flies with relevance to the study of human diseases was described in section 1.5.4. To evaluate how closely TBI may be modelled with *Drosophila*, a few additional features are described here.

The fly brain shares many characteristics, both structural and functional, with the human brain. It is bilaterally symmetrical and is joined to the ventral ganglion that innervates the body. This has been likened to the manner in which the spinal cord innervates the human body[341]. The fly brain has a tripartite organisation and is divided into three regions; the protocerebrum, deutocerebrum, and tritocerebrum, which are homologous to the forebrain, midbrain, and hindbrain, respectively, of humans[342]. The fly brain has a mix of neurons and glial cell types, much like the human brain. Neurons in the fly brain are functionally diverse and possess similar structural features to human neurons, neurotransmitters and ion channels. Similar to humans, glial cells in the fly participate in the innate immune system[343–345]. They also form a BBB equivalent, by sealing off neurons from the surrounding haemolymph, which is the fly equivalent of the blood, as flies do not have a vascular circulatory system. The haemolymph provides nourishment and contains factors important in the immune response[219,346].

Moreover, *Drosophila* are known to have a sophisticated innate immune response but not an adaptive immune system. Their inflammatory response involve pathways that are evolutionarily conserved between humans and rodents such as Toll and Toll-like receptors, TNF α , NF- κ B and JAK/STAT signalling. *Drosophila* Tolls share a cytosolic homology domain with IL-1 receptors in mammals, which interacts with adaptor molecules[222]. In addition, the *Drosophila* immune deficiency (Imd) pathway regulates the activity of a NF- κ B

type protein called Relish. This pathway is often likened to the TNF receptor signalling pathway in mammals, but also shares characteristics with TLR signalling pathways[347,348]. The involvement of Toll and Imd pathways in neurodegenerative processes has been seen in *Drosophila*, similar to the effects seen in mammals, where TNF and NF- κ B cause the release of pro-inflammatory molecules and cell death[348,349]. Furthermore, the neurotransmitters and molecular mechanisms that mediate behaviour such as learning and memory, sleep and circadian patterns are highly conserved between *Drosophila* and higher organisms including humans. Conserved neurotransmitter systems include glutamate, GABA, dopamine, serotonin, acetylcholine, histamine, adenosine and neuropeptides. Both G-protein coupled receptors and ionotropic receptor channels are used[350]. As in mammalian systems, glutamate is a major excitatory neurotransmitter in flies, expressed in the optic lobes, chemosensory neurons and retina. NMDA-type receptors are expressed constitutively throughout the adult brain in all regions, and high levels are detected near ‘mushroom bodies’, which mediate learning and memory processes[351]. Similarly, GABA is the main inhibitory neurotransmitter in *Drosophila*. Ionotropic as well as metabotropic receptors are expressed in the fly CNS while GABA inhibition has been shown to induce seizures[352]. Thus, *Drosophila* exhibit an inflammatory response as well as contain channels involved in excitotoxicity. All of these similarities suggest that the fly brain has the potential to model some of the secondary injury mechanisms seen in humans following a TBI.

7.4.2 Modelling trauma with the spring deflection device

The *Drosophila* model of traumatic injury in this thesis was inspired by the work of Katzenberger and colleagues, who first suggested that it may be possible to model TBI using flies[223]. The assay I have set up was aimed at modelling a blunt trauma. Flies (loaded in vials) were subjected to strike(s) against a hard surface using a metal spring (Figure 2.15). This insult causes the brain to deform against the cuticle, resulting in a traumatic injury to the brain in addition to trauma to the proboscis and thorax. The injury modelled by the device is a mechanical acceleration deceleration type trauma. Flies hit against each other as well as the walls of the hard plastic vial upon the vial contacting the surface of the bench. There is some intrinsic variability to the injury that each fly receives. A fly may hit against another fly before contacting the vial, it may contact the vial head first or not, flies may land on top of each other and so every fly may not suffer the same extent of injury. Each fly’s brain may deform against its cuticle, but the angle or force at which this deformation occurs is not

controlled. Compared to the *in vitro* model of TBI described in this thesis and the *in vivo* CCI model, which are both models where injury parameters are controlled, the fly trauma model has some heterogeneity in it. The heterogeneity in injury on one hand means that examining the effects of treatments on specific pathways and molecules may be challenging because every fly might not have the same extent of injury. However, TBI is a dynamic and heterogeneous disorder, where the primary injury varies in extent, nature and type of damage[3,5]. Thus, the inherent heterogeneity in the *Drosophila* model is actually a key feature of human TBI.

Moreover, the *Drosophila* model is a model of polytrauma because the whole organism is subjected to the mechanical trauma as opposed to other *in vitro* and *in vivo* models of TBI, where only the organism's brain suffers the insult. As the fly can contact the walls of the vial in any position, there is likely to be some injury caused to the proboscis and the thorax. A limitation of this model is that the TBI component of injury is not isolated as yet and it is not clear whether the flies die due to solely brain injury or a combination of other injuries. This would make it difficult to isolate the effects of a neuroprotective agent on brain injury. However, TBI patients typically present with polytrauma due to the accidental and/or violent nature of events that result in a traumatic injury in the first instance[15]. The emergency care team in the hospital must manage these injuries alongside the TBI and thus any drugs or treatments developed must be able to be administered under conditions where patients can be treated for polytrauma. Moreover, as discussed with regards to inflammation in section 7.3.2.4, polytrauma in patients may mask some of the secondary injury mechanisms specific to TBI and to characterise inflammatory responses specific to TBI in a more realistic setting, the development of animal models of polytrauma is encouraged[339]. A polytrauma model in flies presents a simple and cheap first step towards this.

7.4.3 Injury severity is correlated with increased mortality in the short and long term

The first step was to establish a reproducible and controlled injury. In this model, injury severity was assessed as a function of the number of hits or strikes the flies receive and the angle at which the spring is deflected before being released and causing the vial containing flies to strike the hard surface (Figure 2.15). The angle at which the impact device is released is a measure of the tension in the spring and therefore the force of the impact. Some flies

were temporarily incapacitated following the strikes and the time taken to recover mobility was a function of injury severity. The first step in investigating injury severity was to determine whether mortality due to primary (acute) and secondary injury could be isolated. There was no difference between survival at 24 and 48 hours, both when varying the number of hits and the angle of spring deflection (Figure 6.2 and Figure 6.3). Thus, it was concluded that mortality due to the primary injury (number of strikes) was complete within 24 hours and further mortality (i.e., on subsequent days) was due to the development of a secondary injury. Katzenberger and colleagues assessed mortality at 24 hours, the 'MI₂₄', as a measure of primary injury. They found that primary injuries cause death within 24 hours only if the injuries exceed a threshold. A similar pattern was observed in my model. When the vial was held at 90° before releasing the spring, 1 hit did not significantly reduce survival, as survival in this group was 95 ± 2% at 24 hours. However 5 hits, 7 hits and 10 hits significantly reduced survival to 72 ± 3%, 71 ± 5% and 35 ± 7% respectively, compared to sham (100 ± 1%) at 24 hours. The acute mortality (at 24 hours) differs significantly between 1 hit and 5 or 7 hits and between 5 or 7 hits and 10 hits, but not between 5 hits and 7 hits (Figure 6.2). Similarly, retracting the spring back to 180° produced too severe an insult and caused death of all flies within 4 hours of receiving trauma, whereas releasing the spring from 90° and 110° caused a significant decrease in survival to 72 ± 3% and 57 ± 10% respectively, compared to sham (100 ± 7%) at 24 hours. The difference between 90° hits and 110° hits was not significant (Figure 6.3).

To assess survival over the lifetime of the flies, Kaplan Meier analysis was used. Primary injuries that did not cause significant mortality within 24 hours caused a reduction in lifespan. This is exemplified by the percentage survival of flies at 31 days; both the 5 hits group (15 ± 4%,) and 1 hits group (36 ± 6%) were significantly different to the sham group (85 ± 3%). Therefore, the group that received even the mildest injury had a significantly lower survival compared to sham (Figure 6.4). This reduction in survival likely results from the long-term consequences of secondary injuries and is consistent with the findings of Katzenberger and colleagues who found that relative to sham flies, flies that received one strike had a substantially reduced median lifespan[223]. Moreover, hazard ratio analysis indicated that flies who received 5 hits were 2.91 times (95% CI = 2.24 to 3.79) more likely to die than those that received 1 hit. Thus the mortality over a given time interval is positively correlated with injury severity. The survival of flies subjected to 5 hits by releasing the spring from 90° and 110° was also examined (Figure 6.5). The hazard ratio of 110°:90° groups was 1.18 (95%

CI = 0.85 to 1.63), indicating a small numerical difference in the rate of mortality over 31 days between the groups but this difference was not statistically significant. An angle of 90° was chosen on pragmatic grounds because it was easier to standardise. These results show that the model is capable of producing different severities of injury. 1 hit would be mild and 5 hits would be moderate whereas 10 hits would represent a severe injury as it caused 65% mortality within 24 hours. A mild and moderate level of injury were characterised over a longer experimental time frame in the current study. This also highlights the adaptability of the model – it can be used to study severe trauma in the acute phase post injury as well as more chronic effects of trauma in the longer term.

7.4.4 Sensitivity to age and circadian rhythm

Age is a prominent risk factor in human TBI and studies have shown that mortality in the elderly population suffering TBI is approximately double that of younger trauma patients[353]. Studies have shown that older patients (40 years+) show greater decline (assessed by Disability Rating Scale, e-GOS and FIM instrument cognitive score) over the first 5 years following a TBI, compared to younger patients[354]. In the same study, the youngest group of survivors (16 – 26 years) also showed the greatest improvement in disability. In rodent models of TBI, it has been shown that aged rats have a higher risk of death and functional deficits after injury compared to younger rats[355]. In this fly model of TBI, age also increases sensitivity to trauma and is a predictor of poor outcome in the longer term in flies (Figure 6.6). Young (age 1 – 3 days old) and mature (11 – 14 days old) flies were subjected to a mild insult of 1 hit and a moderate insult of 5 hits. When subjected to mild trauma, young flies showed 100% survival over 21 days which was as good as the sham group. However, when mature flies were subjected to mild (and moderate) trauma, their survival was significantly lower than the sham group. The mortality rate in mature flies that received mild trauma was 2.5 times faster than young flies, as indicated by a hazard ratio of 2.45 (95% CI = 1.57 to 3.79). Increasing the severity of trauma further highlighted age at the time of injury as a factor that increases sensitivity to trauma. The mortality rate in mature flies that received moderate trauma was 7 times faster than young flies, as indicated by a hazard ratio of 7.17 (95% CI = 5.38 to 9.55). My results are in agreement with the findings of Katzenberger and colleagues who found an increase in MI_{24} as age increased[223].

Next, the effect of injuring *Drosophila* at different times in their circadian cycle – ‘morning’ (between 1 and 2 hours from the start of subjective day), ‘afternoon’ (between 4 and 6 hours from the start of subject day) and ‘evening’ (more than 8 hours the start of subjective day) was assessed (Figure 6.7). It was found that although percentage survival in injured flies was significantly different to the sham group at all three phases of the day measured, there were some differences between the three groups. The mortality rate in flies injured in the morning was 13 times greater than sham flies as indicated by a hazard ratio of 12.87 (95% CI = 8.33 to 19.88). In contrast, the mortality rate in flies injured in the afternoon was 1.7 times greater than sham flies (hazard ratio = 1.70, 95% CI = 0.59 to 4.86) and the mortality rate in flies injured in the evening was 4.7 times greater than sham flies (hazard ratio = 4.69, 95% CI = 2.49 to 8.83). These results indicate that *Drosophila* are more sensitive to trauma in the early phase of their circadian day, there is a period of lower sensitivity in the afternoon followed by a trend towards increased sensitivity in the evening. A possible explanation for this may involve trends in locomotor behaviour. *Drosophila* are known to show two peaks of activity – one in the morning and one in the evening, under constant environmental conditions[253]. This effect appears to translate into increased sensitivity towards trauma in the morning and evening phases of the *Drosophila* circadian rhythm perhaps due to flies moving more vigorously in the vial and contacting the walls with higher energy. Further, studies have suggested that the circadian clock may be implicated in the regulation of neuroprotective proteins, preventing cerebral oxidative stress and synaptic damage[356]. Elucidating these effects may be of relevance when using genetic and molecular techniques in a *Drosophila* model of trauma. Further, establishing the effect of circadian time on sensitivity to trauma could be useful when studying disturbances to the sleep wake cycle following TBI.

7.4.5 Hypothermia treatment is neuroprotective

This study found that 30 minutes of cooling at 4°C significantly decreased the effect of the primary injury (5 hits at 90°) on fly survival at 24 hours, compared to control injury (72 ± 3% versus a control value of 92 ± 4%) In fact, survival at 24 hours in the hypothermia treated injured group was not significantly different to that of the uninjured sham group, indicating that cooling at 4°C increased survival to the level of uninjured, sham flies at 24 hours (Figure 6.8A). This effect persisted at 14 days post trauma, at which point there was still no difference in survival between the hypothermia treated injured group (90 ± 6%) and sham group (100 ± 8%), but survival in the control injury group had declined significantly (55 ±

2%) in comparison (Figure 6.8B). The hazard ratio (2.55, 95% CI = 1.73 to 3.73) indicated that mortality rate in the injured group was 2.6 times greater than mortality in the hypothermia treated injured group. Moreover, the protective effect of hypothermia in this model is increased at lower temperatures. Treatment with 10°C cooling increased survival at 24 hours compared to the untreated injured group ($86 \pm 16\%$ versus $72 \pm 19\%$) but this difference was not significant (Figure 6.9A). However, over the course of 14 days post trauma (Figure 6.9), the mortality rate in the injured group was 1.6 times greater than the rate in the hypothermia treated injured group (hazard ratio = 1.59, 95% CI = 1.09 to 2.33). This indicates a subtle improvement in long-term survival but not to the level of improvement seen with 4°C treatment.

These findings are interesting because prophylactic hypothermia has been proposed as an option to combat the early stages of TBI pathology by lowering cerebral metabolic rates. Although the exact mechanism is unclear, Diller and colleagues[357] have proposed that there is a 5-7% drop in brain oxygen consumption per degree Celsius decrease in temperature, which is capable of decreasing brain energy expenditure while maintaining blood oxygenation levels. Other mechanisms include a decrease in excitatory amino acid levels, blood brain barrier stabilisation and subsequent reduction in infiltration of inflammatory cytokines and free radicals that also reduces cerebral oedema[357–359]. A *Drosophila* model of trauma would enable the study of mechanisms of hypothermia at a cell and molecular level. For example, the neurotransmitter histamine has been implicated in thermal information processing and Hong *et al.* have suggested that multiple regions of the CNS may be involved in temperature sensation and processing[360]. Additionally, genetic experiments have revealed that an array of TRP channel genes in particular, *painless*[361], *pyrexia*[362] and *trpA1*[363] are involved in thermosensation. Additionally, a protective effect of hypothermia on brain potassium homeostasis was found in a *Drosophila* model of repetitive anoxia. Low temperatures of 17°C slowed the Na⁺/K⁺ pump (but did not affect K⁺ gradient maintenance) and increased tolerance to reperfusion, allowing the fly to recover faster in this ‘stroke’ model[364]. This information could guide experiments to understand the mechanism of hypothermia protection.

So far, prophylactic hypothermia has received mixed results in the literature because of the challenges associated with its implementation, including the rate and initial onset of cooling, desired temperature, temperature at the time of injury and administration of cooling.

However, there is an increasing interest in its neuroprotective use, both alone and as a combination treatment in various experimental models of neurological injury, OHCA, neonatal asphyxia and hypoxia/ischemia[106,110–112,114,365,366]. A *Drosophila* model could through genetic manipulations and the use of knock-in or knockout lines, enable the screening of different compounds in combination with hypothermia to understand mechanisms of action. Finally, the observed efficacy of hypothermia treatment validates the ability of the model to be used as a HTS assay of polytrauma to study treatments.

7.4.6 Refining the *Drosophila* model; considerations and future directions

The results presented are an initial characterisation of a model of polytrauma including head injury in *Drosophila*. The model requires a more definite isolation of the brain injury component of polytrauma. One option could be to design an injury model where similar to rodents, anaesthetised or incapacitated flies receive a precise and controlled brain injury. This could be done using a weight drop device similar to the *in vitro* TBI device used in this thesis. However, while this would more closely simulate a brain injury, the model would no longer be representative of polytrauma, which is becoming more important in TBI research. In order to use the intrinsic heterogeneity that the fly model offers alongside its ability to model polytrauma, another option to confirm that flies do in fact suffer a brain injury component is to use various markers to confirm aspects of injury development. For example, Katzenberger and colleagues noted neurodegeneration in brains by the appearance of vacuolar lesions in the brain neuropil which is a region of axons, synaptic terminals and glial cells[223]. They also observed activation of the innate immune response by quantifying antimicrobial peptide genes, which are implicated in the toll pathway and Imd (analogous to the mammalian TLR and TNF pathways, see section 7.4.1). Another specific way to confirm brain damage is to use a fluorescence assay to examine the blood retinal barrier[344], as a simulation of BBB integrity. This involves injecting a chemical fluor dye into the abdomen of a fly and measuring its penetration into the CNS coarsely *via* epifluorescence microscopy or more finely by imaging dissected brain sections (finely). Other options include behavioural assays like mazes to assess locomotor activity, social interaction, memory and learning.

Ultimately, it is also important to consider the differences between flies and humans and drug delivery issues when developing the model further to study neuroprotection. Firstly, while fundamental pathways can be shared, their implementation can be different. Fly models are

extremely useful when the underlying cause of a human disorder is due to the dysfunction of a single gene or protein. However, TBI is indeed a multifactorial process and before the effects of a drug can be assessed, the particular metric (eg. downregulation of a particular cytokine) must be well defined in flies. Other issues, particularly with regards to drug delivery include the pharmacokinetic and pharmacodynamics profiles of drugs, BBB permeability issues (the fly has an open haemolymph), toxicity and most importantly the actual drug delivery process. Noble gases are inhaled and treatments like hypothermia are straightforward to administer, but the use of soluble drugs may pose a problem. Pandey *et al.* suggest that the drug can be dissolved in food or sucrose, it can be injected into the exposed nerve cord of decapitated flies or the abdomen[222]. Overall, while the *Drosophila* model presents a promising opportunity to develop a high throughput assay, it requires further refinement and recognition of the intrinsic limitations.

7.5 Conclusion and future perspectives for TBI treatment

This thesis was structured as a study of models of TBI and an investigation of drugs (both individually and in combination) to treat the condition. The process began with a series of experiments to refine an *in vitro* model of TBI through developing an injury quantification methodology. Following this, the noble gases xenon and argon, the pleiotropic drug progesterone and the anti-inflammatory meloxicam were explored both individually and in combination to treat TBI *in vitro* (see Table 4.1 for main conclusions). 25% argon in combination with 3 μ M, 10 μ M and 25 μ M progesterone has an increased and additive protective effect at 72 hours. The protective effect of 25% argon increased from $29 \pm 6\%$ to abolishing injury (to sham level) when co-administered with 25 μ M progesterone. With 50% argon ($41 \pm 5\%$ protective alone), co-administration with 3 μ M progesterone abolished injury resulting in a synergistic effect. Co-administration of 50% argon with 10 μ M and 25 μ M progesterone abolished injury in an additive manner. 25% xenon in combination with 3 μ M, 10 μ M and 25 μ M progesterone also had an increased and additive protective effect at 72 hours. The protective effect of 25% xenon increased from $44 \pm 5\%$ to abolishing injury (to sham level) when co-administered with 25 μ M progesterone. Moreover, 25% argon in combination with 0.1 μ M and 1 μ M meloxicam has an additive protective effect at 72 hours and protection with the combination 25% argon and 1 μ M was double that of 25% argon alone. Although the combination of 25% xenon and 0.1 μ M meloxicam was less than

additive, an additive effect was seen with 25% xenon and 1 μ M at 72 hours, which abolished injury (to sham level) unlike the sub-maximally effective 25% xenon. Moreover, isobologram analysis suggests that argon acts synergistically with both progesterone and meloxicam while xenon acts additively to confer neuroprotection with the two drugs.

Next, work undertaken to develop and characterise a CCI model of *in vivo* TBI was presented. Key findings were that contusion volume at 24 hours post injury varied as a function of impact depth, the cytokines TNF and IL-6 are markers of inflammation detectable in the brain and plasma following CCI injury and that the concentration of these cytokines in the plasma correlates positively with injury severity while CCL-2 may be a marker of inflammation after CCI injury, detectable in the brain only. Finally, a series of experiments were conducted to set up a *Drosophila melanogaster* model of polytrauma as a high throughput assay, given that pre-clinical evaluation of drugs is a lengthy and expensive process. *Drosophila* were more sensitive to trauma in the early phase of their circadian day and there was a trend towards increased sensitivity in the evening. While 10⁰C cooling caused a subtle improvement in survival over 14 days post trauma, 4⁰C cooling increased survival to the level of uninjured sham flies at 24 hours and more than doubled the survival over 14 days.

The most interesting finding of this thesis, and the first study of its kind, is that combination treatment with noble gases and progesterone or meloxicam can be more effective than a sub-maximal noble gas treatment and at some concentrations in combination, more effective than progesterone and meloxicam acting alone as well. A more directed approach to developing new treatments for TBI as well as strategies to use existing treatments in combination to increase efficacy are required. The work in this thesis had a translational focus towards this goal. In terms of commercial viability, a 10-year projection of costs and benefits analysis estimated that more than 470,000 quality-adjusted life years were gained at a cost of \$3.6 billion from new therapeutic strategies for TBI, resulting in a net benefit to society of \$17 billion[13,367]. Thus, besides the improved efficacy that may be seen in the clinic with combination therapies, these figures point towards a long-term social and economic benefit. Interestingly, xenon was awarded ‘orphan’ drug status for its use in cardiac arrest by the FDA and in perinatal asphyxia by the EMA in 2015. Orphan status is awarded to drugs that have shown to be safe and effective but are not expected to recover the costs of developing and marketing the treatment. Orphan status comes with lucrative regulatory incentives such as

fast track approvals and up to 10 years of exclusivity if approved. These benefits have triggered a rush of investment and innovation in the last 5 years. Orphan status for xenon is an encouraging step towards increased investment in xenon's use in clinical trials for various disorders, the insights from which will ultimately drive studies on neuroprotection for brain trauma.

The promising results seen with combinations of progesterone, meloxicam and noble gases *in vitro* constitute a solid foundation on which to translate combination drug testing in *in vivo* animal studies, for which the CCI model is an obvious choice as a widely used model of blunt trauma with features similar to clinical TBI. Testament to this, the National Institutes for Health funded 5 preclinical combination studies in adult and immature rat CCI models from 2013 onwards[75]. Using *in vivo* models, it would be possible to vary the treatment regimen and order (noble gas followed by anti-inflammatory or vice versa) more realistically. Although administering xenon requires an expensive closed circuit delivery system, administering argon does not, which makes it easier to test different dose regimen *in vivo*. An important advantage of argon as discussed previously, is that it does not cause general anaesthesia at normobaric pressures, making it useful in clinical scenarios where sedation is not required. Indeed that isobologram analysis revealed that argon acts synergistically with both progesterone and meloxicam is important and warrants further study. Therefore, there is a need to improve the efficacy of argon after TBI while making xenon a more cost effective treatment. The results presented have shown that this is possible *in vitro*, with the use of adjunct therapies like progesterone and meloxicam, and warrants future investigation. In conclusion, the results of this thesis contribute to the growing body of evidence that combination therapies may be the future of TBI treatment.

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References

1. World Health Organisation. *Projections of mortality and burden of disease to 2030: deaths by income group*. Proj Mortal Burd Dis to. 2002;
2. Menon DK, Schwab K, Wright DW, Maas AI. *Position statement: definition of traumatic brain injury*. Arch Phys Med Rehabil. 2010;91(11):1637–40.
3. Reis C, Wang Y, Akyol O, Ho WM, Li RA, Stier G, et al. *What's New in Traumatic Brain Injury: Update on Tracking, Monitoring and Treatment*. Int J Mol Sci. 2015;16(6):11903–65.
4. Langlois JA, Sattin RW. *Traumatic brain injury in the United States: research and programs of the Centers for Disease Control and Prevention (CDC)*. J Head Trauma Rehabil. 20(3):187–8.
5. Maas AIR, Stocchetti N, Bullock R. *Moderate and severe traumatic brain injury in adults*. Lancet Neurol. 2008;7(8):728–41.
6. Dams-O'Connor K, Cuthbert J, Whyte J, Corrigan JD, Faul MG, Harrison-Felix CL. *Traumatic Brain Injury among Older Adults at Level I and II Trauma Centers*. J Neurotrauma. 2013;
7. Ghajar J, Hariri RJ, Narayan RK, Iacono LA, Firlik K, Patterson RH. *Survey of critical care management of comatose, head-injured patients in the United States*. Crit Care Med. 1995;23(3):560–7.
8. Tagliaferri F, Compagnone C, Korsic M, Servadei F, Kraus J. *A systematic review of brain injury epidemiology in Europe*. Acta Neurochir (Wien). 2005;148(3):255–68.
9. Puvanachandra P, Hyder A a. *the Burden of Traumatic Brain Injury in Asia : a Call for Research*. Pak J Neurol Sci. 2009;4(1):27–32.
10. Lu J, Marmarou A, Choi S, Maas A, Murray G, Steyerberg EW. *Mortality from traumatic brain injury*. Acta Neurochir Suppl. 2005;95:281–5.
11. Seifert J. *Incidence and economic burden of injuries in the United States*. J Epidemiol Community Heal. 2007;61(10):926–926.
12. Tosetti P, Hicks RR, Theriault E, Phillips A, Koroshetz W, Draghia-Akli R, et al. *Toward an international initiative for traumatic brain injury research*. J Neurotrauma. 2013;30(14):1211–22.
13. Sobocki P, Olesen J, Jönsson B. *Brain research has high returns but Europe is lagging behind*. Eur J Neurol. 2007;14(6):708–10.
14. Algattas H, Huang JH. *Traumatic Brain Injury pathophysiology and treatments: early, intermediate, and late phases post-injury*. Int J Mol Sci. 2014;15(1):309–41.
15. Ghajar J. *Traumatic brain injury*. Lancet. 2000;356(9233):923–9.
16. Walker KR, Tesco G. *Molecular mechanisms of cognitive dysfunction following traumatic brain injury*. Front Aging Neurosci. 2013;5:29.
17. McAllister TW. *Neurobiological consequences of traumatic brain injury*. Dialogues Clin Neurosci. 2011;13(3):287–300.
18. Hunt RF, Boychuk J a, Smith BN. *Neural circuit mechanisms of post-traumatic epilepsy*. Front Cell Neurosci. 2013;7(89).
19. Roozenbeek B, Maas AIR, Menon DK. *Changing patterns in the epidemiology of traumatic brain injury*. Nat Rev Neurol. 2013;9(4):231–6.
20. Werner C EK. *Pathophysiology of traumatic brain injury*. Br J Anaesth. 2007;99(1):4–9.
21. Palmer AM, Marion DW, Botscheller ML, Swedlow PE, Styren SD, DeKosky ST. *Traumatic brain injury-induced excitotoxicity assessed in a controlled cortical impact model*. J Neurochem. 1993;61(6):2015–24.
22. Faden AI, Demediuk P, Panter SS, Vink R. *The role of excitatory amino acids and NMDA receptors in traumatic brain injury*. Science. 1989;244(4906):798–800.
23. Zhang H, Zhang X, Zhang T CL. *Excitatory amino acids in cerebrospinal fluid of patients with acute head injuries*. Clin Chem. 2001;47(8):1458–62.
24. Baker A, Moulton R, MacMillan V SP. *Excitatory amino acids in cerebrospinal fluid following traumatic brain injury in humans*. J Neurosurg. 1993;79(3):369–72.

25. Chamoun R, Suki D, Gopinath S, Goodman J, Robertson C et. al. *Role of extracellular glutamate measured by cerebral microdialysis in severe traumatic brain injury*. J Neurosurg. 2010;113(3):564–70.
26. Blennow K, Hardy J, Zetterberg H. *The Neuropathology and Neurobiology of Traumatic Brain Injury*. Neuron. 2012;76(5):886–99.
27. Obrenovitch TP, Urenjak J. *Extracellular Glutamate the Key to Traumatic Brain Injury? Excitotoxicity*. 1997;14(10):677–98.
28. Mark LP, Prost RW, Ulmer JL, Smith MM, Daniels DL, Strottmann JM, et al. *Pictorial review of glutamate excitotoxicity: fundamental concepts for neuroimaging*. AJNR Am J Neuroradiol. 2001;22(10):1813–24.
29. Luo P, Fei F, Zhang L, Qu Y, Fei Z. *The role of glutamate receptors in traumatic brain injury: implications for postsynaptic density in pathophysiology*. Brain Res Bull. 2011;85(6):313–20.
30. Niswender CM, Conn PJ. *Metabotropic glutamate receptors: physiology, pharmacology, and disease*. Annu Rev Pharmacol Toxicol. 2010;50:295–322.
31. Beppu K, Sasaki T, Tanaka KF, Yamanaka A, Fukazawa Y, Shigemoto R, et al. *Optogenetic countering of glial acidosis suppresses glial glutamate release and ischemic brain damage*. Neuron. 2014;81(2):314–20.
32. Arundine M, Tymianski M. *Molecular mechanisms of glutamate-dependent neurodegeneration in ischemia and traumatic brain injury*. Cell Mol Life Sci. 2004;61(6):657–68.
33. Das M, Mohapatra S, Mohapatra SS. *New perspectives on central and peripheral immune responses to acute traumatic brain injury*. J Neuroinflammation. 2012;9(1):236.
34. Ziebell JM, Morganti-Kossmann MC. *Involvement of pro- and anti-inflammatory cytokines and chemokines in the pathophysiology of traumatic brain injury*. Neurotherapeutics. 2010;7(1):22–30.
35. Kumar A, Loane DJ. *Neuroinflammation after traumatic brain injury: Opportunities for therapeutic intervention*. Brain Behav Immun. 2012;26(8):1191–201.
36. Aertker BM, Bedi S, Cox CS. *Strategies for CNS repair following TBI*. Exp Neurol. 2015;
37. Rosenfeld J V, Maas AI, Bragge P, Morganti-Kossmann MC, Manley GT, Gruen RL. *Early management of severe traumatic brain injury*. Lancet. 2012;380(9847):1088–98.
38. Krishnamurthy K, Laskowitz DT. *Cellular and Molecular Mechanisms of Secondary Neuronal Injury following Traumatic Brain Injury*. Translational Research in Traumatic Brain Injury. CRC Press/Taylor and Francis Group; 2016.
39. Loane DJ, Faden AI. *Neuroprotection for traumatic brain injury: translational challenges and emerging therapeutic strategies*. Trends Pharmacol Sci. 2010;31(12):596–604.
40. Gentleman SM, Leclercq PD, Moyes L, Graham DI, Smith C, Griffin WST, et al. *Long-term intracerebral inflammatory response after traumatic brain injury*. Forensic Sci Int. 2004;146(2–3):97–104.
41. Tajiri N, Hernandez D, Acosta S, Shinozuka K, Ishikawa H, Ehrhart J, et al. *Suppressed cytokine expression immediately following traumatic brain injury in neonatal rats indicates an expeditious endogenous anti-inflammatory response*. Brain Res. 2014;1559:65–71.
42. Loane DJ, Byrnes KR. *Role of microglia in neurotrauma*. Neurotherapeutics. 2010;7(4):366–77.
43. Lynch MA. *The multifaceted profile of activated microglia*. Mol Neurobiol. 2009;40(2):139–56.
44. Block ML, Hong J-S. *Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism*. Prog Neurobiol. 2005;76(2):77–98.
45. Rothwell NJ. *Cytokines - killers in the brain?* J Physiol. 1999;514(1):3–17.
46. Lucas S-M, Rothwell NJ, Gibson RM. *The role of inflammation in CNS injury and disease*. Br J Pharmacol. 2006;S232–40.
47. Wang CX, Shuaib A. *Involvement of inflammatory cytokines in central nervous system injury*. Prog Neurobiol. 2002;67(2):161–72.
48. Fan L, Young PR, Barone FC, Feuerstein GZ, Smith DH, McIntosh TK. *Experimental brain injury induces expression of interleukin-1 beta mRNA in the rat brain*. Brain Res Mol Brain Res. 1995;30(1):125–30.

49. Kielian T, Hickey WF. *Proinflammatory cytokine, chemokine, and cellular adhesion molecule expression during the acute phase of experimental brain abscess development.* Am J Pathol. 2000;157(2):647–58.
50. Semple BD, Kossmann T, Morganti-Kossmann MC. *Role of chemokines in CNS health and pathology: a focus on the CCL2/CCR2 and CXCL8/CXCR2 networks.* J Cereb Blood Flow Metab. 2010;30(3):459–73.
51. Gaddam SSK, Buell T, Robertson CS. *Systemic manifestations of traumatic brain injury.* Handb Clin Neurol. 2015;127:205–18.
52. Hernandez-Ontiveros DG, Tajiri N, Acosta S, Giunta B, Tan J, Borlongan C V. *Microglia activation as a biomarker for traumatic brain injury.* Front Neurol. 2013;4:30.
53. Tanno H, Nockels RP, Pitts LH, Noble LJ. *Breakdown of the blood-brain barrier after fluid percussion brain injury in the rat: Part 2: Effect of hypoxia on permeability to plasma proteins.* J Neurotrauma. 1992;9(4):335–47.
54. Schouten JW. *Neuroprotection in traumatic brain injury: a complex struggle against the biology of nature.* Curr Opin Crit Care. 2007;13(2):134–42.
55. Brain Trauma Foundation, American Association of Neurological Surgeons (AANS), Congress of Neurological Surgeons (CNS) AJS on N and CC. *Guidelines for the Management of Severe Traumatic Brain Injury (3rd edition).* J Neurotrauma. 2007 May;24(supplement 1):i–vi.
56. McHugh GS, Engel DC, Butcher I, Steyerberg EW, Lu J, Mushkudiani N, et al. *Prognostic value of secondary insults in traumatic brain injury: results from the IMPACT study.* J Neurotrauma. 2007;24(2):287–93.
57. Murray CJ, Lopez AD. *Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study.* Lancet (London, England). 1997;349(9063):1436–42.
58. Teasdale G, Jennett B. *Assessment of coma and impaired consciousness. A practical scale.* Lancet (London, England). 1974;2(7872):81–4.
59. Balestreri M, Czosnyka M, Chatfield DA, Steiner LA, Schmidt EA, Smielewski P, et al. *Predictive value of Glasgow Coma Scale after brain trauma: change in trend over the past ten years.* J Neurol Neurosurg Psychiatry. 2004;75(1):161–2.
60. Stevens RD, Sutter R. *Prognosis in severe brain injury.* Crit Care Med. 2013 Apr;41(4):1104–23.
61. Masel BE, DeWitt DS. *Traumatic brain injury: a disease process, not an event.* J Neurotrauma. 2010;27(8):1529–40.
62. Narayan RK, Michel ME, Ansell B, Baethmann A, Biegon A, Bracken MB, et al. *Clinical trials in head injury.* J Neurotrauma. 2002;19(5):503–57.
63. Maas AIR, Roozenbeek B, Manley GT. *Clinical trials in traumatic brain injury: past experience and current developments.* Neurotherapeutics. 2010;7(1):115–26.
64. Stein DG, Geddes RI, Sribnick EA. *Chapter 28 – Recent developments in clinical trials for the treatment of traumatic brain injury.* In: Handbook of Clinical Neurology. 2015. p. 433–51.
65. Döppenberg EMR, Choi SC, Bullock R. *Clinical trials in traumatic brain injury: lessons for the future.* J Neurosurg Anesthesiol. 2004;16(1):87–94.
66. Pohl D, Bittigau P, Ishimaru MJ, Stadthaus D, Hübner C, Olney JW, et al. *N-Methyl-D-aspartate antagonists and apoptotic cell death triggered by head trauma in developing rat brain.* Proc Natl Acad Sci U S A. 1999;96(5):2508–13.
67. McCormick DA, Contreras D. *On the cellular and network bases of epileptic seizures.* Annu Rev Physiol. 2001;63:815–46.
68. Chen H-SV, Lipton S a. *The chemical biology of clinically tolerated NMDA receptor antagonists.* J Neurochem. 2006;97(6):1611–26.
69. Ikonomidou C, Turski L. *Personal view Why did NMDA receptor antagonists fail clinical trials for stroke and traumatic brain injury? Personal view.* 2002;1:383–6.
70. Diaz-Arrastia R, Kochanek PM, Bergold P, Kenney K, Marx CE, Grimes CJB, et al. *Pharmacotherapy of traumatic brain injury: state of the science and the road forward: report of the Department of Defense Neurotrauma Pharmacology Workgroup.* J Neurotrauma. 2014;31(2):135–58.
71. Bergold PJ. *Treatment of traumatic brain injury with anti-inflammatory drugs.* Exp Neurol.

- 2016;275:367–80.
72. Kochanek PM, Jackson TC, Ferguson NM, Carlson SW, Simon DW, Brockman EC, et al. *Emerging therapies in traumatic brain injury*. Semin Neurol. 2015;35(1):83–100.
73. Kabadi S V, Faden AI. *Neuroprotective strategies for traumatic brain injury: improving clinical translation*. Int J Mol Sci. 2014;15(1):1216–36.
74. Raghupathi R. *Cell Death Mechanisms Following Traumatic Brain Injury*. Brain Pathol. 2004;14(2):215–22.
75. Margulies S, Anderson G, Atif F, Badaut J, Clark R, Empey P, et al. *Combination Therapies for Traumatic Brain Injury: Retrospective Considerations*. J Neurotrauma. 2016;33(1):101–12.
76. Dixon CE, Clifton GL, Lighthall JW, Yaghmai a a, Hayes RL. *A controlled cortical impact model of traumatic brain injury in the rat*. J Neurosci Methods. 1991 Oct;39(3):253–62.
77. Marklund N, Hillered L. *Animal modelling of traumatic brain injury in preclinical drug development: where do we go from here?* Br J Pharmacol. 2011;164(4):1207–29.
78. Morales DM, Marklund N, Lebold D, Thompson HJ, Pitkanen A, Maxwell WL, et al. *Experimental models of traumatic brain injury: do we really need to build a better mousetrap?* Neuroscience. 2005;136(4):971–89.
79. Morganti-Kossmann MC, Yan E, Bye N. *Animal models of traumatic brain injury: is there an optimal model to reproduce human brain injury in the laboratory?* Injury. 2010;41 Suppl 1:S10–3.
80. Kline AE, Leary JB, Radabaugh HL, Cheng JP, Bondi CO. *Combination therapies for neurobehavioral and cognitive recovery after experimental traumatic brain injury: Is more better?* Prog Neurobiol. 2016;142:45–67.
81. Margulies S, Hicks R, Combination Therapies for Traumatic Brain Injury Workshop Leaders. *Combination therapies for traumatic brain injury: prospective considerations*. J Neurotrauma. 2009;26(6):925–39.
82. Lawrence JH, Loomis WF, Tobias CA, Turpin FH. *Preliminary observations on the narcotic effect of xenon with a review of values for solubilities of gases in water and oils*. J Physiol. 1946;5:97–204.
83. Cullen SC, Gross EG. *The Anesthetic Properties of Xenon in Animals and Human Beings, with Additional Observations on Krypton*.
84. Nakata Y, Goto T, Ishiguro Y, Terui K, Kawakami H, Santo M, et al. *Minimum alveolar concentration (MAC) of xenon with sevoflurane in humans*. Anesthesiology. 2001;94(4):611–4.
85. Goto T, Saito H, Shinkai M, Nakata Y, Ichinose F, Morita S. *Xenon provides faster emergence from anesthesia than does nitrous oxide-sevoflurane or nitrous oxide-isoflurane*. Anesthesiology. 1997;86(6):1273–8.
86. Sanders RD, Ma D, Maze M. *Xenon: elemental anaesthesia in clinical practice*. Br Med Bull. 2004;71(1):115–35.
87. Rossaint R, Reyle-Hahn M, Schulte Am Esch J, Scholz J, Scherpereel P, Vallet B, et al. *Multicenter randomized comparison of the efficacy and safety of xenon and isoflurane in patients undergoing elective surgery*. Anesthesiology. 2003;98(1):6–13.
88. Preckel B, Müllenheim J, Moloschavij A, Thämer V, Schlack W. *Xenon administration during early reperfusion reduces infarct size after regional ischemia in the rabbit heart in vivo*. Anesth Analg. 2000;91(6):1327–32.
89. Lachmann B, Armbruster S, Schairer W, Landstra M, Trouwborst A, Van Daal GJ, et al. *Safety and efficacy of xenon in routine use as an inhalational anaesthetic*. Lancet (London, England). 1990;335(8703):1413–5.
90. Lynch C, Baum J, Tenbrinck R. *Xenon anesthesia*. Anesthesiology. 2000;92(3):865–8.
91. Bedi A, Murray JM, Dingley J, Stevenson MA, Fee JPH. *Use of xenon as a sedative for patients receiving critical care*. Crit Care Med. 2003;31(10):2470–7.
92. Franks NP, Dickinson R, de Sousa SL, Hall AC, Lieb WR. *How does xenon produce anaesthesia?* Nature. 1998 Nov 26;396(6709):324.
93. Harris K, Armstrong SP, Campos-Pires R, Kiru L, Franks NP, Dickinson R. *Neuroprotection against Traumatic Brain Injury by Xenon, but Not Argon, Is Mediated by Inhibition at the N-Methyl-D-Aspartate Receptor Glycine Site*. Anesthesiology. 2013;119(5):1137–48.

94. Gruss M, Bushell TJ, Bright DP, Lieb WR, Mathie A, Franks NP. *Two-pore-domain K⁺ channels are a novel target for the anesthetic gases xenon, nitrous oxide, and cyclopropane.* Mol Pharmacol. 2004 Feb;65(2):443–52.
95. Miller KW. *The nature of sites of general anaesthetic action.* Br J Anaesth. 2002;89(1):17–31.
96. de Sousa SL, Dickinson R, Lieb WR, Franks NP. *Contrasting synaptic actions of the inhalational general anesthetics isoflurane and xenon.* Anesthesiology. 2000;92(4):1055–66.
97. Dickinson R, Peterson BK, Banks P, Simillis C, Martin JCS, Valenzuela CA, et al. *Competitive inhibition at the glycine site of the N-methyl-D-aspartate receptor by the anesthetics xenon and isoflurane: evidence from molecular modeling and electrophysiology.* Anesthesiology. 2007;107(5):756–67.
98. Armstrong SP, Banks PJ, McKittrick TJW, Geldart CH, Edge CJ, Babla R, et al. *Identification of two mutations (F758W and F758Y) in the N-methyl-D-aspartate receptor glycine-binding site that selectively prevent competitive inhibition by xenon without affecting glycine binding.* Anesthesiology. 2012;117(1):38–47.
99. Patel AJ, Honoré E. *Anesthetic-sensitive 2P domain K⁺ channels.* Anesthesiology. 2001;95(4):1013–21.
100. Patel AJ, Honoré E. *Properties and modulation of mammalian 2P domain K⁺ channels.* Trends Neurosci. 2001;24(6):339–46.
101. Patel AJ, Honoré E, Lesage F, Fink M, Romey G, Lazdunski M. *Inhalational anesthetics activate two-pore-domain background K⁺ channels.* Nat Neurosci. 1999 May;2(5):422–6.
102. Plested AJR, Wildman SS, Lieb WR, Franks NP. *Determinants of the sensitivity of AMPA receptors to xenon.* Anesthesiology. 2004;100(2):347–58.
103. Coburn M, Maze M, Franks NP. *The neuroprotective effects of xenon and helium in an in vitro model of traumatic brain injury.* Crit Care Med. 2008 Feb;36(2):588–95.
104. Banks P, Franks NP, Dickinson R. *Competitive inhibition at the glycine site of the N-methyl-D-aspartate receptor mediates xenon neuroprotection against hypoxia-ischemia.* Anesthesiology. 2010;112(3):614–22.
105. Dingley J, Tooley J, Porter H, Thoresen M. *Xenon provides short-term neuroprotection in neonatal rats when administered after hypoxia-ischemia.* Stroke. 2006;37(2):501–6.
106. Ma D, Hossain M, Chow A, Arshad M, Battson RM, Sanders RD, et al. *Xenon and hypothermia combine to provide neuroprotection from neonatal asphyxia.* Ann Neurol. 2005;58(2):182–93.
107. Wilhelm S, Ma D, Maze M, Franks NP. *Effects of xenon on in vitro and in vivo models of neuronal injury.* Anesthesiology. 2002;96(6):1485–91.
108. Campos-Pires R; Armstrong, SP.; Sebastiani, Anne; Luh, Clara; Gruss, Marco; Konstantin Radyushkin, Hirnet, Tobias; Werner, Christian; Kristin, Engelhard; Nicholas P Franks, Serge C Thal RD. *Xenon improves neurological outcome and reduces secondary injury following trauma in an in vitro model of traumatic brain injury.* Crit Care Med. 2015;43(1):149–58.
109. Azzopardi D, Robertson NJ, Kapetanakis A, Griffiths J, Rennie JM, Mathieson SR, et al. *Anticonvulsant effect of xenon on neonatal asphyxial seizures.* Arch Dis Child Fetal Neonatal Ed. 2013;98(5):F437-9.
110. Azzopardi D, Robertson NJ, Bainbridge A, Cady E, Charles-Edwards G, Deierl A, et al. *Moderate hypothermia within 6 h of birth plus inhaled xenon versus moderate hypothermia alone after birth asphyxia (TOBY-Xe): a proof-of-concept, open-label, randomised controlled trial.* Lancet Neurol. 2015;15(2):145.
111. Laitio R, Hynninen M, Arola O, Virtanen S, Parkkola R, Saunavaara J, et al. *Effect of Inhaled Xenon on Cerebral White Matter Damage in Comatose Survivors of Out-of-Hospital Cardiac Arrest: A Randomized Clinical Trial.* JAMA. 2016;315(11):1120–8.
112. Ji X, Luo Y, Ling F, Stetler RA, Lan J, Cao G, et al. *Mild hypothermia diminishes oxidative DNA damage and pro-death signaling events after cerebral ischemia: a mechanism for neuroprotection.* Front Biosci. 2007;12:1737–47.
113. Martin J., Ma D, Hossain M, Xu J, Sanders RD, Franks NP, et al. *Asynchronous administration of xenon and hypothermia significantly reduces brain infarction in the neonatal rat.* Br J Anaesth. 2007;93(2):236–40.
114. Thoresen M, Hobbs CE, Wood T, Chakkarapani E, Dingley J. *Cooling combined with*

- immediate or delayed xenon inhalation provides equivalent long-term neuroprotection after neonatal hypoxia-ischemia.* J Cereb Blood Flow Metab. 2009;29(4):707–14.
115. Chakkarapani E, Dingley J, Liu X, Hoque N, Aquilina K, Porter H, et al. *Xenon enhances hypothermic neuroprotection in asphyxiated newborn pigs.* Ann Neurol. 2010;68(3):330–41.
 116. Fries, Michael MD, PhD; Brücken, Anne MD; Çizen, Aysegül MD; Westerkamp, Maren MD; Löwer, Céline MD; Deike-Glindemann, Jan MD; Schnorrenberger, Nora K. MD; Rex, Steffen MD, PhD; Coburn, Mark MD, PhD; Nolte, Kay W. MD; Weis, Joachim MD, PhD; Rossaint, Rolf MM. *Combining xenon and mild therapeutic hypothermia preserves neurological function after prolonged cardiac arrest in pigs.* Crit Care Med. 2012;40(4):1297–303.
 117. Jordan BD, Elizabeth Laura Wright M. *Xenon as an Anesthetic Agent.* AANA J B. 2010;78(5).
 118. Cavendish H. *Experiments on Air. By Henry Cavendish, Esq. F.R.S. and A.S.* Philos Trans R Soc London. 1785;75(0):372–84.
 119. Rayleigh, Lord, Ramsay W. *Argon, a New Constituent of the Atmosphere.* Proc R Soc London. 1894;57:1894–5.
 120. Behnke AR, Thompson RM ME. *The psychologic effects from breathing air at 4 atmospheres pressure.* Am J Physiol Leg Content. 1935;112(3):554–8.
 121. Jawad N, Rizvi M, Gu J, Adeyi O, Tao G, Maze M, et al. *Neuroprotection (and lack of neuroprotection) afforded by a series of noble gases in an in vitro model of neuronal injury.* Neurosci Lett. 2009 Sep 4;460(3):232–6.
 122. Ezzeddine MA. *From xenon to argon: A more clinically accessible neuroprotectant?**. Crit Care Med. 2011 Jun;39(6):1589–90.
 123. Behnke AR YO. *Respiratory resistance, oil–water solubility, and mental effects of argon, compared with helium and nitrogen.* Am J Physiol Leg Content. 1939;126(2):409–15.
 124. Nowrangi DS, Tang J, Zhang JH. *Argon gas: a potential neuroprotectant and promising medical therapy.* Med Gas Res. 2014;4(1):3.
 125. Brücken A, Kurnaz P, Bleilevens C, Derwall M, Weis J, Nolte K, et al. *Dose dependent neuroprotection of the noble gas argon after cardiac arrest in rats is not mediated by K(ATP)-channel opening.* Resuscitation. 2014;85(6):826–32.
 126. Abraini JH, Kriem B, Balon N, Rostain J-C, Risso J-J. *Gamma-aminobutyric acid neuropharmacological investigations on narcosis produced by nitrogen, argon, or nitrous oxide.* Anesth Analg. 2003;96(3):746–9, table of contents.
 127. Zhuang L, Yang T, Zhao H, Fidalgo AR, Vizcaychipi MP, Sanders RD, et al. *The protective profile of argon, helium, and xenon in a model of neonatal asphyxia in rats.* Crit Care Med. 2012;40(6):1724–30.
 128. Fahlenkamp A V, Rossaint R, Haase H, Al Kassam H, Ryang Y-M, Beyer C, et al. *The noble gas argon modifies extracellular signal-regulated kinase 1/2 signaling in neurons and glial cells.* Eur J Pharmacol. 2012;674(2–3):104–11.
 129. Loetscher PD, Rossaint J, Rossaint R, Weis J, Fries M, Fahlenkamp A, et al. *Argon: Neuroprotection in in vitro models of cerebral ischemia and traumatic brain injury.* Crit Care. 2009;13(6):R206.
 130. Ryang Y-M, Fahlenkamp A V, Rossaint R, Wesp D, Loetscher PD, Beyer C, et al. *Neuroprotective effects of argon in an in vivo model of transient middle cerebral artery occlusion in rats.* Crit Care Med. 2011;39(6):1448–53.
 131. Brücken A, Kurnaz P, Bleilevens C, Derwall M, Weis J, Nolte K, et al. *Delayed argon administration provides robust protection against cardiac arrest-induced neurological damage.* Neurocrit Care. 2015;22(1):112–20.
 132. Brücken A, Cizen A, Fera C, Meinhardt A, Weis J, Nolte K, et al. *Argon reduces neurohistopathological damage and preserves functional recovery after cardiac arrest in rats.* Br J Anaesth. 2013;110 Suppl:i106–12.
 133. Zhao H, Mitchell S, Koumpa S, Cui YT, Lian Q, Hagberg H, et al. *Heme Oxygenase-1 Mediates Neuroprotection Conferred by Argon in Combination with Hypothermia in Neonatal Hypoxia–Ischemia Brain Injury.* Anesthesiology. 2016 Jul;125(1):180–92.
 134. Broad KD, Fierens I, Fleiss B, Rocha-Ferreira E, Ezzati M, Hassell J, et al. *Inhaled 45-50% argon augments hypothermic brain protection in a piglet model of perinatal asphyxia.* Neurobiol Dis. 2016;87:29–38.

135. Coburn M, Rossaint R. *Argon in the fast lane: noble gases and their neuroprotective effects*. Crit Care Med. 2012;40(6):1965–6.
136. Weill-Engerer S, David J-P, Sazdovitch V, Liere P, Eychenne B, Pianos A, et al. *Neurosteroid quantification in human brain regions: comparison between Alzheimer's and nondemented patients*. J Clin Endocrinol Metab. 2002;87(11):5138–43.
137. Yen, Samuel; Strauss, Jerome; Barbieri R. *Yen and Jaffe's reproductive endocrinology: physiology, pathophysiology, and clinical management*. 7th ed. Philadelphia: Saunders; 2004.
138. Hammond GL, Hirvonen J, Vihko R. *Progesterone, androstenedione, testosterone, 5 alpha-dihydrotestosterone and androsterone concentrations in specific regions of the human brain*. J Steroid Biochem. 1983;18(2):185–9.
139. Stefanick ML. *Estrogens and progestins: background and history, trends in use, and guidelines and regimens approved by the US Food and Drug Administration*. Am J Med. 2005;118(12):64–73.
140. Kaore SN, Langade DK, Yadav VK, Sharma P, Thawani VR, Sharma R. *Novel actions of progesterone: what we know today and what will be the scenario in the future?* J Pharm Pharmacol. 2012 Aug;64(8):1040–62.
141. Radley E, Akram A, Grubb BD, Gibson CL. *Investigation of the mechanisms of progesterone protection following oxygen-glucose deprivation in organotypic hippocampal slice cultures*. Neurosci Lett. 2012;506(1):131–5.
142. De Nicola AF, Gonzalez SL, Labombarda F, González Deniselle MC, Garay L, Guennoun R, et al. *Progesterone treatment of spinal cord injury: Effects on receptors, neurotrophins, and myelination*. J Mol Neurosci. 2006;28(1):3–15.
143. Singh M, Su C. *Progesterone and neuroprotection*. Horm Behav. 2013;63(2):284–90.
144. Roof RL, Duvdevani R, Braswell L, Stein DG. *Progesterone facilitates cognitive recovery and reduces secondary neuronal loss caused by cortical contusion injury in male rats*. Exp Neurol. 1994 Sep;129(1):64–9.
145. Taraborrelli S. *Physiology, production and action of progesterone*. Acta Obstet Gynecol Scand. 2015;94(S161):8–16.
146. Bullock MR, Lyeth BG, Muizelaar JP. *Current status of neuroprotection trials for traumatic brain injury: lessons from animal models and clinical studies*. Neurosurgery. 1999;45(2):207-17-20.
147. Lancel M, Faulhaber J, Holsboer F, Rupprecht R. *Progesterone induces changes in sleep comparable to those of agonistic GABAA receptor modulators*. Am J Physiol. 1996;271(4 Pt 1):E763-72.
148. Wright DW, Ritchie JC, Mullins RE, Kellermann AL, Denson DD. *Steady-state serum concentrations of progesterone following continuous intravenous infusion in patients with acute moderate to severe traumatic brain injury*. J Clin Pharmacol. 2005;45(6):640–8.
149. Guennoun R, Labombarda F, Gonzalez Deniselle MC, Liere P, De Nicola AF, Schumacher M. *Progesterone and allopregnanolone in the central nervous system: Response to injury and implication for neuroprotection*. J Steroid Biochem Mol Biol. 2015;146:48–61.
150. Wright DW, Bauer ME, Hoffman SW, Stein DG. *Serum progesterone levels correlate with decreased cerebral edema after traumatic brain injury in male rats*. J Neurotrauma. 2001;18(9):901–9.
151. Stein DG, Wright DW. *Progesterone in the clinical treatment of acute traumatic brain injury*. Expert Opin Investig Drugs. 2010;19(7):847–57.
152. Kato J, Hirata S, Nozawa A, Yamada-Mouri N. *Gene expression of progesterone receptor isoforms in the rat brain*. Horm Behav. 1994;28(4):454–63.
153. Meffre D, Labombarda F, Delespierre B, Chastre A, De Nicola AF, Stein DG, et al. *Distribution of membrane progesterone receptor alpha in the male mouse and rat brain and its regulation after traumatic brain injury*. Neuroscience. 2013;231:111–24.
154. Roof RL, Duvdevani R, Stein DG. *Progesterone treatment attenuates brain edema following contusion injury in male and female rats*. Restor Neurol Neurosci. 1992;4(6):425–7.
155. Guo Q, Sayeed I, Baronne LM, Hoffman SW, Guennoun R, Stein DG. *Progesterone administration modulates AQP4 expression and edema after traumatic brain injury in male rats*. Exp Neurol. 2006;198(2):469–78.

156. Limmroth V, Lee WS, Moskowitz MA. *GABAA-receptor-mediated effects of progesterone, its ring-A-reduced metabolites and synthetic neuroactive steroids on neurogenic oedema in the rat meninges*. Br J Pharmacol. 1996;117(1):99–104.
157. Roof RL, Hoffman SW, Stein DG. *Progesterone protects against lipid peroxidation following traumatic brain injury in rats*. Mol Chem Neuropathol. 1997;31(1):1–11.
158. Moorthy K, Sharma D, Basir SF, Baquer NZ. *Administration of estradiol and progesterone modulate the activities of antioxidant enzyme and aminotransferases in naturally menopausal rats*. Exp Gerontol. 2005;40(4):295–302.
159. Pettus EH, Wright DW, Stein DG, Hoffman SW. *Progesterone treatment inhibits the inflammatory agents that accompany traumatic brain injury*. Brain Res. 2005;1049(1):112–9.
160. Djebaili M, Hoffman SW, Stein DG. *Allopregnanolone and progesterone decrease cell death and cognitive deficits after a contusion of the rat pre-frontal cortex*. Neuroscience. 2004;123(2):349–59.
161. VanLandingham JW, Cekic M, Cutler S, Hoffman SW, Stein DG. *Neurosteroids reduce inflammation after TBI through CD55 induction*. Neurosci Lett. 2007;425(2):94–8.
162. He J, Evans C-O, Hoffman SW, Oyesiku NM, Stein DG. *Progesterone and allopregnanolone reduce inflammatory cytokines after traumatic brain injury*. Exp Neurol. 2004;189(2):404–12.
163. Smith SS. *Progesterone administration attenuates excitatory amino acid responses of cerebellar Purkinje cells*. Neuroscience. 1991;42(2):309–20.
164. Luoma JI, Kelley BG, Mermelstein PG. *Progesterone inhibition of voltage-gated calcium channels is a potential neuroprotective mechanism against excitotoxicity*. Steroids. 2011;76(9):845–55.
165. Shen H, Gong QH, Yuan M, Smith SS. *Short-term steroid treatment increases delta GABAA receptor subunit expression in rat CA1 hippocampus: pharmacological and behavioral effects*. Neuropharmacology. 2005;49(5):573–86.
166. González SL, Labombarda F, González Deniselle MC, Guennoun R, Schumacher M, De Nicola AF. *Progesterone up-regulates neuronal brain-derived neurotrophic factor expression in the injured spinal cord*. Neuroscience. 2004;125(3):605–14.
167. Cekic M, Johnson SJ, Bhatt VH, Stein DG. *Progesterone treatment alters neurotrophin/proneurotrophin balance and receptor expression in rats with traumatic brain injury*. Restor Neurol Neurosci. 2012;30(2):115–26.
168. Djebaili M, Guo Q, Pettus EH, Hoffman SW, Stein DG. *The neurosteroids progesterone and allopregnanolone reduce cell death, gliosis, and functional deficits after traumatic brain injury in rats*. J Neurotrauma. 2005;22(1):106–18.
169. Cutler SM, VanLandingham JW, Murphy AZ, Stein DG. *Slow-release and injected progesterone treatments enhance acute recovery after traumatic brain injury*. Pharmacol Biochem Behav. 2006;84(3):420–8.
170. Cutler SM, Cekic M, Miller DM, Wali B, VanLandingham JW, Stein DG. *Progesterone improves acute recovery after traumatic brain injury in the aged rat*. J Neurotrauma. 2007;24(9):1475–86.
171. Wali B, Sayeed I, Stein DG. *Improved behavioral outcomes after progesterone administration in aged male rats with traumatic brain injury*. Restor Neurol Neurosci. 2011;29(1):61–71.
172. Hua F, Reiss JI, Tang H, Wang J, Fowler X, Sayeed I, et al. *Progesterone and low-dose vitamin D hormone treatment enhances sparing of memory following traumatic brain injury*. Horm Behav. 2012;61(4):642–51.
173. Jones NC, Constantin D, Prior MJW, Morris PG, Marsden CA, Murphy S. *The neuroprotective effect of progesterone after traumatic brain injury in male mice is independent of both the inflammatory response and growth factor expression*. Eur J Neurosci. 2005;21(6):1547–54.
174. O'Connor CA, Cernak I, Johnson F, Vink R. *Effects of progesterone on neurologic and morphologic outcome following diffuse traumatic brain injury in rats*. Exp Neurol. 2007;205(1):145–53.
175. Wright DW, Kellermann AL, Hertzberg VS, Clark PL, Frankel M, Goldstein FC, et al. *ProTECT: a randomized clinical trial of progesterone for acute traumatic brain injury*. Ann Emerg Med. 2007;49(4):391–402, 402-2.

176. Xiao G, Wei J, Yan W, Wang W, Lu Z. *Improved outcomes from the administration of progesterone for patients with acute severe traumatic brain injury: a randomized controlled trial.* Crit Care. 2008;12(2):R61.
177. Skolnick BE, Maas AI, Narayan RK, van der Hoop RG, MacAllister T, Ward JD, et al. *A Clinical Trial of Progesterone for Severe Traumatic Brain Injury.* N Engl J Med. 2014;371(26):2467–76.
178. Wright DW, Yeatts SD, Silbergleit R, Palesch YY, Hertzberg VS, Frankel M, et al. *Very Early Administration of Progesterone for Acute Traumatic Brain Injury.* N Engl J Med. 2014;371(26):2457–66.
179. Stein DG. *Embracing failure: What the Phase III progesterone studies can teach about TBI clinical trials.* Brain Inj. 2015;29(11):1259–72.
180. Schumacher M, Denier C, Oudinet J-P, Adams D, Guennoun R. *Progesterone neuroprotection: The background of clinical trial failure.* 2016;160:53–66.
181. Fleischmann R, Iqbal I, Slobodin G. *Meloxicam.* Expert Opin Pharmacother. 2002 Oct 25;3(10):1501–12.
182. Vane JR, Bakhle YS, Botting RM. *Cyclooxygenases 1 AND 2.* Annu Rev Pharmacol Toxicol. 1998;38(1):97–120.
183. Dash PK, Mach SA, Moore AN. *Regional Expression and Role of Cyclooxygenase-2 Following Experimental Traumatic Brain Injury.* J Neurotrauma. 2000;17(1):69–81.
184. Strauss KI. *Antiinflammatory and neuroprotective actions of COX2 inhibitors in the injured brain.* Brain Behav Immun. 2008;22(3):285–98.
185. Hakan T, Toklu HZ, Biber N, Ozevren H, Solakoglu S, Demirturk P, et al. *Effect of COX-2 inhibitor meloxicam against traumatic brain injury-induced biochemical, histopathological changes and blood–brain barrier permeability.* Neurol Res. 2010;32(6):629–35.
186. Strauss KI, Barbe MF, Marshall RM, Raghupathi R, Mehta S, Narayan RK. *Prolonged cyclooxygenase-2 induction in neurons and glia following traumatic brain injury in the rat.* J Neurotrauma. 2000;17(8):695–711.
187. FitzGerald GA, Patrono C. *The coxibs, selective inhibitors of cyclooxygenase-2.* N Engl J Med. 2001;345(6):433–42.
188. Everts B, Währborg P, Hedner T. *COX-2-Specific inhibitors--the emergence of a new class of analgesic and anti-inflammatory drugs.* Clin Rheumatol. 2000;19(5):331–43.
189. Wang Y, Qin Z. *Molecular and cellular mechanisms of excitotoxic neuronal death.* Apoptosis. 2010;15(11):1382–402.
190. Candelario-Jalil E, Ajamieh HH, Sam S, Martínez G, León Fernández OS. *Nimesulide limits kainate-induced oxidative damage in the rat hippocampus.* Eur J Pharmacol. 2000;390(3):295–8.
191. Iadecola C, Niwa K, Nogawa S, Zhao X, Nagayama M, Araki E, et al. *Reduced susceptibility to ischemic brain injury and N-methyl-D-aspartate-mediated neurotoxicity in cyclooxygenase-2-deficient mice.* Proc Natl Acad Sci U S A. 2001;98(3):1294–9.
192. Llorente IL, Landucci E, Pellegrini-Giampietro DE, Fernández-López A. *Glutamate receptor and transporter modifications in rat organotypic hippocampal slice cultures exposed to oxygen–glucose deprivation: The contribution of cyclooxygenase-2.* Neuroscience. 2015;292:118–28.
193. Girgis H, Palmier B, Croci N, Soustrat M, Plotkine M, Marchand-Leroux C. *Effects of selective and non-selective cyclooxygenase inhibition against neurological deficit and brain oedema following closed head injury in mice.* Brain Res. 2013;1491:78–87.
194. Morrison B, Saatman KE, Meaney DF, McIntosh TK. *In Vitro Central Nervous System Models of Mechanically Induced Trauma: A Review.* J Neurotrauma. 1998;15(11):911–28.
195. Morrison B, Elkin BS, Dollé J-P, Yarmush ML. *In vitro models of traumatic brain injury.* Annu Rev Biomed Eng. 2011;13:91–126.
196. Bahr B a. *Long-term hippocampal slices: a model system for investigating synaptic mechanisms and pathologic processes.* J Neurosci Res. 1995;42(3):294–305.
197. Buchs PA, Stoppini L, Muller D. *Structural modifications associated with synaptic development in area CA1 of rat hippocampal organotypic cultures.* Brain Res Dev Brain Res. 1993;71(1):81–91.

198. Gutiérrez R, Heinemann U. *Synaptic reorganization in explanted cultures of rat hippocampus*. Brain Res. 1999;815(2):304–16.
199. Zimmer J, Gähwiler BH. *Cellular and connective organization of slice cultures of the rat hippocampus and fascia dentata*. J Comp Neurol. 1984;228(3):432–46.
200. Gähwiler BH. *Organotypic cultures of neural tissue*. Trends Neurosci. 1988;11(11):484–9.
201. De Simoni A, Yu LMY. *Preparation of organotypic hippocampal slice cultures: interface method*. Nat Protoc. 2006;1(3):1439–45.
202. Renkawek K, Murray MR, Spatz M, Klatzo I. *Distinctive histochemical characteristics of brain capillaries in organotypic culture*. Exp Neurol. 1976;50(1):194–206.
203. Moser K V., Schmidt-Kastner R, Hinterhuber H, Humpel C. *Brain capillaries and cholinergic neurons persist in organotypic brain slices in the absence of blood flow*. Eur J Neurosci. 2003;18(1):85–94.
204. Humpel C. *Organotypic brain slice cultures: A review*. Neuroscience. 2015;305:86–98.
205. Stoppini L, Buchs P a, Muller D. *A simple method for organotypic cultures of nervous tissue*. J Neurosci Methods. 1991 Apr;37(2):173–82.
206. Cho S, Wood A, Bowlby MR. *Brain slices as models for neurodegenerative disease and screening platforms to identify novel therapeutics*. Curr Neuropharmacol. 2007;5(1):19–33.
207. Adamchik Y, Frantseva MV, Weisspapir M, Carlen PL, Perez Velazquez JL. *Methods to induce primary and secondary traumatic damage in organotypic hippocampal slice cultures*. Brain Res Protoc. 2000;5(2):153–8.
208. Roehl AB, Hein M, Loetscher PD, Rossaint J, Weis J, Rossaint R, et al. *Neuroprotective properties of levosimendan in an in vitro model of traumatic brain injury*. BMC Neurol. 2010;10:97.
209. Adembri C, Massagrande A, Tani A, Miranda M, Margheri M, De Gaudio R, et al. *Carbamylated erythropoietin is neuroprotective in an experimental model of traumatic brain injury*. Crit Care Med. 2008;36(3):975–8.
210. Lighthall JW. *Controlled cortical impact: a new experimental brain injury model*. J Neurotrauma. 1988;5(1):1–15.
211. Smith DH, Soares HD, Pierce JS, Perlman KG, Saatman KE, Meaney DF, et al. *A model of parasagittal controlled cortical impact in the mouse: cognitive and histopathologic effects*. J Neurotrauma. 1995;12(2):169–78.
212. Manley GT, Rosenthal G, Lam M, Morabito D, Yan D, Derugin N, et al. *Controlled Cortical Impact in Swine: Pathophysiology and Biomechanics*. J Neurotrauma. 2006;23(2):128–39.
213. King C, Robinson T, Dixon CE, Rao GR, Larnard D, Nemoto CEM. *Brain temperature profiles during epidural cooling with the ChillerPad in a monkey model of traumatic brain injury*. J Neurotrauma. 2010;27(10):1895–903.
214. Hall ED, Sullivan PG, Gibson TR, Pavel KM, Thompson BM, Scheff SW. *Spatial and temporal characteristics of neurodegeneration after controlled cortical impact in mice: more than a focal brain injury*. J Neurotrauma. 2005;22(2):252–65.
215. Osier ND, Dixon CE. *The Controlled Cortical Impact Model: Applications, Considerations for Researchers, and Future Directions*. Front Neurol. 2016;7:134.
216. Bramlett HM, Dietrich WD. *Long-Term Consequences of Traumatic Brain Injury: Current Status of Potential Mechanisms of Injury and Neurological Outcomes*. J Neurotrauma. 2014;15:150127063150005.
217. Bramlett HM, Dietrich WD. *Quantitative structural changes in white and gray matter 1 year following traumatic brain injury in rats*. Acta Neuropathol. 2002;103(6):607–14.
218. Loane DJ, Kumar A, Stoica BA, Cabatbat R, Faden AI. *Progressive neurodegeneration after experimental brain trauma: association with chronic microglial activation*. J Neuropathol Exp Neurol. 2014;73(1):14–29.
219. Lessing D, Bonini NM. *Maintaining the brain: insight into human neurodegeneration from Drosophila melanogaster mutants*. Nat Rev Genet. 2009 Jun;10(6):359–70.
220. Reiter LT, Potocki L, Chien S, Gribskov M, Bier E. *A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster*. Genome Res. 2001;11(6):1114–25.
221. Lloyd TE, Taylor JP. *Flightless flies: Drosophila models of neuromuscular disease*. Ann N Y Acad Sci. 2010;1184:e1–20.

222. Pandey UB, Nichols CD. *Human disease models in Drosophila melanogaster and the role of the fly in therapeutic drug discovery*. Pharmacol Rev. 2011;63(2):411–36.
223. Katzenberger RJ, Loewen CA, Wassarman DR, Petersen AJ, Ganetzky B, Wassarman DA. *A Drosophila model of closed head traumatic brain injury*. Proc Natl Acad Sci U S A. 2013;110(44):E4152-9.
224. Hezel M, Ebrahimi F, Koch M, Dehghani F. *Propidium iodide staining: a new application in fluorescence microscopy for analysis of cytoarchitecture in adult and developing rodent brain*. Micron. 2012 Oct;43(10):1031–8.
225. Morton DB, Griffiths PH. *Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment*. Vet Rec. 1985 Apr 20;116(16):431–6.
226. Lee H-F, Lee T-S, Kou YR. *Anti-inflammatory and neuroprotective effects of triptolide on traumatic brain injury in rats*. Respir Physiol Neurobiol. 2012;182(1):1–8.
227. Dalgard CL, Cole JT, Kean WS, Lucky JJ, Sukumar G, McMullen DC, et al. *The cytokine temporal profile in rat cortex after controlled cortical impact*. Front Mol Neurosci. 2012;5:6.
228. Chen S-F, Hsu C-W, Huang W-H, Wang J-Y. *Post-injury baicalein improves histological and functional outcomes and reduces inflammatory cytokines after experimental traumatic brain injury*. Br J Pharmacol. 2008;155(8):1279–96.
229. Papp EA, Leergaard TB, Calabrese E, Johnson GA, Bjaalie JG. *Waxholm Space atlas of the Sprague Dawley rat brain*. Neuroimage. 2014;97:374–86.
230. Türeyen K, Vemuganti R, Sailor KA, Dempsey RJ. *Infarct volume quantification in mouse focal cerebral ischemia: a comparison of triphenyltetrazolium chloride and cresyl violet staining techniques*. J Neurosci Methods. 2004;139(2):203–7.
231. Indiana University. *Fly Food - Bloomington Protocol* [Internet]. 2007 [cited 2016 Aug 22]. Available from: http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/media-recipes.htm
232. Sayeed O, Benzer S. *Behavioral genetics of thermosensation and hygrosensation in Drosophila*. Proc Natl Acad Sci U S A. 1996;93(12):6079–84.
233. Hamada FN, Rosenzweig M, Kang K, Pulver SR, Ghezzi A, Jegla TJ, et al. *An internal thermal sensor controlling temperature preference in Drosophila*. Nature. 2008;454(7201):217–20.
234. Garrity PA, Goodman MB, Samuel AD, Sengupta P. *Running hot and cold: behavioral strategies, neural circuits, and the molecular machinery for thermotaxis in C. elegans and Drosophila*. Genes Dev. 2010;24(21):2365–82.
235. Foucquier J, Guedj M. *Analysis of drug combinations: current methodological landscape*. Pharmacol Res Perspect. 2015;3(3):e00149.
236. Greco WR, Bravo G, Parsons JC. *The search for synergy: a critical review from a response surface perspective*. Pharmacol Rev. 1995;47(2).
237. Geary N, Afeltra J, Vitale R, Mouton J, Verweij P, Air E, et al. *Understanding synergy*. Am J Physiol Endocrinol Metab. 2013;304(3):E237-53.
238. Bliss CI. *The toxicity of poisons applied jointly*. Ann Appl Biol. 1939;26(3):585–615.
239. Zhao W, Sachsenmeier K, Zhang L, Sult E, Hollingsworth RE, Yang H. *A New Bliss Independence Model to Analyze Drug Combination Data*. J Biomol Screen. 2014 Jun;19(5):817–21.
240. Yadav B, Wennerberg K, Aittokallio T, Tang J. *Searching for Drug Synergy in Complex Dose–Response Landscapes Using an Interaction Potency Model*. Comput Struct Biotechnol J. 2015;13:504–13.
241. Kline AE, Leary JB, Radabaugh HL, Cheng JP, Bondi CO. *Combination therapies for neurobehavioral and cognitive recovery after experimental traumatic brain injury: Is more better?* Prog Neurobiol. 2016;142:45–67.
242. Janowitz T, Menon DK. *Exploring new routes for neuroprotective drug development in traumatic brain injury*. Sci Transl Med. 2010;2(27):27rv1.
243. Pleasant JM, Carlson SW, Mao H, Scheff SW, Yang KH, Saatman KE. *Rate of neurodegeneration in the mouse controlled cortical impact model is influenced by impactor tip shape: implications for mechanistic and therapeutic studies*. J Neurotrauma. 2011;28(11):2245–62.
244. Osier ND, Korpon JR, Dixon CE. *Controlled Cortical Impact Model*. Brain Neurotrauma:

- Molecular, Neuropsychological, and Rehabilitation Aspects. CRC Press/Taylor & Francis; 2015.
245. Deng P, Xu ZC. *Contribution of Ih to neuronal damage in the hippocampus after traumatic brain injury in rats*. J Neurotrauma. 2011;28(7):1173–83.
 246. Plesnila N, Friedrich D, Eriskat J, Baethmann A, Stoffel M. *Relative cerebral blood flow during the secondary expansion of a cortical lesion in rats*. Neurosci Lett. 2003;345(2):85–8.
 247. Carter M, Shieh JC. *Guide to Research Techniques in Neuroscience*. Guide to Research Techniques in Neuroscience. Elsevier; 2010. 297-329 p.
 248. Goodman JC, Cherian L, Bryan RM, Robertson I CS. *Lateral Cortical Impact Injury in Rats: Pathologic Effects of Varying Cortical Compression and Impact Velocity*. J Neurotrauma. 1994;11(5).
 249. Kaplan EL, Meier P. *Nonparametric Estimation from Incomplete Observations*. J Am Stat Assoc. 1958;53(282):457.
 250. Rich JT, Neely JG, Paniello RC, Voelker CCJ, Nussenbaum B, Wang EW. *A practical guide to understanding Kaplan-Meier curves*. Otolaryngol Head Neck Surg. 2010;143(3):331–6.
 251. Jager KJ, van Dijk PC, Zoccali C, Dekker FW. *The analysis of survival data: the Kaplan–Meier method*. Kidney Int. 2008;74(5):560–5.
 252. Stromnes O, Kvelland I. *Sexual activity of Drosophila Melanogaster males*. Hereditas. 1962;
 253. Peschel N, Helfrich-Förster C. *Setting the clock – by nature: Circadian rhythm in the fruitfly Drosophila melanogaster*. FEBS Lett. 2011;585(10):1435–42.
 254. Kotapka MJ, Graham DI, Adams JH, Gennarelli TA. *Hippocampal pathology in fatal non-missile human head injury*. Acta Neuropathol. 1992;83(5):530–4.
 255. Ariza M, Serra-Grabulosa JM, Junqué C, Ramírez B, Mataró M, Poca A, et al. *Hippocampal head atrophy after traumatic brain injury*. Neuropsychologia. 2006;44(10):1956–61.
 256. Maxwell WL, Dhillon K, Harper L, Espin J, MacIntosh TK, Smith DH, et al. *There is differential loss of pyramidal cells from the human hippocampus with survival after blunt head injury*. J Neuropathol Exp Neurol. 2003;62(3):272–9.
 257. Watanabe M, Fukaya M, Sakimura K, Manabe T, Mishina M, Inoue Y. *Selective scarcity of NMDA receptor channel subunits in the stratum lucidum (mossy fibre-recipient layer) of the mouse hippocampal CA3 subfield*. Eur J Neurosci. 1998;10(2):478–87.
 258. Hörtnagl H, Tasan RO, Wieselthaler A, Kirchmair E, Sieghart W, Sperk G. *Patterns of mRNA and protein expression for 12 GABAA receptor subunits in the mouse brain*. Neuroscience. 2013;236:345–72.
 259. Drexel M, Puhakka N, Kirchmair E, Hörtnagl H, Pitkänen A, Sperk G. *Expression of GABA receptor subunits in the hippocampus and thalamus after experimental traumatic brain injury*. Neuropharmacology. 2015;88:122–33.
 260. Gähwiler BH, Capogna M, Debanne D, McKinney RA, Thompson SM. *Organotypic slice cultures: a technique has come of age*. Trends Neurosci. 1997;20(10):471–7.
 261. De Simoni A, Griesinger CB, Edwards FA. *Development of rat CA1 neurones in acute versus organotypic slices: role of experience in synaptic morphology and activity*. J Physiol. 2003;550(Pt 1):135–47.
 262. Li Q, Han X, Wang J. *Organotypic Hippocampal Slices as Models for Stroke and Traumatic Brain Injury*. Mol Neurobiol. 2015;
 263. Adembri C, Bechi A, Meli E, Gramigni E, Venturi L, Moroni F, et al. *Erythropoietin attenuates post-traumatic injury in organotypic hippocampal slices*. J Neurotrauma. 2004;21(8):1103–12.
 264. Sieg F, Wahle P, Pape H-C. *Cellular Reactivity to Mechanical Axonal Injury in an Organotypic in Vitro Model of Neurotrauma*. J Neurotrauma. 1999;16(12):1197–213.
 265. Church AJ, Andrew RD. *Spreading Depression Expands Traumatic Injury in Neocortical Brain Slices*. J Neurotrauma. 2005;22(2):277–90.
 266. Azevedo A, Leao P. *Spreading depression of activity in the cerebral cortex. Remarkable Reaction of the Brain Gray Matter*. J Neurophysiol Department Physiol Harvard Med Sch Boston MA. 1944;7(21):359–90.
 267. Lauritzen M, Dreier JP, Fabricius M, Hartings JA, Graf R, Strong AJ. *Clinical relevance of cortical spreading depression in neurological disorders: migraine, malignant stroke,*

- subarachnoid and intracranial hemorrhage, and traumatic brain injury. *J Cereb Blood Flow Metab.* 2011;31(1):17–35.
268. Laake JH, Haug FM, Wieloch T, Ottersen OP. *A simple in vitro model of ischemia based on hippocampal slice cultures and propidium iodide fluorescence.* *Brain Res Brain Res Protoc.* 1999;4(2):173–84.
 269. Kochanek PM, Clark RSB, Ruppel R a., Adelson PD, Bell MJ, Whalen MJ, et al. *Biochemical, cellular, and molecular mechanisms in the evolution of secondary damage after severe traumatic brain injury in infants and children: Lessons learned from the bedside.* *Pediatr Crit Care Med.* 2000;1(1):4–19.
 270. Lamprecht MR, Morrison B. *A Combination Therapy of 17 β -Estradiol and Memantine Is More Neuroprotective Than Monotherapies in an Organotypic Brain Slice Culture Model of Traumatic Brain Injury.* *J Neurotrauma.* 2015;32(17):1361–8.
 271. Barth A, Barth L, Newell DW. *Combination Therapy with MK-801 and α -Phenyl-tert-butyl-nitron Enhances Protection against Ischemic Neuronal Damage in Organotypic Hippocampal Slice Cultures.* *Exp Neurol.* 1996;141(2):330–6.
 272. David HN, Haelewyn B, Degoulet M, Colomb DG, Risso J-J, Abraini JH, et al. *Ex Vivo and In Vivo Neuroprotection Induced by Argon When Given after an Excitotoxic or Ischemic Insult.* *PLoS One.* 2012;7(2):e30934.
 273. Goss CW, Hoffman SW, Stein DG. *Behavioral effects and anatomic correlates after brain injury: a progesterone dose–response study.* *Pharmacol Biochem Behav.* 2003;76(2):231–42.
 274. Tang H, Hua F, Wang J, Yousuf S, Atif F, Sayeed I, et al. *Progesterone and vitamin D combination therapy modulates inflammatory response after traumatic brain injury.* *Brain Inj.* 2015;29(10):1165–74.
 275. Barha CK, Ishrat T, Epp JR, Galea LAM, Stein DG. *Progesterone treatment normalizes the levels of cell proliferation and cell death in the dentate gyrus of the hippocampus after traumatic brain injury.* *Exp Neurol.* 2011;231(1):72–81.
 276. Tallarida RJ. *Quantitative methods for assessing drug synergism.* *Genes Cancer.* 2011;2(11):1003–8.
 277. Tallarida RJ, Kimmel HL, Holtzman SG. *Theory and statistics of detecting synergism between two active drugs: cocaine and buprenorphine.* *Psychopharmacology (Berl).* 1997;133(4):378–82.
 278. Tallarida RJ, Raffa RB. *The application of drug dose equivalence in the quantitative analysis of receptor occupation and drug combinations.* *Pharmacol Ther.* 2010;127(2):165–74.
 279. Codd EE, Martinez RP, Molino L, Rogers KE, Stone DJ, Tallarida RJ. *Tramadol and several anticonvulsants synergize in attenuating nerve injury-induced allodynia.* *Pain.* 2008 Feb;134(3):254–62.
 280. López-Villodres JA, De La Cruz JP, Muñoz-Marin J, Guerrero A, Reyes JJ, González-Correa JA. *Cytoprotective effect of nonsteroidal antiinflammatory drugs in rat brain slices subjected to reoxygenation after oxygen–glucose deprivation.* *Eur J Pharm Sci.* 2012;45(5):624–31.
 281. Brinton RD, Thompson RF, Foy MR, Baudry M, Wang J, Finch CE, et al. *Progesterone receptors: form and function in brain.* *Front Neuroendocrinol.* 2008;29(2):313–39.
 282. Adams J, Collaço-Moraes Y, de Belleruche J. *Cyclooxygenase-2 induction in cerebral cortex: an intracellular response to synaptic excitation.* *J Neurochem.* 1996;66(1):6–13.
 283. Chen J, Marsh T, Zhang JS, Graham SH. *Expression of cyclo-oxygenase 2 in rat brain following kainate treatment.* *Neuroreport.* 1995;6(2):245–8.
 284. Strauss KI, Marini AM. *Cyclooxygenase-2 inhibition protects cultured cerebellar granule neurons from glutamate-mediated cell death.* *J Neurotrauma.* 2002;19(5):627–38.
 285. Caggiano AO, Breder CD, Kraig RP. *Long-term elevation of cyclooxygenase-2, but not lipoxygenase, in regions synaptically distant from spreading depression.* *J Comp Neurol.* 1996;376(3):447–62.
 286. Gopez JJ, Yue H, Vasudevan R, Malik AS, Fogelsanger LN, Lewis S, et al. *Cyclooxygenase-2-specific inhibitor improves functional outcomes, provides neuroprotection, and reduces inflammation in a rat model of traumatic brain injury.* *Neurosurgery.* 2005;56(3):590–604.
 287. Cernak I, O'Connor C, Vink R. *Activation of cyclo-oxygenase-2 contributes to motor and cognitive dysfunction following diffuse traumatic brain injury in rats.* *Clin Exp Pharmacol*

- Physiol. 2001;28(11):922–5.
288. Loewe S. *The problem of synergism and antagonism of combined drugs.* Arzneimittelforschung. 1953;3(6):285–90.
 289. Tallarida RJ. *Revisiting the isobole and related quantitative methods for assessing drug synergism.* J Pharmacol Exp Ther. 2012;342(1):2–8.
 290. Tallarida RJ. *Drug Combinations: Tests and Analysis with Isoboles.* Curr Protoc Pharmacol. 2016;72:9.19.1–19.
 291. Grabovsky Y, Tallarida RJ. *Isobolographic analysis for combinations of a full and partial agonist: curved isoboles.* J Pharmacol Exp Ther. 2004;310(3):981–6.
 292. Rawat S, Dingley J. *Closed-Circuit Xenon Delivery Using a Standard Anesthesia Workstation.* Anesth Analg. 2010;110(1):101–9.
 293. Atif F, Sayeed I, Ishrat T, Stein DG. *Progesterone with vitamin D affords better neuroprotection against excitotoxicity in cultured cortical neurons than progesterone alone.* Mol Med. 2009;15(9–10):328–36.
 294. Cekic M, Cutler SM, VanLandingham JW, Stein DG. *Vitamin D deficiency reduces the benefits of progesterone treatment after brain injury in aged rats.* Neurobiol Aging. 2011;32(5):864–74.
 295. Aminmansour B, Nikbakht H, Ghorbani A, Rezvani M, Rahmani P, Torkashvand M, et al. *Comparison of the administration of progesterone versus progesterone and vitamin D in improvement of outcomes in patients with traumatic brain injury: A randomized clinical trial with placebo group.* Adv Biomed Res. 2012;1:58.
 296. Thal SC, Timaru-Kast R, Wilde F, Merk P, Johnson F, Frauenknecht K, et al. *Propofol impairs neurogenesis and neurologic recovery and increases mortality rate in adult rats after traumatic brain injury.* Crit Care Med. 2014;42(1):129–41.
 297. Hoane MR, Swan A a., Heck SE. *The effects of a high-fat sucrose diet on functional outcome following cortical contusion injury in the rat.* Behav Brain Res. 2011;223(1):119–24.
 298. Budde MD, Janes L, Gold E, Turtzo LC, Frank J a. *The contribution of gliosis to diffusion tensor anisotropy and tractography following traumatic brain injury: Validation in the rat using Fourier analysis of stained tissue sections.* Brain. 2011;134(8):2248–60.
 299. Griebenow M, Casalis P, Woiciechowsky C, Majetschak M, Thomale U-W. *Ubiquitin reduces contusion volume after controlled cortical impact injury in rats.* J Neurotrauma. 2007;24(9):1529–35.
 300. Yang L, Afroz S, Michelson HB, Goodman JH, Valsamis H a, Ling DSF. *Spontaneous epileptiform activity in rat neocortex after controlled cortical impact injury.* J Neurotrauma. 2010;27(8):1541–8.
 301. Clark RS, Kochanek PM, Marion DW, Schiding JK, White M, Palmer a M, et al. *Mild posttraumatic hypothermia reduces mortality after severe controlled cortical impact in rats.* J Cereb Blood Flow Metab. 1996;16(2):253–61.
 302. Cherian L, Robertson CS, Contant CF, Bryan RM. *Lateral cortical impact injury in rats: cerebrovascular effects of varying depth of cortical deformation and impact velocity.* J Neurotrauma. 1994;11(5):573–85.
 303. Scheff SW, Baldwin SA, Brown RW, Kraemer PJ. *Morris water maze deficits in rats following traumatic brain injury: lateral controlled cortical impact.* J Neurotrauma. 1997;14(9):615–27.
 304. Stover JF, Beyer TF, Unterberg AW. *Riluzole Reduces Brain Swelling and Contusion Volume in Rats Following Controlled Cortical Impact Injury.* J Neurotrauma. 2000;17(12).
 305. Turtzo LC, Budde MD, Gold EM, Lewis BK, Janes L, Yarnell A, et al. *The evolution of traumatic brain injury in a rat focal contusion model.* NMR Biomed. 2013;26(4):468–79.
 306. Shohami E, Ginis I, Hallenbeck JM. *Dual role of tumor necrosis factor alpha in brain injury.* Cytokine Growth Factor Rev. 1999;10(2):119–30.
 307. Morganti-Kossmann MC, Satgunaseelan L, Bye N, Kossmann T. *Modulation of immune response by head injury.* 2008;38(12):1392–400.
 308. Breder CD, Tsujimoto M, Terano Y, Scott DW, Saper CB. *Distribution and characterization of tumor necrosis factor-alpha-like immunoreactivity in the murine central nervous system.* J Comp Neurol. 1993;337(4):543–67.

309. Morganti-Kossmann MC, Rancan M, Otto VI, Stahel PF, Kossmann T. *Role of cerebral inflammation after traumatic brain injury: a revisited concept*. Shock. 2001;16(3):165–77.
310. Allan SM, Rothwell NJ. *Cytokines and acute neurodegeneration*. Nat Rev Neurosci. 2001;2(10):734–44.
311. Chao CC, Hu S, Ehrlich L, Peterson PK. *Interleukin-1 and tumor necrosis factor- α synergistically mediate neurotoxicity: involvement of nitric oxide and of N-methyl-D-aspartate receptors*. Brain Behav Immun. 1995;9(4):355–65.
312. Harting MT, Jimenez F, Adams SD, Mercer DW, Cox CS. *Acute, regional inflammatory response after traumatic brain injury: Implications for cellular therapy*. Surgery. 2008 Nov;144(5):803–13.
313. Ross SA, Halliday MI, Campbell GC, Byrnes DP, Rowlands BJ. *The presence of tumour necrosis factor in CSF and plasma after severe head injury*. Br J Neurosurg. 1994;8(4):419–25.
314. Stover JF, Schöning B, Beyer TF, Woiciechowsky C, Unterberg AW. *Temporal profile of cerebrospinal fluid glutamate, interleukin-6, and tumor necrosis factor- α in relation to brain edema and contusion following controlled cortical impact injury in rats*. Vol. 288, Neuroscience Letters. 2000.
315. Kim KS, Wass CA, Cross AS, Opal SM. *Modulation of blood-brain barrier permeability by tumor necrosis factor and antibody to tumor necrosis factor in the rat*. Lymphokine Cytokine Res. 1992;11(6):293–8.
316. Shohami E, Gallily R, Mechoulam R, Bass R, Ben-Hur T. *Cytokine production in the brain following closed head injury: dexamethasone (HU-211) is a novel TNF- α inhibitor and an effective neuroprotectant*. J Neuroimmunol. 1997;72(2):169–77.
317. Sullivan PG, Bruce-Keller AJ, Rabchevsky AG, Christakos S, Clair DK, Mattson MP, et al. *Exacerbation of damage and altered NF- κ B activation in mice lacking tumor necrosis factor receptors after traumatic brain injury*. J Neurosci. 1999;19(15):6248–56.
318. Scherbel U, Raghupathi R, Nakamura M, Saatman KE, Trojanowski JQ, Neugebauer E, et al. *Differential acute and chronic responses of tumor necrosis factor-deficient mice to experimental brain injury*. Neurobiology. 1999;96:8721–6.
319. Knobloch SM, Fan L, Faden AI. *Early neuronal expression of tumor necrosis factor- α after experimental brain injury contributes to neurological impairment*. J Neuroimmunol. 1999;95(1–2):115–25.
320. Stahel PF, Shohami E, Younis FM, Kariya K, Otto VI, Lenzlinger PM, et al. *Experimental closed head injury: analysis of neurological outcome, blood-brain barrier dysfunction, intracranial neutrophil infiltration, and neuronal cell death in mice deficient in genes for pro-inflammatory cytokines*. J Cereb Blood Flow Metab. 2000;20(2):369–80.
321. Hammacher A, Ward LD, Weinstock J, Treutlein H, Yasukawa K, Simpson RJ. *Structure-function analysis of human IL-6: Identification of two distinct regions that are important for receptor binding*. Protein Sci. 1994;3:2280–93.
322. Hopkins SJ, Rothwell NJ. *Cytokines and the nervous system I: expression and recognition*. Trends Neurosci. 1995;18(2):83–8.
323. Woiciechowsky C, Schöning B, Cobanov J, Lanksch WR, Volk H-D, Döcke W-D. *Early IL-6 plasma concentrations correlate with severity of brain injury and pneumonia in brain-injured patients*. J Trauma. 2002;52(2):339–45.
324. Woodroffe MN, Sarna GS, Wadhwa M, Hayes GM, Loughlin AJ, Tinker A, et al. *Detection of interleukin-1 and interleukin-6 in adult rat brain, following mechanical injury, by in vivo microdialysis: evidence of a role for microglia in cytokine production*. J Neuroimmunol. 1991;33(3):227–36.
325. Shohami E, Novikov M, Bass R, Yamin A, Gallily R. *Closed Head Injury Triggers Early Production of TNF α and IL-6 by Brain Tissue*. J Cereb Blood Flow Metab. 1994;14:615–9.
326. Volkmar, Hans HJ, Kossmann T, Lenzlinger TM, Probstmeier R, Imhof H-G, et al. *Experimental Axonal Injury Triggers Interleukin-6 mRNA, Protein Synthesis and Release Into Cerebrospinal Fluid*. J Cereb Blood Flow Metab. 1999;19(1):184–94.
327. McClain C, Cohen D, Phillips R, Ott L, Young B. *Increased plasma and ventricular fluid interleukin-6 levels in patients with head injury*. J Lab Clin Med. 1991;118(3):225–31.

328. Kossmann T, Hans VH, Imhof HG, Stocker R, Grob P, Trentz O, et al. *Intrathecal and serum interleukin-6 and the acute-phase response in patients with severe traumatic brain injuries.* Shock. 1995 Nov;4(5):311–7.
329. Winter CD, Pringle AK, Clough GF, Church MK. *Raised parenchymal interleukin-6 levels correlate with improved outcome after traumatic brain injury.* Brain. 2004;127(Pt 2):315–20.
330. Penkowa M, Giralt M, Carrasco J, Hadberg H, Hidalgo J. *Impaired inflammatory response and increased oxidative stress and neurodegeneration after brain injury in interleukin-6-deficient mice.* Glia. 2000;32(3):271–85.
331. Glabinski AR, Balasingam V, Tani M, Kunkel SL, Strieter RM, Yong VW, et al. *Chemokine monocyte chemoattractant protein-1 is expressed by astrocytes after mechanical injury to the brain.* J Immunol. 1996;156(11):4363–8.
332. Banisadr G, Quéraud-Lesaux F, Boutterin MC, Pélaprat D, Zalc B, Rostène W, et al. *Distribution, cellular localization and functional role of CCR2 chemokine receptors in adult rat brain.* J Neurochem. 2002;81(2):257–69.
333. Rankine EL, Hughes PM, Botham MS, Perry VH, Felton LM. *Brain cytokine synthesis induced by an intraparenchymal injection of LPS is reduced in MCP-1-deficient mice prior to leucocyte recruitment.* Eur J Neurosci. 2006;24(1):77–86.
334. Galasso JM, Miller MJ, Cowell RM, Harrison JK, Warren JS, Silverstein FS. *Acute excitotoxic injury induces expression of monocyte chemoattractant protein-1 and its receptor, CCR2, in neonatal rat brain.* Exp Neurol. 2000;165(2):295–305.
335. Ho L, Zhao W, Dams-O'Connor K, Tang CY, Gordon W, Peskind ER, et al. *Elevated plasma MCP-1 concentration following traumatic brain injury as a potential predisposition factor associated with an increased risk for subsequent development of Alzheimer's disease.* J Alzheimers Dis. 2012;31(2):301–13.
336. Hughes PM, Allegrini PR, Rudin M, Perry VH, Mir AK, Wiessner C. *Monocyte chemoattractant protein-1 deficiency is protective in a murine stroke model.* J Cereb Blood Flow Metab. 2002;22(3):308–17.
337. Helmy A, Carpenter KLH, Hutchinson PJ. *Microdialysis in the human brain and its potential role in the development and clinical assessment of drugs.* Curr Med Chem. 2007;14(14):1525–37.
338. Hinson HE, Rowell S, Schreiber M. *Clinical evidence of inflammation driving secondary brain injury: a systematic review.* J Trauma Acute Care Surg. 2015;78(1):184–91.
339. Woodcock T, Morganti-Kossmann MC. *The role of markers of inflammation in traumatic brain injury.* Front Neurol. 2013;4:18.
340. Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, Amanatides PG, et al. *The genome sequence of Drosophila melanogaster.* Science. 2000;287(5461):2185–95.
341. Boyan GS, Reichert H. *Mechanisms for complexity in the brain: generating the insect central complex.* Trends Neurosci. 2011;34(5):247–57.
342. Reichert H. *A tripartite organization of the urbilaterian brain: developmental genetic evidence from Drosophila.* Brain Res Bull. 2005;66(4–6):491–4.
343. Littleton JT, Ganetzky B. *Ion Channels and Synaptic Organization: Analysis of the Drosophila Genome.* Neuron. 2000;26(1):35–43.
344. DeSalvo MK, Mayer N, Mayer F, Bainton RJ. *Physiologic and anatomic characterization of the brain surface glia barrier of Drosophila.* Glia. 2011;59(9):1322–40.
345. Petersen AJ, Rimkus SA, Wassarman DA. *ATM kinase inhibition in glial cells activates the innate immune response and causes neurodegeneration in Drosophila.* Proc Natl Acad Sci U S A. 2012;109(11):E656–64.
346. Piyankarage SC, Featherstone DE, Shippy SA. *Nanoliter hemolymph sampling and analysis of individual adult Drosophila melanogaster.* Anal Chem. 2012;84(10):4460–6.
347. Hetru C, Hoffmann JA. *NF-kappaB in the immune response of Drosophila.* Cold Spring Harb Perspect Biol. 2009;1(6):a000232.
348. Myllymäki H, Valanne S, Rämet M. *The Drosophila imd signaling pathway.* J Immunol. 2014;192(8):3455–62.
349. Valanne S, Wang J-H, Rämet M. *The The Drosophila Toll Signaling Pathway.* J Immunol Ref. 2016;186:649–56.

350. Nichols CD. *Drosophila melanogaster neurobiology, neuropharmacology, and how the fly can inform central nervous system drug discovery*. Pharmacol Ther. 2006;112(3):677–700.
351. Xia S, Miyashita T, Fu T-F, Lin W-Y, Wu C-L, Pyzocha L, et al. *NMDA Receptors Mediate Olfactory Learning and Memory in Drosophila*. Curr Biol. 2005;15(7):603–15.
352. Leal SM, Kumar N, Neckameyer WS. *GABAergic modulation of motor-driven behaviors in juvenile Drosophila and evidence for a nonbehavioral role for GABA transport*. J Neurobiol. 2004;61(2):189–208.
353. Nomellini V, Gomez CR, Gamelli RL, Kovacs EJ. *Aging and animal models of systemic insult: trauma, burn, and sepsis*. Shock. 2009;31(1):11–20.
354. Marquez de la Plata CD, Hart T, Hammond FM, Frol AB, Hudak A, Harper CR, et al. *Impact of age on long-term recovery from traumatic brain injury*. Arch Phys Med Rehabil. 2008;89(5):896–903.
355. Hamm RJ, Jenkins LW, Lyeth BG, White-Gbadebo DM, Hayes RL. *The effect of age on outcome following traumatic brain injury in rats*. J Neurosurg. 1991 Dec;75(6):916–21.
356. Musiek ES, Xiong DD, Holtzman DM. *Sleep, circadian rhythms, and the pathogenesis of Alzheimer disease*. Exp Mol Med. 2015;47(3):e148.
357. Diller K, Zhu L. *Hypothermia Treatment for Brain Injury*. Annu Rev Biomed Eng. 2009;11:135–62.
358. Dietrich WD, Bramlett HM. *The evidence for hypothermia as a neuroprotectant in traumatic brain injury*. Neurotherapeutics. 2010 Jan;7(1):43–50.
359. Song SS, Lyden PD. *Overview of therapeutic hypothermia*. Curr Treat Options Neurol. 2012;14(6):541–8.
360. Hong S-T, Bang S, Paik D, Kang J, Hwang S, Jeon K, et al. *Histamine and its receptors modulate temperature-preference behaviors in Drosophila*. J Neurosci. 2006 Jul 5;26(27):7245–56.
361. Tracey WD, Wilson RI, Laurent G, Benzer S. *painless, a Drosophila gene essential for nociception*. Cell. 2003 Apr 18;113(2):261–73.
362. Lee Y, Lee Y, Lee J, Bang S, Hyun S, Kang J, et al. *Pyrexia is a new thermal transient receptor potential channel endowing tolerance to high temperatures in Drosophila melanogaster*. Nat Genet. 2005 Mar;37(3):305–10.
363. Rosenzweig M, Brennan KM, Tayler TD, Phelps PO, Patapoutian A, Garrity PA. *The Drosophila ortholog of vertebrate TRPA1 regulates thermotaxis*. Genes Dev. 2005 Feb 15;19(4):419–24.
364. Rodríguez EC, Robertson RM. *Protective effect of hypothermia on brain potassium homeostasis during repetitive anoxia in Drosophila melanogaster*. J Exp Biol. 2012;215(23).
365. Thoresen M, Satas S, Puka-Sundvall M, Whitelaw A, Hallström A, Løberg EM, et al. *Post-hypoxic hypothermia reduces cerebrocortical release of NO and excitotoxins*. Neuroreport. 1997 Oct 20;8(15):3359–62.
366. Cilio MR, Ferriero DM. *Synergistic neuroprotective therapies with hypothermia*. Semin Fetal Neonatal Med. 2010 Oct;15(5):293–8.
367. Johnston SC1, Rootenberg JD, Katrak S, Smith WS EJ. *Effect of a US National Institutes of Health programme of clinical trials on public health and costs*. Lancet. 2006;367(9519):1319–27.