Mechanism of noble gas neuroprotection in an \textit{in vitro} model of traumatic brain injury

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Statement of originality

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ABSTRACT

Background: The noble gases xenon and argon are neuroprotective in models of brain injury. This thesis investigated the neuroprotective mechanisms of the inert gases xenon, argon, krypton, neon and helium in *in vitro* models of traumatic brain injury and ischemia.

Methods: This study used an *in vitro* model of focal mechanical trauma and oxygen and glucose deprivation using mouse organotypic hippocampal brain slices. Injury was quantified by propidium iodide fluorescence.

Results: Xenon (50.6 kPa) and, to a lesser extent, argon (50.6 kPa) are neuroprotective against traumatic injury when applied after injury (xenon 0.43 ± 0.03 protection at 72 h after injury [N = 104]; argon 0.30 ± 0.0% protection [N = 44] vs control injury 1.0±0.05 [N=144]; mean ± SEM). Helium, neon, and krypton are devoid of neuroprotective effect. Xenon (50.6 kPa) prevents development of secondary injury after trauma. Argon (50.6 kPa) attenuates secondary injury, but is less effective than xenon (xenon 0.50 ± 0.05 reduction in secondary injury at 72 h after injury [N = 104]; argon 0.34 ± 0.08 reduction [N = 44] vs control 0.86±0.05 [N = 144]; mean ± SEM). Glycine reverses the neuroprotective effect of xenon, but not argon in both models of TBI and OGD, consistent with competitive inhibition at the N-methyl-D-aspartate receptor glycine site mediating xenon neuroprotection against traumatic brain injury.

Conclusions: Xenon neuroprotection against traumatic and ischemic brain injury can be reversed by elevated concentrations of glycine, indicating a key role of inhibition of the NMDA receptor glycine co-agonist site in mediating neuroprotection against these injuries. Argon does not appear to have any effects on NMDA receptors and is neuroprotective *via* a mechanism distinct to that of xenon. Krypton and neon are devoid of neuroprotective effects in either injury model.
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LIST OF ABBREVIATIONS

AMPA  α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP  Adenosine triphosphate
BMJ  British Medical Journal
CA1  Cornu ammonis area 1
CA3  Cornu ammonis area 3
CaMKII  Calmodulin-activated kinase II
CBF  Cerebral blood flow
CCI  Controlled cortical impact
CNS  Central nervous system
CT  Computed tomography
DG  Dentate gyrus
DIV  Days in vitro
DNA  Deoxyribonucleic acid
EAA  Excitatory amino acid
ENF  Enflurane
ERK  Extracellular signal regulated kinase
FPI  Fluid percussion injury
GABA  γ-aminobutyric acid
GlyR  Glycine receptor
HIF-1α  Hypoxia-inducible factor 1 alpha
HSA  Human serum albumin
ICP  Intracranial pressure
IL  Interleukin
MAC  Minimum alveolar concentration
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCAO</td>
<td>Middle cerebral artery occlusion</td>
</tr>
<tr>
<td>MOA</td>
<td>Mechanism of action</td>
</tr>
<tr>
<td>MPTP</td>
<td>Mitochondria permeability transition pore</td>
</tr>
<tr>
<td>MTT</td>
<td>(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthetase</td>
</tr>
<tr>
<td>OGD</td>
<td>Oxygen and glucose deprivation</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>TBI</td>
<td>Traumatic brain injury</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>TREK</td>
<td>TWIK-related K⁺ channel</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet light</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

1.1 Traumatic Brain Injury

1.1.1 Traumatic brain injury as a clinical condition

Traumatic brain injury (TBI) occurs as a result of an external force causing mechanical disruption of brain tissue and delayed pathogenic events which collectively exacerbate the damage [1]. The requirement of an external force is what differentiates TBI from other types of brain injury, such as neurodegenerative brain conditions. Common examples of an external force include the head being struck or penetrated by a foreign object, rapid acceleration or deceleration and exposure to blast forces [2].

TBI is considered a global health concern as it is one of the leading causes of death and disability in people aged 15-44 in economically developed countries [3]. TBIs occur most commonly in men where they make up 75% of cases [4]. The main causes of TBI include motor vehicle accidents, falls and assaults. Epidemiological data from the European Union quotes the incidence of hospitalisation for serious or fatal TBI to be an estimated 235 per 100,000 of the population per year [3]. Survivors of TBI face a lifetime of care and rehabilitation due to physical, cognitive and psychological consequences of the injury. As a result many survivors suffer negative impacts on their relationships, independence and employment [5]. Worryingly, incidence rates for TBI worldwide are increasing but overall mortality rates have only slightly improved since 1990 [5]. One reason for this rising incidence is due to the increased use of motor vehicles in lower and middle-income countries [4].

TBI is included among the top three causes of injury-related medical costs in the USA and Europe with approximately 10-15% of patients that incur a TBI requiring long-term specialist care and treatment. The financial costs of TBI are great; in the USA alone they have been estimated at over $60 billion annually. However in his letter to the BMJ Ian Roberts is quoted
as saying trauma research has received ‘less funding than any other cause of human misery’ [6]. Indeed, TBI research is known to have one of the highest unmet financial needs within neuroscience research [4, 7].

There is a requirement for continued research into markers of pathogenic processes after TBI and targeted treatments in order to refine diagnosis and improve prognosis for the patient. The lack of understanding of the pathophysiological mechanisms following severe head injury is a major reason for the slow progress in this field, which has been hindered further by the highly heterogeneous nature of head injuries. It is clear that the worldwide burden of TBI is vast, and the lack of available treatments only seeks to exacerbate this problem.

1.1.2 Pathophysiology of traumatic brain injury

1.1.2.1 Primary injury

TBI is a highly heterogeneous disorder that presents differently depending on the type and force of impact. However, in all cases of TBI two major phases of injury can be identified, known as primary and secondary injury. A primary mechanical injury occurs abruptly upon impact and may be accompanied by skull fracture and disruption of brain parenchyma with the tearing of the brain tissue and surrounding blood vessels [4, 8]. Mechanical perturbation of neurones is associated with a massive release of neurotransmitters, particularly glutamate and other excitatory amino acids, that contribute significantly to the secondary phases of injury [2]. Focal cerebral contusions are the most common form of primary injury [4].

1.1.2.2 Secondary injury

The sequence of pathological events that occur in the hours, days and weeks following the primary injury is known as secondary injury, and is considered in many cases to represent the major burden of injury. Support for the concept of a ‘delayed’ secondary damage has been provided by findings that 30-40% of patients who died after sustaining a head injury had a duration whereby they could obey commands or speak, known as a ‘lucid interval’ [9].
Secondary injury is an evolving, active process that involves many biochemical pathways. These pathways, often act concurrently and synergistically and include excitotoxicity pathways, ischemia, free-radical generation and inflammatory responses [4]. The figures below attempt to simplify these processes firstly by the order of occurrence after the primary injury (figure 1.1) secondly by where these processes occur within various neuronal and glial cells (figure 1.2) and lastly by the specific role of the NMDA receptor (figure 1.3)

Figure 1.1: Events that occur following the initial primary injury that eventually culminate in cell death after a period of time that can span weeks. Figure adapted from McAllister (2011) [2]
Figure 1.2: The location within neurones and glial cells where pathogenic processes relating to secondary injury take place. Figure taken from Rosenfeld et al. (2011) [5]
Figure 1.3: The specific role of the NMDA receptor in mediating downstream excitotoxic pathways following traumatic brain injury.
It is clear that the development of secondary injury involves a complex interplay between many biochemical processes. The following sections will focus on the excitotoxic component of secondary injury development since this is most relevant to the current study, however other factors involved in its development will also be mentioned.

1.1.2.3 Excitotoxicity

A major component of the catastrophic neuronal death that occurs after TBI is believed to be mediated via the activation of excitatory ionotropic glutamate receptor ion channels, in a process known as excitotoxicity.

*NMDF receptors; structure and function*

Glutamate receptor ion channels are classified into \(\text{N}-\text{methyl}-\text{D}-\text{aspartate} \) (NMDA) receptors, \(\alpha\)-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate acid receptors (AMPA) and kainate receptors. All are permeable to positively charged ions; however AMPA and kainate receptors mediate fast excitatory transmission whereas NMDA receptors mediate the slow phase of excitatory transmission. NMDA receptors are usually located postsynaptically and require an initial rapid depolarisation mediated by AMPA/kainate receptors in order to be activated to remove the magnesium that blocks the channels at resting membrane potentials. An additional requirement of NMDA receptor activation is the presence of two agonists, glutamate and glycine, which bind at separate and distinct sites. Once activated NMDA receptors allow influx of calcium ions. Structurally NMDA receptors are tetrameric channels, made up of four subunits in what has been described as a ‘dimer of dimers’. The most abundant NMDA receptor subunit arrangement consists of two NR1 and two NR2 subunits [10]. Four isoforms of the NR2 subunit exist, known as NR2A, NR2B, NR2C and NR2D, each with a different intracellular C-terminal domain. NR2B subunits predominate in the postnatal brain but this switches to NR2A subunit predominance during development. Glutamate or NMDA binds to the NR2 subunit in a three dimensional structure formed by the extracellular N-terminus and the loop between the third and fourth transmembrane domains [10]. Glycine binds to a similar site on the NR1 subunit. A third NMDA receptor subunit, NR3 has been described, but its role (if any) in the brain is as yet uncharacterised [11]. Permeability to calcium ions underlies the physiological role of the NMDA receptor in mediating synaptic plasticity, an essential component of memory formation.
receptors are also involved in pathological processes. Glutamate is released in an
uncontrolled manner during ischemic and traumatic injury leading to excessive NMDA
receptor activation in a process known as excitotoxicity [8].

**NMDA receptors and excitotoxicity**

Numerous studies in clinical patients and animal models have shown that a traumatic impact
to the brain is immediately followed by a massive extracellular release of glutamate [12, 13].
Glutamate levels in the brain increase after impact through a number of sources, including via
enhanced exocytosis, via structural damage to the synaptic terminal, through a disrupted
blood brain barrier from the blood and by astrocytic glutamate transporter impairment or
reversal [14]. This increase in the levels of glutamate in the brain cause an over-stimulation
of glutamate receptors (AMPA, kainate and NMDA), leading to prolonged depolarization and
a resultant increase in intracellular calcium. Due to the higher permeability to calcium ions,
NMDA receptors are thought to play an important role in excitotoxic development (see figure
1.3) [13].

Calcium overload and the activation of calcium-activated neutral proteases (calpains) is one
of the key events in excitotoxicity. Calpain activation leads to the proteolysis of a number of
intracellular proteins, including those of the cytoskeleton [15]. In addition to calpain
activation, elevated intracellular calcium can also lead to an increase in oxidative stress and
the activation of lipases and endonucleases which can damage DNA, cell proteins and lipids
[8] and ultimately leads to death of the cell (see figure 1.3).

**Cortical spreading depression**

A process known as cortical spreading depression is thought to play a role in the spread of
injury following traumatic impacts and involves a wave of depolarisation that propagates
throughout the brain. The wave of depolarisation causes a massive release in excitatory
amino acids including glutamate. Furthermore the voltage dependant magnesium block of
NMDA receptors is removed by the depolarisation wave. This sensitises the NMDA
receptors to small increases in extracellular glutamate which contributes to further neuronal
depolarisation which propagates to neighbouring regions and continues the process [16].
1.1.2.4 Ischemia and free radical generation

Following a traumatic injury the area around the direct trauma site often suffers reduced blood flow due to damaged blood vessels, haemorrhage and vasoconstriction, creating conditions of ischemia (see section 1.2.2). Under ischemic conditions the amount of nitric oxide (NO) in the brain increases due to upregulation of the enzyme that produces NO; neuronal nitric oxide synthetase (nNOS). The NO produced reacts with reactive oxygen species (ROS), for example, the superoxide anion, to produce peroxynitrite. A further source of free radical generation occurs after prolonged elevation of intracellular calcium (part of the excitotoxic response, see figure 1.3) whereby superoxide anions are formed by the respiratory chain. ROS production leads to lipid peroxidation and oxidation of thiol groups on critical proteins and ultimately cell death. The brain is particularly vulnerable to oxidative stress as it has a high rate of metabolic activity (which generates ROS), low antioxidant capacity, lack of cellular repair/regeneration mechanisms, a high membrane surface to cytoplasm ratio and high concentrations of transition metals, which are redox-active and capable of generating ROS through the Haber-Weiss reaction [8]. The Haber Weiss reaction occurs in cells and involves the generation of highly reactive hydroxyl radicals from hydrogen peroxide and superoxide anions in a reaction catalysed by iron.

1.1.2.5 Inflammation

The neuroinflammatory response to TBI occurs minutes after the initial injury and involves the activation of glial cells (microglia and astrocytes) and the infiltration of blood leukocytes [5, 17]. Immune system mediators are secreted from glial cells following injury. These include inflammatory cytokines (IL-1, IL-6, TNF), the anti-inflammatory cytokines (IL-4, IL-10, TGF-beta) and the chemokines which drive the infiltration of activated blood leukocytes into the brain [5, 17]. These infiltrated leukocytes (neutrophils and monocytes) sustain the immune response to injury, causing an impairment in the integrity of the blood brain barrier. This in turn leads to an increase in extracellular fluid which, combined with cell swelling, leads to oedema and an increase in intracranial pressure. Despite their fast induction after injury, the activation of microglial cells can be sustained for several months following injury [17].
In excess the inflammatory response is harmful and can contribute to secondary injury spread, but is also necessary for the ‘clean up’ of cellular debris after injury and may be a trigger for cellular regeneration [18]. It is unclear whether anti-inflammatory therapies could improve outcome after TBI in humans [19].

1.1.3 Cell death mechanisms following TBI

Secondary injury following TBI leads to cell death via apoptotic or necrotic mechanisms. There is evidence for both necrotic and apoptotic cell death in the brain following trauma, with apoptosis estimated to be responsible for around 50% of trauma induced cell death. Whether a cell undergoes apoptosis or necrosis has been postulated to involve factors such as the nature/severity of the injury itself and ATP availability, as apoptosis is an active, energy dependant process [15]. The following table summarises some of the morphological characteristics of cells undergoing apoptotic or necrotic cell death.

<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single cells or small clusters of cells</td>
<td>Often contiguous cells</td>
</tr>
<tr>
<td>Cell shrinkage and convolution</td>
<td>Cell swelling</td>
</tr>
<tr>
<td>Pyknosis and karyorrhexis</td>
<td>Karyolysis, pyknosis and karyorrhexis</td>
</tr>
<tr>
<td>Intact cell membrane</td>
<td>Disrupted cell membrane</td>
</tr>
<tr>
<td>Cytoplasm retained in apoptotic bodies</td>
<td>Cytoplasm released</td>
</tr>
<tr>
<td>No inflammation</td>
<td>Inflammation usually present</td>
</tr>
</tbody>
</table>

*Table 1.1: Comparison of the morphological characteristics of cells following apoptotic and necrotic cell death. Adapted from Elmore, 2007 [20]*
Apoptotic ‘markers’ have been identified following trauma for both the intrinsic (mitochondrial) apoptotic pathway and the extrinsic death receptor pathway [15]. The extrinsic pathway involves the activation of death domains and caspase recruitment via TNF super family receptors. The intrinsic apoptotic pathway is triggered by mitochondrial dysfunction and involves a number of signalling pathways, which can include the NFκB dependant pathway, the p53 dependant pathway and activation of the pro-apoptotic bcl-2 proteins (e.g. bak, bax, bcl-2). Induction of these factors leads to cleavage of the proenzyme caspases to their active form, subsequent release of cytochrome-c from mitochondria and activation of executioner caspases 3 and 9 (‘the executioner’). These activate DNA breaking enzymes such as endonucleases which leads to apoptotic cell death [8]. Once caspases are activated there appears to be an irreversible commitment to cell death, which occurs rapidly after activation [20].

However, the brain does possess a number of endogenous protective mechanisms against a traumatic insult which attempt to counteract the damage and improve neuronal survival. These include heat shock proteins [21], anti-inflammatory cytokines and growth factors [18], antioxidants (e.g. superoxide dismutase and glutathione) [22], endocannabinoids [23], erythropoietin [24] and sex hormones [25]. However the actions of these survival promoting processes are limited both temporally and spatially, so in the case of a serious injury are often overwhelmed by the death promoting processes.

1.1.4 Long-term pathophysiological consequences of TBI

The debilitating aftermath of a traumatic brain injury extends far beyond the injury itself. Sustaining a TBI is considered to be a risk factor in the development of a number of health conditions that can persist throughout life or occur during the aging process. Epidemiological studies have identified a number of conditions that TBI patients are at increased risk of suffering from in the first three years following injury. These include an 11 fold increase in developing epilepsy, a 1.5 fold increased risk of developing depression, a 1.8 fold increased likelihood of binge drinking, a 2.5 to 4.5 fold increased risk of developing Alzheimer’s disease (from moderate and severe TBI respectively) and overall a 7.5 fold increased risk of death [26]. TBI is quoted as being “the best established environmental risk factor for
dementia’’ [27]. A meta-analysis conducted in 2003 investigating the relationship between TBI and dementia concluded that sustaining a TBI of sufficient severity to lose consciousness carried a 50% greater risk of developing dementia compared with others [28]. One study has identified a significant relationship between TBI and the development of schizophrenia, with the largest effect seen in people who are genetically predisposed to psychiatric disorders [29]. It is estimated that up to one third of TBI survivors experience adverse effects on their mental health following the injury [30].

1.1.5 Clinical Treatment of TBI

1.1.5.1 Current treatment strategies for TBI patients

TBI patients often present with numerous pathologies, known as ‘polytrauma’, due to the accidental and/or violent nature of events that result in a TBI. Where these injuries are potentially life threatening, they have to be dealt with by the emergency care team before medical management of the TBI becomes the priority.

The medical management of TBI includes specialised prehospital care, in-hospital intensive care and for serious cases, long term rehabilitation [31]. Prehospital treatment guidelines recommend endotracheal intubation with ventilation assistance and blood-pressure treatment for shock (hypotension) where required. Patients with major trauma should be transported as soon as possible to a specialised trauma centre for further treatment [32].

In the hospital TBI is traditionally classified by mechanism (closed or penetrating), by assessment of structural damage using neuroimaging techniques and by clinical severity using the Glasgow Coma Scale (GCS) [4, 33]. The GCS (table 1.2) is a graded assessment of the level of consciousness of the patient ranging from mild to severe that was once a universal classification system, but more recently its use is becoming limited due to the increasing awareness of additional factors that may influence level of consciousness and therefore overall score [34].
Table 1.2: Glasgow coma scale. Mild traumatic brain injury (GCS 13-15) usually presents as a concussion and in most cases patients make a full neurological recovery albeit with some memory loss. In moderate TBI (GCS 9-13) the patient presents as being lethargic and in a state of near unconsciousness or insensibility. In severe TBI (GCS 3-8) the patient is in a comatose state and is unable to open their eyes or follow commands. These patients have a high risk of brain swelling, hypotension or hypoxaemia [32].

<table>
<thead>
<tr>
<th>Eye Opening</th>
<th>Motor Response</th>
<th>Verbal Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous</td>
<td>Obeys</td>
<td>Oriented</td>
</tr>
<tr>
<td>To speech</td>
<td>Localises</td>
<td>Confused</td>
</tr>
<tr>
<td>To pain</td>
<td>Withdraws</td>
<td>Inappropriate</td>
</tr>
<tr>
<td>None</td>
<td>Abnormal flexion</td>
<td>Incomprehensible</td>
</tr>
<tr>
<td></td>
<td>Extensor response</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Within the hospital environment the major focus is to prevent ongoing brain damage inflicted by secondary injury by reducing brain swelling and treating raised ICP by sedation and artificial ventilation. Raised ICP can also be managed by osmotherapy, where rapid infusion of mannitol creates an osmolar gradient and improves ICP, however this treatment cannot be used for long periods [4]. A craniotomy to reduce pressure from swelling is performed in around 30% of patients [31]. Blood perfusion, temperature and oxygen and glucose levels are monitored as in normal intensive care treatment and prophylactic antiseizure steps may be taken. After these emergency steps patients are simply monitored for development of progressive damage and ICP and cerebral perfusion pressure managed as well as possible. Continuous EEG monitoring takes place to identify asymptomatic seizure activity which occurs in 15-18% of patients [4]. A more recently proposed neuroimaging method using a CT scan to focus on the presence or absence of a mass lesion has been described [35]. Furthermore using this method diffuse injuries can be differentiated by signs of increased intracranial pressure. However this technique is not without limitations, as CT can only capture a brief momentary snapshot of the dynamic and evolving processes following head injury and certain processes such as ischemic injury cannot be visualised. There are currently
no pharmacological therapies for the treatment of TBI that specifically target the secondary injury cascades.

1.1.5.2 What does the future hold for TBI treatment?

As far as treating TBI pharmacologically is concerned there are a number of compounds currently undergoing preclinical research for the treatment of secondary damage following a TBI. These include AMPA receptor antagonists, apoptosis inhibitors (e.g. caspase inhibitors), immunophilin ligands (e.g. MPTP suppressors; cyclosporine A [36]), ovarian hormones (e.g. oestrogen) and erythropoietin (for a review of these treatments see [37]).

A lot of interest has been paid in recent years as to the effectiveness of hypothermia as a treatment for TBI, although its actual efficacy as a treatment is controversial. It has shown promise in a variety of animal species and models and is believed to work via multiple mechanisms. These include a reduction in oxygen consumption and ATP depletion, a decrease in EAA levels, blood brain barrier stabilisation and subsequent reduction in infiltration of inflammatory cytokines and free radicals which also reduces cerebral oedema [38-42]. Numerous clinical meta-analyses’ addressing the effectiveness of hypothermia following TBI have been carried out, which have resulted in inconclusive results [43-45]. One study concluded that hypothermic therapy provides no significant benefit 6 months after treatment [45] whereas another found hypothermic therapy to reduce mortality and unfavourable outcomes (e.g. severe disability) but was also associated with a higher risk of developing pneumonia [44]. It still remains to be determined which types of TBI hypothermia treatment may show efficacy for and which is the best technique for inducing hypothermia in injured patients (surface cooling vs endovascular cooling) [39].

No new treatments for TBI have been approved for more than 30 years due to a number of factors. The most significant of these is that most drugs that make it through to phase III of clinical trials fail due to intrinsic neurotoxicity, failure to reach the site of injury and/or inappropriate design of clinical trials [31, 46, 47] (for an extensive list of these compounds see [9]). From a clinical perspective there is often much uncertainty over the stage of injury development the patient is at or whether the pathophysiological mechanism being targeted by a specific drug is actually present in the patient [4]. Despite this somewhat bleak prognosis for the future of TBI treatment in March 2010 progesterone entered phase 3 clinical trials for
the treatment of TBI. It is believed to exert its neuroprotective effect via reduction of cerebral oedema [48, 49]. Results of this study are not expected until 2014-2016. This, along with the various other compounds (mentioned above) with potential for treating this devastating condition, provides hope for researchers in the search for an effective treatment for TBI.

In particular, potent neuroprotection by the noble gases xenon and argon has been demonstrated in in vitro models of TBI although only a single study for each has been published [50, 51]. It is therefore of great interest to investigate these gases, and the other noble gases, further in order to further characterise their neuroprotective potential and investigate their mechanism of action.
1.2 Ischemic Brain injury

1.2.1 Ischemic brain injury as a clinical condition

Cerebral ischemia occurs when an interruption of cerebral blood flow (CBF) leads to the deprivation of oxygen and glucose to brain tissues [8, 52]. Cerebral ischemia can arise via a number of mechanisms; via blockage of cerebral and extracerebral blood vessels, following cardiac arrest or after a period of prolonged hypotension [53]. Cerebral ischemia is responsible for 88% of strokes. The duration of ischemia largely determines the extent of tissue damage and death of neuronal cells and thus the extent of the neurological deficit [52]. Brain cells at the ischemic core are at the highest risk of cell death, which occurs within the first few minutes of ischemia [8]. The tissue surrounding the core, known as the prenumbra, also suffers reduced blood perfusion. The cells in this region are at risk from various pathological processes that can be fatal to the cells. As such they only have a limited time frame for survival, however this does create an opportunity for therapeutic intervention [8].

Like TBI, cerebral ischemia represents a major global public health problem. A stroke is the second most common cause of death (after heart disease) in most economically developed countries and is responsible for the deaths of approximately 6.2 million people (WHO statistics, 2011) worldwide each year [53]. Stroke is more common in men than women, although the risk of mortality is higher in women [53]. Risk factors for stroke include hypertension, diabetes mellitus and a family history, although the greatest risk factor is old age [52].

The only currently approved stroke therapy is tissue plasminogen activator (tPA), which has been proven effective if administered to a subpopulation of patients within 3 hours of showing neurological symptoms. TPa is a thrombolytic drug which acts by enzymatically catalysing the conversion of plasminogen to plasmin, which is the principal enzyme involved in breaking down clots [53].
1.2.2 Shared mechanisms of injury between ischemic and traumatic brain injury

Ischemic brain injury and traumatic brain injuries can occur in very different circumstances or concurrently (due to disruption of cerebral blood flow following TBI). A number of the molecular and cellular mechanisms that leads to cell death are shared between the two conditions [53]. These include excitotoxicity, reactive oxygen species generation, inflammation and apoptotic cell death.

Following a reduction in cerebral blood flow, energy failure occurs due to a shortage of oxygen and glucose. These are the main sources of energy production via oxidative phosphorylation [54]. Reduced ATP levels impede the functioning of Na-K ATPase resulting in a loss of normal ionic gradients. Cytosolic Na\(^+\) increases in concentration and K\(^+\) decreases, causing a depolarisation of the neuronal membrane which activates voltage gated Ca\(^{2+}\) channels [54]. A rapid influx of calcium results in the release of glutamate which binds NMDA and AMPA receptors leading to further calcium entry. Under normal conditions the excess glutamate is cleared by astrocytes and presynaptic uptake systems but this response is disrupted following ischemia [54]. Excessive calcium influx leads to the activation of numerous downstream signalling pathways, including activation of enzymes such as phospholipases and proteases and endonucleases which decreases cellular integrity and ultimately affects survival of the cell [54]. This process of excitotoxicity progresses similarly to that seen following a TBI.

Oxidative stress occurs after ischemia when reactive oxygen species are produced by dysfunctional mitochondria [55]. The excess calcium entering cells during ischemia enters mitochondria and triggers the opening of the permeability transition pore and cytochrome c release; an early event in the apoptotic pathways. Production of reactive oxygen species leads to peroxidation of plasma membranes and organelle membranes, damage to the endoplasmic reticulum and mitochondria and induces DNA fragmentation [54, 55].

In terms of the inflammatory reaction, ischemic onset leads to a rapid accumulation of microglia, followed by an influx of inflammatory cells such as granulocytes, leukocytes, T cells and monocytes and proinflammatory mediators such as cytokines and chemokines are released from the damaged tissue [54]. Infiltration of leukocytes complicates the injury
further by causing the release of additional cytokines and chemokines when in the brain tissue. This leads to an oxidative stress reaction and activation of matrix metalloproteinases (a family of endopeptidases involved in apoptosis) [54]. Activation of matrix metalloproteinases enhances the breakdown of the blood brain barrier and allows further entry of inflammatory cells into the damaged brain tissue. As with TBI there is also evidence that the inflammatory reaction can also be helpful as well as harmful, however to what extent is unclear [54].

Apoptotic cell death is a feature shared by both ischemic and traumatic brain injury. As with a trauma, the cells at the core of the injury where ATP is heavily depleted most likely die via a necrotic mechanism (as with primary injury in TBI) [56]. The cells in the ischemic prenumbra die predominantly by apoptotic cell death. One pathway of apoptosis involves the previous mentioned opening of the mitochondrial permeability transition pore and cytochrome c release. Cytochrome c release triggers a series of events that ultimately leads to activation of proteolytic caspases. A second pathway of apoptosis involves the activation of pro-apoptotic proteins of the Bcl-2 family, such as bax. The extrinsic death receptor pathway of apoptosis involving the fas death receptor is also involved in cell death following ischemia [54]

When considering shared pathways of injury it is important to remember that ischemic injury itself can form part of the secondary injury following a TBI. In conditions of a severe TBI, local damage to the vasculature and subsequent reductions in cerebral blood flow have been reported to result in ischemia [53].

1.2.3 What does the future hold for treatment of ischemic brain injury

There are generally two therapeutic approaches that are considered when seeking to improve outcome following ischemic brain injury. The first aims to improve blood flow to affected areas of the brain to target the metabolic issues that arise by increasing local concentration of oxygen and glucose available to the tissue. The second approach is via pharmacologic neuroprotection, by targeting a pathological process and selectively blocking it to protect tissue from further damage [57]
Previous attempts to translate experimental efficacy into clinically beneficial effects have so far not been hugely successful. Drugs that target excitotoxic cascades, antioxidants, anti-apoptotic and anti-inflammatory therapies have all failed to produce clinically significant improvements in patients [8]. However there could be numerous reasons for their failure, including inadequate drug delivery, unrealistic therapeutic time windows and poor study designs. Combination therapy has been gaining interest, as it is thought targeting multiple mechanisms may provide a greater benefit than targeting a single pathway, as blocking one pathway only may allow the other death promoting pathways to continue unhindered [8]. Good progress in the experimental setting has been made with combination therapy, for example combining the NMDA receptor antagonist xenon with hypothermia [58]. Other avenues to explore for neuroprotection includes further characterising the glial cell response to ischemia to make those cells (e.g. astrocytes) a possible target for neuroprotection or shifting attention towards repair of damaged tissues with the use of stem cells [54].

Along with neuroprotective strategies, advances in brain imaging techniques, such as magnetic resonance imaging and PET will allow for more accurate determination of tissues that are at risk of impaired blood supply following an ischemic attack which will therefore assist in the selection of appropriate neuroprotective strategies [57].

Given the many shared mechanisms of injury progression between ischemia and traumatic brain injury, neuroprotective strategies that are found to be useful following one type of injury may hopefully prove beneficial for use in both conditions if certain pathogenic pathways are targeted.
1.3 Xenon as a Neuroprotectant

1.3.1 The history and clinical profile of xenon

Xenon is the 54th element in the Periodic Table and exists as a monoatomic gas. It is categorised as a Noble gas due to its full outer shell of electrons. Xenon was first discovered in 1898 by the British chemists William Ramsay and Morris Travers. Its biological effects were reported many years later by Lawrence and colleagues in 1946 who reported behavioural effects including sedation and ataxia when mice were exposed to 0.4-0.8 atm xenon [59].

Xenon was first used clinically as a general anaesthetic agent in the early 1950s [60] and has been used experimentally in clinical anaesthetic practise for more than 60 years. In humans the published minimum alveolar concentrations of xenon for immobilisation (MAC_{immobility}) are between 63% vol [61] and 71% vol [62]. Xenon possesses many of the properties characteristic of an ‘ideal’ anaesthetic. These include a rapid and smooth inhalational induction and emergence (regardless of anaesthetic duration) due to its very low blood/gas partition coefficient of 0.115 and low solubility in lipids (oil/gas partition coefficient; 1.9). Xenon is known to have an excellent cardiovascular stability profile (no change in heart rate or peripheral vascular resistance). It is also known to possess profound analgesic properties and seemingly no toxic, foeto-toxic, carcinogenic, mutagenic or allergic properties. Xenon is not metabolised by the kidney or liver and is excreted unchanged via the lungs [63]; this is advantageous as it eliminates the potential risk of toxic metabolite production and reduces usage costs as xenon can be recycled. Xenon’s safety and efficacy profile as an anaesthetic has been described as “unequalled” [64]. Xenon is manufactured by fractional distillation of air at a cost of £25 a litre which has thus far limited its widespread clinical use.

Xenon has been shown to exert organoprotective properties, notably in the brain and heart. Indeed there is considerable interest in the use of xenon as a neuroprotectant after ischemic or traumatic brain injuries [50, 58, 65-68]. Its potential use in this setting is described more fully in section 1.3.3.
1.3.2 Molecular targets of xenon; binding properties and sites of action

Noble gases are described as being ‘inert’ due to their inability to form covalent bonds with other molecules, thus it might be thought that noble gases would be incapable of biological interactions. However, some noble gases are capable of producing a biological effect via interactions with amino acids in the active site of enzymes or receptors [69]. The electron shell of xenon is capable of being polarised by surrounding molecules which induces a dipole, thereby enabling interactions with proteins via Van Der Waals interactions. This type of weak intermolecular force is also known as London dispersion forces or an ‘induced dipole-dipole attraction’, and underlies xenons ability to exert pharmacological effects despite its lack of reactivity.

Many inhalational and intravenous anaesthetics potentiate inhibitory synaptic transmission via allosteric modulation of the ligand-gated GABA_\text{A} receptor. Numerous studies have agreed that xenon has no substantial effects at GABA_\text{A} receptors or other inhibitory (glycine) receptors [70-73]. Xenon also has no effect on N-type voltage-gated calcium channels [71, 74, 75] and appears to target the NMDA receptor component of glutamatergic transmission [71, 76]. Based on this evidence it appears that xenon exerts its actions predominantly postsynaptically.

The NMDA receptor was first discovered to be one of the targets of xenon when in 1998 it was found to inhibit NMDA evoked currents in hippocampal neurones by around 60% when applied at a clinically relevant concentration of 80% xenon [71]. Mechanistically, xenon is thought to exert its effects at the NMDA receptor via competitive antagonism at the glycine binding site [68, 71]. Electrophysiological studies identified the co-agonist glycine site as the target of xenon binding, and it is believed that xenon stabilises the open confirmation of the glycine binding domain, preventing the closure of these domains and subsequent NMDA receptor channel opening [76]. Consistent with competitive antagonism at the glycine site, xenon inhibits NMDA receptors more potently at low glycine concentrations than at high glycine concentrations (see figure 1.2a) [76]. Further work in this lab identified an amino acid (phenylalanine 758) that is essential for the interaction by xenon at the glycine site of NMDA receptors [77]. Lineweaver Burk analysis of NMDA receptor inhibition by xenon identified an additional non-competitive component of xenon inhibition (see figure 1.2b).
Figure 1.4 (a) Inhibition by 80% xenon in HEK cells transfected with the NR1/NR2A NMDA receptor subunits increases as glycine concentration is reduced. (b) Lineweaver-Burk analysis shows a mixed competitive and non-competitive inhibition by xenon. White circles indicates 80% xenon, black circles indicate no xenon present. The insets show example traces of the current produced in the absence and presence of 80% xenon at glycine concentration of 100µM (left) and 1µM (right). Both figures from Dickinson et al. (2007) [76]

Additional studies have identified possible non-NMDA receptor targets of xenon as the two-pore domain K+ channel TREK-1 [78] Channels of this type act normally to modulate neuronal excitability by providing a background leak potassium conductance. Activation of these channels can cause hyperpolarisation of the cells and therefore reduce excitability [74]. Other targets include the ATP sensitive K+ channel (K\textsubscript{ATP}) [79] which is thought to play a role in the preconditioning neuroprotective effect of xenon. A study in 2009 by Trapp and colleagues used neuronal-glial co-cultures exposed to 75% xenon in a preconditioning paradigm (15 minutes prior exposure) before being subjected to 75 minutes of oxygen and glucose deprivation 24 hours later. Exposure to xenon prevented cell death after the OGD procedure as measured by the MTT reduction test and this neuroprotection was reversed by the presence of 0.1mM of the K\textsubscript{ATP} channel inhibitor tolbutamide. Patch clamp electrophysiology experiments on HEK293 cells expressing the Kir6.2/SUR1 K\textsubscript{ATP} channel revealed that xenon acts as a K\textsubscript{ATP} channel opener as shown by an increased K\textsuperscript{+} current [79]. Further work by this group demonstrated that xenon targets the Kir6.2 pore forming subunit of the K\textsubscript{ATP} channel [80]. Some subtypes of nicotinic acetylcholine receptors [81] and 5-HT\textsubscript{3}
receptors [82] are also thought to be targets of xenon, however the role (if any) of these channels in xenon’s pharmacological properties remains to be fully determined.

Inhibition of NMDA receptors is thought to underlie a number of xenons pharmacological properties, including general anaesthesia and neuroprotection following brain injury (which is explained in greater depth in the following section). NMDA receptor glycine site antagonists are known to be well tolerated in patients.

1.3.3 The neuroprotective properties of xenon and its potential use following traumatic brain injuries

NMDA receptors are known to play a pivotal role in the progression of injury after a traumatic or ischemic brain injury (as outlined in section 1.1.2.3), hence it has long been postulated that NMDA receptor antagonists may be useful as neuroprotective agents. Xenon has been shown to be neuroprotective in in vitro [47, 68, 79, 83-86] and in in vivo [58, 65-67, 87, 88] models of ischemic brain injury and in an in vitro model of TBI [50].

A recent study by Banks et al. [68] investigated the mechanism of xenon neuroprotection in an in vitro model of ischemic brain injury, using hippocampal organotypic slice cultures exposed to OGD for 30 minutes followed by incubation with 50.6 kPa xenon for 24 hours. They found application of xenon to provide around 68% protection from OGD induced injury; which was not significantly different from a slice that received no injury. This neuroprotective effect was mediated by xenon’s competitive inhibition at the co-agonist glycine site of the NMDA receptor as addition of 100μM glycine abolished xenon neuroprotection entirely. Numerous studies have confirmed xenon’s efficacy as a neuroprotectant in in vivo models of ischemia, including middle cerebral artery occlusion [84, 89, 90] and carotid artery ligation [66, 67, 91]) and direct injection of NMDA [47] with a reported lack of neurotoxic effects compared to other NMDA receptor antagonists (e.g. MK-801) [87]. A number of studies have additionally investigated the synergistic neuroprotective effects of combining hypothermia with xenon administration in models of ischemia [58, 65, 66, 69]. It is plausible that xenon binding is increased at lower temperatures which would correlate with its increased potency during hypothermia as it has been established that the
binding of volatile general anaesthetics increases at lower temperatures due to favourable enthalpic reactions [74, 92-94].

In models of ischemic injury the therapeutic time window of xenon has been approximated at around 3 hours post injury [38, 67, 86, 97]. This is highly encouraging for the clinical implications for the potential use of xenon to treat these conditions, as it allows a window of opportunity for emergency services to reach the patient, assess their condition and move them to an appropriate location for treatment. From a further clinical perspective xenon’s pharmacological properties (outlined in section 1.3.1), particularly its lack of metabolism and good cardiovascular stability, allow for prolonged and repetitive dosing in the critical period after injury has been sustained. This provides xenon with an advantage over other agents that require IV infusion into the body which may require constant dosing.

More recently xenon has entered into phase III clinical trials as a neuroprotectant following ischemic brain injuries, including cardiopulmonary bypass, neonatal asphyxia and brain damage following cardiac arrest. Previous clinical trials involving xenon have only investigated its safety and efficacy as a general anaesthetic [74]. As yet none of the neuroprotection trials involving xenon have been completed or have reported results. Any clinical benefits that xenon may possess would greatly outweigh the initial cost of xenon treatment in terms of financial costs of long term rehabilitation following brain injuries.

A study by Coburn et al. [50] using in vitro model of focal traumatic brain injury found 75% xenon provide marked neuroprotection against the development of secondary injury. The mechanism of this neuroprotection is currently unknown, although it may involve interference with NMDA receptor signalling similar to that observed in ischemic injury. As yet only a single in vitro study and no in vivo studies investigating xenon’s neuroprotective potential after TBI have been carried out.

1.4 Other noble gases as potential neuroprotectants

The discovery of xenon’s neuroprotective properties has prompted researchers to investigate the potential of other noble gases helium, neon, argon and krypton as neuroprotectants [95] (radon is excluded as it is radioactive). As mentioned previously, the binding forces created by the formation of a temporary dipole relies on the ability of the gas to be polarised, which in itself it determined by the electronic configuration. It is therefore reasonable to predict that
other noble gases aside from xenon to have the capability of binding to proteins, receptors or molecules and thus exert some kind of biological effect.

Helium and neon are known as non-immobilisers [96], that is, they are unable to cause anaesthesia even at elevated pressures. Argon and krypton possess anaesthetic properties, albeit at elevated pressures (4.5 atm krypton and 15 atm argon [74]). The difference in (or lack of) anaesthetic activity between the noble gases may be due to the ability of the gas to become polarised. The ability of the gas to interact with proteins arises from London dispersion forces and/or charge induced dipole interactions. Both of these are proportional to the polarizability. The binding energy must be sufficiently large to overcome the unfavourable (repulsive) entropy term associated with binding [74, 97]. Helium and neon can be distinguished from the other noble gases by the unfavourable balance between the binding forces and the repulsive forces [97]. Table 1.3 lists some of the key properties of the noble gases.
<table>
<thead>
<tr>
<th>Physical Property</th>
<th>Helium</th>
<th>Neon</th>
<th>Nitrogen</th>
<th>Argon</th>
<th>Krypton</th>
<th>Xenon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atomic number</td>
<td>2</td>
<td>10</td>
<td>7</td>
<td>18</td>
<td>36</td>
<td>54</td>
</tr>
<tr>
<td>Atomic mass (g/mol)</td>
<td>4.0</td>
<td>20.2</td>
<td>14.0</td>
<td>39.9</td>
<td>83.8</td>
<td>131.3</td>
</tr>
<tr>
<td>Density (g/l) (0°C)</td>
<td>0.1785</td>
<td>0.900</td>
<td>1.251</td>
<td>1.784</td>
<td>3.736</td>
<td>5.887</td>
</tr>
<tr>
<td>Thermal conductivity (W/m/K) (300k)</td>
<td>0.1499</td>
<td>0.0491</td>
<td>0.0260</td>
<td>0.0178</td>
<td>0.0094</td>
<td>0.0056</td>
</tr>
<tr>
<td>Polarizability α (Å³)</td>
<td>0.21</td>
<td>0.39</td>
<td>1.74</td>
<td>1.64</td>
<td>2.48</td>
<td>4.04</td>
</tr>
<tr>
<td>Water/gas partition coefficient at 25⁰C</td>
<td>0.0085</td>
<td>0.010</td>
<td>0.015</td>
<td>0.031</td>
<td>0.053</td>
<td>0.095</td>
</tr>
<tr>
<td>Oil/gas partition coefficient at 25⁰C</td>
<td>0.016</td>
<td>0.019</td>
<td>0.07</td>
<td>0.14</td>
<td>0.44</td>
<td>1.9</td>
</tr>
<tr>
<td>General anesthesia (atm)</td>
<td>N/a</td>
<td>N/a</td>
<td>39</td>
<td>15.2</td>
<td>4.5</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td>(mouse)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.6-0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(human)</td>
</tr>
</tbody>
</table>

Table 1.3: Physical properties of the inert gases and nitrogen. Reproduced from Dickinson and Franks 2010 [74].

1.4.1 Helium as a neuroprotectant

Helium is widely considered to be inert and lacking any intrinsic pharmacological activity and is often used in experiments to test the effects of pressure on biological systems [50, 74, 96]. With regard to the neuroprotective properties of helium, numerous studies in different models and preparations of brain injury have yielded contrasting results. One in vitro study of ischemia found 50.6 kPa helium to have no effect on cell survival or death [68], whereas another in vitro study of ischemia found 75% helium to be detrimental to cell survival [95].
To further complicate the matter, an in vivo model of focal ischemia found 75% helium to reduce infarct volume and improve functional outcome 24 hours post injury [98]. It is currently unknown why these differing results were found. David and colleagues [99] found that the neuroprotection afforded by helium following MCAO was temperature dependent, that is, when the inspired temperature was 25°C neuroprotection was seen, but when the temperature of the inspired helium was raised to 33°C this effect was abolished. They therefore propose that helium causes hypothermia when inspired, which is responsible for the neuroprotection. The high thermal conductivity of helium compared to nitrogen (0.1499 W/m/k vs 0.0260 W/m/k) makes this theory entirely plausible [74], as breathing in helium below normal body temperature will cause a reduction in core temperature. This may also provide an explanation for differing findings in different models of brain injury if temperature of the model during experimentation was not strictly monitored or regulated [74].

1.4.2 Neon as a neuroprotectant

Neon has received very little attention with regard to biological activity, as it appears to possess none. Only a single in vitro study of ischemic brain injury has been carried out involving neon, which found it to have no effect [95]. Neon has a thermal conductivity that is twice that of nitrogen (0.0491 W/m/k) therefore may induce hypothermia upon inhalation, although to a much lesser degree than helium. Further studies in different models of brain injury are required to assess neon’s potential as a neuroprotective gas.

1.4.3 Argon as a neuroprotectant

Argon, another of the noble gases capable of causing anaesthesia (at elevated pressures) has been found to be neuroprotective in certain in vitro and in vivo models of ischemic and traumatic brain injury at both elevated and normobaric pressures [51, 95, 100-102]. Argon has been found to be less potent as a neuroprotectant than xenon [95]. However argon has an advantage over xenon in that it is more abundant in the atmosphere (0.93%) and is thus
substantially cheaper to obtain and would not require a closed circuit delivery system [103]. The molecular targets of argon and its mechanism of neuroprotective action are currently unknown.

### 1.4.4 Krypton as a neuroprotectant

Krypton is another of the noble gases that has received very little attention thus far, despite the fact it does appear to possess some biological properties. Krypton is an anaesthetic under hyperbaric conditions (4.5 atm) although no molecular targets for anaesthesia have been identified [74]. To date only a single study has investigated the neuroprotective effects of krypton. Jawad and colleagues [95] used pure neuronal cultures exposed to OGD for 90 minutes in order to determine whether noble gases, including krypton, displayed any neuroprotective properties. They found exposure to krypton (at the same effective concentration of xenon, 75%) did not have a protective effect, but instead krypton was found to be injurious to uninjured cells.

Given the conflicting results on the efficacy of inert gases (other than xenon) as neuroprotectants, one aim of this study was to investigate the effects of all the inert gases under identical conditions.
1.5 Aims of research

1.5.1 Research outline

It is clear from the literature that there is a critical need for effective compounds to treat secondary injury development following a traumatic brain injury, as there are currently no treatments approved for this condition. The aim of this thesis is to investigate the neuroprotective efficacy of the noble gases xenon, argon, krypton and neon in an in vitro model of TBI, the first of its kind. Once a neuroprotective effect has been established, this project aims to investigate the mechanism by which this is achieved focusing on the involvement of the glutamatergic NMDA receptor. Knowing the mechanism of a pharmacological compound is important for a number of reasons. It can answer some important questions as to when administration of the drug is required in order to achieve maximum efficacy, what potential side effects the drug may have, how long administration is required for, which combination therapies may enhance its effects and what other pathologies it may be useful for which share a similar injury mechanism, among others.

The method chosen to model TBI in vitro uses organotypic hippocampal slice cultures subjected to a weight-drop injury to create a primary focal contusion injury. These cultures allow long term maintenance of the slices to monitor the development of secondary injury. Injury in the slices will be assessed via fluorescence imaging in the days following the initial injury. Treatment with gases will be applied via a custom built chamber following the initial injury and maintained throughout the experimental protocol.

Further to addressing neuroprotection following TBI, this project will also investigate the mechanism of neuroprotection by the noble gases in an in vitro model of ischemic brain injury. Targeted treatments for the reduction of secondary injury development following ischemic brain injury represents another unmet clinical need thus research into neuroprotective strategies is of great interest. The same hippocampal organotypic slice culture model will be utilised for this investigation, with injury inflicted via 30 minutes of oxygen and glucose deprivation prior to treatment.
1.5.2 Hypotheses

Given previous findings and the available evidence the hypotheses for this project are as follows:

1. Given the wealth of data regarding the efficacy of xenon as a neuroprotectant in various models of acquired brain injury it is hypothesised that xenon will provide neuroprotection against secondary injury development following TBI in vitro.

2. Glutamate excitotoxicity is a well-established mechanism involved in the development of secondary injury following TBI. Given that xenon acts as an antagonist of the NMDA receptor at its glycine binding site [76] it is reasonable to hypothesise that this mechanism may also be responsible for the neuroprotection observed after TBI.

3. I will test the hypothesis that the other noble gases helium, neon, argon and krypton are neuroprotective following traumatic brain injury in vitro.

4. If these gases are found to be neuroprotective, then I will test the hypothesis that the other gases provide neuroprotection via the same mechanism as xenon.
CHAPTER 2
MATERIALS AND METHODS

2.1 Preparation and maintenance of neonatal C57BL/6 mouse hippocampal organotypic slice cultures

Organotypic cultures are slices of tissue which are cultured whole, without dissociation of the individual cells. Organotypic slice cultures maintain normal structural, organisational, and functional features of native brain tissue with preserved synaptic and chemical signalling, which allows for functional analysis of the properties of nervous tissue [104-106]. Organotypic slices maintained in culture from neonatal mice closely mimic developmental events that occur in vivo [106]. The spatial orientation and complex electrophysiological pathways between cell types of particular brain regions are maintained in three dimensions in this system, with greater fidelity to the in vivo state than is possible with other systems. (A detailed description of the similarities between organotypic slices and the in vivo brain appears in Discussion section 7.1.1).

The hippocampus was chosen as the brain region under investigation for a number of reasons. Bilateral loss of hippocampal neurons has been observed in 85% of fatal head injury cases as soon as 48 hours following the initial traumatic event [107]. This is suspected to be due to the high density of NMDA receptors in this brain region, making it particularly at risk of excitotoxic damage especially in the CA1 region where density is highest. A study by Watanabe et al. of adult wild type C57BL/6 mouse brain found distribution of the NR2 subunit of the NMDA to have the highest levels of expression in the hippocampus (see figure 2.1). High levels are also detected in the cerebral cortex and granular layer of the cerebellum [108]. The hippocampus is therefore an obvious choice of brain region when investigating a pharmacological compound that possibly targets the NMDA receptor.
Hippocampal slices were obtained from 5-6 day old neonatal C57/BL6 mouse pups using a modified interface method of Stoppini et al. [109]. All experiments were performed in compliance with the United Kingdom Animals (Scientific procedures) Act of 1986 and have been approved by the Ethical Review Committee of Imperial College London. Mice were purchased as a dam with a litter averaging 5 pups (Charles River, UK) and all efforts were used to minimise suffering and the number of animals used.

2.1.1 Preparation of laboratory prior to dissection.

It was not possible to carry out the dissection and slicing procedure in a laminar flow hood so every care was taken to sterilise the environment in which the slices were prepared and a number of steps were taken to prevent contamination. A labcoat, latex gloves, hairnet and PPE mask were worn and all surfaces wiped down with 70% ethanol. Sterile autoclaved paper towels were laid down in all areas of the bench and culture hood and all dissection equipment treated with Virkon (VWR International, Leicestershire, UK) before being autoclaved and sprayed with 70% ethanol before use. Dissection dishes (150mm glass petri dishes containing a base of Sylgard elastomer (Dow Corning, MI, USA)) and individual razor blades (Wilkinson Sword, Bucks, UK) were autoclaved and sprayed with 70% ethanol before use. The plastic chopping disk for the tissue chopper was also sterilised by immersion.
in 70% ethanol for at least 1 hour. All equipment was allowed to dry fully before use. Solutions necessary for the procedure (dissection media and growth media) were kept on ice and all petri dishes that would contain brain tissue at any point were placed on a large metal heat-sink on ice to ensure temperatures were kept as low as possible throughout. Unless indicated all solutions were purchased from Sigma Aldrich (Dorset, UK).

### 2.1.2 Dissection of mouse brain, slicing and isolation of hippocampus.

Organotypic cultures were prepared from 2 or 3 pups at a time. Any more than 3 would extend the time taken to complete the procedure which may compromise the health of the hippocampal slices. Pups were culled by schedule 1 euthanasia (cervical dislocation) followed by decapitation with the head handled using sterile paper towels. Surgical scissors were used to cut the skin from the neck to the nose along the midline to allow the peeling back of the skin to access the skull. Using Vannas scissors an incision was made at the caudal end of the skull above the cerebellum across the left and right hemispheres. A second incision was then made along the midline in a rostral direction before a third incision was made above the olfactory bulbs. This ‘I’ shape that was cut created two panels of the skull which were pulled back carefully using forceps to reveal the brain beneath. The brain was gently removed using a small spatula and transferred to a dissection dish containing ice cold dissection media (Gey’s balanced salt solution with 5g\text{L}^{-1} \text{ D-glucose (Sigma G8270) and 1\% antibiotic-antimycotic suspension (Amphotericin B, penicillin, streptomycin, Sigma A5955)}). Using a razor blade (Wilkinson Sword, Bucks, UK) a coronal cut was made at the caudal end of the brain removing the cerebellum and a second cut was made along the midline to separate the two hemispheres. These were then transferred to a 35mm petri dish on a cold heat-sink and taken to the culture hood. In the hood the hemispheres were transferred again to a dissection dish under a light microscope and the olfactory bulbs removed with a razor blade. This created a flat surface on which to stand the hemispheres on allowing for easily removal of the midbrain by gently teasing apart the midbrain from the cortex using ultra-fine forceps. Flipping the cortex back into its side revealed the hippocampus running obliquely through the middle.
The pre-cooled plastic chopping disk was then attached to the cutting table of the McIlwain Tissue Chopper (Mickle Laboratory Engineering Co., Surrey, UK) and an excess of ice cold dissection medium added to its surface. A clean sterile razor blade was affixed to the blade holder and dropped against the chopping disk to confirm they sat flush against one another during slicing. The brain tissue was transferred using a spatula and orientated so that the axis of the hippocampus was parallel to the chopper blade. The excess medium was removed with a fine tipped Pasteur pipette and the tissue chopper switched on immediately. Tissue was sliced at 400μm thick at 50% chopping speed and force (arbitrary units). After slicing ice cold dissection media was re-applied to the slices before transferring them to the dissection dish with the flat of a scalpel blade. Using ultra-fine forceps the slices were gently teased apart (with care being taken to avoid touching the hippocampus) and the hippocampus removed from the surrounding cortex with ultra-fine vannas scissors. A cut off Pasteur pipette was used to transfer the individual hippocampal slices to a fresh 35mm petri dish containing ice-cold dissection media.

A p5 mouse brain generated on average 10-12 hippocampal slices per hemisphere.

Figure 2.2: Bright field photograph of a typical p5 hippocampal slice prior to plating on membrane.

2.1.3 Plating of slices onto membrane

The petri dish containing the hippocampal slices was transferred to a sterile laminar flow hood where it was placed in a pre-cooled heat sink to prevent warming of the slices during plating. A cut-off Pasteur pipette was used to transfer 5-6 slices to each tissue culture insert.
(Millicell CM, Merck Millipore, Darmstadt, Germany) which were themselves placed inside sterile 35mm petri dishes, with care being taken to distribute the slices evenly around the membrane and with suitable distance from the membrane edge. The excess dissection medium was removed with a fine-tipped Pasteur pipette. Around 1ml of ice cold growth media (50% Minimum essential media with Earle’s salts, L-glutamine and bicarbonate Sigma M4655, 25% Hank’s balanced salt solution Sigma H9269, 25% heat-inactivated horse serum Sigma H1138, 2mM L-glutamine Sigma G7513, 5mg ml⁻¹ D-glucose Sigma G8270, 10mM HEPES VWR 441485H, 1% Antibiotic-antimycotic suspension Sigma A5955; pH 7.2) was then added to the petri dish; enough so that it completely covered the base of the membrane but without overflow or ‘floating’ of the insert. Following plating all dishes were transferred to a humidified incubator at 37°C with 5% CO₂.

![Figure 2.3: Schematic diagram of hippocampal organotypic slices plated on a membrane at the ‘air-medium’ interface, allowing the slices access to both growth media and oxygen.](image)

2.1.4 Maintenance of organotypic slices

Medium was replaced with prewarmed (37°C) growth medium the day after plating, and at least every 3 days thereafter for around 10-14 days before experiments were began. Old media was removed from beneath the membrane using a fine-tipped pipette and replaced with fresh growth media. Care was taken not to disturb or stretch the membrane in any way during
media changing, as a ‘stretch’ of the slice itself is considered an injury paradigm for TBI [110].

2.2 Traumatic brain injury in vitro

In vitro models of TBI provide researchers with a platform on which to perform well controlled, repeatable and environmentally isolated experiments [111] whilst allowing investigation of specific pathways without systemic or vascular influences. Numerous in vitro models of TBI exist all of which aim to recreate distinct components of injury cascades following injury. This study aimed to recreate conditions of a focal contusion (compression) injury, which in itself is characterised by a region of primary injury followed by a secondary spread of injury. Primary injury was inflicted upon the slices by dropping a weight onto them using a custom built impact device.

2.2.1 Building the traumatic impact device

The traumatic impact device was custom built in house. The design was inspired by the in vitro TBI device used by Coburn et al. [50] and others [112, 113] to model primary injury followed by secondary injury development after TBI. The device was designed to drop a small weight (hereafter referred to as a ‘stylus’) onto the slice at a defined energy to inflict a focal injury in the CA1 region. The mechanism of the weight drop device was simple; an electrical current (4 x 6V batteries) was delivered to an electromagnet upon which the stylus would suspend due to the magnetic force. By switching a relay switch momentarily the current was interrupted and the stylus was allowed to make brief contact with the surface of the hippocampal slice with a selected energy (see figure 2.4). Impact energy (J) was determined by the height above the slice and calculated using the equation mass (kg) x acceleration (m/sec$^2$) x height (m) [113].
Figure 2.4: Schematic diagram of electrical circuit for impact device. Current supplied to an electromagnet suspends the stylus above the surface of the slice. The relay switch allows for a brief interruption to the current allowing the stylus to drop.

The stylus had a diameter of 1.3 mm and mass of 70 mg with the distal part of the stylus smooth and rounded to prevent perforation of the slice. The stylus was held in a glass capillary for support which was mounted within a plastic support allowing for the easy removal of the stylus from the electromagnet for sterilisation. The electromagnet was attached to a three-axis micromanipulator for easy positioning of the stylus over the CA1 region and for accurate determination of drop height (see figure 2.5). After building the circuit and stylus and mounting them on a micromanipulator the supporting structure of the TBI device was built. This included a stereomicroscope to visualise regions of the slices and monitor impact, and a support platform for the slices which consisted of an upturned large glass petri dish (see figure 2.4) under which a fibre optic light source was positioned to enable illumination of the slices from beneath (not pictured).
2.2.2 Preparation of slices for experimental procedures

After 10-14 days in culture the slices were ready to begin experimentation. Each experiment consisted of 6 membranes per 6-well plate, half of which were assigned into a sham group and the other half were assigned to receive a TBI. This was done so that sham slices received exactly the same conditions (besides TBI injury) as the TBI slices in the same 6-well plate. All membranes contained slices that were dissected on the same day from the same litter and each membrane contained on average 5-6 slices. Assignment into sham or TBI groups was carried out before initial imaging to avoid potential bias when the health status of the slices was already known. The membranes were transferred into a 6 well plate with custom-made metal rings inserted into the wells to raise the membrane into the magnification range of the 2x objective of the florescence microscope. Each well contained \( \approx 8 \text{ mL} \) of pre-warmed \((37^\circ\text{C})\) experimental media containing Propidium iodide (PI) (75% Minimum essential media with Earle’s salts, L-glutamine and bicarbonate, 25% Hank’s balanced salt solution, 2mM L-glutamine, 5mg mL\(^{-1}\) D-glucose, 10mM HEPES, 1% Antibiotic-antimycotic suspension, 4.5μM propidium iodide; pH 7.2). PI is a membrane impermeable dye that only enters cells with damaged cell membranes, where it forms an irreversible bond principally to DNA [114]. When bound it becomes highly fluorescent with a peak emission in the red region of the

Figure 2.5: (A) Traumatic impact device including supporting structures and power supply and (B) a close up of the electromagnet with the stylus (circled) suspended above the slices.
visible spectrum. PI is known to be non-toxic to neurons (as interaction with DNA can only occur in a cell with a compromised membrane) and is used frequently as an indicator of neuronal integrity and cell viability [115].

Membranes were transferred with the aid of forceps and the level of experimental media beneath the membrane topped up using a fine tipped pipette to ensure no bubbles remained. Slices were incubated for 1 hour at 37°C in this medium to allow PI to equilibrate, as previous studies have found PI binding to reach maximum after 30 minutes exposure [50]. For experiments where exogenous glycine and strychnine were added to the experimental media, these agents were present in the media at the time of transfer to completion of the experiment.

![Graph showing PI uptake over time](image)

**Figure 2.6**: A study by Coburn et al. [50] found PI binding following permeabilisation with 70% ethanol to be rapid, with a half time of 7 minutes and complete by 30 minutes. Image from Coburn et al. 2008 [50]

### 2.2.3 Fluorescence imaging of organotypic slices

One hour after transfer into experimental media slices were transferred in an insulated polystyrene box to the fluorescence microscope for imaging (Nikon Eclipse 80). Imaging was carried out before experimentation (referred to as t=-1 imaging) to identify any slices whose
viability was compromised in order to exclude them from experimental analysis (see section 2.4 for exclusion criteria).

Slices were imaged using a Nikon Eclipse 80 florescence microscope with a super high pressure mercury lamp. Due to the lamp intensity dropping over time, the exposure time of each set of slices per session was adjusted using a glass slide standard (Fluor-Ref Texas red, Omega Optical, VT, USA) as a reference. Exposure time was adjusted depending on the mean intensity of the red channel in the glass slide standard which was exposed for 4ms under a 10x objective. The text on the slide was used to gain focus. The ratio of the maximum intensity of 126.6789 (previously measured) to the intensity measured in each session was multiplied by the exposure time for the slices (1.2 seconds) to calculate the relative exposure time for that session \((\frac{126.6789}{\text{measured intensity}}) \times 1.2\). This exposure time was selected as it did not cause a saturation of the image at any level of injury (be it uninjured t-1 slices or maximally damaged 70% ethanol treated slices). Slices were imaged using the calculated exposure time for that session. A hairdryer was used to gently heat the lid of the plate if condensation built up inside which would attenuate the signal from the PI. The hairdryer was held at a distance of around 2 ft to avoid damaging the slices. After imaging slices were transferred back into the incubator for 1 hour to equilibrate temperature back to 37°C.

### 2.2.4 Traumatic impact procedure

Before starting the impacting procedure the hood and equipment (figure 2.5) were cleaned and sterilised with 70% ethanol. During the procedures a face mask, hair net and gloves were worn to avoid contamination of the slices. The stylus was pre-sterilised by spraying liberally with 70% ethanol before being placed under a UV lamp for 1 hour before impacting. It was essential that the stylus be sterilised thoroughly as it comes into direct physical contact with the slices. The slices were transported from the incubator to the impactor in an insulated polystyrene box, the lid removed and the slices placed on the glass platform. The well which was to be impacted was positioned below the stylus with a cold light source focused beneath it. Using the micromanipulator the stylus was lowered and positioned under the microscope so that it just touched the membrane in a region where there were no slices in close proximity. This was assigned the 0’ position and the height of this read off the micromanipulator and recorded. The stylus was then raised 1-2mm from the surface of the
slice and positioned above the CA1 region using the microscope; this allowed accurate
determination of where the stylus would drop. The stylus was then raised to the full height
(5mm) and the relay switch pressed briefly to allow the stylus to drop and then retract
instantly. This process was monitored under the microscope to ensure all slices were hit in the
correct region. Once all slices in a well had been impacted the entire procedure was repeated
for each well until all slices (except shams) had been impacted. Previous experiments during
calibration of the device determined that the 0’ position did not change within each well
(ensuring all slices were impacted from the 5mm height) but did change between wells, thus
its position was determined for each individual well. The impacting procedure was carried
out with both accuracy and efficiency in mind; typical impacting experiments took less than
10 minutes to complete fully. Half of the wells in each 6 well plate was assigned as a sham
group, and half of the wells the TBI group. Assignment into sham or TBI groups was carried
out before the initial t0 imaging.

2.2.5 Hyperbaric chamber procedure and application of experimental gases following TBI

Immediately after impacting slices were carried to the custom made hyperbaric gas chamber
in the heat box and sealed inside. The chamber (figure 2.7) had a volume of 0.925 litres and
was capable of maintaining pressures of up to 6 atm for several days. With the inlet and outlet
valves both open the chamber was flushed with humidified gas (20.2 kPa O2/76.0 kPa
N2/5.0kPa CO2) for 5 minutes at 5 l min⁻¹ (determined by a flow meter); this ensured better
than 99.99% gas replacement. The gas was humidified by bubbling through a sealed beaker
of autoclaved H2O. Following flushing the outlet valve was sealed and 50.6 kPa experimental
gas (helium/neon/argon/krypton/xenon) was added slowly over >1minute before sealing the
inlet valve. The chamber had an integrated pressure gauge for accurate determination of
pressure within the chamber. Following addition of experimental gas the high speed chamber
fan was switched on for 5 minutes to allow for rapid mixing of gases. Some concentration
response experiments involving argon and xenon required 30.4 kPa or 70.9 kPa of
experimental gas added. No blinding took place to the treatment condition as analysis of the
injury levels in the slices was purely quantitative from this point on, that is, no part of the
analysis had any subjective element that could be affected by subconscious bias.
The chamber was housed inside an incubator set at 37°C. This temperature was maintained throughout all experiments. Previous experiments using the chamber (conducted by Paul Banks) found that the flushing and pressurisation procedures had negligible effects on temperature within the chamber. Opening of the chamber was found to cause a transient 1°C drop in temperature and pressurisation with 50.6 kPa experimental gas was found to cause a transient temperature increase of 0.5°C (when pressurisation was carried out >1 minute) which returned back to 37°C within 30 minutes. Temperature was maintained at 37°C inside the chamber throughout all experiments.

Figure 2.7: Custom made hyperbaric chamber with integrated pressure gauge and mixing fan.

After 24 hours of exposure to experimental gases, slices were removed from the chamber by slowly releasing the pressure (over >1 minute) and carried to the florescence microscope in an insulated polystyrene box. Slices were imaged as outlined in section 2.2.3 before being immediately returned to the chamber. Flushing, addition of experimental gas and mixing was repeated every time the slices were removed from the chamber. Slices were imaged again at 48 and 72 hours to monitor the development of injury. Injury was quantified and
subsequently analysed as outlined in section 2.4. A full schematic representation of the TBI protocol is shown in figure 2.8.

Figure 2.8: Schematic timescale of TBI protocol. After 14 days in culture slices are transferred to experimental medium containing PI and reincubated for 1 hour to allow PI binding to equilibrate. Slices are then imaged to assess general viability before another reincubation period of 1 hour to equilibrate temperature back to $37^\circ$. Slices are then impacted and sealed in the chamber for 24 hours before imaging and returned to the chamber. Subsequent imaging takes place at 48 and 72 hours.
2.3 Oxygen and glucose deprivation in vitro

Preparation of organotypic slices for the oxygen and glucose deprivation (OGD) protocol were prepared as outlined in section 2.1. Additionally, the preparation of slices for experimental procedures and the fluorescence imaging of slices before experimentation were carried out as described in sections 2.2.2 and 2.2.3.

Immediately after imaging slices were taken to a laminar flow hood and transferred to Ringers solution (120mM NaCl, 5mM KCl, 1.25mM NaH$_2$PO$_4$, 2mM MgSO$_4$, 2mM CaCl$_2$, 25mM NaHCO$_3$, 10mM sucrose, 20mM HEPES, pH 7.25). For sham experiments sucrose was replaced with 10mM D-glucose but all other ingredients remained the same. Ringers solution was deoxygenated for OGD experiments by bubbling for 45 minutes at 50 mL min$^{-1}$ with 96.3 kPa N$_2$/5.0 kPa CO$_2$. The sham rings was bubbled with (20.2 kPa O$_2$/76.0 kPa N$_2$/5.0kPa CO$_2$) for the same time and at the same flow rate. After bubbling, the solution was transferred to a sterile laminar flow hood where it was immediately drawn into a 50mL syringe using a 5” kwill filling tube (Smiths medical supplies, Kent, UK), the tube was then detached and filtered through a 0.2µm cartridge filter and injected into the bottom of a 15ml falcon tube using another 5” kwill filling tube. Kwill tubes were used to limit reoxygenation of the solution by exposure to air. Falcon tubes were filled to the brim and inverted to check for bubbles. Ringer’s solution was always bubbled and filtered on the day of use, usually around 60 minutes before required and pre-warmed to 37°C.

For the transfer, a 6 well plate that had been prepared previously with rings warmed to 37°C was removed from the incubator and one individual 15ml Falcon tube of deoxygenated Ringers solution was poured into a well. Using forceps the membrane was carefully removed from the plate containing experimental media and the underside of the membrane briefly washed in a petri dish containing Ringers solution to remove residual experimental media that might contain traces of D-glucose. The membrane was transferred into the well containing Ringers solution and topped up to remove any bubbles beneath. This process was repeated for the remaining 5 wells. The slices were immediately transferred to the chamber which was then flushed with 96.2 kPa N$_2$/5.0 kPa CO$_2$ for 5 minutes at 5 L min$^{-1}$ (or (20.2 kPa O$_2$/76.0 kPa N$_2$/5.0kPa CO$_2$) for sham experiments). After flushing the inlet and outlet valves were sealed and a timer started for 30 minutes. After this period of OGD the slices
were removed from the chamber and transferred back into the plate containing experimental media which had been stored in the incubator at $37^\circ$C. No extra wash steps took place. After transfer the slices were sealed back inside the chamber which was flushed for 5 minutes at 5 L min$^{-1}$ with (20.2 kPa $\text{O}_2$/76.0 kPa $\text{N}_2$/5.0kPa $\text{CO}_2$). After flushing 50.6 kPa of experimental gas was added to the chamber over $>$1 minute. The high speed mixing fan was switched on for 5 minutes to allow rapid mixing of the gases. After 24 hours the slices were removed from the chamber and imaged as outlined in section 2.2.3.

For OGD experiments it was important that all 6 wells were filled with media and a membrane, in order to avoid empty wells acting as a reservoir for oxygen during the procedure. A full schematic representation of the OGD protocol is shown in figure 2.9.

![Figure 2.9: Schematic timescale of OGD protocol. After 14 days in culture slices are transferred to experimental medium containing PI and reinsulated for 1 hour to allow PI binding to equilibrate. Slices are then imaged to assess general viability before transfer to Ringers solution. Slices are then sealed in the chamber for 30 minutes with 96.2 kPa $\text{N}_2$/5.0kPa $\text{CO}_2$ or 20.2 kPa $\text{O}_2$/76.0 kPa $\text{N}_2$/5.0kPa $\text{CO}_2$ (for sham experiments). Slices are then transferred back to experimental media and sealed in the chamber for 24 hours before imaging.](image-url)
2.4 Data analysis and statistical testing

During the imaging procedure images of the slices were captured as an RGB 24-bit TIF image using a digital video camera and software (Micropublisher 3.3 RTV camera and Qcapture Pro software, Burnaby, BC, Canada). Each image contained a total of 3145728 pixels distributed across 256 intensity levels (0-255). Using ImageJ software it was possible to split the images into the red, green and blue channels. Using only the red channel, histograms were plotted showing pixel distribution over 256 intensity levels. The background of the image contained a large number of pixels in the 0-25 range of intensity level, creating a large peak in the intensity histogram. PI fluorescence in the slices caused a shift in the number of pixels towards the higher intensity levels. By integrating the number of pixels above a certain threshold intensity level, injury in the slices could be quantified (see figure 2.10). For the TBI experiments a threshold of 50 was selected. The reason for this choice of threshold is explained in further detail in chapter 3.

Slices were assessed for viability before experimentation in order to exclude any slices from analysis whose health was already compromised. Reasons for exclusion from analysis included any slices where the distribution of pixels reached 20 or more pixels at intensities above a threshold of 80 (a reliable indicator of poor survival throughout the experimentation procedure) or where a qualitative assessment deemed them unhealthy (e.g. visible focal lesion present). Additionally slices that had other slices/cell debris visible in frame or where the impact was not in the CA1 region at t=24 were also excluded from analysis. Approximately 10-20% of slices were excluded from analysis in each experiment due to a combination of the above factors.

Sample sizes for each condition were from a minimum of 3 experiments per condition. It was kept in mind that larger sample sizes yield more accurate results but cost and time constraints meant there were limitations in the number of experiments which could be performed. Three repeats yielding the same result was considered suitable to demonstrate reproducibility of the result. Statistical analysis of the data was carried out using 2-way ANOVA with Bonferroni’s post-hoc test with significance defined as a P value of less than 0.05. Factor one was treatment (helium, xenon, glycine addition etc.) and factor 2 was the time after injury (24 hours, 48 hours and 72 hours). A repeated measures ANOVA was used with factor 1 as the repeated factor. Significance was defined as a P value of less than 0.05. All TBI data
presented is normalised to the helium control injury at 72 hours after injury, unless otherwise stated.

For measurements of the area of the slice and the area of the primary injury site ImageJ software was used. A graticule was used to apply an appropriate scale to each image. Images were split into the red channel and the colours inverted. The impact area or slice was manually drawn around using a drawing tool and the area filled using a paintbrush tool. The images were then converted to binary images and an analysis macro applied which allowed an outline to be drawn and the area calculated using the area tool.

For the OGD experiments a threshold of 100 was used as this allowed for a clear distinction between sham and OGD injury. Since OGD experiments were only carried out for 24 hours a one-way ANOVA with post-hoc Bonferroni’s test was used to test significance between groups, with significance defined as a P value of less than 0.05. All OGD data presented is normalised to helium (control) injury at 24 hours, unless otherwise stated.

Statistical tests were implemented using the SigmaPlot (Systat Software Inc., California) for two-way repeated measures ANOVA tests and the Origin (OriginLab Inc., Northampton, MA) software package for one-way ANOVA tests. All graphs were produced using the Origin software.
Figure 2.10: Example histogram traces of an impacted (red line) and sham (black line) slices. A scale of red intensity from 0-255 is shown underneath the traces. Threshold of 50 has been indicated and the shaded section represents the area under the curve that would be integrated to obtain quantification of injury in a particular slice. Images are actual images of the slices that the individual histogram traces were obtained from. Both sham and TBI slices were exposed to 50.6 kPa helium and injury quantified at 72 hours.
CHAPTER 3

Modelling traumatic brain injury *in vitro*; calibration of the custom built impact device

3.1 Impact energy

The device used to impact the slices was custom built in the laboratory and required calibration in order to achieve an injury in the slices that would meet certain criteria. These criteria were determined so that the *in vitro* injury modelled characteristics of *in vivo* TBI. The criteria were:

- The injury should not cause catastrophic cell death in the entire slice but instead have a distinct primary injury site observable after impact.
- The primary injury itself should be robust and reproducible.
- Injury should develop significantly over a period of time that mirrors secondary injury development in *in vivo* models and in clinical TBI.
- The injury produced must be distinct from levels of damage observed in the sham slices due to manipulation of the slices.

With these criteria in mind the calibration of the device primarily assessed the size of the primary injury and its subsequent development over 72 hours. Both of these factors were influenced by the energy that the slices were impacted with. As the mass of the stylus and the acceleration of the stylus under gravity remained constant, the variable factor in determining impact energy was the height the stylus was dropped from. From a technical perspective the impact energy chosen had to occur from a drop height whereby the stylus could be easily retracted back to the electromagnet when current was restored and did not rebound or stick to the slices upon impact. It was also taken into consideration that the higher the drop height the lower the error in measuring an accurate distance from the slice. However the maximum distance was in turn limited by the retractability of the stylus. Preliminary experiments using the impact device determined that the maximum drop height whereby it was possible for the
stylus to retract back towards the electromagnet was 6mm. It was noted that using a higher
drop height caused the stylus to stick to the slices and on manual retraction excessive damage
was caused to the slices by tearing. Since a manual retraction of the stylus was not a
condition that could be controlled stringently, that was deemed to be not ideal for use in
subsequent experiments. The stylus was designed in order for the maximum achievable drop
height (5mm) to produce an impact energy of ≈3.5µJ as this impact energy had previously
been shown to produce reproducible injury in similar in vitro models of TBI [50]. This was
achieved by constructing the stylus at a specific mass of 70mg. The three drop heights
investigated were 2mm, 4mm and 5mm which gave impact energies of 1.4µJ, 2.7µJ and
3.5µJ respectively. Had these energies not been ideal for subsequent experiments then the
mass of the stylus could have been modified in order to achieve higher or lower impact
energies.

As expected, there was a correlation between impact energy and damage inflicted in the
slices; such that the larger the impact energy the greater the progression of injury as shown in
figure 3.1. A primary impact site is visible in all three impacted groups, however secondary
injury is most prominent in the slice impacted at 3.5µJ. Secondary injury is visible
surrounding the primary injury site and at regions distinct to the primary site which is absent
in the other treatment groups. At each time point the level of damage in the 3.5µJ impacted
slices was significantly higher than in the sham treated slices (p<0.001) as shown in figure
3.2. Although at the lower energies of 2.7µJ and 1.4µJ the mean level of injury was greater
than the sham, this difference was not significant at any time point.

With regard to injury progression over time, the 3.5µJ injury developed significantly between
24 and 48 and 48 and 72 hours (p<0.001) reflecting conditions observed in vivo. However
this development did not occur in the 2.7µJ, 1.4µJ, or sham treated slices as shown in figure
3.2. Given these results it was decided that a 3.5µJ injury was ideal for the purposes of the
investigation as it met the specific criteria for the injury that was being modelled.
Figure 3.1: Representative slices from the sham and each of the impact energy groups at 72 hours post injury. The site of primary injury is approximated in each image by the location of the hatched circle. Secondary injury is interpreted as PI fluorescence outside of this region.
Figure 3.2: Development of injury with different traumatic impact energies. Uninjured sham slices are shown as white bars, compared with injured slices with impact energies of 1.4µJ (light grey bars), 2.7µJ (grey bars) and 3.5µJ (dark grey bars). Data have been normalised to 3.5µJ impact 72 hours after injury. The error bars are standard errors. * indicates value significantly different (P<0.001) from sham slices at each time point (N=33: sham; N=43: 3.5µJ; N=7: 1.4µJ and 2.7µJ).
3.2 Time course of injury development

With the ideal impact energy determined, further experiments were carried out in collaboration with Dr Rita Campos-Pires to investigate the development of the secondary injury at earlier time points post impact: 30 minutes, 1 hour, 6 hours and 12 hours (followed by standard imaging at 24, 48 and 72 hours) as shown in figure 3.3. Dr Campos-Pires was a fellow PhD student in the lab who carried out these experiments in the early stages of her PhD. It was my responsibility at this time to train and supervise her during these experiments. Data analysis was carried out by Dr Campos-Pires. In these experiments the number of imaging sessions for a given slice set was limited to 4 to avoid having slices out of the stable temperature and humidity of the chamber for extended periods. The earliest time point that injury could be observed in the slices was 30 minutes after impact, which is likely due to the time it takes for PI binding to equilibrate. The earliest time point where TBI was found to be significantly different from sham treated slices was at 6 hours, and at all subsequent time points afterwards (P=0.004). At all time points investigated the injury developed significantly from initial t-1 (before injury) imaging (P<0.001), however a significant difference between sham treated slices and t-1 slices was only apparent by 24 hours and this was true at all subsequent time points. By 1 hour after trauma, the injury had developed to 0.32±0.04 of total injury, and by 6 hours the injury development was 0.51±0.08 of the total injury, increasing at 12 hours to 0.67±0.10 of the total injury present at 72 hours. The lower panel of figure 3.3 shows the same TBI injury data as the upper panel but plotted on a linear time axis to allow a visualisation of the rate of progression of the injury (this interpretation of the data was carried out by myself). From this graph it is clear that the greatest rate of injury development occurs in the first 12 hours following injury, after which it develops steadily but at a less rapid rate, as shown by a decrease in the slope.

![Figure 3.3a: Propidium iodide fluorescent images of slices at 1h before injury, and 30 minutes, 24h, 48h and 72h after injury.](image)
Figure 3.3b: Development of injury (grey bars) compared to sham (white bars) quantified by propidium iodide florescence intensity after injury with impact energy 3.5µJ. The error bars are standard errors. Data has been normalised to TBI injury at 72 h. *Indicates value significantly different (P<0.01) from injured slices at each time point, ** Indicates value significantly different (P<0.001) at each time point. (N=48: traumatic brain injury (TBI), t=-1, 24, 48 and 72h; N=33: sham, t=-1, 24, 48 and 72h; N=5: TBI 0.5h; N=4: sham 0.5h; N=11: TBI 1h; N=13: sham 1h; N=19: TBI 6h; N=8: sham 6h; N=13: TBI 12h; N=8: sham 12h. (lower panel) Development of injury in TBI slices plotted over time to indicate the rate of injury development in the slices. Data is the same as in upper panel. Data is courtesy of Dr Rita Campos Pires who kindly gave her permission for the data to be included.
In parallel to the above experiments the histograms of slices before and immediately after injury were studied in order to determine the ideal threshold for pixel integration that accurately represented injury levels in the slices. Figure 3.4 shows that before injury almost all the pixels in each slice image are distributed within the intensity level range of approximately 0-40 (left panel). This sharp peak therefore represents the background of the image and does not represent PI fluorescence. 30 minutes after the impact when the PI fluorescence at the primary injury site is visible by eye, the histogram reflects this with a subtle yet clear shift in the number of pixels between 50 and 100 which appears to be the range in which PI fluorescence is detectable. It was therefore concluded that a threshold of 50 accurately captured all the PI fluorescence in the slice at a single time point and no information was lost which may have been the case if a higher threshold was chosen. Furthermore the right panel of figure 3.4 demonstrates that this threshold allows a clear distinction between impacted and sham slices.

Figure 3.4: (left panel) Histogram showing pixel distribution of slices 1 hour before (black line) and 30 minutes after traumatic impact (red line). (right panel) Histogram showing pixel distribution 30 minutes after traumatic impact (red line) compared with non-injured sham slices (black line) at the same time point. In both panels the blue line indicates the threshold above which the numbers of pixels were totalled to provide a quantitative measure of injury levels. Error bars are standard errors. Lines represent mean data from 28 slices before impact and at 30 minutes post impact and 22 slices for sham.
A further element of the calibration process was to characterise the size of the primary impact and subsequent development of injury with regard to the maximum injury possible to inflict in the hippocampal slices. Maximum cell death was achieved in the slices by submersion in 70% ethanol for several hours and incubation overnight. At 72 hours after traumatic injury the level of traumatic injury was 25±1% of the maximum possible injury, indicating that the degree of injury inflicted upon the slices was not ‘catastrophic’. Results are shown in figure 3.5. Primary injury compromised 3.5±0.2% of the total possible injury. Measurement of the area of the primary injury in slice images in relation to the total area of the slice showed the primary injury to be 0.34±0.01mm$^2$ compared to the average total area of a hippocampal slice of 4.80±0.08mm$^2$ as measured in 50 slices. This indicates that the area of primary injury occupies approximately 7% of the total slice area.

Figure 3.5: Injury development in the slices (grey bars) compared to the maximum achievable fluorescence when slices are permeabilised with ethanol (red bar). Primary injury indicates slices imaged at 30 minutes post impact. All TBI data is from control (50.6 kPa helium) data. Data is shown as a percentage of maximum fluorescence. The error bars are standard errors. (N=28: TBI primary injury; N=141: TBI 24, 48, 72h; N=6: maximum death).
3.3 Sentinel slice experiments

The final process in calibrating the traumatic brain injury protocol was to investigate whether repeated impacts on a single membrane could exacerbate the damage already received from the primary injury. Each membrane contained on average 6 slices, so it was unknown whether the primary impact may cause vibrations within the membrane that could be damaging. These ‘aftershocks’ could potentially be problematic for experimental design, as repeated injuries would not reflect the *in vivo* state of TBI and would also alter the level of damage inflicted on the slices depending on how many slices were plated in each membrane. An experiment was designed whereby on each membrane three slices were impacted, and the remaining three slices on the membrane were left untouched; these were termed the ‘sentinel’ slices. In parallel normal sham treated wells were included in the same experiment so comparisons could be made between sham treated and sentinel slices. Figures 3.6 and 3.7 show that no significant difference was found between the sham treated and sentinel slices at any time point. A significant difference was found between impacted slices and both sham and sentinel slices at each time point (P<0.001). It was therefore concluded that repeated impacts on the membrane had no additional effect on slice injury.

![Sham, Sentinel, TBI slices](image)

*Figure 3.6: Representative slices from the sham, sentinel and TBI (3.5µJ) groups at 72 hours post injury. Sham and sentinel slices show a distinct lack of injury compared with traumatically injured slices.*
Figure 3.7: Development of injury in sentinel slices (hatched bars) compared to sham (white bars) and TBI injured slices (grey bars). Data have been normalised to 3.5µJ TBI injury at 72h. The error bars are standard errors. **Indicates value significantly different (P<0.001) from sham slices at each time point. N=33: sham; N=15: sentinel; N=43 TBI.
3.4 Effects of added pressure

To avoid flushing the chamber with 50% gas/air mixtures (and the subsequent high costs of this) gases were added as 50.6 kPa of added pressure, in addition to (20.2 kPa O\textsubscript{2}/76.0 kPa N\textsubscript{2}/5.0kPa CO\textsubscript{2}). It first was established that 50.6 kPa of additional pressure would have no significant effect on injury development by using helium as a control gas. Helium was chosen because it is unlikely to exert any pharmacologic effect of its own at these low pressures and any effect observed can be attributed to pressure alone. Figure 3.8 demonstrates that 50.6 kPa of helium had no significant effect (P>0.9) on sham or traumatic injury at any time point. All subsequent control experiments were carried out in the presence of 50.6 kPa helium gas.

![Figure 3.8](image_url)

*Figure 3.8: The addition of 50.6 kPa helium had no effect on the injured or sham-treated slices. Sham treated slices are shown as white hatched bars (no helium) or white bars (helium), and injured slices are shown as grey hatched bars (no helium) or grey bars (helium). The error bars are standard errors. The data have been normalised to traumatic injury with 50.6 kPa helium at 72h after injury. **Indicates value significantly different (P<0.001) from injured slices at each time point (N=141: helium TBI; N=105: helium sham; N=23: no helium TBI; N=25: no helium sham.*
For concentration response studies with the inert gases two additional concentrations were investigated: 30.4 kPa and 70.9 kPa. It was deemed unnecessary to investigate concentrations above 70.9 kPa as a concentration higher than that would be unlikely to be used in clinical medicine as it does not allow for sufficient oxygen. It was first investigated whether these pressures would affect control levels of injury, as shown in figure 3.9. It was found that neither concentration, 30.4 kPa nor 70.9 kPa had any significant effect on injury development at any time point (P=0.413).

Figure 3.9: Pressures of 30.4 kPa (light grey bars) and 70.9 kPa helium (dark grey bars) have no effect on the development of injury compared to the control pressure of 50.6 kPa (grey bars) at any time point. The error bars shown are standard errors. The data have been normalised to traumatic injury with 50.6 kPa helium at 72h after injury (N=14: 50.6 kPa helium; N=15: 30.4 kPa helium; N=11: 70.9 kPa helium).
CHAPTER 4

Effect of noble gases on injury development following traumatic brain injury \textit{in vitro}

4.1 Effects of noble gases on traumatic injury

Following calibration of the traumatic impact device and characterisation of the injury, experiments investigating the neuroprotective effects of the noble gases xenon, argon, krypton and neon were carried out. Representative images of slices exposed to 50.6 kPa xenon, argon, krypton and neon are shown in figure 4.1. Figure 4.2 shows normalised data of the neuroprotective effect of the inert gases. Only xenon and argon showed a significant degree of neuroprotection compared to control. Xenon was found to be a more potent neuroprotectant than argon. Xenon reduced injury by $0.57\pm0.03$ at 24 hours, $0.56\pm0.03$ at 48 hours and $0.43\pm0.03$ by 72 hours compared to the control injury slices. Argon reduced injury by $0.38\pm0.07$ at 24 hours, $0.43\pm0.07$ at 48 hours and $0.30\pm0.07$ by 72 hours compared to the control injury slices. Krypton and neon (like helium) were devoid of any neuroprotective effect.
Figure 4.1: Representative images from each treatment group; sham, control injury (50.6 kPa helium), 50.6 kPa xenon, 50.6 kPa argon, 50.6 kPa krypton and 50.6 kPa neon. Each slice is from the 72h time point. Slices were selected on the basis of having a level of injury that is most similar to the mean for the group.
Figure 4.2: Effect of 50.6 kPa of the inert gases xenon (red bars), argon (blue bars), krypton (purple bars) and neon (green bars) on control traumatic injury (grey bars) at 24, 48 and 72h after injury. Sham treated slices (white bars) were not subject to trauma. Xenon and argon provided significant neuroprotection at all time points. Xenon was more effective than argon, with xenon treated slices being 0.57±0.03 of control injury compared with 0.70±0.07 of control injury for argon treated slices, 72h after injury. None of the other inert gases provided significant protection against injury at any of the time points. The error bars are standard errors. The data have been normalised to control injury at 72h after injury. # Indicates value significantly different (P<0.05) from control injury at each time point, **Indicates value significantly different (P<0.001) from control injury at each time point. (N=141: control traumatic injury; N=105: sham; N=104: xenon; N=44: argon; N=45: krypton; N=22: neon).
4.2 Effects of xenon and argon on secondary injury

Having shown that xenon and argon are neuroprotective, the next stage was to characterise this property further. The data shown in figure 4.2 represents total injury, that is, both primary and secondary injury are included. By definition it is not possible to protect against primary injury, only the secondary injury that begins in the moments directly after the primary injury has been sustained. With this in mind the neuroprotective potencies of xenon and argon were re-addressed by investigating the specific effects on secondary injury. To do this, injury was measured at 30 minutes post impact to determine the magnitude of the primary injury. This was then subtracted from the total injury at 24, 48 and 72h leaving only the secondary injury. Figure 4.3 shows the primary injury at 0.5hr after impact and shows schematically how it was subtracted from the total injury to give the secondary injury.

![Graph showing secondary injury](image)

**Figure 4.3:** Primary injury measured at 30 minutes post impact was subtracted from total injury (indicated by the red line) in impacted (not sham treated) slices to give a measure of secondary injury shown in light grey. The error bars are standard errors. The data have been normalised to control injury at 72h after injury.
When secondary injury was isolated in this way it was found that xenon was particularly effective at reducing secondary injury development. This is shown in figure 4.4. With the primary component removed xenon treated injured slices were found to have the same degree of injury as uninjured sham slices, as demonstrated by a lack of significance difference between the two data sets. At 24 hours sham injury was 0.30±0.03 of the secondary injury in the controls at the same time point, compared with xenon which was 0.28±0.04. At 48 hours sham injury was 0.34±0.03 of control and xenon was 0.33±0.03. By 72 hours sham injury was 0.35±0.03 of the control secondary injury at 72hrs whereas xenon was higher at 0.50±0.05 however this difference was not found to be significant. At all time points xenon demonstrated a significant degree of protection compared to control injury slices (P<0.001), reducing injury by 0.72±0.04, 0.67±0.03 and 0.50±0.04 compared to control at 24, 48 and 72 hours respectively. Xenon therefore effectively abolished the development of secondary injury in the slices over this time period.

Figure 4.4: Effect of xenon on secondary injury development. At the 24, 48 and 72h time points, xenon treated slices (red bars) were not significantly different to uninjured sham slices (white bars). At the 72h time point, secondary injury in the xenon treated slices was 0.50±0.05 of secondary injury in untreated injured slices (grey bar). The error bars are standard errors. Data have been normalised to the control injury at 72h after injury. *Indicates value significantly different (P<0.001) from control injury at each time point (N=141: control traumatic injury; N=105 sham; N=104: xenon.)
Argon was also found to reduce secondary injury (figure 4.5) although not to the same extent as xenon. Argon reduced secondary injury significantly when compared to secondary injury controls by 0.48±0.09 at 24h, by 0.51±0.08 at 48h and by 0.34±0.08 at 72 hours (P<0.05). No significant difference was found between argon secondary injury and sham treated slices at 24 and 48 hours after injury, however by 72 hours this difference had become significant (P=0.026). Argon could therefore be said to be attenuating secondary injury development, unlike xenon which abolished it.

**Figure 4.5: Effect of argon on secondary injury development.** At the 24 and 48h time points, argon treated slices (blue bars) were not significantly different to uninjured sham slices (white bars). At the 72h time point, secondary injury in the argon treated slices was 0.66±0.08 of secondary injury in untreated injured slices (grey bar). The error bars are standard errors. Data have been normalised to the control injury at 72h after injury. # Indicates value significantly different (P<0.05) from control injury at each time point, ** Indicates value significantly different (P<0.001) from control injury at each time point. (N=141: control traumatic injury; N=105 sham; N=44: argon.)
4.3 Concentration response relationships of xenon and argon

The experiments with the series of noble gases used concentrations of 50% (50.6 kPa) of each gas. This concentration was chosen because it if often necessary to give additional oxygen to the brain trauma patients, and a concentration of 50% would allow for this. Nevertheless, we wished to determine whether higher concentrations of xenon and argon would be more effective and in addition, whether their efficacy would be retained at concentrations below 50.6 kPa. The concentrations investigated were 30.4 kPa and 70.9 kPa. It was previously established that these pressures had no significant effect on control injury development (figure 3.9, previous chapter). At a concentration of 30.4 kPa xenon provided a significant level of neuroprotection (P=0.026) at 72 hours compared to argon which was without protective effect (figure 4.6). Injury in 30.4 kPa xenon treated slices was 0.54±0.10, 0.62±0.10 and 0.60±0.13 of the control injury at 24, 48 and 72 hours respectively and injury in the 30.4 kPa argon treated slices was 0.78±0.06, 0.80±0.06 and 0.94±0.07 of the control injury at 24, 48 and 72 hours respectively.

![Figure 4.6](image)

Figure 4.6: The neuroprotective effect of 30.4 kPa xenon (red bars) and lack of neuroprotection by 30.4 kPa argon (blue bars) on injury progression following traumatic injury compared to control injury slices (grey bars) at the same pressure. The error bars are standard errors. Data are normalised to 30.4 kPa helium injury at 72h. # Indicates value significantly different (P<0.05) from control injury at each time point. (N=6: 30.4 kPa xenon TBI; N=14: 30.4 kPa argon TBI; N=15: 30.4 kPa helium TBI).
In contrast, at a 70.9 kPa concentration of both xenon and argon provided neuroprotection which was significant at the 72 hour time point (P=0.023 xenon and P=0.035 argon). Injury in 70.9 kPa xenon treated slices was 0.62±0.11, 0.59±0.13 and 0.48±0.12 of the control injury at 24, 48 and 72 hours respectively and injury in the 70.9 kPa argon treated slices was 0.55±0.12, 0.54±0.11 and 0.60±0.10 of the control injury at 24, 48 and 72 hours respectively.

![Figure 4.7](image)

*Figure 4.7: The neuroprotective effect of 70.9 kPa xenon (red bars) and 70.9 kPa argon (blue bars) on injury progression following traumatic injury compared to control injury slices (grey bars) at the same pressure. The error bars are standard errors. Data are normalised to 70.9 kPa helium injury at 72h. # Indicates value significantly different (P<0.05) from control injury at each time point. (N=6: 70.9 kPa xenon TBI; N=10: 70.9 kPa argon TBI; N=11: 70.9 kPa helium TBI).*

Figure 4.8 summarises the data shown in figures 4.6 and 4.7 with the addition of the 50.6kPa concentrations at the 72 hour time point. A concentration of 30.4 kPa, xenon provided protection of 0.40±0.13 whereas argon was without protective effect (0.06±0.07) at the same concentration. At 50.6 kPa xenon provided 0.43±0.03 protection whereas argon provided 0.30±0.07, and at 70.9 kPa xenon provided protection of 0.52±0.12, whereas argon protected by 0.40±0.10. These results show that a concentration-response relationship exists with regard to neuroprotection by argon whereas xenon appears to be almost equally as potent across a range of concentrations. Xenon is more potent than argon across a range of clinically relevant concentrations.
Figure 4.8: Concentration-response relationship for neuroprotection by xenon and argon. Xenon (filled circles) exhibited a neuroprotective effect at a concentration of 30.4 kPa, with 40±8% protection, increasing to 52±13% protection at 70.9 kPa xenon. In contrast argon, open circles was not neuroprotective at 30.4 kPa but did exhibit a protective effect at concentrations of 50.6 kPa and 70.9 kPa argon. Lines shown were drawn by eye and have no theoretical significance. Error bars are standard errors (N=104: 50.6 kPa xenon; N=6: 30.4 kPa and 70.9 kPa xenon; N=44: 50.6 kPa argon; N=14: 30.4 kPa argon; N=10: 70.9 kPa argon).
CHAPTER 5

The mechanism of xenon and argon neuroprotection following traumatic brain injury in vitro

Previous findings in our lab have shown that inhibition of NMDA receptors by xenon is competitive. Xenon has been found to inhibit NMDA receptors more strongly at low glycine concentrations (1µM) than at high glycine concentrations (100µM) [76]. This finding was used as a tool to investigate whether it was possible to modulate the neuroprotective effect of xenon in the same manner by manipulating the glycine concentration in the experimental media. Additionally it was investigated whether increased glycine could modulate the degree of neuroprotection by argon, as its molecular targets within the brain are unknown.

5.1 Effect of added glycine and strychnine on control injury

The first stage of investigation was to investigate whether adding a saturating concentration of glycine (100µM) in the experimental media altered the degree of injury observed in control impacted or sham treated slices. This concentration was chosen because it is a saturating concentration for the GluN1/GluN2A and GluN1/GluN2B subunit concentrations that predominate in the hippocampus [76, 77]. Figure 5.1 shows that 100µM glycine had no effect on sham or control injury at any time point as shown by a lack of significance (P>0.9) between slices exposed to glycine and control slices. Injury in the glycine treated slices was 0.93±0.11, 0.85±0.11 and 0.98±0.09 of control injury at 24, 48 and 72h respectively.
Figure 5.1: The addition of 100µM glycine has no effect on injury development or sham treated slices. Sham slices (white bars) were not significantly different in the presence of 100µM glycine (white hatched bars) at any time point. Similarly, the development of control injury (grey bars) was not significantly different in the presence of 100µM glycine (grey hatched bars) at any time point. Data are normalised to control injury at 72h. The error bars are standard errors (N=141: control traumatic injury; N=105: sham; N=39: glycine traumatic injury; N=35: glycine sham).
To rule out any involvement of the ligand gated inhibitory glycine receptors in neuroprotective mechanistic studies, experiments with additional glycine were also performed in the presence of strychnine, a high affinity competitive antagonist of the inhibitory glycine receptor. First it was investigated whether strychnine had any effect on injury development or on sham treated slices by itself at a concentration of 100nM. This concentration was chosen as it has previously been demonstrated that this abolishes inhibitory glycine receptor responses at glycine concentrations up to 300µM [116] Figure 5.2 demonstrates that no significant difference (P>0.9) was found between sham or injured slices in the presence or absence of 100nM strychnine at any time point. Injury in strychnine exposed slices was 1.04±0.08, 0.95±0.08 and 1.12±0.12 of control injury at 24, 48 and 72h respectively.

**Figure 5.2:** The addition of 100nM strychnine has no effect on injury development or sham treated slices. Sham slices (white bars) were not significantly different in the presence of 100nM strychnine (white crosshatched bars) at any time point. Similarly, the development of control injury (grey bars) was not significantly different in the presence of 100nM strychnine (grey crosshatched bars) at any time point. Data are normalised to control injury at 72h. The error bars are standard errors (N=141: control traumatic injury; N=105: sham; N=18: strychnine traumatic injury; N=19: strychnine sham).
Next the combined effects of both 100µM glycine and 100nM strychnine were investigated (figure 5.3) and this too had no significant effect on either sham injury or control injury at any time point (P>0.9). Injury in glycine and strychnine exposed slices was 0.95±0.08, 0.98±0.09 and 0.93±0.08 of control injury at 24, 48 and 72h respectively.

Figure 5.3: The addition of 100µM glycine and 100nM strychnine in combination has no effect on injury development or sham treated slices. Sham slices (white bars) were not significantly different in the presence of 100µM glycine and 100nM strychnine (white squared bars) at any time point. Similarly, the development of control injury (grey bars) was not significantly different in the presence of 100µM glycine and 100nM strychnine (grey squared bars) at any time point. Data are normalised to control injury at 72h. The error bars are standard errors (N=141: control traumatic injury; N=105: sham; N=37: glycine and strychnine traumatic injury; N=21: glycine and strychnine sham).
5.2 Effect of added glycine and strychnine on xenon neuroprotection

Having established that neither glycine nor strychnine had any effect on control injury development on their own or in combination, this allowed the next stage of mechanistic investigations using xenon and argon to be carried out. Figure 5.4 shows the effects of 100μM added glycine on xenon’s neuroprotective effect. In the absence of added glycine, xenon significantly (P<0.001) reduces injury to 0.43±0.03, 0.44±0.03 and 0.57±0.03 of control injury at 24, 48 and 72h after injury respectively. The addition of glycine abolished xenon’s neuroprotective effect. In the presence of 100μM glycine the xenon treated slices were not significantly different to the untreated injury controls at all time points, being 0.82±0.09, 1.03±0.11 and 1.13±0.14 of the control at 24, 48 and 72h.

Figure 5.4: Glycine reverses the neuroprotective effect of 50.6 kPa xenon. In the absence of glycine, 50.6 kPa xenon (red bars) protects against trauma (grey bars). The addition of 100μM glycine abolishes the protective effect of 50.6 kPa xenon. There was no significant difference between the injured slices in the absence (grey hatched bars) and presence (red hatched bars) of 100μM glycine at any time point. Data are normalised to control injury in the absence of glycine at 72h. The error bars are standard errors. *Indicates value significantly different (P<0.001) from control injury at each time point. (N=141: control traumatic injury; N=104: xenon; N=32: xenon glycine; N=39: glycine traumatic injury).
In order to carry out further glycine experiments in the presence of strychnine first it was investigated whether strychnine alone at a concentration of 100nM altered the level of neuroprotection provided by xenon. Figure 5.5 demonstrates that strychnine present in the media throughout experimentation did not significantly alter the level of neuroprotection at any time point. Slices treated with xenon alone significantly (P<0.001) reduced injury to 0.43±0.03, 0.44±0.03 and 0.57±0.03 of control injury at 24, 48 and 72h after injury respectively. When strychnine was present in the media, xenon treatment reduced injury to 0.47±0.09, 0.48±0.11 and 0.43±0.16 of the untreated control injury at 24, 48 and 72 h after injury respectively.

Figure 5.5: The addition of 100nM strychnine has no effect on injury development or on the level of neuroprotection observed in xenon treated slices. Neuroprotection by 50.6 kPa xenon (red bars) was not significantly different in the presence of 100nM strychnine (red crosshatched bars) at any time point. Similarly, the development of control injury (grey bars) was not significantly different in the presence of 100nM strychnine (grey crosshatched bars) at any time point. Data are normalised to control injury in the absence of strychnine at 72h. The error bars are standard errors (N=141: control traumatic injury; N=104: xenon; N=45: xenon strychnine; N=18: control strychnine traumatic injury).
Having established that the presence of 100nM strychnine did not alter the level of neuroprotection by xenon the next stage was to expose injured slices to both 100µM glycine and 100nM strychnine in combination, in order to fully exclude any possible involvement of inhibitory glycine receptors in xenon’s mechanism of neuroprotection. Figure 5.6 shows the effects of 100µM added glycine and 100nM added strychnine on the neuroprotective effect of xenon. The addition of glycine and strychnine in combination abolished xenon’s neuroprotective effect. In the presence of 100µM glycine and 100nM strychnine the xenon treated slices were not significantly different to the injury controls (with glycine and strychnine) at all time points, being 1.05±0.11, 1.07±0.14 and 1.18±0.11 of the control at 24, 48 and 72h. A significant difference was found between slices treated with xenon alone and slices treated with xenon and glycine at all time points (P<0.05).

A key feature of these experiments is that at each time point, when glycine was present it reversed the effects of xenon to the level of the untreated slices at the same time point, as demonstrated visually in figure 5.7. Taken together with the lack of effect of glycine on the control injury, this means that the effects of glycine cannot be explained by simply exacerbating the injury itself. In addition the lack of effect of strychnine (figures 5.5 and 5.6) in both the absence and presence of glycine indicates that inhibitory glycine receptors have no role in both injury development or in neuroprotection by xenon. The reversal of xenon’s protective effect by glycine is consistent with xenon neuroprotection at the NMDA receptor glycine site.
Figure 5.6: Glycine and strychnine in combination reverses the neuroprotective effect of 50.6 kPa xenon. In the absence of glycine and strychnine, 50.6 kPa xenon (red bars) protects against trauma (grey bars). The addition of 100µM glycine and 100nM strychnine abolishes the protective effect of 50.6 kPa xenon. There was no significant difference between the injured slices in the absence (grey squared bars) and presence (red squared bars) of 100µM glycine and 100nM strychnine in combination at any time point. Data are normalised to control injury in the absence of glycine at 72h. The error bars are standard errors. **Indicates value significantly different (P<0.001) from control injury at each time point. (N=141: control traumatic injury; N=104: xenon; N=19: xenon glycine strychnine; N=37: glycine strychnine traumatic injury).
Figure 5.7: Addition of 100µM glycine reverses neuroprotection by xenon to the level of control injury slices. Glycine itself has no effect on injury progression. Primary injury is visible in all slices.
5.3 Mechanism of argon neuroprotection

To investigate whether xenon and argon act via the same mechanism, investigations into the effect of 100µM added glycine were conducted on argon treated slices. Figure 5.8 shows that in the presence of 100µM glycine, 50.6 kPa argon treatment resulted in significant neuroprotection at all time points (P<0.05). With no glycine present, argon treatment significantly reduced injury in the slices to 0.62±0.07, 0.57±0.07 and 0.70±0.07 of control injury slices at 24, 48 and 72 h respectively. When glycine was present argon treatment reduced injury in the slices to 0.67±0.09, 0.68±0.09, 0.58±0.07 of control injury at 24, 48 and 72h respectively. No significant difference was found between slices treated with argon in the absence or presence of 100µM glycine at all time points. Figure 5.9 demonstrates visually the lack of reversal of neuroprotection by argon in the presence of glycine. This is not consistent with argons neuroprotective effect being mediated via the NMDA receptor glycine site and indicates that argon has a different mechanism of action to xenon.

![Figure 5.8: Glycine does not reverse the neuroprotective effect of 50.6 kPa argon. In the absence of glycine, 50.6 kPa argon (blue bars) protects against trauma (grey bars). The level of injury in argon treated slices is unchanged regardless of the presence (hatched blue bars) or absence (blue bars) of 100µM glycine. Data are normalised to control injury at 72h. #Indicates value significantly different (P<0.05) from control injury at each time point. **Indicates value significantly different (P<0.001) from control injury at each time point. The error bars are standard errors (N=141: control traumatic injury; N=44: argon TBI; N=37: argon glycine; N=39: control glycine traumatic injury).](image-url)
Figure 5.9: Addition of 100µM glycine has no effect on injury development in both control injury slices and slices treated with 50.6 kPa argon. Neuroprotection is shown in the slices by a lack of secondary injury development compared to control treated slices. Primary injury is visible in all slices. Each experiment contained ‘internal control’ wells whereby no glycine was present as an additional control measure.
Unlike xenon, where a few molecular targets have been identified that could mediate its neuroprotective effect, in the case of argon thus far no molecular targets have been clearly identified. In order to clarify this situation electrophysiological experiments were performed by Scott Armstrong alone (and reproduced here with his kind permission) on HEK-293 cells transfected with GluN1/GluN2A NMDA receptors and TREK-1 potassium channels. Figure 5.10 shows electrophysiological recordings in the absence and presence of argon. 80% argon had no effect on NMDA-receptor mediated currents at both high (100µM) and low (1µM) concentrations of glycine. In contrast to xenon, these results are not consistent with argons neuroprotective effect being mediated via inhibition of the NMDA receptor glycine site, indicating a different mechanism of action to xenon.

Figure 5.10: 80% argon has no effect on NMDA-activated currents at high glycine (100µM) and low glycine (1µM). The traces show typical currents activated by 100µM NMDA in HEK cells expressing NMDA receptors containing the GluN1/GluN2A subunit combination. Data courtesy of Scott Armstrong.
In addition, it was shown that the other noble gases; krypton, neon and helium, also had no effect on NMDA receptor currents (figure 5.11) at high (100µM) and low (1µM) concentrations of glycine. This shows NMDA receptor inhibition to be a unique property of xenon among the noble gases and, besides from argon, may explain a lack of neuroprotective effect seen after treatment with these gases.

Figure 5.11: NMDA receptor currents were unaffected by 80% argon (light blue bars), 80% krypton (brown bars), 80% neon (green bars) or 80% helium (black bars). The error bars are standard errors. (100µM glycine; N=10: argon; N=9: krypton; N=5: neon; N=9: helium. 1µM glycine; N=12: argon; N=9: krypton; N=6: neon; N=6: helium). Data courtesy of Scott Armstrong.
The data collected regarding the mechanism of argon neuroprotection in studies using the organotypic slices and electrophysiology experiments by Scott Armstrong strongly suggests that the NMDA receptor is not a molecular target of argon. Therefore an alternate mechanism was investigated. The target of investigation was the two-pore domain K+ channel TREK-1. This channel acts normally to modulate neuronal excitability by providing a background leak potassium conductance. Activation of channels of this type can cause hyperpolarisation of the cells and therefore reduce excitability, and possibly lead to neuroprotection [74]. To investigate how the noble gases alter activation of TREK-1 channels electrophysiological experiments were performed by Scott Armstrong and reproduced here with his kind permission.

Cells that were not transfected with TREK-1 channels passed only a few picoamperes or less of current when clamped at -50mV (N=3, data not shown). Cells that expressed TREK-1 channels had an outwardly rectifying current that reversed at -80mV. Halothane was used as a positive control in these experiments (figure 5.12) to activate TREK-1 currents. 0.82mM halothane was found to potentiate TREK-1 currents by 152±20%. Figure 5.12 shows that 80% xenon also activates TREK-1 currents by 39±5% of the current measured at -50mV. In contrast to this effect by xenon, none of the other noble gases (helium, argon, krypton and neon) were found to potentiate TREK-1 currents at a concentration of 80% (figure 5.13). Figure 5.13 summarises the results of the TREK-1 experiments using all noble gases. These results suggest that TREK-1 channels are not a molecular target of argon and therefore this is not a mechanism of neuroprotection. In contrast the potentiation TREK-1 channel currents by xenon may or may not contribute to the neuroprotective properties of this gas.

*For details on the methods used for electrophysiology experiments see Appendix section A*
Figure 5.12: Upper panel: Halothane (0.82mM) activates TREK-1 potassium channel currents. Traces are recordings from voltage ramps (-120 to 0mV in 250ms) performed in the presence (hatched line) and absence (solid line) of halothane. Activation by halothane was used as a positive control for the noble gases. Halothane potentiates the current measured at -50mV by 152±20% (n=10). Lower panel: 80% xenon activates TREK-1 potassium channel currents. Traces are recordings from voltage ramps (-120 to 0mV in 250ms) performed in the presence (hatched line) and absence (solid line) of xenon. Data were sampled at 20 kHz, with each trace containing 3,000 data points. Lines are through these individual points. Data courtesy of Scott Armstrong.
Figure 5.13: 80% helium, 80% argon, 80% krypton and 80% neon have no effect on TREK-1 potassium channel currents. Traces are recordings from voltage ramps (-120 to 0mV in 250ms) performed in the presence (hatched line) and absence (solid line) of noble gases. Data were sampled at 20 kHz, with each trace containing 3,000 data points. Lines are through these individual points. Lower panel: 80% xenon potentiates TREK-1 potassium channel currents, whereas 80% helium, neon, argon, and krypton do not. Currents were measured at -50mV. Bars are mean values, error bars are standard errors (n=3, helium; n=4, neon; n=3, argon; n=9, krypton; n=5, xenon). Data courtesy of Scott Armstrong.
CHAPTER 6

Neuroprotection by xenon and argon in an *in vitro* model of ischemic brain injury

6.1 Establishing the OGD protocol

There is a lack of previously published data with regard to neuroprotection by noble gases following TBI [50] as the main focus of interest in the past decade has been on neuroprotection following ischemic brain injury (particularly focusing on xenon *in vitro* [47, 68] and *in vivo* [58, 65-67, 87, 88]). This final results chapter investigates neuroprotection by xenon and argon in an ischemic *in vitro* model of brain injury, in order to determine if the mechanism of neuroprotection is the same as for TBI. Data presented in this chapter was collected with the assistance of Mariia Koziakova who was a Masters student under my supervision. Approximately half of the data in this chapter was collected by me alone, and the other half was collected with the assistance of Ms Koziakova, i.e. experiments were either performed by me whilst she observed, or performed by Ms Koziakova whilst I supervised.

First the OGD protocol was established. Slices were exposed to deoxygenated Ringers solution and sealed inside the chamber with 96.3 kPa N₂/5.0 kPa CO₂ for 30 minutes to produce a level of injury at 24 hours as seen in figures 6.1 and 6.2. Sham treated slices were exposed to normal Ringers solution and sealed inside the chamber with 20.2 kPa O₂/76.0 kPa N₂/5.0kPa CO₂ for 30 minutes. This procedure resulted in a small amount of injury (figure 6.1 and 6.2) that was 0.27±0.07 of the OGD injury. A significant difference was seen between OGD slices and sham treated slices (P=3E-6). Both OGD slices and sham treated slices were exposed to 50.6 kPa helium gas in the 24 hour incubation period following injury, to control for hyperbaric conditions in the experiments with argon and xenon.

Generally fewer initial experiments were required for the OGD protocol than the TBI protocol as it had already been established in the lab prior to these experiments by Paul Banks (PhD thesis 2010, Imperial College London). He established a number of conditions:
- 30 minutes of OGD is sufficient to produce a significant level of injury in the slices

- Injury after 30 minutes of OGD does not develop further after 24 hours following injury

- Addition of 50.6 kPa helium does not significantly alter the level of injury in either sham treated or OGD slices compared to slices exposed to no added pressure.

A threshold of 100 was selected for analysis of the data. This was selected by studying the histogram (figure 6.2) of sham and OGD treated slices. The histogram shows very few pixels distributed above an intensity level of 50 in healthy, uninjured slices. Following OGD and sham procedures, a greater number of pixels are distributed towards higher intensity levels. The point at which sham treated and OGD treated slices could be distinguished as different from one another occurs at an intensity level of approximately 75. The threshold was chosen to be 100, as this is above the 75 threshold for distinguishing condition and was the point of the histograms where the line became smooth with less sharp peaks. Furthermore, this threshold is consistent with the OGD study by Paul Banks [68].

![Figure 6.1](image)

*Figure 6.1: 30 minutes of oxygen and glucose deprivation in the slices causes a significant level of damage compared to sham treated slices. Sham damage (white bar) is 0.27±0.07 of control injury (grey bar). Error bars are standard errors. Data is normalised to control OGD injury at 24 hours. **indicates value significantly different (P<0.001) from control OGD injury. (Sham: N=31; OGD: N=83).*
Figure 6.2: Histogram showing pixel distribution of slices immediately before OGD (black line), 24 hours after OGD (blue line), and sham treated slices at 24h (red line). The purple line indicates the threshold above which the numbers of pixels were summed to provide a quantitative measure of injury levels. Error bars are standard errors. (T0: N=114; Sham: T=31; OGD: N=83).
6.2 Mechanism of xenon and argon neuroprotection following OGD

With control conditions established the effects of 50.6 kPa xenon and 50.6 kPa argon on OGD injury was investigated. Figure 6.3 shows low levels of injury in xenon and argon treated slices compared to control OGD slices. Control OGD slices show high levels of damage in the CA1 region in particular, with further sensitivity to damage in the DG and CA3 regions. Treatment with xenon and argon attenuates the high levels of injury in the CA1 and CA3, although some sensitivity to DG damage is still apparent. This is also the case for sham treated slices. Figure 6.4 shows that both xenon and argon provided a significant \((P=5\times10^{-8})\) level of neuroprotection when compared to control levels of injury. Xenon reduced injury to 0.32±0.05 of control injury, and similarly argon reduced injury to 0.29±0.07 of control injury. Both xenon and argon treatment reduced levels of injury in the slices to the level of sham treated slices, shown by a lack of significance when compared to sham treated slices \((P=0.98 \text{ xenon } P=0.99 \text{ argon})\). Both xenon and argon showed remarkably similar levels of neuroprotection in this model, in contrast to the TBI model in which xenon was more potent as a neuroprotectant.
Figure 6.3: High levels of injury can be seen in slices following 30 minutes of oxygen and glucose deprivation (top right panel) compared to sham treated slices. Treatment with both 50.6 kPa xenon and 50.6 kPa argon provides robust neuroprotection against OGD. Slices are typical examples from each group, selected on the basis of having an average injury value close to the mean for that group.
Figure 6.4: 50.6 kPa xenon and 50.6 kPa argon both provide significant neuroprotection following oxygen and glucose deprivation. Xenon treatment (red bar) reduces injury to 0.32±0.05 of control injury. Similarly argon treatment (blue bar) reduces injury to 0.29±0.07 of control injury. Error bars are standard errors. Data is normalised to control OGD injury at 24 hours. ** indicates value significantly different (P<0.001) from control OGD injury. (Sham: N=31; OGD: N=83; Xenon: N=84; Argon: N=36).

In order to investigate the mechanism of neuroprotection, that is, the involvement of the NMDA receptor in neuroprotection, the same technique was adopted as in the TBI model. The slices were exposed to 100µM glycine in the experimental media for the 24 hour duration of the experiment during xenon and argon treatment. Previous experiments by Paul Banks had established that this strategy of reversing xenon neuroprotection following ischemic injury was successful in organotypic slices [68]. These experiments were repeated in order to confirm that the model being used was sensitive to these changes. Figure 6.5 shows that the presence of glycine in the media was found to not have any significant effect on the extent of injury in the slices (P=0.6), with control injury with glycine present 1.18±0.17 of the control injury without glycine. When 100µM glycine is present in the media a significantly (P=3E-4) higher level of damage occurs in xenon treated slices. Injury
in slices treated with xenon alone was 0.32±0.05 of the control injury, whereas when glycine was present this increased to 0.78±0.09 of the control injury. There was no significant difference found between slices treated with xenon and glycine compared to control injury (P=0.24), however there was a difference found between control slices with glycine present and slices treated with xenon and glycine present (P=0.039). The presence of 100µM glycine therefore reversed the neuroprotective effects of xenon almost fully to control levels. Further experiments involving strychnine were not carried out for the OGD model as it had been previously shown that the inhibitory glycine receptor did not have a role in xenons mechanism of neuroprotection following OGD [68].

**Figure 6.5**: Addition of 100µM glycine reverses neuroprotection by 50.6 kPa xenon. Glycine has no significant effect on control levels of injury (grey hatched bars). When glycine is present the level of injury in xenon treated slices (red hatched bar) is 0.78±0.09 of injury seen in control slices (grey bar). With no glycine present the level of injury in xenon treated slices (red bars) is 0.32±0.05 of control injury. Error bars are standard errors. Data is normalised to control OGD injury at 24 hours. ** indicates value significantly different (P<0.001) from control OGD injury. (OGD: N=83; Xenon: N=84; Xenon + glycine: N=73; Helium + glycine: N=35).
Following confirmation that NMDA receptor glycine site inhibition was a shared mechanism of xenon neuroprotection in both traumatic brain injury and ischemia, the same was investigated for argon. Figure 6.6 shows that neuroprotection by argon is not significantly (P=0.69) changed regardless of the presence of glycine in the media. With no glycine present the level of injury in argon treated slices is 0.29±0.08 of control injury. When 100µM glycine is present the level of injury in argon treated slices is 0.38±0.09 of injury seen in control slices. This result provides further evidence that inhibition of the NMDA receptor is not a mechanism of argon neuroprotection following either ischemic or traumatic brain injury.

Figure 6.6: Addition of 100µM glycine has no significant effect on neuroprotection by 50.6 kPa argon. Glycine has no significant effect on control levels of injury (grey hatched bars). When glycine is present the level of injury in argon treated slices (blue hatched bar) is 0.38±0.09 of injury seen in control slices (grey bar). With no glycine present the level of injury in argon treated slices (blue bars) is 0.29±0.08 of control injury. Error bars are standard errors. Data is normalised to control OGD injury at 24 hours. **indicates value significantly different (P<0.001) from control OGD injury. (OGD: N=83; Argon: N=36; Argon + glycine: N=32; Helium + glycine: N=35).
CHAPTER 7
DISCUSSION

The results presented in this thesis describe the neuroprotective properties of the noble gases xenon and argon following *in vitro* TBI and OGD, and the lack of neuroprotection by helium, neon and krypton following *in vitro* TBI. Furthermore, this study describes the mechanism by which neuroprotection is achieved by xenon and rules out a number of neuroprotective mechanisms of argon. This discussion will explore the suitability for the model to mimic a human head injury, the consistency of these findings with previously published results and most importantly, the significance of these findings with regard to future work in this area.

7.1 The *in vitro* weight drop device as a model of human traumatic brain injury

Before embarking on a comprehensive discussion of the experimental results presented in this thesis it is important to first consider the use of the models themselves to mimic traumatic brain injury and ischemia. The initial aim of the current study was to establish a protocol whereby the effects of noble gases in a model of TBI and ischemia could be investigated in order to prove or disprove the hypothesis challenged in this thesis. Creating a model of a complex neurodegenerative disorder such as TBI is not without difficulty, so extreme care had to be taken when designing and building the equipment and establishing the protocols necessary to model TBI, with the main outcome focused on producing a robust and reproducible level of injury. The current study used hippocampal organotypic slice cultures from neonatal C57BL/6 mice which were traumatically impacted using a weight drop device to create a focal contusion injury or deprived of oxygen and glucose for 30 minutes.
7.1.1 Organotypic slice cultures as an in vitro representation of the in vivo brain

There are a number of choices of the tissue surrogates used for in vitro studies of brain injury, including dissociated primary cultures, acute slices and organotypic slice cultures, which can critically determine the translatability of results to the in vivo state [111]. Dissociated cultures are cell cultures that are mechanically and enzymatically isolated from brain tissue. Acute slices are thin slices of brain tissue that have been freshly isolated, and organotypic slice cultures are slices of brain tissue that have been maintained in culture for an extended period of time (>7 days). Both acute slices and organotypic slices preserve the anatomical structure, neuronal circuitry and heterogeneous cell populations found in an in vivo brain whereas dissociated cell cultures typically contain a single cell type [111]. A limitation of acute slice preparations is the effect of tissue explantation on the response to a subsequent trauma, as experiments have to be performed on the day of explantation and slicing whereas organotypic slices are cultured for 14 days before experimentation so they have the opportunity to recover and return to a stable state [111]. Organotypic slice cultures allow for longer-term investigations of the neuroprotective effects of compounds (in this case, three days post injury), whereas acute slices and dissociated cell cultures typically only remain viable for experiments for 8 and 24 hours respectively, which was a significant advantage for investigations in this study. Organotypic slices maintain the normal structural, organisational, and functional features of native brain tissue with preserved synaptic and chemical signalling, which allows for functional analysis of the properties of nervous tissue [104-106].

In this study slices were prepared from neonatal P5 C57BL/6 pups. C57s are one of the most widely used mice strains in medical research and have been described as a ‘general multipurpose model’. Advantageous to this study was their ability to be good breeders, as a regular steady supply of pups was required. In addition, C57BL/6 mice have had their entire genome sequenced which makes it an ideal choice for knockout and transgenic models. It is now of particular interest following this study and the study by Scott Armstrong (which discovered an amino acid mutation that eliminated xenons interaction at the glycine site without affecting glycines affinity for the receptor) to pursue a transgenic route to dissect out the contribution of glycine site inhibition to anaesthesia and neuroprotection by xenon, thus it is more suitable to use the same breed in in vitro experiments as subsequent in vivo
experiments. Neonatal mice were used as at this age the tissues show a high degree of resistance to the mechanical trauma suffered during dissection and have already established basic synaptic connections [117]. During the two week incubation period after explantation, organotypic slices mature similarly to the in vivo schedule of development, with the basic synaptic connections forming mature synaptic networks. [106, 117]. A study by De Simoni and colleagues in 2003 [106] found that in terms of total length and primary branches of neurones, outgrowth of apical dendrites and spine density, the organotypic slices cultured for varying times in vitro were the developmental equivalent of slices taken from the corresponding age of pup in vivo. They also found that there were no differences between organotypic slices and their age matched acute slices in the frequency of action potentials and miniature synaptic activities [106, 117].

Glial cell populations are also maintained in organotypic slice cultures [105, 109, 118]. Oligodendrocyte populations are present in organotypic slices and axons become myelinated over the first 14 DIV [105]. Microglial cells become activated during the explantation procedure but return to the resting state after 3 DIV [119]. Morphological characteristics of astrocytes are maintained throughout the entire culture period [120], with the typical glial scar of hypertrophic astrocytes found from 5DIV onwards [119]. When modelling complex brain pathologies it is especially important that all the native cell populations within the brain are represented accurately within the model and their relative contributions to injury or recovery allowed to contribute to the final outcome.

For this study organotypic slice cultures were chosen over alternative in vitro models and indeed over an in vivo model of TBI as they confer a number of advantages over the alternatives. An advantage of this technique over alternative in vitro models (such as dissociated cell cultures) is the more accurate replication of the distribution of extracellular signalling molecules, since their local concentration may be elevated in the intercellular spaces within the tissue as opposed to just diffusing away freely after release [121]. As mentioned above, OSCs also maintain populations of glial cells [105, 109, 118] which is a feature often overlooked in simpler in vitro models. Despite representing a very basic model of the in vivo brain, in certain scenarios OSCs can confer an advantage over in vivo models. One key example of this is that the extracellular environment can be precisely controlled, that is, when investigating the mechanism of action of drugs one can control the concentration of
compounds in the surrounding media in order to correlate molecular changes with neuropathological outcomes [117]. This was especially useful in the current study as it allowed direct control over glycine concentrations in the slice environment, thus allowing a saturating concentration to be achieved for competition binding experiments. Direct injection of glycine into animals would not have been possible as glycine itself is a major inhibitory neurotransmitter in the CNS and thus injection of a bolus dose could have a major impact on CNS function. It would also have been difficult to control or determine the extracellular concentration of glycine in vivo to know if adequate concentrations for competition experiments with xenon had been reached. Another advantage over whole animal models is that a single slice can be monitored at multiple time points over an extended period of time, whereas in vivo multiple measurements are mostly restricted to behavioural studies and pathological assessment of injury can only be carried out once the animal is sacrificed, restricting the analysis to only a single time point following injury. Other important advantages of OSCs over animal models are the lower associated costs, and lower requirement for the animals themselves (one pup typically yields 15-20 slices) which confers an ethical advantage to their use.

The main limitation of the OSCs are, quite obviously, the extent to which they are capable of reproducing events that occur in vivo, as is the case with any in vitro model (discussed in more detail in section 7.1.2). Currently organotypic slices culture techniques are limited to tissue explanted from neonatal pups, usually p5-7 in the case of mice and p8-15 in rats [109]. Organotypic slices prepared from adult tissue exhibit poor viability, with 90% of cells dead after 15DIV [122]. Further investigations have identified that during the culturing phase adult organotypic slices (at 5DIV) display neurons with a severe loss of volume and shrunken, pyknotic nuclei and a failure of glial cells to return to a resting state after initial slicing [119]. This limitation should be of consideration when translating results from this study into in vivo experiments on adult mice and indeed when translating these results to human head injury scenarios since the majority of TBI cases occurs in adult humans. Unfortunately as it stands OSCs from adult mice exhibit poor viability so neuroprotection experiments on adult models will be limited to in vivo models of TBI. A further issue with the use of OSCs was highlighted by the aforementioned study by De Simoni who found that there was an increased number of branches and higher order dendrites in OSCs which corresponded to an increase in glutamatergic miniature synaptic currents, which means the contribution of excitotoxicity to outcomes after traumatic or ischemic injury could possibly be over-accentuated [106].
Taken together the breadth of published studies which use organotypic slice cultures to mimic neurodegenerative events and neuroprotection in vitro justified their use to investigate the neuroprotective potential of noble gases.

7.1.2 How well does the weight drop device model traumatic brain injury in vivo?

Models of traumatic and ischemic brain injury can be used as a platform on which to test the neuroprotective potential of new compounds in a system that is repeatable, environmentally isolated and tightly-controlled [111] as well as being more cost effective and higher throughput than whole animal models [121]. There are a number of different in vitro models of TBI that each targets a different injury mechanism. These include weight drop and impact models to mimic focal contusion injuries, stretch injury, hydrostatic pressure, shear strain and fluid shear stress to model diffuse axonal injury as a result of rapid deceleration, transection to model penetration injuries and blast to model primary blast injury. For the study presented in this thesis a weight drop model was used as it was possible to create a focal primary injury which allowed for observation of the development of secondary injury. Secondary injury development was a key characteristic for this study to investigate how neuroprotective gases may alter its development.

The weight drop model can be used in both in vitro [50] and in vivo systems [123, 124] to model TBI. It is used to produce a largely focal injury via the impact of a free falling weight on a slice of brain. This model has the advantage of having control over the severity of the insult by adjusting the impact force, shape of impact device and duration of impact [111]. Using this model in this study it was possible to create an area of primary injury which lay directly beneath the area weight dropped upon and a surrounding secondary injury, making this a good choice for investigating agents that may aid in halting the spread of injury following TBI. A limitation of the weight drop model is that the impact energy can be calculated theoretically but is difficult to measure forces accurately, so a complete characterisation of the biomechanics of injury is difficult [111, 121]. Oxygen and glucose deprivation is a widely used model to study acute stroke pathology [117]. Region specific death occurs in slices exposed to OGD, with selective death seen specifically in the CA1 region, similar to what is observed in vivo [125]. A number of characteristics of ischemia induced changes in brain activity have been successfully reproduced in organotypic models.
of OGD (where oxygen and glucose deprivation is brief at around 30 minutes). These include neurotransmitter release (particularly glutamate), delayed injury development, involvement of caspase pathways and post translational modification of proteins [117, 125, 126].

Some aspects of an in vitro model of a complex injury that could be seen as beneficial to pharmacological experiments could also be a limitation of the model itself. For example, the extracellular environment can be stringently controlled in vitro; however it may not be an accurate representation of the extracellular environment in vivo. Indeed this is almost impossible to model in the case of TBI and ischemia as the exact status of the extracellular environment at the time of injury is difficult to measure [111]. Certain other systemic aspects such as inflammatory response, temperature regulation, oxygenation and local ionic concentrations are also not present in in vitro models of injury. The lack of these however allows for simpler studies without the complication of such factors.

The hippocampus was chosen in this model to be cultured and receive a primary injury for a number of reasons. From a practical aspect the hippocampus is easy to identify and remove, and slices prepared are all of a similar size, meaning quantitative analysis could not be skewed by larger slices giving off larger signals. From an experimental aspect the hippocampus has a high distribution of NMDA receptors and thus is highly sensitive to excitotoxic damage. However in the case of a human head injury, a direct impact to the hippocampus is unlikely, which represents a major limitation to this model. Hippocampal damage is observed in up to 85% of human head injury cases. This is likely due to a process known as cortical spreading depression, which involves a wave of depolarisation that propagates throughout the brain. The wave of depolarisation causes a massive release in excitatory amino acids including glutamate which could spread to the hippocampus and lead to secondary injury development in this region of the brain. Therefore the development of secondary injury in the hippocampus is of clinical relevance, whereas primary injury in the hippocampus is less relevant to the real life scenario. It is also important to note that due to the high density of NMDA receptors in this particular brain region, results obtained using this model could be bias toward NMDA receptor mediated mechanisms of action and other neuroprotective targets could be seen as being less dominant.

Another limitation to consider, not with the model itself but with this particular study, is highlighted in a review paper by Morrison and colleagues [111] who state that “the underlying mechanism that is addressed by a protective compound can be shown to be
relevant by comparing in vitro results with in vivo results”. That is, one can validate the model chosen to mimic injury development in vitro by confirming that a compound that is successful in one model translates to the animal models of injury too. This is a simple way to confirm that similar, if not the same, mechanisms are occurring both in vitro and in vivo. A wealth of in vivo data exists for both xenon and argon confirming their efficacy as neuroprotectants following ischemic injury [51, 58, 65-67, 87, 88, 95, 100-102] which provides a degree of confidence that the same injury mechanisms are present in the in vitro model used in this study and in vivo ischemia. There is currently no published data regarding the in vivo neuroprotective effects of xenon and argon following TBI to assist in validating the in vitro model chosen, however the work in this thesis was carried out in order to provide more information on this emerging area of research, so a lack of previously published data represents simply a limitation and not a weakness.

7.1.3 Using a weight drop device it is possible to model secondary injury development following a TBI.

The first part of this study involved the characterisation of an in vitro model of traumatic brain injury that was capable of being utilised for neuroprotection studies. Although similar models had been previously described, it was essential for the subsequent experiments that the model was reliable and reproducible. The equipment for creating a focal injury in vitro is not commercially available and had be custom built in the lab, with the design aided by previously published studies [50, 112, 113]. It would not suffice to simply create a weight drop model similar to a published model and begin neuroprotection studies straight away without rigorous testing of the reproducibility of the model first, as this was essentially a new protocol that had not been previously tested by this or other labs and thus few comparisons between this and other studies of neuroprotection following in vitro TBI could be performed. It was crucial that all parameters that were possible to measure were investigated and well defined, so that changes observed in the slices following impact and following treatment with different agents could be attributed to a single manipulation with a high degree of confidence. After the device was built, the calibration process aimed to answer the following questions:

- Does a relationship exist between the energy the slices are impacted with and the extent to which secondary injury develops?
- Where should the threshold is set in order to accurately quantify the level of injury in the slices?
- Can primary and secondary injury be separated into two distinct components of injury development?
- What are the characteristics of the injury development?
- To what extent does the impact damage the slice with regards to the maximum damage that is possible to inflict?
- Do repeated impacts on the same membrane create an additional element of injury?

The first stage of calibration involved investigating a range of impact energies to assess the relationship between impact energy and secondary injury development. The impact energies selected for investigation were 1.4µJ, 2.7µJ and 3.5µJ, which corresponded with drop heights of 2mm, 4mm and 5mm. With the mass of the stylus fixed at 70mg and the acceleration under gravity constant the drop height represented the variable for different impact energies. The energies used to inflict damage are somewhat arbitrary, as it would be extremely difficult to extrapolate impact forces used in in vivo TBI models to in vitro models involving a thin slice of brain tissue. Instead, the ‘ideal’ impact energy for experimentation would be that which best models the characteristics of an in vivo TBI, that is, a clear measurable area of primary injury that occurs immediately upon impact, followed by subsequent secondary injury that develops significantly in the hours and days following the initial impact. With this in mind, 3.5µJ was selected as the impact energy for subsequent experiments, as impacts of 1.4µJ and 2.7µJ produced a level of injury that was greater than in the sham slices, but not to a level of a significance, and injury did not develop over the 72 hour experimentation procedure. In comparison slices impacted at 3.5µJ exhibited levels of injury that were significantly different from sham treated slices at all time points and secondary injury developed significantly from 24-72 hours (figure 3.2). The choice of impact energies to investigate was based on previous published descriptions of similar weight-drop models of TBI in organotypic slice cultures [50, 113]. A study by Adembri and colleagues [113] investigated impacts ranging from less than 3µJ to 9µJ. They found that at impacts of 3µJ or below, an area of primary injury was apparent but this did not develop significantly between 24 and 48 hours. In addition, impacts of 9µJ produced a large lesion that was maximal by 24 hours which did not allow for observation of injury development.
The level of injury at 30 minutes post impact was considered a measure of primary injury in this study. For subsequent experiments where only secondary injury was analysed, the mean value of the primary injury was subtracted from the total injury to give a measure of secondary injury (figure 4.3). Primary injury was calculated to make up 14±1% (n=28) of the total injury. A fixed value of primary injury made two assumptions: that each impact was of the same severity, and each slice did not possess different intrinsic resistance to mechanical injury. Drop height and mass of the stylus was the same in each experiment, so theoretically the impacts would be of the same severity each time, slices were prepared from pups of the same age and breed, and days in culture was kept within a narrow time frame of 10-14 days.

Coburn et al. [50] used an alternative method to calculate secondary injury in impacted slices. They masked the area of primary injury with a circle with a diameter of 1000µm and integrated only the pixels that were outside of this masked area. This technique however raises a concern that the primary injury is overestimated as any secondary injury that develops close to the primary injury in the region of the mask would be excluded from secondary injury analysis. Given the nature of secondary injury development it is highly likely that a significant proportion of the secondary injury would occur adjacent to the primary site. Indeed this may be the case in the study by Coburn [50] who estimated that secondary injury made up approximately 40% of the total injury. 50% of the total injury was reached in 24 hours, so by definition this could be taken to assume that all injury developing within the first 24 hours could be classed as a primary injury. In an ideal world, the exact value of primary injury in each individual slice would be determined to allow for a more tailored analysis of secondary injury development. However this was not considered practical, as imaging the slices at 30 minutes post impact may introduce an element of hypothermia to the slices which has been found to be neuroprotective, and would undoubtedly interfere with subsequent neuroprotection studies.

To characterise injury development in the slices in the first 24 hours following impact, Rita Campos Pires investigated additional time points of 1, 6 and 12 hours post impact (figure 3.2a) and kindly gave her permission for this data to be used in this thesis. The development of injury in the slices was found to increase rapidly in the first 12 hours following injury, with 51% of the total injury occurring by 6 hours post impact. Injury continued to develop steadily after 12 hours but at a reduced rate. The rapid rate of injury development in the first 12 hours following injury found in this study most likely reflects the massive release of neurotransmitters (including glutamate) and prolonged depolarisation that follows from
mechanical perturbation of neurones [2]. Secondary injury developed in a characteristic manner in the slices, specifically affecting the CA1, CA3 and DG regions with the densest PI stain seen in the CA1 region. This pattern of selective vulnerability of these hippocampal regions is known to occur following in vivo TBI [127, 128] and is thought to be attributed in part to the high density of NMDA receptors found in these regions [129]. A study by Kristenson et al. [130] found sensitivity to excitotoxic injury induced by NMDA application in organotypic slice cultures to be highest in the CA1 region, followed by the DG and the CA3 which is in agreement with the findings of this study.

A further aspect of the calibration process involved characterising the extent of injury in the slices that is produced by impacting in relation to the maximum possible injury. If the injury inflicted by the impact was similar to the maximum, this would translate to a lack of secondary injury development as the slice would already be dead, making neuroprotection studies difficult. Maximum injury was inflicted by submerging the slices in 70% ethanol. It was found that primary injury represented 3.5% of the total possible injury (figure 3.5). The total TBI injury increased rapidly to 18% of the maximum by 24 hours and reached 25% of the maximum injury by 72 hours.

The final stage of calibration focused on determining whether repeated impacts on the same membrane could create an additional element of injury that would affect the development of secondary injury. This would be an issue for the current study as the model was designed whereby the primary injury occurred only once and thus secondary injury could be isolated entirely to a single impact. It was considered that perhaps impacting the slice may cause a distension of the membrane beneath, creating a form of stretch injury which in itself is another form of traumatic brain injury [110]. Experiments were devised whereby on each membrane half of the slices were impacted, and half were not; the unimpacted slices were termed ‘sentinel slices’. It was found that the sentinel slices did not exhibit a level of injury that was significantly different to that of sham treated slices (figure 3.6), thus concerns regarding repeated impacts effect could be eliminated.

With the characteristics of the injury fully explored in this model it was possible to proceed to experiments involving neuroprotection by the noble gases.
7.2 Neuroprotection by the noble gases

7.2.1 Xenon and argon provide neuroprotection following TBI and ischemia in vitro

This study investigated the potential neuroprotective effects of the noble gases helium, neon, argon, krypton and xenon under identical conditions. Only xenon and argon possessed neuroprotective properties (figures 4.1 and 4.2). Xenon was found to be more efficacious as a neuroprotectant than argon in this study (figures 4.4 and 4.4).

In this study helium was used as a control gas, to control for the hyperbaric conditions (50.6 kPa added pressure) that slices were subjected to for experiments involving the other noble gases. Helium was chosen because it is considered inert and lacking in any pharmacological effect; no molecular targets have been identified and it is often used as a pressuring gas in experiments investigating the effects of pressure [74, 131, 132]. It was found that the addition of helium at this pressure had no significant effect on injury development when compared to TBI slices and sham treated slices under normobaric conditions (figure 3.8). For concentration response studies involving xenon and argon, a range of pressures was investigated using helium to identify any effects of pressure per se. It was found that over a range of added pressures; 30.4 kPa, 50.6 kPa and 70.9 kPa no effect on injury development was observed on TBI or sham treated slices (figure 3.9). Further discussion of helium in the literature can be found in the following section 7.2.2.

Neuroprotection by xenon

In this study, 50.6 kPa xenon was found to be potently neuroprotective following traumatic and ischemic brain injury in vitro (figures 4.2 and 6.4). This effect was sustained through the 72 hour experimentation period for TBI, and the 24 hour experimentation period for ischemia. At 72 hours 50.6 kPa xenon reduced total injury (primary and secondary injury included) by 0.43±0.03. When the primary injury component was removed from analysis, it was calculated that xenon reduced secondary injury by 0.50±0.05 at 72 hours. Most remarkably the extent of damage xenon treated injured slices was not found to be significantly different from sham treated slices at any time point (figure 4.4). This data suggests that this concentration of xenon completely abolished the development of secondary
injury in the slices when slices were exposed to xenon for the entire 72 hour experimentation procedure. The work produced in this thesis found that exposure to 50.6 kPa xenon was found to reduce OGD induced injury by approximately 0.69±0.05, which is comparable to that found by Paul Banks [68] using the same in vitro model. Similarly, Jawad et al. found a 75% concentration of xenon following ischemic injury in dissociated cells afforded more than 92% protection [95] and David et al. [84] found that treatment with xenon at concentrations ranging from 50%-75% reduced injury levels of brain slices subjected to OGD to the level of control uninjured slices.

*Concentration response effects of xenon*

This study in this thesis also addressed possible concentration response effects of xenon across a range of concentrations from 30.4 kPa to 70.9 kPa (figure 4.8). At a concentration of 30.4 kPa xenon provided protection of 0.40±0.13, at 50.6 kPa xenon provided 0.43±0.03 protection which rose to 0.52±0.12 protection at 70.9 kPa. Overall the concentration studies of xenon yielded encouraging results, as it appears that even at lower concentrations the neuroprotection afforded by xenon is robust. These results suggest that potent neuroprotection may be observed at concentrations below the ones investigated in this study. This has clinical advantages in terms of allowing for sufficient oxygen levels during treatment, and economic advantages as lower effective concentrations results in a lower overall cost of treatment. Concentration dependence of neuroprotection by xenon has been investigated in a number of previous studies [47], Encouragingly, neuroprotection has been observed in in vitro models of brain injury at concentrations as low as 10.3 kPa. However an in vivo study found 20% xenon to be ineffective as a neuroprotectant whereas 70% xenon provided a significant level of protection following carotid artery ligation [85]. This may reflect the relative complexity of whole animal models compared to simple in vitro models where other factors such as inflammation and blood-brain barrier breakdown may play a significant role in the spread of damage.

*Neuroprotection by argon*

In this study argon was also identified as possessing neuroprotective properties following traumatic and ischemic brain injuries. Following trauma, 50.6 kPa argon was found to reduce injury development by 0.30±0.07 (figure 4.2). When secondary injury alone was examined argon was observed to reduce secondary injury development by 0.34±0.08 (figure 4.5). In this model argon was less effective than xenon at reducing secondary injury development, as
secondary injury in argon treated slices was not significantly different from sham treated slices at the 24 and 48 hour time points but by 72 hours it was significantly higher than in the shams, indicating an attenuation or a delay in the development of secondary injury. When neuroprotection by 50.6 kPa argon was investigated following OGD, it reduced injury development to 0.29±0.08 of control injury (figure 6.4) which was not significantly different to the level of protection afforded by xenon, thus they appear to have similar potencies following OGD. However OGD injury was only assessed up to 24 hours, whereas TBI injury was assessed up to 72 hours, so it is possible that if a longer experimental period had been implemented following OGD then differences between the potencies of xenon and argon may have been observed.

A number of publications have reported similar neuroprotective effects of argon following traumatic and ischemic injury both in vitro and in vivo. Loetscher et al. [51] investigated argon neuroprotection in a similar model of in vitro TBI to the current study. They found 50% argon to reduce injury by approximately 86% compared to control injury 72 hours after the TBI. The reason for the higher potency observed by argon following TBI in their study compared to the data in this thesis may be due to methodological factors; Loetschers study used an impact force of 5.26µJ, a threshold for pixel integration of 100 and normobaric conditions. On the other hand, their study observed a lower potency of argon following OGD than in this thesis (44% Loetscher study vs. 71% in this study) despite a similarity between the protocols, although the OGD slices in Loetschers study were observed for 72 instead of 24 hours. In vivo studies of middle cerebral artery occlusion [97], neonatal asphyxia [135] and NMDA injection [101] exposed to argon concentrations ranging from 37.5-70.9 kPa argon (respectively) observed reduced infarct volumes and improved functional recovery following treatment. These results indicate that in vitro neuroprotection observed by argon has been successfully translated to the in vivo setting when given at the investigated concentrations, which is encouraging.

Concentration response effect of argon

This study investigated the concentration response effect of argon following TBI, by investigating the neuroprotection afforded by argon at concentrations of 30.4 kPa, 50.6 kPa and 70.9 kPa (figure 4.8). It was found that argon was devoid of neuroprotective effects at a lower concentration of 30.4 kPa, with a reduction in injury of only 0.06±0.07 (figure 4.6). A significant neuroprotective effect of argon was observed at concentrations of 50.6 kPa and
70.9 kPa, providing 0.30±0.07 and 0.40±0.10 neuroprotection respectively at 72 hours. In comparison to xenon there appears to be a concentration dependence of argon neuroprotection. Concentration dependence of argon neuroprotection has recently been demonstrated by Brucken and colleagues [133] who reported that rats receiving 70.9 kPa argon following cardiac arrest had significantly less neurological impairment than rats receiving 40% argon. David and colleagues [101] found that both in an in vivo model of excitotoxic injury, and an in vitro model of OGD injury, argon provided a significant level of neuroprotection when administered at concentrations of 37.5 kPa or above (50.6 kPa and 70.9 kPa was also investigated), but injury returned to control levels when argon was administered at a concentration of 25.3 kPa. Interestingly, they found that in vivo, argon was not neuroprotective when administered at a 70.9 kPa concentration, whereas it was significantly neuroprotective at this concentration in vitro [101]. This may be of concern, as the target of argon neuroprotection may be one which is easily overwhelmed by other death promoting mechanisms in vivo compared with in vitro studies.

Comparing the relative potencies of xenon and argon

The reasons for the differing concentration dependence between xenon and argon may be due to different potencies between the gases, which are most likely accounted for by the different mechanism of neuroprotection (see section 7.3.1). In terms of potency, this study found that following TBI xenon was more potent at a 50.6 kPa concentration when assessing the reduction of secondary injury development, whereas following OGD both xenon and argon were equally as effective as neuroprotectants although the OGD procedure was limited to assessing cell injury at 24 hours post injury. The reason for limiting the experimental procedure to 24 hours was based on observations by Paul Banks who reported no further injury development after 24 hours using this model of OGD. There are two published studies in which xenon and argon have both been assessed in the same model; the in vitro dissociated cell model of OGD by Jawad et al. [95], and the in vivo model of neonatal asphyxia by Zhuang et al. [134]. These studies found conflicting results, with Jawad’s study concluding that at a 75% concentration xenon was more effective as a neuroprotectant, whereas Zhuang’s study found that whereas xenon and argon (at a 70.9 kPa concentration) both decreased infarct volume to the same extent, only argon improved cell survival to naïve levels, indicating argon was more effective. Coburn and colleagues used an in vitro model of TBI to assess both argon [51] and xenon [50] in separate studies, although the methods are very similar. In these studies argon was found to be a more effective neuroprotectant following...
TBI, reducing total cell death to 14% of control, whereas xenon reduced it to 50% (both at a 75% concentration). There are a number of subtle differences between the two studies however, including different impact energies (5.26µJ argon study and 3.5µJ xenon study) and different thresholds for pixel quantification (100 argon study, 150 xenon study). It is clear that there is a requirement for more studies investigating both xenon and argon in the same controlled models both \textit{in vitro} and \textit{in vivo} in order to further assess the relative potencies of both. In terms of economic costs, if argon were found to be equally as effective as xenon but only when given at higher concentrations, then this would potentially make argon a more clinically feasible option as it would negate the requirement for expensive closed circuit delivery systems.

### 7.2.2 Helium, neon and krypton do not provide neuroprotection following TBI \textit{in vitro}

The work described in this thesis found that helium, neon and krypton were all devoid of neuroprotective effects when administered for 72 hours following TBI \textit{in vitro}.

**Helium**

The general consensus regarding heliums neuroprotective effect, or lack of, is disputed in other publications, most of which focus on ischemic brain injury. The findings of the current study are in agreement with that of Banks \textit{et al.} [68] who determined that 50.6 kPa of additional helium had no significant effect on injury development in organotypic hippocampal slices in an \textit{in vitro} model of ischemic brain injury (the same model as employed in chapter 6). Another \textit{in vitro} model of ischemic brain injury [95] using dissociated cortical cells exposed to 90 minutes of oxygen and glucose deprivation found that exposure to 75% helium actually worsened the injury when cells were assessed 24 hours later. In \textit{in vivo} studies of ischemia helium appears to have consistently neuroprotective effects. A study by Pan \textit{et al.} in 2007 [135] found 75% helium to reduce infarct volume and improved neurological deficits 24 hours after focal ischemia. Another \textit{in vivo} study of MCAO by Pan \textit{et al.} [136] several years later found 70.9 kPa helium to reduce infarct size and improve neurological deficit scores but only if it was administered immediately after the injury. The neuroprotective effect was lost if treatment with helium was delayed by 30 minutes or longer [136]. Interestingly one \textit{in vivo} study [134] found that 70.9 kPa helium was
protective after 90 minutes of ischemia in rats, but this neuroprotection was lost in a more severe insult of 120 minutes of ischemia (in comparison to xenon and argon that were significantly protective in both).

The reasons for the discrepancies between different studies concerning helium neuroprotection are unclear, although it can most likely be attributed to the different models employed in each study and differing concentrations of helium. Helium is very unlikely to be exerting neuroprotective effects via a pharmacological mechanism (due to unfavourable binding enthalpies) and is more likely to be due to a physical mechanism \[74\]. David and colleagues [99] propose that the neuroprotection observed by helium in vivo is due to the induction of hypothermia on account of the high specific heat in comparison to normal air (thermal conductivity of helium and nitrogen are 0.1499 and 0.0260 W/m/K respectively \[74\]). They found that when 75% helium was administered following MCAO at 25°C neuroprotection was observed, whereas when the temperature of the inspired helium was adjusted to 33°C then this neuroprotective effect was abolished. This does provide a plausible explanation for neuroprotection by a gas that is seemingly devoid of biological targets, and goes some way towards explaining the lack of effect observed by helium in this study as temperature was controlled at 37°C throughout the experimental procedure. Despite the conflicting evidence in the literature regarding potentially neuroprotective effects of helium, this study found both helium and hyperbaric conditions to have no significant effect on injury development. It was assumed that the effects of helium and pressure were not exactly equal and opposite, and helium was considered a suitable control gas for this investigation.

**Neon**

No effect was observed following treatment with 50.6 kPa neon following TBI in this study (figure 4.2). Similar to helium, the unfavourable balance between binding energies and repulsive forces means neon is unlikely to interact with binding cavities in proteins [103] and thus unlikely possess any biological activity. Although never investigated before in a model of TBI, this finding is in agreement with that of Jawad and colleagues [95] who found neon to be devoid of neuroprotective effects in a dissociated cell culture model of oxygen and glucose deprivation. A thorough literature search found a single paper suggesting neon possessed biological activity [137]. Pagel and colleagues found that preconditioning with 70.9 kPa neon significantly reduced myocardial infarct size by 20±3% after 30 minutes of left anterior coronary artery occlusion in rabbits, which they suggest is due to activation of prosurvival
signalling kinases and inhibition of the MPTP opening [137]. These exact mechanisms remain unclear, as does the existence of any biological activity of neon in brain tissues.

**Krypton**

This study found that krypton was devoid of neuroprotective effects in an *in vitro* model of TBI (figure 4.2). As a molecule krypton possesses a polarizability that is favourable for the formation of London dispersion forces and thus the interaction with protein binding cavities [74, 97]. In contrast to the findings of this study Jawad and colleagues [95] found that the presence of 75% krypton was actually detrimental to cell survival in a dissociated neuronal cell culture model in the naïve state, and not after OGD, as shown by a reduction in MTT reducing ability [95], suggesting an intrinsic toxic effect of krypton to cells in their normal, uncompromised state. However experiments carried out for this thesis found that krypton treated sham slices did not show a significantly higher level of damage than sham slices treated with any other noble gas (data not shown). However, despite a lack of observed neuroprotection, krypton may possess biological targets and the capability to exert a physiological response, but these may not be involved in neuroprotection.
7.3 Mechanism of xenon and argon neuroprotection

7.3.1 Xenon provides neuroprotection via inhibition of the NMDA receptor at the glycine co-agonist site

Following identification of xenon and argon as neuroprotectants following in vitro TBI and OGD, subsequent experiments were devised to investigate the mechanism of neuroprotection. The primary focus of investigation was the involvement of the glycine co-agonist site of the NMDA receptor. The reason for this was twofold. Firstly, the NMDA receptor is known to have an important role in excitotoxic cell death following traumatic brain injuries, and secondary, the NMDA receptor is a known target of xenon, with glycine site inhibition believed to form a major component of the neuroprotective profile of xenon following ischemic brain injury [68].

To investigate the role of glycine site inhibition in the neuroprotective effects of xenon and argon, experiments were devised whereby 100µM glycine was present in the media throughout the experimental period, with the rationale that it may compete with xenon and argon at the glycine binding site and reduce the effectiveness of these agents. This concentration of glycine was used as it has been shown electrophysiologically to maximally inhibit the competitive component of NMDA receptor inhibition by 80% xenon in concentration response experiments [76]. Furthermore, this concentration of glycine was used in the experiments by Banks et al. [68] to demonstrate reversal of neuroprotection by xenon following in vitro OGD. It was found that addition of glycine at this concentration had no significant effect on traumatic injury development or on sham injury (figure 5.1 and 5.3). This is in agreement with previous published reports that this concentration of glycine is not toxic to cells in vitro [138-142] or in vivo [143], with glycine toxicity only reported at concentration of 10mM and higher [138, 139] To rule out any involvement of the ligand gated inhibitory glycine receptors (GlyRs) on injury development or neuroprotection control experiments were performed in the presence of 100nM strychnine. This concentration was chosen as it has been shown to completely inhibit GlyRs even in the presence of high concentrations of glycine [116]. The presence of strychnine at this concentration had no
significant effect on traumatic injury development or sham injury when applied alone (figure 5.2) or in combination with 100µM glycine (figure 5.2c).

Reversal of neuroprotection by glycine site antagonists with the addition of glycine is a technique that has been employed successfully in a number of previous studies using glycine concentrations ranging from 10µM-1mM [141, 144-147]. Of note is the study by Paul Banks [68] which reversed the neuroprotective effects of 50.6 kPa xenon and 0.5µM and 5µM gavestinel (a competitive NMDA receptor glycine site antagonist) by addition of 100µM glycine in a model of ischemic injury in organotypic hippocampal slice cultures. D-serine has been proposed as an alternative endogenous modulator for the glycine site of the NMDA receptor. Glycine and d-serine are regulated by distinct uptake and release pathways and both are believed to have a role in activating synaptic NMDA receptors, and in the development of excitotoxic events [142]. In studies where the effects of d-serine depletion are investigated, the effects can be rescued by the addition of glycine [148, 149]. In addition knockout mice lacking serine racemase (an enzyme that generates d-serine from l-serine) have been observed to retain normal functioning of synaptic NMDA receptors [149, 150]. In this study it was proposed that xenon competes for the same site as glycine and/or d-serine and not a direct interaction with the molecule themselves so, providing the receptors respond in the same way to the co-agonist, its endogenous identity is of less relevance. The primary reasons for selecting glycine over d-serine in this study is due to the fact that concentration response relationships between glycine concentration and xenon inhibition of the NMDA receptor have previously been performed [76] and reversal of xenon neuroprotection by glycine has previously been observed following OGD in hippocampal organotypic slices [68].

This study found that when 100µM glycine was present in the media the neuroprotective effect of xenon was abolished (figure 5.4). This effect was sustained when slices were exposed to 100µM glycine and 100nM strychnine in combination (figure 5.6), with the presence of 100nM strychnine alone not having a significant effect on the level of neuroprotection by xenon (figure 5.5). These observations lead to two conclusions. Firstly, inhibition of NMDA receptors via the glycine site forms a major component of the neuroprotective profile of xenon following TBI, and secondly, inhibitory glycine receptors play no role in xenons neuroprotective profile. It was also observed that addition of 100µM glycine reversed the neuroprotection by 50.6 kPa xenon in a model of in vitro OGD (figure...
6.5). This had been previously established by Banks and colleagues [68], but in this experiment it was performed to act as a control for the argon experiments, that is, to prove that elevated glycine is capable of reversing a neuroprotective effect.

Given the diversity of NMDA receptors it is of interest to consider whether subunit composition or location of the NMDA receptor may affect xenon's ability to interact with the receptor. The glycine binding site is found on the NR1 subunit, however the NR2 subunit is differentially expressed during neuronal development and is found predominantly in extrasynaptic NMDA receptors. In early developmental stages the NR2B subunit is predominantly expressed and the NR2A/NR2B ratio increases during development with an NR2A predominant expression in mature neurons [151]. A study by Dickinson and colleagues [76] established in electrophysiological experiments that 80% xenon inhibited NMDA receptor currents to the same extent in HEK cells transfected with the NR1-NR2A and NR1-NR2B subunits (see figure 7.1). Further studies by Haseneder et al. [152] and Kratzer et al. [153] have confirmed that inhibition of NMDA receptors by xenon is not subunit-selective for NR2A or NR2B containing receptors. Synaptic NMDA receptors are activated by the release of glutamate from the presynaptic membrane. In contrast extrasynaptic NMDA receptors are located outside of synapses and do not respond to synaptically released glutamate [153]. Extrasynaptic NMDA receptors have been proposed to play an important role in neuronal degeneration as they respond to increased ambient glutamate concentrations, such as those experienced during ischemic and traumatic brain injury [154]. Work by Kratzer and colleagues [153] investigating the effect of 65% xenon on mouse hippocampal slices found that xenon reduced NMDA receptor mediated excitatory postsynaptic currents by approximately 65% and extrasynaptic NMDA currents by approximately 59%, thus they concluded that xenon antagonises both synaptic and extrasynaptic NMDA receptors with the same potency.
Figure 7.1: Concentration–response curves for activation of receptors by N-methyl-D-aspartate (NMDA) in the absence (black circles) and presence (white circles) of 80% xenon for NR1/NR2A receptors (A) and NR1/NR2B receptors (B). The concentration of the co-agonist glycine was 100µM throughout. Each point represents the mean value from, on average, 7 cells (NR2A), or 6 cells (NR2B). The error bars are SEs; where not shown, these are smaller than the symbol. The curves are fitted to the Hill equation, as described in the text. (C) There was no significant difference (P > 0.05) in the inhibition of NR1/NR2A or NR1/NR2B receptors by 80% xenon. The data shown are at an NMDA concentration of 100 µM. The values are means from 11 cells (NR2A) and 8 cells (NR2B). The error bars are SEs. Figures and legend reproduced from Dickinson et al. 2007 [76].

That glycine site inhibition makes up a significant proportion of the neuroprotective properties of xenon provides an encouraging basis for its use following in vivo TBI. A microdialysis study by Stoffel and colleagues investigated levels of glutamate and glycine release in the first 24 hours following focal cortical lesion in rats [155]. They measured glycine concentrations of 0.84±0.11µM glycine at baseline, which then peaked at 3.82±0.56µM directly after the primary injury and fell rapidly back to baseline levels. Glutamate levels were measured at 1.45±0.61µM at baseline, which rose to 9.16±3.32µM following trauma and remained elevated for around two hours before returning back to baseline (figure 7.2). This result is particularly interesting for a number of reasons. Firstly, a concern with the use of xenon in human patients would be that extracellular glycine levels would rise to a high enough concentration following injury which could potentially eliminate xenon binding at the glycine site, in the same manner as observed in this study. Stoffels work shows that the peak in glycine concentration is very rapid and that the baseline levels of glycine would be sufficiently low to allow xenon inhibition of the NMDA receptor. Secondly, the rise in glutamate levels that persisted for several hours following injury may provide an explanation for why xenon has been observed to have a 3 hour therapeutic
window for neuroprotection. That is, xenon is at its most effective when glutamate levels are at a toxic high.

Figure 7.3: Course of extracellular glycine (top) and glutamate (bottom) levels in the depth of the parietal cortex directly adjacent to the traumatic tissue necrosis (open circles; sham group; filled circles; trauma group). The first sample after trauma revealed a sharp increase of these excitotoxins in the extracellular space of the penumbra zone. Glycine showed a sudden increase in the sample immediately after injury and went back to baseline thereafter. Glutamate remained on a somewhat elevated level for more than 2 hours. Figures and legend reproduced from Stoffel et al. 2002 [155]
7.3.2 Additional targets of xenon neuroprotection

In addition to NMDA receptor glycine site inhibition a number of other targets of xenon have been identified which may contribute to xenon’s neuroprotective properties, including $K_{\text{ATP}}$ channels, TREK-1 channels and apoptotic and inflammatory pathways. The ATP sensitive K+ channel ($K_{\text{ATP}}$) is thought to play a significant role in the preconditioning neuroprotective effect of xenon. [79]. $K_{\text{ATP}}$ channels are widely distributed in the brain and are activated by metabolic stresses such as ischemia leading to a reduction in excitability and increased resistance to ischemia [156] A study in 2009 by Trapp and colleagues found that exposure to xenon prevented cell death following OGD in vitro, which could be reversed by the presence of the $K_{\text{ATP}}$ channel inhibitor tolbutamide. Patch clamp electrophysiology experiments on HEK293 cells expressing the Kir6.2/SUR1 $K_{\text{ATP}}$ channel revealed that xenon acts as a $K_{\text{ATP}}$ channel opener as shown by an increased $K^+$ current [79]. Further work by this group demonstrated that xenon targets the Kir6.2 pore forming subunit of the $K_{\text{ATP}}$ channel [80]. The role of $K_{\text{ATP}}$ channels in neuroprotection was further demonstrated in a study by Yamada and colleagues [157] who showed that knockout mice lacking the Kir6.2 gene were more sensitive to seizures following ischemia. Xenon’s actions at the $K_{\text{ATP}}$ channel may have important implications in its neuroprotective profile following ischemic and traumatic brain injuries. A reduction in excitability of the brain would be extremely beneficial in limiting the propagation of waves of depolarisation that occur following brain injuries and thus a reduction in secondary injury development. Further to this, xenon may provide neuroprotection via this mechanism where NMDA receptor involvement is minimal, for example where the distribution of these receptors is low, or when glutamate levels have returned to normal physiological concentrations.

In this and other studies it has been demonstrated that xenon activates the two pore domain potassium channel TREK-1 [78]. Channels of this type act normally to modulate neuronal excitability by providing a background leak potassium conductance. Activation of these channels can cause hyperpolarisation of the cells and therefore reduce excitability which may have a role in neuroprotection [74]. Studies by Heurteaux and colleagues [158] using TREK-1 knockout mice found they have a reduced sensitivity to volatile anaesthetics (higher concentrations required for loss of righting reflex and longer time to induction of anaesthesia). Similarly to $K_{\text{ATP}}$ channels, a reduction in excitability via xenons actions at
TREK-1 channels would be extremely beneficial to reduce spreading waves of depolarisation following injury, and thus represents another clinically important mechanism of xenon neuroprotection.

Additional studies have proposed that xenon is capable of modulating the immune and inflammatory systems following injury which may contribute to its neuroprotective effects. Fahlenkamp and colleagues [159] observed that exposure to 74% xenon in cultured microglial cells caused an increase in LPS-stimulated IL-1β mRNA and protein levels, induced a rapid and persisting ERK 1/2 phosphorylation and inhibited phosphatases. Inhibition of phosphatases is likely to underlie the observed ERK 1/2 activation [159] and also affect multiple signalling pathways [160]. In support of this is the observation by Fries et al. [88] that exposure to 70.9 kPa xenon following cardiac arrest in pigs caused a decrease in perivascular inflammation in the caudate and putamen nucleus which translated to an improved functional outcome. Xenon has also been proposed to target apoptotic pathways by regulating the calmodulin-activated kinase II (CaMKII) complex [86]. The CaMKII complex is involved in the control of presynaptic Ca\(^{2+}\) associated events and its activation is associated with over release of neurotransmitters and ultimately cell death [86].

In this study it was observed that when glycine site inhibition by xenon is prevented, the neuroprotective effect is abolished. This could be interpreted as glycine site inhibition being the sole target of xenon neuroprotection, however this is unlikely given the convincing evidence for other targets. An alternative hypothesis to glycine site inhibition being the only important target of xenon neuroprotection is that xenon acts at more than one target, but removing the inhibition of NMDA receptor component from neuroprotection is sufficient for the injurious pathways to overwhelm other neuroprotective mechanisms. This model used the hippocampus as a platform for neuroprotective studies which has a high NMDA receptor density compared to other brain regions. It is therefore reasonable to suggest that NMDA receptor inhibition will be the dominant mechanism of neuroprotection in this model, but this may not be the case for other brain regions \textit{in vivo}, where other targets such as the K\textsubscript{ATP} channel and TREK-1 channels are likely to have a much more dominant role in the neuroprotective profile of xenon. This study therefore can only conclude that NMDA receptor inhibition by xenon forms a portion of the neuroprotective profile of xenon, and is not the sole mechanism, as the model used is somewhat bias towards NMDA receptor mediated cell death. Further work investigating the contribution of NMDA receptor inhibition
and K+ channel opening on xenon neuroprotective profile in other cultured brain regions which are lower in NMDA receptor density would be of great interest. In addition, *in vivo* neuroprotection studies involving genetically altered rodents could aid in determining the relative contributions of each mechanism in the neuroprotective profile of xenon, for example TREK-1 deficient mice, or mice with selective point mutations rendering the NMDA receptor glycine site insensitive to xenon while retaining its affinity for glycine [77]. Whilst this would provide more information about xenons mechanism of action, it could also answer important questions as to when xenon may be most effective, for how long it should be administered for and which patients could be at most benefit from the treatment. This is explained further in section 7.4.

Given the evidence presented in this thesis, and previous evidence of xenons targets in the brain the figure below presents a proposed mechanism of xenon neuroprotection.

*Figure 7.3: Schematic representation of neuroprotective mechanisms of xenon*
7.3.3 Mechanism of argon neuroprotection

Argon also possessed potent neuroprotective properties in this in vitro model of TBI (figure 4.2) albeit less potently than that of xenon. No molecular targets of argon have been fully identified or understood, so at this stage of the investigation it was simplest to rule out (or in) targets that xenon possesses, notably the NMDA receptor glycine site and TREK channels. Whether xenon and argon shared the same mechanism of neuroprotection was a complete unknown so experiments involving the addition of 100µM glycine to the experimental media were performed for argon. It was found that additional glycine had no significant effect on the neuroprotection afforded by 50.6 kPa argon following both TBI and OGD (figure 5.8 and 6.6), indicating that glycine site inhibition of the NMDA receptor was not a key mechanism of argon neuroprotection following both types of brain injury. To strengthen these conclusions electrophysiological experiments were performed by Scott Armstrong on HEK-293 cells expressing NMDA receptors (NR1-NR2A) to assess whether exposure to argon had any effect on NMDA induced currents. These experiments revealed that 80% argon had no effect on NMDA currents at high (100µM) and low (1µM) glycine concentrations, providing further evidence that the glycine site is not a target of argon, or indeed the NMDA receptor itself (at the concentrations investigated). The next target to be investigated was the 2-pore domain K\(^+\) TREK-1 channels. TREK-1 channels are known to be activated by xenon (figure 5.12) however their contribution to xenons neuroprotective profile is currently not fully understood. Electrophysiological techniques were employed (and performed by Scott Armstrong) on HEK-293 expressing TREK-1 channels. It was found that 80% argon (and the other noble gases helium, neon and krypton) had no effect on TREK-1 channel currents (figure 5.13). These results suggested that TREK-1 channels are not a molecular target of argon and therefore this is not a mechanism of neuroprotection. Thus the mechanism of argon neuroprotection remains elusive. An obvious future avenue of investigation will be to investigate whether argon, like xenon, has any effects at K\(_{\text{ATP}}\) channels.

A number of studies have proposed molecular targets of argon that may underlie its neuroprotective effect. A recent paper by Fahlenkamp and colleagues identified the extracellular signal regulated kinase (ERK) 1/2 as a possible target for argon neuroprotection in neurons and glial cells [161]. Activation of ERK signalling can affect many cellular functions, such as cell proliferation and differentiation and activation of gene transcription.
programs. Exposure to 50% argon caused activation of the ERK pathway in neurons and glial cells (microglia and primary astrocytes) after 15-30 minutes of exposure [161]. Zhuang and colleagues [134] found that following neonatal asphyxia treatment with argon increased the expression of the pro-survival proteins bcl-2 and bcl-xL and decreased expression of the pro-apoptotic protein bax. Abraini and colleagues [162] suggest that argon may act at the benzodiazepine site of the GABA_A receptor. This may have an effect on NMDA receptor function as GABA receptor stimulation can lead to inhibition of NMDA receptors, and GABA receptor activation has been shown to be neuroprotective in in vitro and in vivo models of ischemia [163, 164]. Furthermore Zhang et al. [165] provided evidence that GABA agonists can attenuate the phosphorylation of the NR2A subunit, decreasing its functionality and thus exerting a neuroprotective effect. Other studies investigating the neuroprotective effects of GABA agonists unrelated to NMDA receptor function have identified activation of the phosphoinositide 3-kinase/protein kinase b cascade [166] and attenuation of cytochrome c release [167] as possible mechanisms, although their relationship to argon's mechanism of neuroprotection is unknown. The reduced ability to form dipoles by argon compared to xenon is also likely to limit its number of available protein binding sites compared to xenon [103]. Xenon is known to possess multiple targets that are thought to contribute to its neuroprotective profile. If argon were to possess fewer targets (or a single target), then it may be expected that it would be less effective as a neuroprotectant as alternative injury pathways could continue unhindered following injury, although without a full characterisation of all the molecular targets of argon and xenon that contribute to neuroprotection, this remains just speculation.
7.4 What is the clinical importance of these findings?

It has been demonstrated in this thesis and in numerous other pre-clinical studies that xenon and argon are neuroprotective following brain injury. This thesis studied the specific mechanism of both xenon and argon following identification of them as neuroprotectants after *in vitro* traumatic brain injury. On the surface, efficacy in human brain injury appears to be the most important factor with regards to identifying drugs that are neuroprotective, with their specific mechanisms considered a curiosity. However, scratch the surface and there is a lot more that mechanistic studies can tell us. These include optimum dosing parameters and therapeutic time windows, diagnostic tools that may be useful in determining the success of a certain drug in an individual, combination therapies that may produce a beneficial effect, other brain pathologies that the drug may be useful for treating and may aid in the design of novel compounds that target the same mechanism.

Previous studies of both xenon and argon in *in vitro* and *in vivo* models of have estimated that neuroprotection is only achieved if the gases are administered within the first 3 hours after the initial ischemic or traumatic injury [50, 51, 68, 84, 100] with the greatest level of neuroprotection observed within the shortest time frame to administration (ideally, directly after the insult). Time between injury onset and treatment with neuroprotective agents has created complications in human clinical trials of NMDA receptor antagonists. This may be due to the fact that administration of these agents is too many hours later following the peak in glutamate that occurs immediately after the injury where downstream cell death processes have already begun, and thus administration of an NMDA receptor blocker after this time is almost a case of ‘shutting the gate after the horse has bolted’. By knowing that xenon targets the NMDA receptor and the early excitotoxic events after injury this could improve the design of clinical trials whereby xenon (or other drugs acting at the same target) must be given within a very limited time frame if it is possible. However, this may be extremely difficult to implement clinically especially in the case of stroke and milder head injuries which usually don’t present in the first 3 hours after injury. Calculation of the time between injury and first dosing may be simpler in the case of severe TBIs as they often occur in accidental settings requiring emergency medical treatment, such as in the case of road traffic accidents, where witnesses to the injury can approximate when it occurred and relay this information to the emergency response team. There is an increasing realisation that mild and
moderate head injuries do worse than predicted but they do not present as quickly as severe head injuries. NMDA receptor antagonists may not be particularly useful for this subset of patients as NMDA receptor over activation is an early event in the secondary injury cascade and thus requires early intervention. This could effectively rule out xenon as a treatment option in these patients. However the other targets of xenon that contribute to neuroprotection, notably the K\(^+\) channels (TREK-1 and K\(_{\text{ATP}}\)) may be useful in the treatment of these patients, particularly with regard to cortical spreading depression, as drugs that target these channels would reduce the overall excitability of the brain. Perhaps it is this target of xenon neuroprotection that could provide it with a more effective use across a wider subset of head injury patients. In addition, imaging techniques that could identify cortical spreading depression in patients that present with a head injury later than 3 hours post injury could aid in determining who may be most suitable to receive this treatment.

Xenons properties of providing neuroprotection after preconditioning with the gas also provide a potential opportunity in some clinical scenarios. Neonatal asphyxia represents a major cause of death and disability worldwide, with an estimated 2-4 per 1000 full-term infants experiencing an asphyxial episode either antenatally or perinatally, of which an estimated 15-20% die, with 25% of the survivors experiencing severe disability [168]. Studies have found xenon preconditioning to provide neuroprotection in models of ischemia [79, 169] and neonatal asphyxia [170] which gives rise to a number of possible clinical uses of xenon preconditioning, such as before or during birth to provide some pharmacological protection to the unborn child against neurological injury caused by asphyxia or in patients undergoing surgery where risk of an ischemic episode is high, such as during heart surgery or carotid endarterectomy [79]

Combination therapies have been suggested in some publications as ‘the future’ of treating TBI and ischemic brain injuries [8], as different drugs that target different aspects of the secondary injury cascades could be given in parallel to the patient. Obviously this form of treatment requires an understanding of the mechanism being targeted (so the same pathway isn’t targeted), so the use of xenon in combination therapies is realistic option. There is a growing body of evidence that xenon and hypothermia in combination provides a synergistic neuroprotective effect in in vivo studies on rats and pigs following ischemic brain injury [65, 66, 85, 91, 171]. The binding of volatile general anaesthetics is increased at lower temperatures to favourable enthalpic interaction conditions [92-94]. Dickinson and Franks
[74] suggested this synergistic effect may be due to increased binding of xenon at lower temperatures, although a study by Maze and colleagues [91] found that when xenon and hypothermia were administered asynchronously the synergistic effect was still maintained, which indicates that the mechanism is not simply physical binding properties. Hypothermia is also thought to act presynaptically to reduce glutamate release and interfere with the apoptotic cascade [91, 172, 173] and to decrease levels of glycine and p53 protein levels in the brain [171, 174, 175]. How these mechanisms specifically account for the synergistic relationship with xenon is unclear, but the combination of the two therapies represents an exciting treatment option for the future. Another interesting combination therapy to consider would be xenon and argon administered together. If xenon and argon act at different targets then it would be plausible to suggest combination therapy of the two agents to have an additive or synergistic effect, however combining the two would require lowering the concentration of both to allow for sufficient oxygen levels. This would in turn reduce the neuroprotective efficacy of both agents if in combination their effect was additive, but could be viable if their effect in combination was synergistic and thus combination therapy of xenon and argon is a possible consideration for the future.

Translation in efficacy across a range of brain injuries is also possible once a mechanism of a drug is known. NMDA receptor inhibition was understood first to be one of the key mechanisms in neuroprotection following ischemic type brain injuries. Armed with this information and the knowledge that glutamate also plays a key role in the development of secondary injury following TBI formed the rationale for testing xenon in a model of TBI, with success. It is therefore reasonable to hypothesise that where glutamate excitotoxicity plays a key role in a progression of a brain injury (e.g. blast injury and neurodegenerative conditions such as Alzheimer’s disease [176]), xenon may provide some clinical benefit. Xenons actions at potassium channels are also of great importance when considering conditions where spreading excitation is a key pathology. Thus mechanistic research can open up exciting new avenues of research.

Finally, knowing the mechanism of xenon may aid in the development of novel pharmacological compounds that target the same mechanisms. These could be cheaper to obtain and administer (i.e. not requiring closed circuit anaesthesia stations). Given that xenon is an inhaled gas, new compounds could be designed with alternative routes of administration that may be easier to administer where a patient does not require sedation or intubation.
Perhaps this is the most important finding of this study; that any drug that possesses a mechanism of action the same as that of xenon (NMDA receptor inhibition at the glycine site combined with opening of K⁺ channels) may provide clinical benefit in patients following head injury.
7.5 Why have drugs with similar mechanisms failed clinical trials and why might noble gases be different?

The evidence so far supporting the clinical use of xenon following brain injuries is very encouraging, with favourable pharmacological properties, lack of toxicity and robust and reproducible neuroprotection in numerous models of brain injury. However this promising tale is not unfamiliar, as over the years every agent that targeted the excitotoxic component of secondary injury development has failed human clinical trials. This could be for a number of reasons, which can be broadly broken down into issues with the pharmacology of the compound itself, conduction of clinical trials, issues with the role of glutamate itself in contribution to brain damage and issues with the pre-clinical models used to model brain injuries in vitro and in vivo.

In term of pharmacology of the compounds, they have failed for numerous suggested reasons, including high toxicity [177], poor pharmacokinetics, inability to rise to effective concentrations in brain tissues (possibly due to metabolism or poor blood brain barrier penetration), short therapeutic time windows and inappropriate receptor subunit selectivity. Poor design and execution of clinical trials with inappropriate and insensitive assessments of neurological function has also been an issue [46]. Gavestinel is a classic example of a drug with a proposed mechanism the same as that of xenon, in that it is a NMDA receptor glycine site antagonist, that failed in clinical trials. The reasons for gavestinels failure have been proposed as failure to reach the site of action in appropriate concentrations and administration outside of the therapeutic time window at 6 hours post ischemia [178-180]. As mentioned in the previous section, administration of a glutamate receptor antagonist hours and hours after the initial peak in glutamate levels could be futile, as the cell death promoting processes have already begun, and using a treatment that targets an early event in the excitotoxic cascade will inevitably have little effect.

Ikonomidou and Turski [46] propose an alternative view that the issue lies within the concept of glutamate excitotoxicity mediating development of injury. They hypothesise that “glutamate may be involved in the acute neurodestructive phase that occurs immediately after traumatic, or ischemic injury, but that after this period it assumes its normal physiological functions
which includes promotion of neuronal survival” [46]. That is, a continued block of NMDA receptor function could be harmful to the damaged brain instead of helpful.

One very important aspect to consider when assessing why so many agents fail human clinical trials following encouraging pre-clinical success in models of brain injury is the fact that pre-clinical models are heavily controlled, that is, injury is usually isolated to the brain. *In vitro* models typically involve isolated brain regions or single cells cultured in conditions that are amenable to survival of the slices/cells but with less relevance to the real-life scenario, lacking influences from inflammation, blood supply and blood brain barrier breakdown. *In vivo* studies are more useful with regard to these, but the nature of a pre-clinical model is often far removed from the clinical scenario. Injury is isolated to the brain, that is, the poly-trauma that occurs in a large number of head injuries is absent, animals are usually anaesthetised at the time of injury and drug treatment is usually began within a time window that would be very difficult to achieve in real life.

With regard to these issues, xenon’s well characterised safety profile and fast induction of anaesthesia indicates a rapid delivery to brain tissues. A lack of metabolism eliminates the risk of toxic metabolites. Failure to reach the brain in effective concentrations is unlikely with xenon, as neuroprotection studies have been conducted at concentrations lower than that known to cause general anaesthesia. Xenon is thought to only be effective if administered within 3 hours following the initial injury, so it seems possible that the failure of gavestinel is due to administration outside of the therapeutic time window. Since we now know that xenon targets the NMDA receptor as one of the key mechanisms of neuroprotection, in which over activation is an early event in the injury pathway, clinical trials can progress with specific time limits between injury and administration. In terms of extended blockade of NMDA receptors being harmful, Dingley and colleagues propose a reason why this issue may be circumvented by the use of xenon, as they hypothesise that although it may be true that complete NMDA receptor blockade can be pro-apoptotic, a dose of 80% xenon only partially antagonises NMDA receptor currents by reducing them to approximately 60% [58, 71]. Xenon also has a number of targets within the brain which are thought to contribute to neuroprotection. This confers another advantage to its use, as pre-clinical models that target limited injury pathways will be biased towards showing robust neuroprotection by agents that target that pathway (for example, NMDA receptor antagonists following direct injection of NMDA), which may not translate well to the clinical scenario where a large number of
chaotic injury pathways are triggered in parallel. It is hopeful that xenons combined NMDA receptor blockade and potentiation of $K^+$ channel currents could provide a unique pharmacological profile to limit secondary injury spread. Possible improvements to the techniques of modelling traumatic injury in vitro in discussed in the following section.

A major limitation to the widespread use of xenon is the high cost due to its rarity in the atmosphere, currently around £25 per litre. However due to its lack of metabolism, xenon can be re-used or recycled if it is delivered to the patient using a closed circuit. Dingley and colleagues have developed a closed circuit xenon anaesthesia delivery system that could also be used for neuroprotection [181]. A 50% gas mixture of xenon was administered for nearly 6 hours at a total cost of around £540 ($148.5/hr). This may seem like a pricey treatment option, but the NHS figures for long term treatment for a young person sustaining a serious head injury to be a staggering £4.89 million over a lifetime for a single patient. This figure takes in account the cost of medical care, educational costs, direct social costs, missed employment, lost tax revenue, and benefits. Given these massive economic costs that are incurred for the long term treatment and rehabilitation of brain injury patients, then the cost of xenon treatment would be negated by longer term reductions in spending.

In comparison to xenon, argon is more abundant in the atmosphere (in fact, the most abundant noble gas in the atmosphere at 0.93% [161]) and as a result is cheaper to obtain, negating the need for a closed circuit delivery system [103]. This is a major advantage of argon. Another advantage lies with its property of not being capable of causing general anaesthesia at normobaric pressures which may be of benefit in clinical scenarios where sedation is not desired, such as acute, focal injury such as stroke [103]. Before its neuroprotective properties were discovered, argon has had very limited use as a pharmacological agent and thus there is a lack of data regarding its specific pharmacological properties, although it is understood to be non-toxic to humans.
7.4 Further Studies

Further experiments and improvements to the model

The work presented in this thesis open up some interesting avenues for further investigation in order to characterise xenon and argons mechanism of action further and address some of the issues highlighted in this discussion. Firstly, the data presented here demonstrates a robust neuroprotection by xenon even at lower partial pressures. It would therefore be of interest to lower the concentration of xenon further in these experiments to see if a similar level of neuroprotection is achieved, which could have economic advantages to its use clinically. Previous studies have suggested that neuroprotection by xenon in vitro can only be achieved if the initial treatment is started within 3 hours post injury, which is hypothesised to correlate to raised levels of glutamate. It would therefore be interesting to see how the injury progresses if the treatment is removed after a certain period of time, for example immediate treatment followed by withdrawal of treatment after 3 hours, will the neuroprotective effect remain or will it continue? A sustained neuroprotective effect could possibly indicate alternative mechanisms of action, for example an inhibition of spreading depression by its actions at $K^+$ channels. In addition this may be more relevant to the clinical scenario where 72 hour administration of a drug may not be feasible (xenon is sedative so would not allow for assessment of improvement using the GCS during this time, argon is not sedative so may be better) and would be expensive to implement.

Another interesting avenue of investigation would be to culture organotypic slices from other brain regions which are lower in NMDA receptor density, to identify the contribution of glycine site inhibition and the contribution of $K^+$ channel opening in different regions of the brain. One possibly is that lower NMDA receptor density would translate to a lower efficacy of xenon as a neuroprotectant. Alternatively, efficacy would be retained but alternative neuroprotective targets of xenon ($K^+$ channels) would have a much more dominant role than in the current study.

From a clinical perspective ischemic brain injury occurs as a consequence of the traumatic brain injury. This model could therefore be improved to include an element of ischemic brain
injury that occurs after the primary injury to see if the neuroprotective effects of these drugs are sustained during a more severe insult that is closer to the clinical scenario.

What is perhaps quite an obvious avenue of investigation following the results of this study is the potential effect of argon on $K_{\text{ATP}}$ channels, as its effects on NMDA receptors and TREK-1 channels have effectively been ruled out. Experiments performed similar to that of Trapp and colleagues [80] could answer this question simply.

*Xenon neuroprotection of oligodendrocytes following in vitro TBI*

With the knowledge that one of the mechanisms of xenon neuroprotection following TBI involves inhibition of the NMDA receptor, this opens up an intriguing line of thought that xenon may be beneficial for the protection of glial cells, such as oligodendrocytes, as well as neurones. Oligodendrocytes are a type of glial cell that are crucial for the function of the brain [182]. They are responsible for the myelination of axons and thus the fast propagation of action potentials, allowing for rapid transmission of information between brain regions [182]. Oligodendrocytes are known to possess functional NMDA receptors at all stages of development, which are thought to contribute to the myelination process in a use-dependant manner [183, 184]. Myelination begins with the extension of multiple processes from the somata of the oligodendrocytes which make contact with the axon. It has been proposed that calcium influx through activated NMDA receptors on these processes governs the decision to proceed with myelination or retract from the axon [183]. NR1, NR2 and NR3 subunits have been detected in myelin [185], however oligodendrocyte NMDA receptors are thought to differ from neuronal NMDA receptors in that they possess an unusual combination of subunits, thought to be NR1, NR2C and NR3 with a weak $Mg^{2+}$ block [184].

A number of studies have shown that the presence of NMDA receptors on oligodendrocyte processes confers a high sensitivity to damage following various forms of brain injury, such as ischemic brain injury, in a calcium dependant manner [183-185]. Under conditions of excess glutamate the levels of $Ca^{2+}$ within the myelin is raised which destroys the myelinating processes of oligodendrocytes [183, 185, 186]. Furthermore, injured axons accumulate intracellular $Na^+$, lose $K^+$, and depolarise which promotes the release of excess glutamate [185]. Oligodendrocyte processes are particularly susceptible to excitotoxic damage due to their small dimension and low resistance to oxidative stress [183]. Studies investigating NMDA receptor blockade as a neuroprotective strategy for oligodendrocytes have found that exposure the MK-801 (an NMDA receptor channel blocker) and 7-
chlorokynurenic acid (a glycine site antagonist) following ischemic injury can prevent calcium mediated damage to oligodendrocyte processes [183, 185, 186].

It has been proposed that the vulnerability of oligodendrocytes to excitotoxicity and the subsequent downstream effects on myelination of axons may contribute to the loss of brain function following a TBI [187]. Given that the evidence in this thesis proposes that xenon provides neuroprotection by antagonism of the NMDA receptor, it seems feasible to hypothesise that it may also provide a degree of protection to oligodendrocytes. A study by Berger and Frotscher in 1994 [188] identified the presence of functional oligodendrocytes in organotypic hippocampal slices from rats, prepared using the Stoppini interface method [109]. This implies that the current technique to model TBI in vitro could be adapted to carry out these investigations. Immunohistochemical techniques could be employed for detecting oligodendrocyte processes. Propidium iodide is not an ideal choice for this type of investigation, as it has been proposed that when the processes are damaged the cell seals of its membrane to protect the soma, with the possibility of regenerating new processes [182]. This would be an interesting and potentially clinically relevant avenue of investigation in the future, and may provide further evidence that xenon will improve functional recovery after a TBI.
7.6 Summary

This study investigated the potential neuroprotective effects of the noble gases helium, neon, argon, krypton and xenon in a controlled \textit{in vitro} model of TBI; the first of its kind. The initial stages of this study involved the design and calibration of a device capable of modelling a traumatic brain injury \textit{in vitro} which possessed the characteristics of a TBI \textit{in vivo}, specifically the development of a secondary injury.

In neuroprotection studies following TBI, only xenon and argon were found to possess neuroprotective properties, with helium, neon and krypton found to possess none. Xenon was found to be more efficacious as a neuroprotectant than argon in this study as it was found to completely abolish the development of secondary injury in comparison to argon which attenuated the injury when both were administered at the same concentration of 50.6 kPa. Following OGD \textit{in vitro} 50.6 kPa xenon and argon were found to be equally potent.

NMDA receptor over-activation forms a key component in the spread of secondary injury following a TBI. Mechanistic studies revealed that xenon mediates neuroprotection following TBI by competitive inhibition of the glycine co-agonist site of the NMDA receptor, as demonstrated by a reversal of its neuroprotective effect when a high concentration of exogenous glycine was added to the experimental culture media. Argon neuroprotection could not be reversed by elevated glycine, indicating that argon does not act at the NMDA receptor glycine site. This finding was confirmed by electrophysiology showing argon does not inhibit NMDA receptors or TREK-1 channels.
7.7 Conclusions

This thesis has addressed the importance of knowing the mechanism of a pharmacological compound as opposed to just whether it works or not. Given that xenon is known to have an excellent safety profile and is already in clinical trials for neuroprotection following ischemic injury it does seem reasonable to postulate that clinical trials administering xenon to trauma patients should be started as soon as possible. However, studies of this kind that seek to determine the mechanism of xenon neuroprotection following TBI could impact on the success of clinical trials. For example, a characteristic of a head injury involves a sudden increase in the extracellular concentration of glutamate that can remain elevated for several hours (as demonstrated in a rodent model of TBI [155]). As we now understand one of the components of xenon’s neuroprotective profile following TBI involves inhibition of glutamatergic NMDA receptors and this property then indicates a maximum effect by xenon may only be achieved whilst glutamate levels remain elevated, that is, within the first few hours following the initial injury. When extracellular glutamate returns to normal physiological levels treatment may no longer be required, although xenon has other pharmacological targets that may provide neuroprotective benefits after glutamate levels have decreased via actions at potassium channels. Perhaps the most interesting aspect of determining the mechanism of a neuroprotective drug is the fact that it opens up avenues for development of compounds that act at the same targets.

Adverse effects are another concern for patients, especially those very vulnerable following a severe trauma and glycine site antagonists (such as gavestinel) have been previously shown to be well tolerated in patients. Generally, the pharmacological properties of xenon are favourable for its use clinically, with a lack of reported toxicity, lack of metabolism, potent analgesia and no known adverse effects. Knowing the mechanism of xenon neuroprotection may also open up exciting avenues for where combination therapy may be beneficial. Indeed, the knowledge that argon targets a separate pathway of injury that does not involve the NMDA receptor suggests that the two gases in combination may have enhanced benefits. The high costs of xenon which have so far hindered its use as a general anaesthetic can be circumvented by the use of closed circuit delivery systems which are commercially available. In conclusion, the results of this study form a solid foundation in which to continue to in vivo
studies of xenon and argon neuroprotection following traumatic brain injury and to further investigate potential other mechanisms of xenon and argon neuroprotection.
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This thesis is dedicated to Bruce Porter

Firstly I would like to thank you, the reader, for making it this far. I hope this thesis has instilled some hope in you that the treatment for brain injuries is within our sights.

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Appendix A

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Neuroprotection against Traumatic Brain Injury by Xenon, but Not Argon, Is Mediated by Inhibition at the N-Methyl-D-Aspartate Receptor Glycine Site


ABSTRACT

Background: Xenon, the inert anesthetic gas, is neuroprotective in models of brain injury. The authors investigate the neuroprotective mechanisms of the inert gases such as xenon, argon, krypton, neon, and helium in an in vitro model of traumatic brain injury.

Methods: The authors use an in vitro model using mouse organotypic hippocampal brain slices, subjected to a focal mechanical trauma, with injury quantified by propidium iodide fluorescence. Patch clamp electrophysiology is used to investigate the effect of the inert gases on N-methyl-D-aspartate receptors and TREK-1 channels, two molecular targets likely to play a role in neuroprotection.

What We Already Know about This Topic

* Xenon has been shown to be neuroprotective in ischemic brain injury models
* The cellular mechanisms of this effect are not well understood

What This Article Tells Us That Is New

* Given after traumatic injury to hippocampal slices, xenon (and argon to a lesser degree) halves the secondary neuronal injury
* N-methyl-D-aspartate antagonism is an important component of this protective effect

Results: Xenon (50%) and, to a lesser extent, argon (50%) are neuroprotective against traumatic injury when applied after injury (xenon 43 ± 1% protection at 72h after injury [N = 104]; argon 30 ± 6% protection [N = 44]; mean ± SEM). Helium, neon, and krypton are devoid of neuroprotective effect. Xenon (50%) prevents development of secondary injury up to 48h after trauma. Argon (50%) attenuates secondary injury, but is less effective than xenon (xenon 50 ± 5% reduction in secondary injury at 72h after injury [N = 104]; argon 34 ± 8% reduction [N = 44]; mean ± SEM). Glycine reverses the neuroprotective effect of xenon, but not argon, consistent with competitive inhibition at the N-methyl-D-aspartate receptor glycine site mediating xenon neuroprotection against traumatic brain injury. Xenon inhibits N-methyl-D-aspartate receptors and activates TREK-1 channels, whereas argon, krypton, neon, and helium have no effect on these ion channels.

Conclusions: Xenon neuroprotection against traumatic brain injury can be reversed by increasing the glycine concentration, consistent with inhibition at the N-methyl-D-aspartate receptor glycine site playing a significant role in xenon neuroprotection. Argon and xenon do not act via the same mechanism.

T RAUMATIC brain injury (TBI) is a major cause of death and disability throughout the world. In developed countries, TBI is the main cause of death and disability in those who are aged less than 45 yr with falls and motor
vehicle crashes being the leading causes.\textsuperscript{2} Currently, there are no treatments aimed specifically at preventing neuronal loss after TBI.\textsuperscript{1-3} TBI is characterized by a "primary injury" determined by the initial mechanical force, followed by a "secondary injury" developing hours to days after the initial trauma. Secondary injury is thought to underlie the majority of the short- and long-term neurological and cognitive impairments that follow TBI.\textsuperscript{4-6} hence treatments to prevent or limit secondary injury are of clinical importance. The pathophysiology of secondary injury is complex, involving multiple injury cascades,\textsuperscript{7-10} but glutamate excitotoxicity is believed to play a key role.\textsuperscript{11-15}

The anesthetic noble gas xenon inhibits the N-methyl-D-aspartate (NMDA)-subtype of glutamate receptor and has been shown to be neuroprotective in models of brain injury.\textsuperscript{16-24} In this article, we test the hypothesis that, in addition to xenon, other noble gases are neuroprotective in an in vitro model of TBI. After the finding that xenon protects against ischemic injury, other noble gases have been investigated as potential neuroprotectants in models of ischemia.\textsuperscript{25-27} Argon has been found to be protective in in vitro and in vivo models of ischemia.\textsuperscript{27-30} However, a recent study reported a protective effect of argon in vitro but no effect in vivo.\textsuperscript{31} The situation with the other noble gases is even less clear. Some studies report helium is neuroprotective against ischemia,\textsuperscript{19,32,33} whereas others find no effect.\textsuperscript{34} Another study found that helium, neon, and krypton were actually detrimental in ischemic injury.\textsuperscript{23} In traumatic injury, the potential protective effect of the noble gases has been less well studied, but xenon, helium, and argon are reported to be protective in in vitro models of trauma.\textsuperscript{19,35} However, to date, there have been no studies systematically investigating the complete series of noble gases under identical conditions in a model of trauma. Furthermore, there have been few studies that have investigated the effects of inert gases (except xenon) on molecular targets that may be involved in neuroprotection\textsuperscript{26} such as NMDA receptors and two-pore domain potassium channels.

We recently showed that the mechanism of xenon protection against ischemic injury is mediated by inhibition at the NMDA receptor glycine site.\textsuperscript{36} In this study, we test the hypothesis that xenon’s protective effect against TBI is mediated by inhibition at the NMDA receptor glycine-binding site. We also test the hypothesis that xenon and other neuroprotective inert gases act via the same mechanism. We discovered that xenon is an NMDA receptor antagonist and that it acts by competing with the cosynaptic glycine.\textsuperscript{37} This means that xenon inhibits NMDA receptors less at higher glycine concentrations.\textsuperscript{38} This property can be used as a pharmacological tool to investigate the mechanism of xenon neuroprotection. If xenon neuroprotection can be reversed by increased glycine concentration, this is consistent with the effect being due to NMDA receptor glycine site inhibition.\textsuperscript{39} Materials and Methods

Hippocampal Organotypic Slices

All experiments were performed in compliance with the United Kingdom Animals (Scientific Procedures) Act of 1986 and the Ethical Review Committee of Imperial College London, London, United Kingdom. All efforts were made to minimize animal suffering and the number of animals used. Unless otherwise stated, chemicals were obtained from Sigma Chemical Company Ltd. (Poole, Dorset, United Kingdom). Organotypic hippocampal slice cultures were prepared as previously described\textsuperscript{39} with some modifications. In brief, 6-day-old C57BL/6 mouse pups (Harlan Ltd., Bicester, Oxfordshire, United Kingdom) were humanely killed by cervical dislocation, brains were removed and placed in ice-cold "preparation" medium. The preparation medium contained Gey balanced salt solution, 5 mg/ml of d-glucose, and 1% antibiotic–antimycotic suspension. The hippocampi were removed from the brains and 400-μm thick transverse slices were prepared using a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Guildford, Surrey, United Kingdom). Slices were transferred into ice-cold preparation medium, gently separated under a stereomicroscope, and then placed onto tissue-culture inserts (Millicell-CM; Millipore Corporation, Carrigtwohill, Co., Cork, Ireland) that were inserted into 35-mm Petri dishes. The wells contained "growth" medium, which consisted of 50% Eagle minimal essential media, 25% Hank balanced salt solution, 25% inactivated horse serum, 3 μM of L-glutamine, 5 mg/ml of d-glucose, 1% antibiotic–antimycotic suspension, and 10 mM of HEPES titrated to pH 7.2. Slices were incubated at 37°C in a 95% air–5% CO₂ humidified atmosphere. The growth medium was changed every 3 days. Experiments were carried out after 12 days in culture.

Traumatic Injury and Hyperbaric Gas Chamber

After the hippocampal slices had been in culture for 12 days, the inserts were transferred to six-well tissue-culture plates, and the growth medium was changed to "experimental" medium. The experimental medium was serum-free and consisted of 75% Eagle minimal essential media, 25% Hank balanced salt solution, 3 μM of L-glutamine, 5 mg/ml of d-glucose, 1% antibiotic–antimycotic suspension, 4.5 μM of propidium iodide (PI), and 10 mM of HEPES titrated to pH 7.2. One hour after transfer to experimental media, the slices were imaged to assess slice viability before injury. Typically, slices exhibited very little PI fluorescence, an indicator of tissue health. A small number of slices showed regions of dense staining, indicating compromised viability, presumably due to mechanical damage during the slice preparation stage. These slices were excluded from further analysis. One hour after imaging, slices were subjected to traumatic injury. The trauma was produced with a specially designed apparatus based on published descriptions.\textsuperscript{40-44} Under a
stereomicroscope, a stylus was positioned above the CA1 region of the hippocampus using a three-axis micromanipulator. The stylus dropped onto the slice when power to a small electromagnet was switched off. The distal part of the stylus was smooth and rounded to prevent perforation of the slice and the impact produced a focal injury with a diameter of 340 ± 1.2 μm (N = 50 slices).

After traumatizing the CA1 region, the culture plates were immediately transferred to a small pressure chamber, which contained a high-speed fan for rapid gas mixing, housed in an incubator set at 37°C. The chamber (gas volume 0.925 l) was flushed with humidified control gas (75% nitrogen−20% oxygen−5% CO2) for 5 min at 5 l/min, which would ensure better than 99.99% gas replacement. After flushing, the pressure chamber was sealed and 0.5 atm of noble gas was added, giving a total pressure of 1.5 atm. After a given time in the chamber (30 min to 24 h), the slices were imaged using a fluorescent microscope (see ‘Quantifying Cell Injury’). It was previously shown that uptake of PI by slices is complete within 30 min.19 After completing the imaging, the slices were transferred back to the pressure chamber and the appropriate gas mixture reestablished. This procedure was repeated at 48 and 72 h after trauma. Note that, for all gas mixtures and for all pressures, the partial pressures of oxygen and carbon dioxide were fixed at 0.2 and 0.05 atm, respectively.

Quantifying Cell Injury
PI is a membrane-impermeable dye that only enters cells with damaged cell membranes.20 Inside the cells, it binds principally to DNA and becomes highly fluorescent, with a peak emission spectrum in the red region of the visible spectrum. An epi-illumination microscope (Nikon Eclipse 80; Kingston upon Thames, Surrey, United Kingdom) with a low-power (x2) objective was used to visualize the PI fluorescence. A digital video camera and software (MicroPublisher 3.3 RTV camera and QCapture Pro software; Qimaging Inc., Surrey, British Columbia, Canada) were used to capture the images. The images were analyzed using ImageJ software.21 Red, green, and blue channels were recorded, but only the red channel was used and the distribution of intensities was plotted as a histogram with 256 intensity levels. Slices under standard control conditions (incubated in the chamber for 72 h at 37°C with 95% air and 5% CO2) showed a well-defined peak in the intensity distribution which fell rapidly to zero. In contrast, after trauma, the peak in the intensity distribution was lower, broader, and shifted to higher intensity levels. As a measure of trauma, we integrated the number of pixels above a threshold of 50, which under our experimental conditions provided a robust quantitative measurement of PI fluorescence, and hence of cell injury.22 Because the light output from the mercury lamp changed over time, the exposure time was adjusted to take this into account.

This was done by recording fluorescence from a glass slide standard (Fluor-Ref; Omega Optical, Brattleboro, VT) and adjusting the exposure time accordingly.

Electrophysiology
Human embryonic kidney, cells (HEA293) were plated on glass cover slips and transfected with complementary DNA for rat NMDA receptor GluN1-1a and GluN2A receptors or human TREK-1 channels and green fluorescent protein for identification, as described previously.18,62 Whole-cell recordings were made using an Axoclamp 200B amplifier (Axon Instruments, Foster City, CA). Pipettes (3–5 MΩ) were fabricated from borosilicate glass. For NMDA receptor experiments, internal solution contained 110 ms of K-glucuronate, 2.5 mm of NaCl, 10 mm of HEPES, 10 mm of 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, titrated to pH 7.3 using KOH, and the extracellular solution contained: 150 mm of NaCl, 2.5 mm of KCl, 2 mm of CaCl2, 10 mm of HEPES, titrated to pH 7.35 using NaOH. Cells were voltage clamped at −60 mV. For TREK-1 experiments, the intracellular solution contained 120 mm of KCl, 50 mm of NaCl, 1.2 mm of MgCl2, 15 mm of EGTA, 10 mm of HEPES, 3 mm of MgATP, and 0.3 mm of NaGTP, titrated to pH 7.3 with KOH, and the extracellular solution contained 145 mm of NaCl, 2.5 mm of KCl, 1 mm of CaCl2, 2 mm of MgCl2, 10 mm of HEPES, 10 mm of d-Glucose, titrated to pH 7.4 with NaOH. Cells were voltage clamped at −80 mV and voltage ramps from −120 to 0 mV and from 0 to −80 mV were performed over 250 ms. Currents were filtered at 100 Hz (−3 dB) using an eight-pole Bessel filter (model 900; Frequency Devices Inc., Otawa, IL), digitized at 20 kHz (Digidata 1322A; Axon Instruments) and stored on a computer. Solutions containing the inert gases were prepared by bubbling gases through sintered glass bubbles in Dreschel bottles containing extracellular saline, as described previously.27,44

Statistical Analysis
We assessed significance using two-way ANOVA, with Bonferroni post hoc test. Factor 1 was inert gas or treatment (He, Xe, Kr, glycine, and others) and factor 2 was the time after injury (e.g., 24, 48, and 72 h). Our experimental design involved measuring the injury development over time in the same set of slices, for a given experimental condition (e.g., inert gas); in most cases, these times were at 24, 48, and 72 h after injury. We, therefore, used repeated-measures ANOVA with factor 1 as the repeated factor. We used two-tailed hypothesis testing with P values of less than 0.05 taken to indicate a significant difference among groups. The samples sizes (N) are indicated in the figures. For the main inert gas neuroprotection experiments, we aimed to have samples sizes of at least 30–40 slices in each group. In every experiment, the slices were imaged at 1 h before the experiment to assess slice viability before injury; a small number of slices showed regions of dense PI staining.
Fig. 1. (A) Propidium iodide fluorescent images of slices at 1 h before injury, and 30 min, 24 h, 48 h, and 72 h after injury. (B) Development of injury (black bars) compared to sham (white bars) quantified by propidium iodide fluorescence intensity, after injury with impact energy of 3.5 μJ. The error bars are standard errors. *Indicates value significantly different (P < 0.05) from injured slices at each time point (N = 48: traumatic brain injury (TBI); t = –1, 24, 48, and 72 h; N = 33: sham, t = –1, 24, 48, and 72 h; N = 5: TBI 0.5 h; N = 4: sham 0.5 h; N = 11: TBI 1 h; N = 13: sham 1 h; N = 19: TBI 6 h; N = 8: sham 6 h; N = 13: TBI 12 h; N = 8: sham 12 h). (C) Development of injury with different traumatic impact energies. Uninjured sham slices are shown as white bars, compared with injured slices with impact energies of 1.4 μJ (hatched bars), 2.7 μJ (crosshatched bars), and 3.5 μJ (black bars). Data have been normalized to 3.5 μJ at 72 h after injury. The error bars are standard errors. *Indicates value significantly different (P < 0.05) from sham slices at each time point (N = 33: sham; N = 43: 3.5 μJ; N = 7: 1.4 μJ, 2.7 μJ). (D) The addition of 0.5 atm of helium had no effect on the injured or sham-treated slices. Sham-treated slices are shown as white bars (no helium) or white crosshatched bars (helium), and injured slices are shown as grey bars (no helium) or grey crosshatched bars (helium). The error bars are standard errors. The data have been normalized to traumatic injury with 0.5 atm helium at 72 h after injury. *Indicates value significantly different (P < 0.001) from injured slices at each time point (N = 141 helium TBI; N = 105 helium sham; N = 23 no helium TBI; N = 25 no helium sham).
Results

Traumatic Injury and Development of Secondary Injury

We first investigated the development of control injury in our model (fig. 1). To determine the optimum traumatic injury, we performed a series of experiments with impact energies from 1.4 to 3.5 μJ (fig. 1C). We aimed to determine an impact energy that would produce a focal primary lesion and that exhibited a developing secondary injury. We found that an impact energy of 3.5 μJ, corresponding to a stylus drop of 5 mm, produced a consistent and reproducible focal traumatic injury that developed significantly (P < 0.001) over 72 h, compared with uninjured slices, and we chose 3.5 μJ as our standard injury. To determine the role of the NMDA receptor in our injury model, we performed experiments with the NMDA receptor open-channel blocker MK801. We found that 100 μM MK801 reduced injury to 37 ± 12%, 42 ± 4%, and 84 ± 7% of control at 24, 48, and 72 h after injury, respectively (N = 7; data not shown). The large reduction in injury at 24 and 48 h is consistent with a role of NMDA receptor in injury development, whereas the attenuation of the reduction at 72 h most likely reflects the intrinsic toxicity that has been reported for MK801 and other open-channel blockers such as ketamine. We wished to determine how the injury developed early after trauma with the aim of distinguishing the primary lesion and secondary injury development over time. The development of the injury was determined at time intervals from 30 min to 72 h after trauma. Figure 1A shows images of slices 1 h before injury and 30 min, 24 h, 48 h, and 72 h after traumatic injury. In these experiments, we limited the number of imaging sessions for a given slice set to four (to avoid having slices out of the stable temperature and humidity of the chamber for extended periods). Figure 1B shows the development of the injury from 30 min to 72 h after trauma. The earliest time point that we could observe injury compared with sham slices was 30 min after impact. At this point, injury was 8 ± 2% of total injury at 72 h, whereas sham slices not subjected to injury had a barely measurable PI fluorescence. We took this 30-min point as a measure of the "primary injury" in our model. Secondary injury began to develop early in this model. By 1 h after trauma, the injury had developed to 32 ± 4% of total and by 6 h the injury development was 51 ± 8% of the total injury, increasing at 12 h to 67 ± 10% of the total injury present at 72 h.

Because the neuroprotective experiments would use 0.5 atm of noble gases, we determined whether pressure per se would affect the injury. The effect of 0.5 atm helium on both sham-treated and trauma-injured slices was investigated (fig. 1D). Helium was chosen because it is unlikely to exert any pharmacologic effect of its own at these low pressures and any effect observed can be attributed to the effect of pressure alone. Figure 1D shows that 0.5 atm helium had no significant effect (P > 0.3) on either the sham- or trauma-injured slices at any time point. Nevertheless, in the experiments with xenon and the other noble gases, we used 0.5 atm helium as a control for any effect of the added pressure.
Neuroprotection by the Noble Gases

Figure 2A shows the effect of 50% atm of xenon, argon, neon, and krypton on injury development at 24, 48, and 72 h after trauma. Xenon and argon showed a significant neuroprotection ($P < 0.01$) at each time point, but krypton, neon, and helium were without effect. Xenon potently protected against trauma, with injury in xenon-treated slices being reduced by $57 \pm 3\%$ ($P < 0.001$) compared with the untreated injured slices 24 h after injury. A similar degree of protection ($56 \pm 3\%; P < 0.001$) was seen 48 h after injury, whereas at 72 h after injury, the degree of protection by xenon was $43 \pm 3\%$ ($P < 0.001$). Argon was less effective than xenon, but exhibited a similar trend, with injury in argon-treated slices being reduced by $38 \pm 7\%$ ($P < 0.01$) compared with untreated injured slices 24 h after injury, $43 \pm 7\%$ ($P < 0.001$) at 48 h after injury, and $30 \pm 7\%$ ($P < 0.001$) at 72 h after injury. We further investigated the relative efficacy of xenon and argon by determining the concentration–response of these two inert gases (fig. 2B). Xenon was more effective than argon at all concentrations. At a concentration of 30% atm, xenon provided protection of $40 \pm 8\%$, whereas argon was without protective effect ($6 \pm 7\%$) at the same concentration. At 50% atm, xenon provided $43 \pm 3\%$ protection, whereas argon provided $30 \pm 7\%$ protection, and at 70% atm, xenon provided protection of $52 \pm 13\%$, whereas argon protected by $40 \pm 10\%$.

Xenon and Argon Reduce Secondary Injury Development

To understand the mechanism of action of xenon and argon, we determined the effect of these gases on secondary injury development (fig. 3), calculated by subtracting the primary injury measured at 30 min after trauma. Xenon was particularly effective at preventing development of the secondary injury. At 24 h after injury, xenon-treated slices were almost identical to uninjured sham slices, with injury of $14 \pm 2\%$ and $15 \pm 1\%$ of the total injury at 72 h in xenon-treated and sham slices, respectively (fig. 3A). The same was true at 48 h with xenon-treated slices $23 \pm 2\%$ of total injury compared with uninjured sham slices $24 \pm 2\%$ of total injury at 72 h after trauma. Xenon-treated slices exhibited greater injury ($43 \pm 4\%$) compared with uninjured sham slices ($30 \pm 3\%$), but this difference was not significant ($P = 0.8$). Compared to untreated injured slices, xenon reduced secondary injury by $50 \pm 3\%$ at 72 h, and this was highly significant ($P < 0.001$). Argon attenuated secondary injury, but was less effective than xenon (fig. 3B), reducing secondary injury significantly by $48 \pm 9\%$ ($P < 0.01$) at 24 h, $51 \pm 7\%$ ($P < 0.001$) at 48 h, and by $34 \pm 8\%$ ($P < 0.001$) at 72 h after injury.

Mechanism of Neuroprotection by Xenon and Argon

To test the hypothesis that xenon neuroprotection is mediated by competitive inhibition at the NMDA receptor, we performed experiments where we investigated whether increased glycine concentration could reverse xenon’s neuroprotective action. We chose to use glycine rather than serine in these experiments because we had previously established from electrophysiological experiments that glycine attenuated the effects of xenon at the NMDA receptor, and we had established accurate glycine concentration–response relationships for the GluN1/GluN2A and GluN1/GluN2B subunit combinations that predominate in the hippocampus.37,38 We first established whether adding glycine had an effect on injury in our in vitro model. Figure 4A shows the effect of adding 100 μM glycine on trauma-injured or sham-treated slices. We chose a concentration of 100 μM glycine because this is a saturating concentration for the GluN1/ GluN2A and GluN1/GluN2B subunit combinations.37,38

Fig. 3. Effect of xenon and argon on secondary injury development. (A) Xenon is particularly effective at preventing development of secondary injury. At the 24, 48, and 72 h time points, xenon-treated slices (red bar) were not significantly different to uninjured sham slices (white bar). At the 72-h time point, secondary injury in xenon-treated slices was $50 \pm 5\%$ of secondary injury in untreated injured slices (black bar). The error bars are standard errors. (B) Argon attenuates development of secondary injury at 24, 48, and 72 h after injury. At the 72-h time point, argon–treated slices (cyan bar) were $66 \pm 8\%$ of secondary injury in untreated injured slices (black bar). The error bars are standard errors. *Indicates value significantly different ($P < 0.001$) from control injury at each time point. #Indicates value significantly different ($P < 0.01$) from control injury ($N = 141$ control traumatic injury; $N = 105$ sham; $N = 104$ xenon; $N = 44$ argon).
Fig. 4. Glycine reverses neuroprotective effect of xenon but not argon. (A) The addition of glycine or the inhibitory glycine receptor antagonist strychnine has no effect on injury development or sham-treated slices. Sham slices (white bars) were not significantly different in the presence of 100 μM glycine (white crosshatched bars) or 100 nM strychnine (white hatched bars) at any time point. Similarly, the development of control injury (grey bars) was not significantly different in the presence of 100 μM glycine (grey crosshatched bars) or 100 nM strychnine (grey hatched bars) at any time point. The error bars are standard errors (N = 141 control traumatic brain injury (TBI); N = 105 sham; N = 30 glycine TBI; N = 35 glycine sham; N = 18 strychnine TBI; N = 19 strychnine sham). (B) Glycine reverses the neuroprotective effect of 50% xenon. In the absence of glycine, 50% xenon (red bars) protects against trauma (grey bars). The addition of 100 μM glycine abolishes the protective effect of 50% xenon. In the presence of glycine, there was no significant difference between injured slices in the absence (grey crosshatched bars) or presence of xenon (red crosshatched bars) at any time point. The error bars are standard errors (N = 141 TBI; N = 104 xenon; N = 32 xenon glycine; N = 39 glycine TBI). (C) Glycine does not reverse the neuroprotective effect of 50% argon. In the absence of glycine, 50% argon (cyan bars) protects against trauma (grey bars). In the presence of 100 μM glycine, 50% argon (cyan crosshatched bars) retains a protective effect compared with injured slices (crosshatched grey bars). The error bars are standard errors (N = 141 TBI; N = 44 xenon; N = 37 argon glycine; N = 39 glycine TBI). " Indicates value significantly different (P < 0.001) from the control injury at each time point. * Indicates value significantly different (P < 0.05) from control injury at each time point.

We found there was no significant difference in the injured slices at 24, 48, or 72 h (P > 0.6) after injury in the presence of 100 μM glycine. Similarly, sham-control slices in the absence and presence of glycine were not significantly different at 24, 48, or 72 h (P > 0.6). To rule out a possible role of strychnine-sensitive inhibitory glycine receptors in our injury model, we tested the effect of adding 100 nM strychnine, a concentration that has been shown to abolish inhibitory glycine receptor responses at glycine concentrations up to 300 μM. Strychnine had no significant effect on injured (P > 0.6) or sham slices (P > 0.6) at any time point (fig. 4A). Having established that adding glycine did not affect the magnitude of the traumatic injury or its development over time, we investigated the effect of glycine on the neuroprotective effect of xenon. Figure 4B shows that in the absence of added glycine, 50% atm xenon had a significant neuroprotective effect, reducing injury to 43 ± 3%, 44 ± 3%, and 57 ± 3%, of nontreated injured slices at 24, 48, and 72 h after injury (P < 0.001). The addition of 100 μM glycine abolished xenon's neuroprotective effect. At all time points, in the presence of glycine, the xenon-treated injured slices were not significantly different to the nontreated injury controls (fig. 4B), being 82 ± 9% (P > 0.2), 103 ± 11% (P > 0.8), and 113 ± 14% (P > 0.3) of the control at 24, 48, and 72 h.
after injury. A powerful feature of our model is that at each time point, glycine reversed the effect of xenon to the level of the untreated slices at the same time point. Taken together with the lack of effect of glycine on the control injury, this means that the effect of glycine cannot be explained by an exacerbation of the injury. The reversal of xenon’s protective effect is consistent with xenon neuroprotection against traumatic injury being mediated by xenon inhibition at the NMDA receptor glycine site.

Because argon had a similar, although lesser, neuroprotective effect to xenon, we investigated the effect of glycine on argon neuroprotection, to determine whether argon and xenon act via a similar mechanism. Interestingly, as shown in figure 4C, we found that glycine did not reverse the neuroprotective effect of argon, in contrast with its effect on xenon neuroprotection. In the presence of 100 μM glycine, 50% atm argon treatment resulted in significant neuroprotection (P < 0.05) at all time points. There was no difference in argon-treated slices in the absence or presence of glycine at 24, 48, and 72 h. This is not consistent with argon’s protective effect being mediated via the NMDA receptor glycine site and indicates that argon has a different mode of action to xenon.

Electrophysiology

To further investigate the mechanism of argon neuroprotection, we determined whether argon, and the other inert gases, had an effect on NMDA receptors and TREK-1 channels expressed in HEK-293 cells (fig. 5). Figure 5A shows electrophysiological recordings from GluN1/GluN2A NMDA receptors in the absence and presence of argon. Argon (80%) had no effect on NMDA receptor-mediated currents, at either high (100 μM) or low (1 μM) glycine concentrations, in contrast to xenon, which inhibits NMDA receptors, as we have previously shown.36,38,39 Krypton, neon, and helium were also without effect on NMDA receptors as shown in figure 5B. Having ruled out NMDA receptor inhibition as a mechanism of argon neuroprotection, we determined whether argon and the other inert gases activated TREK-1 potassium channels. Untransfected cells passed only a few picoamperes or less when clamped at −50 mV (N = 3 data not shown). In contrast, cells expressing TREK-1 channels exhibited a characteristic large outwardly rectifying current that reversed at −80 mV. We used halothane as a positive control to activate TREK-1 currents; 0.82 μM halothane potentiated TREK-1 currents by 152 ± 20% (N = 10; data not shown). Figure 5C shows recordings from HEK cells expressing TREK-1 channels in the presence and absence of argon. Argon (80%) had no effect on TREK-1 currents. We found that krypton, neon, and helium were also without effect on TREK-1 (fig. 5, D–F and H). In contrast to the other inert gases, xenon (80%) activated TREK-1 currents markedly (fig. 5G), potentiating the current measured at −50 mV by 39 ± 5%, as shown in figure 5H.

Discussion

We investigated the neuroprotective mechanisms of the noble gases such as helium, krypton, neon, argon, and xenon in an in vitro model of TBI. Cultured hippocampal mouse brain slices were subjected to a reproducible mechanical trauma, and injury was quantified by PI fluorescence. Organotypic cultures retain a heterogeneous population of cell types whose synaptic connectivity mirrors that seen in vivo,45–49 and are intermediate between dissociated cell cultures and whole-animal models. The reproducibility of the primary focal trauma, and the progressively developing secondary injury (fig. 1), provides a useful model in which to investigate the efficacy and mechanism of putative treatments.50,51 This in vitro model allows us to control the slice environment, particularly the concentration of glycine. Although the PI fluorescence measure of injury in this model does not distinguish between cell types (e.g., neuronal and nonneuronal cells), it has the advantage of providing a robust quantification of traumatic injury, allowing continuous monitoring of the same slices over time.

Within 30 min of mechanical injury, PI fluorescence was evident, allowing clear distinction from uninjured slices (fig. 1). Secondary injury developed rapidly in the hours after trauma. One hour after injury, the lesion had developed to 32 ± 4% and by 6 h to 51 ± 8% of its extent at 72 h after injury. This secondary injury, developing rapidly in the hours and more slowly in the days after trauma, mirrors what is seen in vivo52 and in clinical traumatic injury progression.53–54 We measured cell death and neuroprotection in the hippocampal slice as a whole, avoiding subjective difficulties associated with precisely defining the boundaries of CA1, CA3, and dentate gyrus in each slice. Although some areas, such as CA1, appear to be more sensitive to traumatic injury (fig. 1A), qualitatively xenon and argon protected equally in different areas (data not shown), in line with studies regarding ischemic injury showing little regional differences in neuroprotection.54,55–57

Effect of Noble Gases on Traumatic Injury

An aim of our study was to evaluate the neuroprotective potential of the series of noble gases against traumatic injury under identical conditions. We found that only xenon and argon exhibited neuroprotection (fig. 2). The lack of effect of 50% atm helium (fig. 1D) on traumatic injury in this model is similar to what we found using the same organotypic preparation in a model of ischemic injury.54 Neon and krypton were also devoid of a protective effect. Xenon was particularly effective, with 50% atm xenon reducing total injury by 57% at 24 h after injury and by 43% at 72 h after injury. The degree of protection against total injury that we observed with 50% xenon was similar to that found by Coburn et al.19 who used 75% xenon. Argon was less effective than xenon, with 50% atm argon reducing total injury by 38% at 24 h after injury and by 30% at 72 h after injury. This contrasts...
Fig. 5. Inert gases such as argon, krypton, neon, and helium have no effect on N-methyl-D-aspartate (NMDA) receptors and TREK-1 channels. (A) Argon (80%) has no effect on NMDA-activated currents at high glycine (100 μM) and low glycine (1 μM) concentrations. Traces show typical currents activated by 100 μM NMDA in HEK cells expressing NMDA receptors containing the GluN1/GluN2A subunit combination. (B) NMDA receptor currents were unaffected by 80% argon (cyan bars), 80% krypton (brown bars), 80% neon (green bars), or 80% helium (black bars). The error bars are standard errors (100 μM glycine: argon N = 10, krypton N = 9, neon N = 5, helium N = 9; 1 μM glycine: argon N = 12, krypton N = 9, neon N = 6, helium N = 6). (C–F) Inert gases argon (80%), krypton (80%), neon (80%), and helium (80%) have no effect on TREK-1 potassium channel currents. (G) Xenon (80%) activates TREK-1 potassium channel currents. Data were sampled at 20 kHz and each trace contains 3,000 data points, lines shown are through these individual points. (H) Xenon (red bar) potentiates TREK-1 currents by 39 ± 5%, whereas argon (cyan bar), krypton (brown bar), neon (green bar), and helium (black bar) do not potentiate TREK-1 currents, measured at −60 mV. The error bars are standard errors and where not shown they are smaller than the bar (xenon, N = 5; argon, N = 3; krypton, N = 9; neon, N = 4; helium, N = 5).
with the findings by Loetscher et al. who reported approximately 80\% reduction in injury by 50\% argon in an in vitro trauma model, but the reason for the difference is not clear.

Xenon strongly inhibited the development of secondary injury (fig. 3A), with 50\% atm xenon preventing secondary injury development at 24 and 48 h after trauma. Our finding that xenon completely arrests secondary injury at 24 and 48 h after trauma is novel and is particularly relevant to xenon’s potential clinical use because clinical lesions may develop significantly in the first 24 h after injury. The reason why xenon appears slightly less effective at 72 h is unclear, but it may reflect the fact that additional injury is occurring (e.g., due to exhaustion of nutrients in the culture media) against which xenon is less effective. Argon attenuated the development of secondary injury at all time points, but was less effective than xenon (fig. 3B). Secondary injury in argon-treated slices was reduced but not prevented, with a maximum reduction of 51 \pm 7\% at 48 h after injury.

The degree of protection against secondary injury that we observed with xenon and, to a lesser extent, argon is notable, given that in our protocol the noble gas is present only after the insult, unlike some models where the neuroprotectant is also present before, during, and after the insult. Our protocol ensures that the insult itself remains a constant, and that any neuroprotection cannot be explained simply by an attenuation of the primary insult. This more closely models the clinical scenario when a patient presents for treatment after the traumatic injury. The fact that under identical conditions, helium, neon, and krypton were found to have no effect indicates that these gases are without neuroprotective effect in this model. The lack of effect of helium, neon, and krypton on NMDA receptor and TREK-1 channels (fig. 5) is consistent with their lack of neuroprotective effect.

**Reversal of Xenon Neuroprotection by Glycine**

Our strategy to determine whether xenon neuroprotection was mediated by inhibition of the NMDA receptor at its glycine-binding site was based on our observation that xenon competes with glycine, and that xenon inhibition of the NMDA receptor is reduced at high glycine concentrations. We investigated whether the degree of neuroprotection in our in vitro model could be modulated by altering the glycine concentration. We have previously validated this approach in a model of ischemic injury. We showed that adding a saturating concentration of glycine had no effect on the control injury or on sham-treated slices (fig. 4A), but that adding glycine abolished xenon neuroprotection completely at all time points (fig. 4B). These results are consistent with xenon neuroprotection against traumatic injury being mediated by inhibition of the NMDA receptor at its glycine site. This finding is important because it clearly identifies the NMDA receptor as a target mediating xenon neuroprotection against traumatic injury. Estimates of normal brain extracellular glycine concentrations, from microdialysis experiments, are approximately 5 \mu M at which concentration xenon will inhibit NMDA receptors. Glutamate excitotoxicity is involved in neuropathologies such as ischemia and TBI. Hence, xenon’s inhibition of NMDA receptors is plausible as a mechanism of neuroprotection. However, it has become clear that there are other potential targets for xenon neuroprotection that are as plausible as the NMDA receptor. For example, the two-pore domain potassium channel TREK-1 is activated by xenon (fig. 5 and see study by Gross). That TREK-1 activation may have a role in ischemic injury is suggested by the finding that genetic ablation of TREK-1 increases sensitivity to ischemia and epilepsy in in vivo models. Whether activation of these TREK-1 channels play a role in xenon neuroprotection against TBI remains to be determined. However, our current findings indicate that xenon neuroprotection in our model of traumatic injury can largely be accounted for by xenon inhibition of the NMDA receptor at its glycine-binding site.

**Mechanism of Argon Neuroprotection against TBI**

Despite growing evidence that argon is neuroprotective, there have been few studies that have investigated mechanisms for argon’s biological actions. Our finding that argon neuroprotection is not reversed by glycine indicates that argon’s neuroprotective effect is not mediated by the NMDA receptor glycine site. This is supported by our electrophysiological results showing that argon has no effect on NMDA receptors at high or low glycine concentrations (fig. 5, A and B). The lack of effect of argon on TREK-1 currents (fig. 5, C and H) indicates that this potassium channel is not involved in argon neuroprotection. The molecular mechanism(s) by which argon exerts its neuroprotective effects merits further investigation. A recent study by Fahlenkamp et al. found that argon transiently increased levels of phosphorylated extracellular signaling kinase 1/2 in vitro, but whether this is involved in argon neuroprotection remains to be determined.

**Relevance to Use of Inert Gases as Neuroprotectants**

We have shown that both xenon and argon protect against TBI in an in vitro model, and we demonstrate that xenon’s neuroprotective effect is largely mediated by inhibition at the NMDA receptor glycine site. Our findings indicate that both these inert gases merit further investigation as neuroprotectants in in vivo models of trauma. In the case of xenon, the identification of the mechanism(s) is particularly relevant to clinical studies. We have shown that xenon acts at two targets likely to play a role in neuroprotection, namely NMDA receptors and TREK-1 channels. It may be that the reason xenon is particularly effective as a neuroprotectant is its action at both these targets. Such a pleiotropic mechanism has been suggested to underlie neuroprotection by other anesthetic agents. In addition, drugs that act at the glycine site of the NMDA receptor (e.g., gavestinel) are well tolerated in patients and devoid of psychomimetic side effects. Xenon is particularly attractive as a neuroprotectant because it rapidly crosses the blood-brain barrier, exhibits cardiovascular effects...
Appendix

Xenon and Argon Protection by Different Mechanisms

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