

Argon as a potential strategy against the neurotoxicity of nitrous oxide and perinatal hypoxia/ischemia induced brain injury

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Abstract

Perinatal hypoxia is the cause of a large proportion of deaths in newborn infants globally, and accounts for up to 20% of cerebral palsy cases clinically. While clinical trials are currently under way to include the noble gas xenon alongside hypothermia as a treatment, there are still disadvantages, hence the search for a novel method to administer neuroprotective agents. By focusing on the noble gas argon, which is available at a fraction of the cost of xenon, and administer this gas before any hypoxic insult, we seek to improve outcomes after perinatal hypoxia.

This study used four model systems to assess the neuroprotective benefits of argon against both N₂O neurotoxicity and hypoxic brain injury. It was found that in primary cortical neurons, 50% N₂O administered for four hours could induce apoptosis, which argon could attenuate. However, this did not prove to also be the case in either immortalised cell lines or naïve pups in the first week of life.

In an *in vivo* model of perinatal hypoxia, pre-treatment with 50% N₂O was associated with similar or worse outcomes than hypoxia alone for most outcome measures, including hippocampal cell damage and mortality, while the addition of argon to N₂O pre-treatment could reverse these neurotoxic effects.

This thesis presents the first data regarding the preconditioning effects of argon gas against both anaesthetic and hypoxic neurotoxicity. As well as this, it provides evidence in a perinatal hypoxia model that just 20% argon can have neuroprotective benefits. This expands the field of work surrounding argon significantly and paves the way to begin experiments in higher model systems and eventually clinical trials. Argon has many distinct advantages so its use clinically would be an excellent addition to medicine.

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Declaration of Originality

I declare that the work presented in the following thesis has been conceived, performed and written by myself, unless stated otherwise.

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Abbreviations

ABC	Avidin Biotin Complex	MAC	Minimum Alveolar Concentration
Ach	Acetylcholine	MFI	Mean Fluorescence Intensity
ADP	Adenosine Diphosphate	MOMP	Mitochondrial Outer Membrane Permeabilisation
AIF	Apoptosis Inducing Factor	MOR	μ -Opioid Receptor
APAF-1	Apoptosis Protease Activating Factor	MPTP	Mitochondrial Permeability Transition Pore
APP	Amyloid Precursor Protein	MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ATP	Adenosine Triphosphate	N/A	Not Applicable
BDNF	Brain Derived Neurotrophic Factor	N+A	N ₂ O plus Argon
BH	Bcl-2 Homology	N ₂ O	Nitrous Oxide
BSA	Bovine Serum Albumin	NARP	Neuropathy, Ataxia and Retinitis Pigmentosa
CD-3	Cluster of Differentiation 3	NGF	Nerve Growth Factor
CS	Caesarean Section	NMDA	N-Methyl-D-aspartate
DAB	Diamino Benzadine	NO	Nitric Oxide
DAPI	4',6-diamidino-2-phenylindole	NOS	Nitric Oxide Synthase
dH ₂ O	Deionised Water	O ₂ ⁻	Superoxide
DIV	Days <i>in vitro</i>	OGD	Oxygen Glucose Deprivation
DMEM	Dulbecco's Modified Eagle Medium	OH	Hydroxyl Radical
DMSO	Dimethyl Sulfoxide	OMM	Outer Mitochondrial Membrane
DPBS	Dulbecco's Phosphate-Buffered Saline	OXPHOS	Oxidative Phosphorylation
DTT	Dithiothreitol	P	Postnatal Day
E	Embryonic Day	PBS	Phosphate-Buffered Saline
ETC	Electron Transport Chain	PC-RSC	Posterior Cingulate and Retrosplenial Cortex
FCS	Foetal Calf Serum	PFA	Paraformaldehyde
FITC	Fluorescein isothiocyanate	PI	Propidium Iodide
GABA	γ -Aminobutyric acid	RIPA	Radioimmunoprecipitation assay
H ₂ O ₂	Hydrogen Peroxide	ROS	Reactive Oxygen Species
Hcy	Homocysteine	SD	Standard Deviation
HI	Hypoxia	SD	Sprague-Dawley
HIE	Hypoxic Ischaemic Encephalopathy	SOD	Superoxide Dismutase
HIF	Hypoxia Inducible Factor	tBID	Translocated Bid
HRP	Horseradish Peroxidase	TBS	Tris-Buffered Saline
HSG	High Sucrose, High Glucose Solution	TdT	Terminal deoxynucleotidyl transferase
IF	Immunofluorescence	TNF- α	Tumour necrosis factor α
IL-1 β	Interleukin-1- β	tPA	Tissue Plasminogen Activator
IL-6	Interleukin-6	TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
IMM	Inner Mitochondrial Membrane	VEGF	Vascular endothelial growth factor
iNOS	Inducible Nitric Oxide Synthase	WB	Western Blot
JNK	c-Jun N-terminal kinases	WM	Morris watermaze
kATP	Potassium ATP Channel		
kDa	Kilodalton		

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Chapter 1.

Introduction

While the health of both mother and child has improved consistently over the past few hundred years, there are still a number of clinical issues that have little to no satisfactory treatment outcomes. Health screening and regular check-ups can identify and allow for the treatment of many disorders such as preeclampsia, rhesus disease and gestational diabetes (Lapolla et al., 2009, Sibai, 2003). Other disorders, such as spina bifida, are being reduced by the supplementation of many common foods with vitamins and minerals essential to a healthy foetus (Williams et al., 2002). However, despite these advances in medicine, it is still difficult to predict and treat a number of serious conditions that occur at or around the time of birth. One such set of conditions are those leading to perinatal hypoxia, which is one of the most common causes of infant mortality in the developing world, and can result in a range of serious infant morbidities in the first world (Kurinczuk et al., 2010). However, despite its common occurrence and high clinical burden, there is little in the way of treatment and no predictors outside of vital signs monitored during the birthing process, leaving no possibility to screen at-risk patients.

Current treatment strategies for perinatal hypoxia are limited to post-injury interventions, which often introduce delays, the need for patient transport, and the allowance for onset of any damage. A prophylactic treatment would allow for an element of preconditioning of any organs which may be damaged by hypoxic incident. While this has been studied *in vivo* with a range of drugs, such as creatine to protect the kidneys from damage (Ellery et al., 2013), many of these pre-treatments are unrealistic in their approach. Since any preconditioning must occur in women who have no indicators of their likelihood of perinatal asphyxia, it must be simple, easily accessible and have no adverse effects on either mother or foetus. Clinically, pretreatment options must be simple, safe and administered directly before birth, not necessary over long periods, due to contraindications, cost, or even lack of comfort for

the mother in taking drugs to reduce the risk of an uncertain problem (Whitelaw and Thoresen, 2000, Doyle et al., 2014). For this reason, it may be possible to perform a short course of preconditioning directly before birth.

This brings into play a treatment many women already get in the period directly before birth; labour analgesia using nitrous oxide gas. Nitrous oxide (N_2O) is a commonly used analgesic agent, given during labour in a 50:50 ratio with oxygen, clinically known as Nitronox™ (Klomp et al., 2012). If a preconditioning agent could be supplied with N_2O treatment, this would allow for minimal intervention and stress, while maximising availability. It is difficult to find a treatment that does not itself have adverse side effects, but one gas which has recently been gaining attention as an organoprotective agent is the noble gas argon (Coburn et al., 2012). Argon has proven protective benefits on the brain, kidney and eye in *in vivo* models, as well as providing protection from a range of toxicities in many cell culture models (Coburn and Rossaint, 2012, Ezzeddine, 2011, Sanders et al., 2010a). Argon has also shown no track record of any adverse effects in human, porcine and rodent studies (Alderliesten et al., 2014, Horrigan et al., 1979). If argon could be safely added to N_2O for the period of analgesia before birth, any subsequent trauma or insult may be reduced or eliminated.

To allow for the clinical investigation into this treatment option, it is of course necessary to lay the foundation using *in vitro* and *in vivo* models to determine the true potential benefits, as well as possible risks, associated with this combination of gases. Nitrous oxide has long been a contentious gas in clinical use due to conflicting reports of possible neurotoxicity, particularly for the young (Savage and Ma, 2014). It may be possible that N_2O itself may induce more damage than a perinatal insult alone could achieve. On the other hand, nitrous

oxide is an N-methyl-D-aspartate (NMDA) antagonist, a class of drug long known to have protective benefits against hypoxic brain damage. It is therefore difficult to predict whether N₂O alone would be detrimental or beneficial to newborns having perinatal asphyxia, while the addition of argon may have positive or negative effects. It is unknown how argon has its protective effects but has not been studied in combination with other gases before, so any possible effect needs to be fully studied.

To allow for the full elucidation of the pathways and molecular mechanisms behind the effects found, a multi-level approach will be undertaken to fully assess the feasibility of this treatment. A combination of primary neuronal culture, immortalised cell lines and postnatal rat pups will be used to assess toxicity alone *in vivo* and *in vitro*, along with a rat model of perinatal asphyxia, all using either (i) no treatment, (ii) nitrous oxide alone or (iii) nitrous oxide in combination with argon. With this data, an analysis of the neuroprotective or neurotoxic potential of argon and N₂O can be achieved. A primary neuronal line was initially used to investigate the actions of N₂O alone or in combination with argon. This was expanded upon with the use of two cell lines, glial and neuronal in origin, with the introduction of a hypoxic insult to more closely mimic the *in vivo* work. Two separate *in vivo* models were then used, the first to determine any neurotoxicity of either treatment in the naïve brain, and finally, to assess the effects of these treatments on perinatal hypoxia. Each stage builds upon published research in the field, and each section will draw on the results of the previous, and inform the direction of the subsequent parts.

Chapter 2.

Background review

2.1. Perinatal hypoxic-ischaemic encephalopathy

Perinatal hypoxia is one of the leading causes of mortality and morbidities of newborn infants worldwide. Defined as the lack of oxygen directly before, during or after the birth process, perinatal hypoxia causes a range of conditions including hypoxic-ischaemic encephalopathy (HIE), the clinical term for the lasting effects of perinatal hypoxia. It is widely reported that between 1 and 3 births per 1000 live births suffer from HIE (Sanders et al., 2010b, Lai and Yang, 2011). However this encompasses data from both high and low income countries (Costello and Manandhar, 1994); in reality, high income countries such as the USA and UK have rates of occurrence of 1 in 1000 live births, while in lower income countries this rate reaches between 5-10 per 1000 live births (McGuire, 2007). This alone should give an idea of how level of care and monitoring can drastically affect neonatal outcomes of this disease. It is difficult to determine whether it is better care during birth which reduces hypoxic incidence, or better identification of a hypoxic incident and treatment of the neonate at an early stage which accounts for such a different outcome profile, but, in all likelihood, both factors play a role. One study from a low socioeconomic area in Nepal found an association between maternal stature and HIE, citing poor nourishment as a possible cause (Ellis et al., 2000). Interestingly, this study compared its data to an unmatched study performed in the higher socioeconomic area of Perth in Western Australia (Badawi et al., 1998). They found that most risk factors were similar between the two areas, such as maternal age and meconium stained amniotic fluid. They also found that total incidence of moderate or severe encephalopathy was the same between the two cohorts, 3.8/1000 live births. In one study from Pakistan, with data obtained from low and middle income areas, it was determined that both delivery by a midwife and giving birth at home or in a private clinic were risk factors for perinatal

asphyxia (Aslam et al., 2014). Comparing risk factors between a different study from Australia and the Pakistani study, giving birth at home was equally a risk factor between both, however the Australian study classed its incidence as higher than Europe and the United States (Bastian et al., 1998), although still present in these countries (Snowden et al., 2015). This highlights the large disparity in data between almost all studies of perinatal hypoxia and hypoxic ischaemic encephalopathy. It is probable that the differences between higher and lower income countries can be explained by clinical care after the incident as opposed to any major differences in risk factors besides those more common to lower socioeconomic areas, such as poorly trained staff and maternal health.

2.1.1. Risk factors for perinatal asphyxia

As discussed above, there are a number of known risk factors associated with perinatal hypoxia. These can generally be categorised into antepartum, intrapartum and foetal risk factors. Antepartum risk factors are those occurring before the birth process and include complications such as pre-eclampsia (Kaye, 2003) or the administration of adrenergic drugs to prevent preterm labour (Aslam et al., 2014). Intrapartum factors refer to those occurring during the birth process and most often refer to conditions suffered by the mother such as prolapsed umbilical cord (Aslam et al., 2014), fever, or placental abruption (Kayani et al., 2003). Foetal risk factors are the most commonly used markers to assess whether a hypoxic incident has occurred. These include a range of outcomes associated with HIE such as poor Apgar score, cord arterial blood pH, the presence of meconium in the amniotic fluid, and premature delivery (Martinez-Biarge et al., 2012). Apgar score is a five point measurement of infant health taken at birth, encompassing information about colour, heart rate, reflex irritability, muscle tone and respiration. Each category has three levels of severity classed as 0 (worst), 1 (less severe) or 2 (normal). A combined Apgar score of 0-3 at five minutes post-

partum is a commonly used measure of perinatal asphyxia (American Academy of Pediatrics, 2006). Interestingly, a new study has determined there is an increased risk of severe perinatal asphyxia associated with maternal obesity (Persson et al., 2014). With the global obesity rates increasing steadily over the past decade, it may be possible that this group of at-risk patients will also increase. However, despite there being a number of risk factors associated with perinatal hypoxia, none of these stands out as a reliable predictor of a hypoxic incident occurring, and there are a far greater number of hypoxic events with no prior indications (Kurjak and Chervenak, 2015).

2.1.2. Mechanisms of cell death in perinatal hypoxia

2.1.2.1. Energy failure

Hypoxia of any form primarily causes its neurotoxic damage *via* energy failure due to insufficient oxygen and glucose supply to cells. This results in disruption in the normal ionic gradients within the brain (Silver et al., 1997). When there is insufficient energy in the system, adenosine triphosphate (ATP)-dependent ion channels become dysfunctional, leading to an imbalance in cellular membrane potential. When these ATP-dependent ion channels, such as ATP-sensitive potassium channel (K_{ATP}) fail, ion gradients become dysregulated (Ballanyi, 2004). This results in depolarisation of the neurons, (Hochachka et al., 1993) which in turn facilitates the release of the excitatory neurotransmitters glutamate and glycine. In hypoxia, it is glutamate which is primarily released to a neurotoxic extent (Nishizawa, 2001), which, in the synaptic cleft leads to excessive activation of postsynaptic neurons. This constant barrage of excitatory neurotransmitters is toxic to cells and is classed as excitotoxicity. While this mechanism of excitotoxicity is present in all types of hypoxic incident, and a common factor in many other brain injuries such as traumatic brain injury, it

is especially prevalent in the neonatal brain (Johnston, 2001). There are a number of factors which account for this increased sensitivity, including different NMDA receptor subunit expression and increased excitatory pathways (Cull-Candy et al., 2001, Jensen, 2006). The neonatal brain has a vast network of synapses between neurons at birth, many of which are removed and modulated in a period of intense cell pruning after birth known as synaptogenesis. One key factor in the process of synaptogenesis is the activation of the N-methyl-D-aspartate (NMDA) receptor, where expression of NMDA receptors mediates the high levels of excitatory signalling necessary to strengthen synapses (Gambrill and Barria, 2011). As glutamate activates the NMDA receptor to cause neuronal activation, an increased level of glutamate in an already NMDA receptor enriched environment leads to a high risk factor for excitotoxicity. This is exacerbated by the specific type of NMDA receptor most highly expressed in the perinatal brain (Mishra et al., 2001). The NMDA receptor is comprised of four subunits and one of these, the NR2B subunit (Wenzel et al., 1997, Furukawa et al., 2005), is particularly highly expressed in the neonatal period. This subunit is known to degrade slower than other subunit types (Yashiro and Philpot, 2008), which increases the time during which glutamate can induce neurotoxicity. Human studies show results in line with, but not identical to rodent studies. Expression patterns appear to be somewhat different between the species, with humans having a more pronounced shift in receptor subunit composition from NR2B to NR2A in the hippocampus (Law et al., 2003) and white matter (Jantzie et al., 2015). This is compounded further by the aberrant glutamate reuptake mechanisms which result from hypoxic insult. As some of the glutamate transporter systems are energy-dependent (Rose et al., 2009), the characteristic energy failure seen in hypoxia impairs the removal of glutamate from the synaptic cleft (Camacho and Massieu, 2006), ensuring a more sustained period of glutamate presence. The

importance of ATP for this process is evidenced when overall brain glucose usage is analysed. It has been found that the highest energy usage in the brain occurs not at the cell body, but at the synaptic cleft (Harris et al., 2012). This implies that in the synaptic cleft, the energy-dependent reuptake of neurotransmitters such as glutamate is a constant and necessary process. Animal and clinical studies have measured glutamate concentrations in neonates who experienced hypoxia during birth, and found an increase in glutamate (Pu et al., 2000, Hagberg et al., 1987). This was extended further when it was discovered, using cerebrospinal fluid measurements, that glutamate levels were proportional to the severity of encephalopathy (Johnston, 2001). This finding has been echoed in the brain, using more advanced proton magnetic resonance spectroscopy techniques (Groenendaal et al., 2001). In addition to a higher level of NMDA receptors in the brain, there is also a bias towards excitatory pathways. This is because the main inhibitory pathway in adults, the gamma-aminobutyric acid (GABA)-ergic pathway, are actually excitatory for the first week of life (Leinekugel et al., 1999). The overall picture, therefore, is a system functioning on a high excitatory output, thus any further excitatory stimulation can easily cause a state of excitotoxicity.

2.1.2.2. Excitotoxicity

NMDA receptor overactivation leads to excitotoxicity, as discussed above, but the glutamate itself is not directly neurotoxic. The reason for increased cell death is due to an imbalance in intracellular ion concentrations, which causes a toxic cascade culminating in cellular apoptosis or necrosis. Excitotoxic cell death can be divided into two stages, an acute stage dependent on Na^+ and Cl^- (Inoue and Okada, 2007), and a delayed stage which is Ca^{2+} dependent (Northington et al., 2001). The two stages are independent and it has been shown that preventing the acute toxicity will not offset the occurrence of delayed toxicity

(Rothman et al., 1987). As mentioned, acute toxicity is dependent on Na^+ and Cl^- , which build up inside the cell when the Na^+/K^+ -ATPase proton pump in the cellular membrane fails due to a loss of intracellular ATP (Weerasinghe and Buja, 2012). As ions move into the cell, water is also passively transported, resulting in cellular oedema (Simard et al., 2007, Inoue and Okada, 2007). This cellular swelling can result in cell lysis, which is a common result of acute glutamate toxicity (Coyle and Puttfarcken, 1993). This type of cell death is known as oncosis, from the Greek word “ónkos” which refers to swelling. The second stage of excitotoxic cell death is the delayed, Ca^{2+} dependent form. Instead of leading to oncosis, this process leads to apoptosis, or programmed cell death. Postsynaptic glutamate receptors allow for the movement of ions such as sodium and calcium into the cell (Kim et al., 2003, Tanaka, 2000). Ca^{2+} can induce an excess activity of enzymes in the cell (Arundine and Tymianski, 2003), which leads to degradation of integral structural proteins. This can also generate free radicals which are detrimental to cell membranes and DNA (Frantseva et al., 2001). Ca^{2+} is capable of inducing nitric oxide synthases to produce nitric oxide (NO) (Sattler and Tymianski, 2000). NO is a well-documented inducer of mitochondrial failure (Brown and Borutaite, 2004, Brown, 2001, Moncada and Erusalimsky, 2002). As mitochondria are the energy producing organelles of the cell, damage to this system will have strong negative effects on cellular viability. As well as via NO, Ca^{2+} can also induce damage to mitochondria more directly. Both high Ca^{2+} and mitochondrial damage can lead to the production of oxygen free radicals such as superoxides (Brechard and Tschirhart, 2008). Again, the neonatal brain has a number of key physiological differences from the adult brain which increases vulnerability. Studies have found that nitric oxide synthases (NOS) are highly expressed during the perinatal period (Zhu et al., 2005). Normally, reactive oxygen species (ROS) and reactive nitrogen species are scavenged before they can cause excess damage.

However these scavenging systems are not fully matured in the neonate (Blomgren and Hagberg, 2006, Davis and Welty, 2014). In addition, newborn infants have high levels of iron in their body, which can act as a catalyst for the production of ROS (Ray et al., 2012). As seen above with glutamate, there is a two part failure, with an increased formation of ROS and a failure in the removal of the ROS, meaning oxidative stress likely plays a larger role in perinatal cases of hypoxic encephalopathy. Reactive oxygen species and the role of mitochondria in cell death will be discussed in greater detail later in the chapter.

2.1.2.3. Inflammation

Inflammation plays an important role in the overall health of the perinatal brain, and foetal infections are well linked to subsequent complications in the perinatal period, including preterm birth, an increased risk of hypoxia, and cerebral palsy. Inflammation is a complex response to infection or tissue damage, mediated primarily by cytokines, which are proteins which act as messengers across short distances (Hanada and Yoshimura, 2002). Immune cells also play a large role in inflammation, although in the brain there are a different class of immune cells than those found in the rest of the body, namely microglia. The release of cytokines and activation of microglia can cause downstream damaging effects such as reactive oxygen species formation, activation of immune cells, and the release of toxic enzymes (Ofek-Shlomai and Berger, 2014). In the perinatal brain in particular, this can lead to cell death and white matter damage (Hagberg et al., 2002, Dammann et al., 2002), leading to irreversible brain damage and cerebral palsy. The link between intrauterine inflammation and perinatal hypoxia has been quite well established (Girard et al., 2009), and the combination of the two appears to be a very strong risk factor for the development of cerebral palsy in infants. The presence of cytokines such as IL-1 β , IL-6 and TNF α have been associated with cerebral palsy after perinatal hypoxia (McAdams and Juul, 2012) and

women with infection during pregnancy, such as chorioamnionitis which affects 10% of pregnancies (Ofek-Shlomai and Berger, 2014), are at higher risk of infants with brain damage. As well as this, after hypoxia-ischaemia in infants, there is an increase in immune cells entering the brain via a permeabilised blood brain barrier (Hagberg et al., 2015). A number of treatments currently under investigation for perinatal hypoxia have anti-inflammatory properties, highlighting the importance of this pathway in long-term consequences of hypoxia. Erythropoietin is a drug primarily used to increase red blood cell numbers; however it has proven effective against perinatal hypoxic brain injury in animal models (Spasojevic et al., 2013, Wu and Gonzalez, 2015). It is known to stimulate growth factors such as vascular endothelial growth factor (VEGF) and brain derived neurotrophic factor (BDNF), while downregulating pro-inflammatory cytokines such as IL-6 and IL-8 (Wu and Gonzalez, 2015). Melatonin, a naturally produced hormone which plays a role in the human circadian rhythm, has also been used to treat the effects of perinatal hypoxia in animal models, and at low doses has shown benefits by reducing white matter inflammation (McAdams and Juul, 2012). Much of the research surrounding inflammation after perinatal hypoxia has highlighted the differences between the adult brain and the neonate, including faster inflammatory responses and, as will be discussed later in this chapter, a higher production of reactive oxygen species. This highlights the pitfalls of trying to directly translate known pathways from adult to neonatal treatments, as the latter is often far more sensitive (Liu and McCullough, 2013).

2.2. Mitochondria in hypoxia and apoptosis

Mitochondria are a vital organelle, found in almost every cell in the body. They are commonly referred to as the “powerhouse” of the cell, as it is in the mitochondria that

adenosine tri-phosphate (ATP), the molecule used as an energy source throughout the body, is made. It is thought that mitochondria were bacteria which became incorporated into larger single-cell organisms, allowing for the development of larger multi-cellular organisms due to an increase in available energy (Henze and Martin, 2003). They have their own mitochondrial DNA which is inherited maternally, and is responsible for a subset of diseases such as neuropathy, ataxia and retinitis pigmentosa (NARP), mitochondrial myopathy, and Leigh disease (Schapira, 2006). Mitochondria have a double membrane structure, and important processes are carried out not only in the mitochondrial matrix (similar to the cytoplasm, see Figure 2.1), but also on both the inner and outer membrane, and in the intermembrane space (Martinou and Youle, 2011). The matrix is where ATP production occurs by a process called oxidative phosphorylation (OXPHOS). For OXPHOS to occur, there first needs to be glycolysis in the cytosol, where glucose is broken down to pyruvic acid (Papandreou et al., 2006). There are then three main stages of oxidative phosphorylation which converts the end products of glycolysis to energy. All three stages occur in some part of the mitochondria, with the citric acid cycle occurring within the matrix, the electron transport chain (ETC) occurring on the interface between matrix and intermembrane space, with the inner membrane necessarily involved, and ATP synthase activity happening in the same region as the electron transport chain, but in the opposite direction, from intermembrane space to matrix (Wallace, 1999). Briefly, as can be seen in Figure 2.1, the electron transport chain consists of three so-called 'complexes' or ion channels which harness the by-products of the citric acid cycle, primarily NADH, to produce protons (H^+) which are transported from the matrix to the intermembrane space. This allows a proton concentration and charge gradient to build up across the inner membrane. It is at this point that O_2 is needed as an electron acceptor, with an end product of H^+ and O_2 coming

together to form H_2O , the oxidative part of the process. As the electron transport chain complexes can only allow protons to move in one direction, there is only one other path for the protons to be transported back across the inner membrane and balance out the system, namely *via* ATPase. As H^+ ions pass through ATPase, this channel converts adenosine diphosphate (ADP) to adenosine triphosphate (ATP) by harnessing the energy released from the rebalancing of the proton gradient in a process called chemiosmosis. This accounts for the “phosphorylation” element of oxidative phosphorylation.

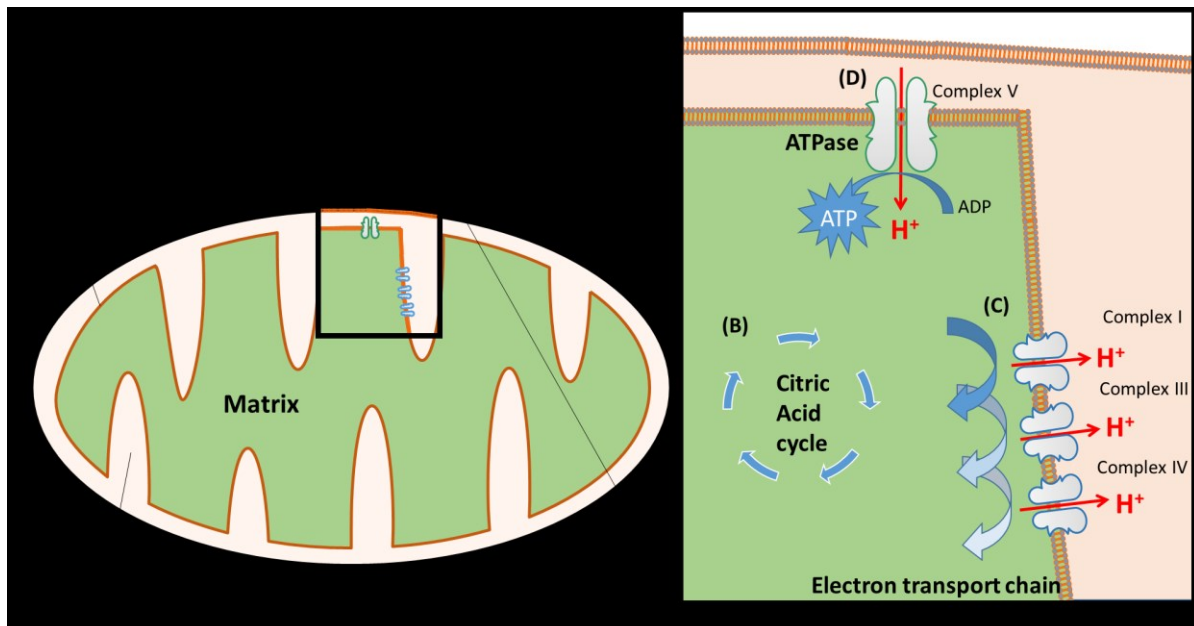


Figure 2.1 **The structure of the mitochondria and a simplified illustration of oxidative phosphorylation.** (a) The structure of mitochondria consists of an inner mitochondrial membrane (IMM) and outer membrane, separated by an intermembrane space. Within the inner membrane, which has an increased surface area due to numerous folds, is the matrix of the mitochondria. The area comprising the IMM, intermembrane space and matrix is the location for oxidative phosphorylation. The citric acid cycle (b) produces NADH which feeds into the electron transport chain (c), pumping H^+ atoms from the matrix to the intermembrane space. This creates a proton imbalance, and to re-balance the system, H^+ atoms must pass back to the membrane via the ATPase channel. While passing through, ATPase uses the energy to convert ADP to ATP. An intact IMM is necessary to build up the charge gradient needed to drive oxidative phosphorylation.

2.2.1. Reactive oxygen species

Oxidative phosphorylation is an essential part of all cellular processes as this is the primary source of energy for all cellular activities. In some cells, mitochondria can account for up to 90% of all O_2 used (Solaini et al., 2010). During this process, reactive oxygen species (ROS) are an inevitable and natural by-product of the use of O_2 . There are three main types of reactive oxygen species: superoxides (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH) (Camello-Almaraz et al., 2006). Superoxides are a by-product of OXPHOS but normally pose little danger due to the presence of superoxide dismutases (SODs); enzymes which act to catalyse O_2^- into either H_2O_2 or O_2 . This, however, leads to the formation of a second ROS, H_2O_2 , which slowly decomposes to OH, the most reactive and dangerous of the oxygen free radicals, in a process which is sped up by the presence of iron (Coyle and Puttfarcken, 1993, Sampaio et al., 2014). The hydroxyl radical is particularly dangerous as it can act to remove H^+ ions from poly-unsaturated fatty acids by a process called lipid peroxidation (Sultana et al., 2013). As these fatty acids compose a large proportion of cell membranes, this lipid peroxidation can cause high levels of membrane damage, which can have disruptive consequences to normal cellular function.

One interesting point to consider is the strong body of evidence which shows that reactive oxygen species formation is increased during periods of hypoxia (Lafemina et al., 2006, Zorov et al., 2014). This can be somewhat counterintuitive, as the lack of oxygen should theoretically halt the formation of ROS as OXPHOS is no longer occurring. However, by looking at a secondary role of ROS as cellular messengers, this increase becomes much clearer (Schieber and Chandel, 2014). It has been discovered that H_2O_2 produced in the mitochondria under conditions of hypoxia can diffuse out from the mitochondria into the cytosol. Once in the cytosol, H_2O_2 can interact with and modulate the activity of hypoxia

inducible factor 1 α (HIF-1 α) (Chang et al., 2008). HIF-1 α is a transcription factor which is capable of activating transcription of a wide range of genes which protect the cell from hypoxic damage (Collins et al., 2012b). This signalling of ROS is called redox signalling (Liu et al., 2005), which could explain the paradoxical increase in reactive oxygen species, despite a decrease in total O₂ availability. As to how the ROS are actually produced without O₂, the evidence appears to suggest that even during hypoxia, during the initial stages there will still be sufficient O₂ throughout cells for ROS formation, but not enough for full cellular respiration (Clanton, 2007).

2.2.2. The role of calcium

Mitochondria play an important role in the propagation and progression of Ca²⁺ dependent cell death as they have the ability to act as transient calcium storage (Schonfeld and Reiser, 2007). Ca²⁺ can freely enter the intermembrane space *via* porins, and can then cross the inner membrane for short term storage in the mitochondrial matrix (Rizzuto et al., 2012). Ca²⁺ can, however, cause a number of negative reactions inside the mitochondria which will eventually lead to cell death. Ca²⁺ can induce the formation of both O₂⁻ and nitric oxide (NO) (Peng and Jou, 2010). The negative effects of O₂⁻ have been discussed above, but it is worth noting that in addition to normal O₂⁻ formation, this excess formation has the possibility of overwhelming SODs and increasing the levels of other downstream ROS. Nitric oxide is a molecule which can severely disrupt normal mitochondrial function as it can compete with cytochrome c, an essential part of the electron transport chain, for O₂ (Giuffre et al., 2005, Brunori, 2001), leading to dysfunction in the ETC and thus OXPHOS. In a hypoxic environment where free oxygen levels are already low, any competition for O₂ will negatively affect cellular respiration. Ca²⁺ is also strongly implicated in the formation of mitochondrial permeability transition pores (Halestrap, 2009), channels in the mitochondrial

outer membrane which allow the release of large molecules from the mitochondria such as cytochrome c (Brookes et al., 2000). This is particularly dangerous as cytochrome c is known to bind the cytosolic molecule apoptotic protease activating factor-1 (APAF-1) to form the apoptosome (Adrain and Martin, 2001), which in turn triggers apoptosis in the cell.

2.2.3. Bcl-2 family proteins

There are a number of proteins which work to promote either the stability of mitochondrial membranes, or exacerbate the formation of membrane pores and mitochondrial destruction. The largest players in this system are the Bcl-2 family of proteins. The first of these to be identified was a B-cell lymphoma oncogene, now known as Bcl-2, hence the “Bcl” nomenclature (Antonsson and Martinou, 2000). There are seventeen proteins in the family, subdivided into three categories based on either function or composition, outlined in Table 2.1. The first two categories contain multiple Bcl-2 homology (BH) domains, BH1-4; and are therefore classified by either their anti-apoptotic or pro-apoptotic properties. The third category contains only the BH3 domain, and can have a range of functions, either inhibiting anti-apoptotic Bcl-2 proteins, or activating pro-apoptotic Bcl-2 proteins, therefore having an overall pro-apoptotic function.

Table 2.1 **Bcl-2 family member proteins, subdivided into their classification groups.** Anti-apoptotic and pro-apoptotic proteins act either to induce or suppress apoptosis in cells, while the BH3 only members of the family can have a range of functions.

Anti-apoptotic proteins	Pro-apoptotic proteins	BH3 only
Bcl-2	Bax	Bid
Bcl-XL	Bak	Bim
Bcl-w	Bok	Bad
A1		Puma
Mcl-1		Noxa
		Bik
		Bmf
		Hrk
		Bnip3

In general, the Bcl-2 family of proteins have specific cellular locations in healthy cells, and some tend to translocate during cellular stress and apoptosis. For example, Bcl-2 itself is most commonly found on the outer mitochondrial membrane (OMM), while Bax is known to move between the cytosol and OMM in healthy cells, but becomes bound to the OMM during apoptosis (Ola et al., 2011, Renault et al., 2013). Bak on the other hand is always bound to the OMM, but is deactivated in healthy cells. This may help to explain their different functions, with Bcl-2 acting as a mitochondrial protector hence the need to be localised where needed. In contrast, Bax must be activated to have its pro-apoptotic effects, therefore requiring movement to the site where it can have its action. However, it has been found that Bax translocation alone is not sufficient to cause mitochondrial outer membrane permeabilisation (MOMP) (Lindsay et al., 2011). It is at this point that the BH3 only proteins may play their largest role. Bid, for example, is cleaved by caspase-8 during FAS receptor mediated cell death (Ola et al., 2011). Once Bid is cleaved, becoming truncated Bid (tBid), it translocates to the OMM where it can induce conformational change of Bax and promote its oligomerization and insertion into the OMM (Martinou and Youle, 2011). Bim can be phosphorylated by c-Jun N-terminal kinases (JNK), whereupon it can translocate and displace Bcl-xL in the OMM and promote Bax translocation (Antonsson and Martinou, 2000). Bad, on the other hand, needs to be dephosphorylated to have its pro-apoptotic effects, where it binds and inactivates Bcl-2 and Bcl-xL. p53 promotes the transcription of the two BH3 proteins Puma and Noxa. Puma has a number of functions at the OMM, both in promoting the dissociation of Bcl-xL from Bax, and causing oligomerization of Bax, a vital step in the progression of MOMP (Ming et al., 2006).

One missing step in the story of how pro-apoptotic Bcl-2 family proteins cause apoptosis is definitive proof of the mechanism by which they cause release of mitochondrial

intermembrane proteins. It is widely known that the permeabilisation of the mitochondrial outer membrane leads to the release of a host of proteins which stimulate apoptotic pathways. These proteins, which in the mitochondria are normally important and cause no harm, can have serious effects once released into the cytosol. These proteins include cytochrome c, an important factor in the electron transport chain, apoptosis inducing factor (AIF), and second mitochondrial activator of caspases/Direct IAP protein with low pI (SMAC/DIABLO) (Halestrap, 2009, Adrain et al., 2001). AIF, for example, causes DNA damage and chromatin condensation (Daugas et al., 2000). Cytochrome c can bind to APAF1 in the cytosol to form the so-called apoptosome (Adrain and Martin, 2001), which induces the cleavage of caspase 9, which itself cleaves and activates caspase 3. Caspase 3 is known as the executor caspase and is one of the final stages of cellular apoptosis due to its ability to cleave a wide range of other proteins which cause cell death. However, there is no consensus as to how these proteins are released from the intermembrane space. Early work speculated that opening of mitochondrial permeability transition pores (MPTP) in the inner membrane led to osmotic imbalance and swelling of the mitochondria (Halestrap, 2009). This swelling could cause disruption of the OMM, releasing pro-apoptotic proteins. However, as described by Antonsson and Martinou (2000), apoptosis can often cause mitochondrial shrinkage as opposed to swelling, and cyclosporine A can not prevent MOMP, despite preventing MPTP formation. This shifts focus instead to the probable formation of pores or opening of channels in the OMM. It has been suggested that cardiolipin, a protein found only in the mitochondrial inner membrane (IMM), is necessary for the oligomerization of Bax in the membrane, most likely at the points where IMM and OMM come into contact (Raemy and Martinou, 2014), resulting in the formation of giant pores. In keeping with this theme, a number of other publications cite Bax oligomerization in the OMM as a causative

factor for the opening of pores. Evidence has shown that oligomerisation of Bax is not only causative but necessary for the formation of pores large enough to release proteins such as cytochrome c (Roucou et al., 2002). In addition, Bax and Bak are known to regulate the opening of voltage dependent anion channel in the OMM, while Bcl-xL can close this channel (Shimizu et al., 1999). However, there is a certain level of controversy whether this alone is responsible for the release of pro-apoptotic proteins (McCommis and Baines, 2012). The one certainty is that the presence of Bax and Bak is essential for permeabilisation as experiments have shown that knockout animals or cells lacking these proteins do not undergo MOMP or apoptosis (Wei et al., 2013). While there is no clear consensus on the general mechanisms underlying the interactions between Bcl-2 proteins and mitochondrial stability, it is clear that they play an important role in general cell health. Examining these proteins can lead to an understanding of the stages before apoptosis, and highlight targets for treatment of a range of disorders.

2.3. Perinatal anaesthesia

In recent years, the risks associated with the clinical use of anaesthetics to the health of the infant brain have become a large area of discussion, exacerbated by a wide range of outcomes reported in rodent, non-human primate and clinical studies. This is an extremely important area for further investigation as anaesthetics are widely used in paediatric procedures. In one study of 10,450 children in New York state, 668 (6.39%) had surgery with anaesthesia before 3 years of age (DiMaggio et al., 2011), while another study in Minnesota (Wilder et al., 2009) had a rate of 4.09% (350 out of 8548) giving some idea of the rate of exposure. In premature infants this number increases dramatically, with one study from California showing rates of 18.2% having one surgery and 13.1% having more than one

surgery before reaching term-equivalent age, i.e. the age at which they would have been born at full term (Gano et al., 2015). A review on the subject cites numbers of children undergoing anaesthesia at 6 million per annum in the USA, with 1.5 million of these being infants, i.e. under 12 months of age (Sun, 2010). To investigate these findings, a number of cohorts have been established to assess the safety of anaesthetics in children including SafeKids (Durieux and Davis, 2010) and SmartTots (Ramsay and Rappaport, 2011), as well as numerous clinical trials which will be discussed below.

2.3.1. Animal data

2.3.1.1. Rodent models

The first hints that anaesthetics have the ability to induce neurotoxicity came from rodent studies. One of the first catalysts to investigate this toxicity came from a study in 1999 which showed that NMDA receptor antagonists could induce neuronal apoptosis (Ikonomidou et al., 1999). This report, along with similar ones which followed (Beals et al., 2003, Jevtovic-Todorovic et al., 2003a, Jevtovic-Todorovic et al., 2003b), caused an immediate reaction from the anaesthetic community. A number of editorials and letters on the subject were published in clinical anaesthesia journals (Davidson and Soriano, 2004, Olney et al., 2004, Todd, 2004, Soriano and Loepke, 2005, Mellon et al., 2007), included a pro/con pair of editorials in one journal (Jevtovic-Todorovic and Olney, 2008, Loepke et al., 2008). In virtually all studies of animals exposed to anaesthetics during the perinatal period, there was evidence of neurotoxicity with or without the presence of developmental disturbances as measured by cognitive and behavioural testing (Fredriksson et al., 2007, Loepke et al., 2009, Viberg et al., 2008). In particular, volatile anaesthetics such as isoflurane and sevoflurane are consistently shown to induce neurotoxicity in young animals (Lu et al., 2010,

Bercker et al., 2009, Kodama et al., 2011, Head et al., 2009, Liang et al., 2010). NMDA antagonists such as ketamine and N₂O also show evidence of neurotoxicity (Viberg et al., 2008, Scallet et al., 2004), and when anaesthetics are used in combination (Yon et al., 2006, Yon et al., 2005, Culley et al., 2004, Shu et al., 2012, Zhen et al., 2009), as is often the case during paediatric surgery, the risk of toxicity increases. However most studies draw fire for what have been classed as clinically non-relevant study designs, with the use of high concentrations, such as over 100% N₂O in hyperbaric conditions (Jevtovic-Todorovic et al., 1998), or prolonged periods of 8 or more hours (Jevtovic-Todorovic et al., 2003a, Zou et al., 2009) which are not reflective of the true paediatric surgical experience. Nonetheless, these studies indicate that anaesthetics are not the relatively harmless agents they were once thought to be, and provide a framework to base future clinical studies.

2.3.1.2. Non-human primate models

While rodent models do show consistent evidence of neurotoxicity in young animals, a number of non-human primate models have been used to assess anaesthetics and have returned results which are generally in line with rodent studies. A number of groups use these models to test a wide range of paediatric anaesthetics, namely ketamine (Brambrink et al., 2010a, Koo et al., 2014, Slikker et al. (2007), Zou et al., 2009, Paule et al., 2011), isoflurane (Brambrink et al., 2012, Brambrink et al., 2010b, Zou et al., 2011), propofol (Creeley et al., 2013) and N₂O (Zou et al., 2011). A summary of these publications has been compiled in Table 2.2. Almost all studies use rhesus macaque monkeys, with the majority exposed to anaesthetics on postnatal day 5 or 6, with some gestational exposures and one exposure at 35 days old. Postnatal day 5 for rhesus monkeys is roughly equivalent to 4-6 months of age in humans (Brambrink et al., 2012). All of these studies found cell death, as evidenced by apoptotic neurons in the cortex of animals culled within 24 hours of gas

exposure. One study, which carried out behavioural testing more than three years after a single ketamine exposure in the first week of life, found significant differences in colour and position discrimination tasks and learning abilities compared to untreated animals, as well as poorer response speed (Paule et al., 2011). These data show that the toxic effects of commonly used anaesthetic agents do carry between species, and give strong evidence that similar effects could be expected in humans.

Table 2.2 **Studies in non-human primates examining neurotoxic potential of anaesthetic agents.** Over the past decade, a number of studies have examined the neurotoxic potential of some of the most commonly administered anaesthetic agents in neonatal non-human primates. As outlined in the table, many of the results are in line with rodent data, where anaesthetics can induce increased neuronal death at relatively long exposure times. *; Studies maintained a light plane of anaesthesia resulting in a varying concentration administered

Reference	Species	Age	Anaesthetic	Concentration	Duration	Test age	Outcome
(Slikker et al., 2007)	Rhesus	G122, P5, P35	Ketamine	20-50mg/kg*	24hr	+6hr	↑ cell death, ↑ NMDA-R NR1subunit RNA in cortex
(Zou et al., 2009)	Rhesus	P5	Ketamine	20-50mg/kg*	3, 9, 24hr	P6	↑ cell death in cortex at 9 or 24hr only
(Brambrink et al., 2010)	Rhesus	P6	Ketamine or Iso	*	5hr	+3hr	↑ cell death of neurons and oligodendrocytes Iso 4x more toxic than ketamine
(Brambrink et al., 2010)	Rhesus	P6	Isoflurane	0.17-1.5%*	5hr	+3hr	↑ cell death of neurons
(Paule et al., 2011)	Rhesus	P5	Ketamine	20-50mg/kg*	24hr	3yr	Poor performance in behavioural testing
(Zou et al., 2011)	Rhesus	P5	N2O ± Iso	70% ± 1%	8hr	+6hr	↑ cell death with N2O+Iso combination only
(Brambrink et al., 2012)	Rhesus	P6	Isoflurane	0.17-1.5%*	5hr	+3hr	↑ cell death of oligodendrocytes
(Creeley et al., 2013)	Rhesus	G120, P6	Propofol	*	5hr	+3hr	↑ cell death of neurons and oligodendrocytes
(Koo et al., 2014)	Cynomolgus	G120	Ketamine or Dex	20-50mg/kg* (Ket) or 3µg/kg/hr(LD Dex) or 30µg/kg/hr(HD Dex)	12hr	+0hr	↑ cell death in cortex for ketamine but not Dex
(Zhou et al., 2015)	Cynomolgus	G6	Sevoflurane	2-2.6%*	5hr	3/7mnths	No effect on behavioural testing or brain structure

2.3.2. Clinical studies

It is only in recent years that clinical studies have been carried out to determine the effects of paediatric anaesthesia on neurodevelopmental outcomes. As the risks associated with anaesthesia in children have only recently been highlighted, the vast majority of clinical data currently available are retrospective studies, where cohorts were extracted from previously collected patient data which were not designed with the question of anaesthetic neurotoxicity in mind. What is interesting is that a surprising number of these studies do actually find some significant differences between infants exposed to general anaesthesia in the first years of life compared with control children. Some of the results have been outlined in Table 2.3. One important trend which comes out of these data is that some neurodevelopmental outcomes measured are limited in their ability to detect differences between infants exposed to anaesthesia or not. In general, behavioural tests do not show differences between groups and IQ tests have variable results, particularly when group testing is administered (Flick et al., 2014). On the other hand, language seems to be affected by exposure to anaesthesia (Ing et al., 2012). However, these retrospective cohort studies all use widely varying inclusion criteria, outcome measures, and statistical analysis, making a true comparison quite difficult. One meta-analysis carried out in 2015 did determine that single exposure to anaesthesia during early childhood was moderately associated with neurodevelopmental issues (Zhang et al., 2015). This probably means that any toxicity in children is relatively subtle and needs robust experimental design to determine the true extent, if any.

Table 2.3 **Clinical studies regarding anaesthetic neurotoxicity in young children.** A number of retrospective studies have been carried out to investigate the potential neurotoxicity of anaesthetics in young children.

Reference	Total	Anaesth	Age	Notes	Tests	Result
DiMaggio et al., 2009	5933	383	<3	Retrospective – Matched peers USA	Diagnosis of behavioural /developmental disorders	Increased risk of diagnosis with behavioural or developmental disorders
Flick et al., 2011	700	350	<2hrs	Retrospective - Matched peers USA	Group administered cognition/achievement, incidence learning disability/emotional or behavioural disorder	Multiple exposures associated with LD and speech/language impairments
Ing et al., 2012	2868	321	<3yrs	Retrospective, from RAINE study	Neuropsychological tests	Deficits in language and abstract reasoning vs. Non anaesth children
Ko et al., 2014	16465	3293	<3yrs	Retrospective - Matched peers Taiwan	Incidence of ADHD	No increased risk of ADHD
Stratmann et al., 2014	56	28	<1yrs	Retrospective - Matched peers USA	Object recognition, familiarity, IQ, child behaviour	Decreased scores in object recognition Study matched with rats to find same result
Backeljauw et al., 2015	106	53	<4yrs	Retrospective - Matched peers USA	Oral/written language, intelligence, MRI	↓ vs controls in listening comprehension/IQ, Lower grey matter density in occipital lobes/cerebellum
Gano et al., 2015	137	43	Premature	Retrospective, from MRI study	IQ, neonatal MRI	Decreased IQ with ≥2 anaesth exposures. No association with MRI sedation

The real test of the effects of general anaesthesia on childhood development will be prospective studies, where subjects are randomised into treatment groups with the express intention of studying the differences between exposure to general anaesthesia and other interventions. There are some such studies already under way, and recently the first of these published secondary outcome measures from children at 2 years of age. The study randomised infants under 60 weeks gestational age (sum of time *in utero* and postnatal age) undergoing inguinal herniorrhaphy to either general anaesthesia (sevoflurane) or spinal anaesthesia (bupivacaine or its enantiomer) (Davidson et al., 2016). The primary outcomes of this study will be at 7 years of age; however the data for 2 years has been collected and published to give an early idea of the effects. This study of 532 children found no association between anaesthesia in early infancy and a developmental deficit as assessed by the Bayley Scales of Infant and Toddler Development III, a set of tests which assess five outcomes; cognition, receptive language, expressive language, fine motor and gross motor. It is worth noting that this developmental scale has been shown to underestimate developmental delays in a similar population previously, so may be underestimating any damage here (Chinta et al., 2014). However it is possible that the effects seen in retrospective studies, although corrected for confounding factors, could still be looking at the wrong cause. When a child undergoes surgery, generally there are other stressors surrounding the event which affect not only the child but their family. These effects could lead to differences in the way the children develop at key stages. One interesting retrospective study did attempt to answer this family effect, and found that having a surgery before 3 years of age was associated with developmental delays. However, when data from matched siblings was compared there was no associated risk of surgery (DiMaggio et al., 2011). This result cannot fully answer the question as it was a retrospective study with no specific behavioural or

developmental tests carried out on children; instead the data were collected from doctor's reports and may not be comprehensive. Only true prospective studies may be able to determine the effects of anaesthesia on children without confounding factors within the family, such as stress, which would be difficult to correct for.

Another factor which may cause damage which is then attributed to anaesthesia is the surgical intervention itself, with mechanical tissue damage and inflammatory responses being some of the effects of surgery. However, much of the animal data finds no difference between anaesthesia alone or in combination with surgery (Davidson, 2012). One study, which used the same test of learning and memory in children and animals, found the same impairments and areas of brain changes between the two species (Stratmann et al., 2014). In the rat, anaesthesia alone was compared with surgical intervention and no change to the damage was found, implying that the drug caused the damage. Since the two pathways were shown to be similar, it is not difficult to expect the same responses take place in humans. The biggest challenge which needs to be addressed when planning studies is using the most appropriate tests. One study only found a difference when comparing subjects to matched controls; when compared to the general population there was no significant difference (Backeljauw et al., 2015). Another problem is the use of group testing versus individual testing. Some reports state that group testing is not sensitive enough to determine subtle changes after anaesthesia exposure. While it might be argued that if they are still within normal boundaries the effects are not relevant, it is still important to discover any adverse effect. This is particularly relevant if the disease, co-morbidities and other biological stressors do cause damage, prompting the question as to whether the use of particular anaesthetics exacerbate this damage in vulnerable children.

2.4. N₂O neurological effects

2.4.1. Clinical use of N₂O

Nitrous oxide (N₂O) is a gaseous agent which, for over 140 years, has been used as an analgesic and anaesthetic agent. Discovered in 1793 by Joseph Priestly, it was first used by Sir Humphrey Davy. He initially investigated the effects on himself, before allowing some acquaintances and patients some use (Goerig and am Esch, 2001). By 1799, nitrous oxide, otherwise known as laughing gas, was being used as a recreational agent, and continued to be used purely as such until Horace Wells, an American dentist, used it as an anaesthetic during a tooth extraction on himself in 1844. Unfortunately, the following year, when exhibiting the anaesthetic properties to medical students, his patient cried out, possibly due to a problem with the administration, and Wells was unable to convince them of the advantages of using inhalational anaesthetics for surgeries (Jacobsohn, 1995). However, around the 1870s, N₂O use in surgery and dentistry became more commonplace (Eger et al., 2014), and now it is one of the most commonly used inhalational anaesthetics, with a place on the World Health Organisations model list of essential medicines (World Health Organisation, 2015).

N₂O has a minimum alveolar concentration (MAC) of 104% (Becker and Rosenberg, 2008). MAC refers to the minimum concentration needed to produce a suppression of motor response in response to surgical stimulus in 50% of patients. As can be inferred then, a MAC of 104% precludes the use of N₂O as an anaesthetic agent at anything other than hyperbaric concentrations. However, N₂O still plays a large role in a clinical setting, used both for analgesia and to allow for a phenomenon called anaesthetic sparing. This refers to the ability of clinicians to use N₂O during anaesthesia to allow for quicker induction (Lee et al.,

2013b) or lower concentrations of volatile anaesthetic agents such as isoflurane or sevoflurane (Jakobsson et al., 1999, Whyte and Booker, 2004). Explained *via* the “second-gas effect”, it is suggested that when N₂O is taken into the lungs, it is absorbed into the bloodstream quickly, leaving the alveolar space at a subatmospheric pressure. This, in turn, can induce the inspiration of a greater volume of air and, in this case, any anaesthetic mixture therein (Peyton et al., 2011). As well as use during surgery as an anaesthetic adjunct, nitrous oxide is also very commonly used as an analgesic agent. In a 50:50 gas mixture with O₂, distributed as Entonox, N₂O can quickly give great pain relief. It has little possibility for suffocation due to the high concentrations of oxygen used and, with the use of a handheld face mask, if a patient does become sedated the hand holding the mas will drop from the face and normal air flow will resume. For this reason, Entonox is widely used as a labour analgesic and does not require the presence of an anaesthetist. In the UK, over 70% of labouring mothers use N₂O as analgesia (Rosen, 2002), and in other European countries such as Finland and Norway, as well as Australia it is used in approximately 50% of labours (Starr and Baysinger, 2013). However, some countries, such as the United States and Germany, have a very low uptake of labour N₂O (Collins et al., 2012a). N₂O has such a widespread usage not solely due to its ease of use, but also because of positive patient feedback and fast onset and offset of action. From inhaling the Entonox mixture, it takes approximately 50 seconds to feel the analgesic effect, with an equally fast cessation of action once breathing the mixture is stopped (Rosen, 2002). If contractions are timed accurately, parturient women can easily adjust their levels of analgesia to suit their needs without continuous administration of an agent. N₂O can also be used during caesarean section either for pain relief (Karasawa et al., 2003), maintenance of anaesthesia (Murdoch et al., 2013), or as an anxiolytic agent (Manouchehrian and Bakhshaei, 2014, Vallejo et al.,

2005). However the use of N₂O in this setting may be liable to change in the near future since it is classed as a greenhouse gas and, as such (Ishizawa, 2011), is heavily regulated on acceptable concentrations released to the atmosphere (Brown and Sneyd, 2015). As well as this, there are concerns over occupational exposure to N₂O as it is not readily metabolised in the body, so the majority will be exhaled by the patient (Yasny and White, 2012). For this reason, N₂O use in surgical settings requires scavenging units which are costly to install and maintain, and it is suggested that its clinical use be decreased (Enlund et al., 2003, Dimpel, 2003).

2.4.2. Biological effects

Nitrous oxide is active as an analgesic, anaesthetic and anxiolytic agent. Each of these functions is brought about by action at a number of different sites throughout the central nervous system. It was discovered in 1998 that N₂O acts as an NMDA receptor antagonist (Jevtovic-Todorovic et al., 1998). Anaesthetic agents can bring about their effects either by dampening down excitatory neuronal circuits, or enhancing inhibitory pathways, with a mean effect of depressing neuronal activity (Rudolph and Antkowiak, 2004). As the NMDA system is one of the major excitatory networks in the central nervous system, antagonising it will lead to profound depression of the CNS, therefore causing anaesthesia. NMDA antagonist anaesthetics, such as N₂O and ketamine, are known as dissociative anaesthetics and can lead to a range of symptoms including hallucination and amnesia. Amnesia is an advantage for an anaesthetic agent as it is one of the aims of general anaesthesia, the others being unconsciousness, analgesia, loss of muscle tone and a loss of reflexes (Brown et al., 2010). However, when taken as sub-anaesthetic doses, these dissociative anaesthetics can be used as recreational drugs due to the hallucinations and euphoric sensations, and

both N₂O and ketamine have a high rate of substance abuse (Substance Abuse and Mental Health Services Administration, 2011, Morgan et al., 2012, Coussaert et al., 2013).

It has been found that the analgesic effects of N₂O are due to a collection of receptor types, including the κ -opioid receptor in mouse abdominal muscle, and the μ - and ϵ -opioid receptors in the peripheral nerves of rats (Hodges et al., 1994). Another study discovered that in the guinea pig brain, N₂O binds the κ -opioid receptor in a non-competitive manner, while the μ -opioid receptor was inhibited competitively (Orestes et al., 2011). In humans, the opioid antagonist naloxone has been found to reverse the analgesic effects of N₂O (Gillman et al., 1980, Yang et al., 1980). There is also some evidence of involvement of the T-type calcium channel, where N₂O acts to indirectly inhibit these channels (Todorovic and Jevtovic-Todorovic, 2011, Orestes et al., 2011). Interestingly, it has been suggested that N₂O has this analgesic effects through T-type calcium channels *via* reactive oxygen species signalling, as described in Section 2.2.1. Finally, the two pore domain TREK-1 potassium channels have been implicated to underlie not only the analgesic properties of N₂O, but also the anaesthetic ones. The role of these receptors in cell death and neuroprotection has previously been investigated, which may help give some context to any biological effect of N₂O. While the δ -opioid receptor has been shown to provide neuroprotective benefits against glutamate excitotoxicity, neither κ - nor μ -opioid receptors could have the same action (Zhang et al., 2000). T-type calcium channel activation by another anaesthetic agent, bupivacaine, has been shown to induce neurotoxicity (Wen et al., 2013), while T-type calcium channel antagonism has neuroprotective benefits (Kopecky et al., 2014, Nikonenko et al., 2005). This work would therefore imply that N₂O is more likely to have neurotoxic effects than neuroprotective. On the other hand, TREK-1 channel activation has been shown to give neuroprotective benefits of volatile anaesthetic gases (Heurteaux et al., 2004) such

as sevoflurane (Tong et al., 2014). This again leaves us with a dual role possibility for the action of N₂O, furthering the need to test neurotoxicity in a range of models to truly understand the complex actions of this agent.

2.4.3. Neurotoxicity

There is intense debate regarding the actual neurotoxicity of nitrous oxide. While there is evidence from a number of animal models that N₂O is toxic either alone or in combination with other anaesthetic agents, it remains to be proven in clinical studies. However, from dissecting the published *in vitro* and *in vivo* data, a clear trend of vulnerable groups emerges which may help to focus future clinical studies in this area.

From animal studies, it is clear that there is an age-based divide in those vulnerable *versus* those insensitive to N₂O neurotoxicity. Most published *in vivo* data regarding N₂O neurotoxicity are in the rat at postnatal day 7 (P7). At P7, rat neurodevelopment is approximately equivalent to term human development. In a number of studies of N₂O in combination with isoflurane and/or midazolam, there was evidence of apoptosis and neurobehavioural deficits (Yon et al., 2006, Shu et al., 2012, Lu et al., 2006). It is of note that the majority of studies regarding the neurotoxicity of N₂O administer the anaesthetic in combination with isoflurane, a volatile anaesthetic agent, and sometimes midazolam, as this is a very clinically relevant mixture of gases, often used particularly in paediatric surgery (Bhaskar, 2013, Murray et al., 1991). Interestingly, in a study of different postnatal aged rats, P7 was found to be the most vulnerable to neurotoxicity by N₂O/isoflurane/midazolam, while by P14 there was little evidence of an effect (Yon et al., 2005). These findings have been extended to a non-human primate model, with N₂O + isoflurane given to rhesus monkeys resulting in increased apoptosis in the brain (Zou et al., 2011). Interestingly, the

brain areas affected differed between rat and rhesus models, with neonatal rats showing cell death through the thalamus and posterior cingulate and retrosplenial cortex (PC-RSC). Rhesus monkeys, on the other hand, showed great sensitivity in the temporal gyrus, hippocampus and frontal cortex. The RSC plays a vital role in memory and navigation in rodents, and there are known connections between the PC-RSC and thalamus such that lesions in the thalamus can cause amnesia by affecting the RSC (Garden et al., 2009). This may give a clue to the memory impairment found in N₂O/isoflurane toxicity. In rhesus monkeys, the brain areas affected are also well documented as being involved with learning and memory, as well as auditory processing, social cognition and processing language; a much broader and socially relevant set of structures. In rhesus monkeys, this N₂O/isoflurane exposure was found to impair learning and memory for up to 15 months after one neonatal exposure (Paule et al., 2015). In humans, a study of infants born with N₂O administration during the birth found these infants deficient in certain neurological areas, including resistant to smiles (Eishima, 1992), which may reflect the findings of social cognition areas affected in non-human primates. It was also found that another NMDA antagonist anaesthetic, ketamine, induces the same pattern of neurotoxicity in the perinatal rhesus monkey, suggesting NMDA antagonism may underlie the damage seen (Wang et al., 2006, Paule et al., 2011, Slikker et al., 2007). As well as evidence of N₂O neurotoxicity in the perinatal brain, there have been studies in the aged rodent brain showing similar trends. In a series of studies with 6 or 18 month old rats exposed to N₂O + isoflurane, it was found that the older animals suffered from impaired ability in the radial arm maze, a learning and memory behavioural test, while the younger animals were less affected (Culley et al., 2003, Culley et al., 2007). Again, this was corroborated by a similar study using another NMDA antagonist, MK801, where rodents grew more susceptible to neurotoxicity from postnatal

day 30 up to postnatal day 60. This would suggest a period between P7 and P30 where the brains were less vulnerable to neurotoxicity from these general anaesthetics. This is roughly equivalent to juvenile and adolescent humans.

There have been a number of clinical studies which, while not directly measuring neurotoxicity in humans, have hinted towards toxic side effects of either brief or occupational exposure to N₂O. N₂O can irreversibly inhibit the actions of methionine synthase, by binding one of the cofactors necessary for methionine synthase action, vitamin B₁₂ (Nunn, 1987). Inhibiting this enzyme results in a reduction of the myelination of nerve fibres, in particular the spinal cord (Safari et al., 2013). This comes about as myelin proteins must be methylated to maintain their structural integrity (Kim et al., 1997). This demethylation can result in myelopathy and neuropathy in patients, presenting as numbness or tingling of the extremities, weakness of the limbs, and impaired balance. While most patients presenting with myelopathies are as a result of chronic N₂O abuse (Hsu et al., 2012, Sotirchos et al., 2012, Cheng et al., 2013, Lin et al., 2011b), there have been a number of cases of clinical exposure in at-risk patients resulting in myelopathies. These include cases such as a patient undergoing routine dental surgery (Singer et al., 2008), and short (30 minute) exposure during surgical anaesthesia (Jordan et al., 2014). In addition, there was a report of a young 3 month old infant who, having 2 exposures to N₂O in the perinatal period, had seizures resulting in death, whereupon histology revealed severe brain atrophy and lesions (Selzer et al., 2003). In all of these cases, vitamin B₁₂ or methionine synthase deficiencies were responsible for the severity of the symptoms seen, either due to long term abuse or genetic or dietary deficiencies. As well as myelopathy, there have also been reports of psychosis associated with N₂O abuse (Sethi et al., 2006, Wong et al., 2014), and have even been implicated during manic relapse in a patient taking N₂O for four hours during

labour analgesia (Tym and Alexander, 2011). In all cases where the patient survived, treatment with vitamin B₁₂ supplementation eventually abated the symptoms over a period of days to weeks.

As well as case studies reporting individual occurrences, a small number of clinical studies have found evidence of N₂O toxicity (Solmaz et al., 2014). In one study of nurses occupationally exposed to N₂O, there were increased levels of reactive oxygen species and DNA damage compared with matched controls who had not been exposed to N₂O (Wronska-Nofer et al., 2012). Another study of patients undergoing 70% N₂O during anaesthesia also found increased evidence of DNA damage, as well as a significant increase in postoperative wound infection (Chen et al., 2013). These cases serve to highlight the possible consequences of nitrous oxide administration. The majority of these cases are not high threat to patients or staff. However, as many of the pathways affected are the same across different categories of patients, it is worth bearing in mind the risk factors associated with N₂O use in normal patients. As this thesis addresses the use of N₂O in vulnerable patients during an already neurotoxic incident, it is necessary to fully understand the mechanisms behind all forms of N₂O toxicity so that limits for its use may be put in place where necessary.

2.4.4. Neuroprotection

In the first paper which outlined the NMDA antagonistic properties of nitrous oxide, the neuroprotective role of N₂O was also investigated. It was discovered that N₂O could provide protection against excitotoxic insult, similar to MK801, another well-known NMDA receptor antagonist (Jevtovic-Todorovic et al., 1998). However, in the same paper, the neurotoxic effects of N₂O were discussed, where vacuolisation of cells was seen, caused by the swelling

of intracellular organelles such as mitochondria. This dual effect of N₂O underlies much of the controversy associated with its continued use in a clinical setting.

There are few papers which try to evaluate N₂O as a neuroprotective agent, and all but one of these deal with hypoxic brain injury. One limitation of this research is that all positive data highlighting the neuroprotective properties of nitrous oxide, with the exception of the original Jevtovic-Todorovic (1998) paper, come from one research group (Abraini et al., 2003, David et al., 2006, Haelewyn et al., 2008). As highlighted in an editorial on a recent research paper (Bracco and Hemmerling, 2008), this research group cites NNOXe and Air Liquide Santé as funding bodies for their work. Air Liquide Santé is a gas company who supply N₂O to both clinical and industrial companies, while NNOXe registered a patent in 2008 for N₂O in combination with a thrombolytic agent for the treatment of stroke (Abraini, 2008). To date, no other group has successfully replicated the neuroprotective benefits of N₂O in hypoxic brain injury models *in vivo*. In other studies regarding N₂O as a treatment for hypoxic brain injury, there has been no amelioration of the insult (Yokoo et al., 2004, Warner et al., 1990). This would suggest that for hypoxic ischaemic brain injury, such as stroke, N₂O may not have the beneficial properties which might be assumed due to its NMDA antagonism.

Besides its possible neuroprotective effects on the brain, there is one other area where N₂O has been researched; that is, as a positive medical strategy for the treatment of depression (Nagele et al., 2015), in the reduction of postoperative hyperalgesia (Echevarria et al., 2011) and, paradoxically, treating drug addiction (Benturquia et al., 2008, David et al., 2006). Much of these data have come from *in vivo* models, but some have been investigated in clinical trials. The main hypothesised methods of action regarding these positive

neurological effects are *via* endogenous opioid release, nitric oxide synthesis (Zarate and Machado-Vieira, 2015), and blockade of drug induce dopamine release. Many of these disorders have been shown to be treatable using ketamine, therefore it is unsurprising that N₂O has a similar effect. As there is no evidence of these treatment options affecting cell survival, they will not be discussed in greater detail here.

2.4.5. Mechanisms

Some of the mechanisms of action of N₂O have been discussed above, but this section will expand upon the general mechanisms of neurotoxicity caused by N₂O. The three main pathways which either cause toxicity or prevent N₂O from being used in clinical scenarios are its NMDA antagonism action, the alteration of homocysteine levels in the body, and its effects on cerebral blood flow. N₂O as an NMDA antagonist was posited as a factor which could enable it to be used as a neuroprotective strategy after excitotoxicity. However, as discussed above, there is little evidence that this intervention has any effect. On the other hand, NMDA antagonists have been shown to cause lesions and vacuoles in cells. This vacuolisation has been identified as mitochondrial swelling. These lesions were named Olney's lesions after John Olney, who first described this phenomenon with the use of ketamine and MK-801 (Olney et al., 1991). Nitrous oxide has been found to induce vacuolisation which can be reversed during short-term exposure, but cannot itself cause full lesions (Jevtovic-Todorovic et al., 1998). It can, however, cause cell death when administered for long periods (8+ hrs) (Jevtovic-Todorovic et al., 2003a). Both of these studies use an anaesthetic concentration of N₂O, 150% in hyperbaric conditions, so are not necessarily relevant to current clinical practice. Nonetheless, they do show clear evidence that N₂O has a similar effect to other neurotoxic NMDA receptor antagonists such as ketamine and MK-801, albeit a much smaller one. This NMDA receptor antagonism leading

to neurotoxicity is thought to be caused not directly by the inhibition of NMDA receptor action, but, instead, by the downstream effects this has. NMDA antagonist lesions are primarily found in the posterior cingulate-retrosplenial cortex (PC-RSC) and have been linked to acetylcholine (ACh) release in this area (Kim et al., 1999). It was discovered that NMDA antagonists, including 75% N₂O for just 1 hour, lead to increased ACh in the PC/RSC (Shichino et al., 1998). This ACh release is associated with a release of inhibitory tone from GABAergic neurons. NMDA receptors on the GABAergic neurons ensure consistent neuronal activation, therefore consistent inhibition of ACh release (Shichino et al., 1998). As N₂O antagonises NMDA receptors, there is a reduction in GABA release from neurons (Wakita et al., 2015), resulting in a loss of inhibition and therefore increased acetylcholine concentrations. This acetylcholine release has been implicated in the increased vomiting experienced by some patients receiving N₂O, as ACh stimulates the area postrema, a major emesis centre (Strominger et al., 2001). N₂O has also been shown to inhibit ACh receptor activity (Suzuki et al., 2003). Both the PC/RSC and the hippocampus, another area affected by N₂O induced ACh increases (Giovannini et al., 1994), are involved in learning and memory pathways, therefore any changes in cell signalling in these areas could have negative effects in vulnerable patients, particularly the very young or old.

Perhaps the most clinically relevant negative effect of N₂O is its interaction with vitamin B₁₂. It has been shown to irreversibly bind to the cobalt atom in vitamin B₁₂, otherwise known as cobalamin, and interrupt normal function (Nunn, 1987). Under normal circumstances, the amino acid methionine is converted to homocysteine (Hcy) as an intermediary step in the synthesis of other essential amino acids such as cysteine (Braekke et al., 2007). Homocysteine, a non-protein α -amino acid, is then either converted to cystathionine, or converted back to methionine by methionine synthase, the enzymatic pathway which

requires cobalamin. During normal physiological events, homocysteine is a transient middle product, and serves no biological purpose itself, however when cobalamin function is compromised, there is a build-up of Hcy which has serious toxic effects throughout the body (Blaise et al., 2007, Daval et al., 2009, Troen et al., 2008, Zhang et al., 2009). The clinical administration of N₂O has been shown to increase levels of Hcy in patients and sometimes staff, including during dental surgery (Levine and Chengappa, 2007, Meyers and Judge, 2008), under general anaesthesia (Badner et al., 1998, Foschi et al., 2001, Myles et al., 2008, Nagele et al., 2011), and even during caesarean section (Zanardo et al., 2003). *In vivo* studies have found that the administration of N₂O to a pregnant dam can affect the Hcy levels of the foetus, and, indeed, the study by Zanardo et al. (2003) of patients undergoing caesarean section with N₂O analgesia, there were elevated Hcy levels in the mother but also in cord blood, which would also result in elevated levels in the foetus. As discussed above, the loss of vitamin B₁₂ can induce neuropathies due to a loss of myelination, but an increase in Hcy is linked with neuronal cell death (Blaise et al., 2007, Ho et al., 2002, Kruman et al., 2000, Wang et al., 2012). Interestingly, Hcy causes much of its neurotoxicity by acting as an agonist of the NMDA receptors (Ho et al., 2002, Kim and Pae, 1996), which is the opposite effect of that seen with N₂O. However, while this might suggest that the two actions could counteract each other, it is important to note that while N₂O acts to antagonise the NMDA receptors, it has a very fast onset and offset of action, while Hcy levels can stay elevated in the blood for hours (Nagele et al., 2011, Badner et al., 1998) or even days (Ermens et al., 1991, Badner et al., 2001), meaning eventually this increased Hcy concentration will lead to increased excitatory output. As well as altering neuronal signalling, high levels of homocysteine are associated with Alzheimer's disease and schizophrenia (Herrmann and Obeid, 2011, Zhuo et al., 2011, Levine et al., 2002, Muntjewerff et al., 2006), although a

causal relationship has not been established. The risks of N₂O inducing excess homocysteine leading to negative side effects are more common in patients already at risk of elevated homocysteine. These include patients with vitamin B₁₂ deficiencies, and those with gene mutations leading to enzyme deficiencies such as MTHFR (Shay et al., 2007), which is an important enzyme in the methionine-homocysteine-cysteine pathway outlined above. As described earlier in the chapter (section 2.6.3), these risk factors can have serious detrimental health effects on patients if not caught before treatment, even leading to death. Considering the use of N₂O in circumstances without full medical tests, such as during dental or emergency surgeries (Heinrich et al., 2015, O'Sullivan and Bengner, 2003), these risk factors could have effects on a wide range of patients.

Finally, N₂O is contraindicated for use under certain clinical circumstances due to its effects on circulation and blood clotting. In particular, for hypoxic incidents, N₂O can increase cerebral oxygen metabolism and blood flow (Bracco and Hemmerling, 2008, Deutsch and Samra, 1990), and can induce an increase in intracranial pressure (Henriksen and Jorgensen, 1973). In addition, N₂O has been shown to inhibit the action of tissue plasminogen activator (tPA) (Haelewyn et al., 2008), one of the first line treatments for strokes caused by blood clots. This evidence shows that N₂O can have serious negative effects on the haemodynamic stability and normal function of the brain, particularly for patients with brain injury (Hancock and Nathanson, 2004).

The large picture of nitrous oxide points towards a widely used gas with a long history of safety in a clinical setting. However, recent advances in molecular biology point towards a subtle yet dangerous element of neurotoxicity. In the general population, this toxicity may not have detrimental effects for patient health, but in vulnerable patient groups, the use of

N₂O could have long term consequences to neurodevelopment and nervous system health. More robust clinical trials are urgently needed to examine the effects of nitrous oxide in these vulnerable groups so that more up-to-date guidelines for the safe use of N₂O can allow this reliable treatment to continue its use without risking the health of patients.

2.5. Argon organoprotection

2.5.1. The history of argon

Argon, a chemical element with symbol Ar, atomic number 18, and atomic mass 40, was discovered in 1894. It is one of 6 noble or inert gases, and is the third most abundant gas in the atmosphere behind nitrogen and oxygen, comprising almost 1% of the air. The high percentage is primarily due to the decay of potassium-40 from the earth's crust to argon-40, causing high levels of argon in the atmosphere. Because of the unreactivity of the gas, as well as its relatively high density, it cannot then easily escape from the atmosphere, as lighter gases such as helium do.

Due to the abundance of argon, it is quite inexpensive (see Table 2.4), and so has a number of uses, both medically and industrially. Argon lasers are often used during ophthalmological surgeries including for diabetic retinopathy and LASIK laser eye surgery, as well as in flow cytometers. In an industrial setting argon is widely used due to its unreactive nature and low cost, for a range of purposes including welding, annealing and even as a filler for air-bags in cars.

2.5.2. Clinical research into argon

In recent years, noble gases have been subject to greater study in clinical scientific research. Xenon has long been used in a clinical setting due to its ability to induce anaesthesia in

humans. A number of review articles have been published, highlighting the main attractions of xenon anaesthesia over many common inhalational anaesthetics, including benefits such as greater analgesia, rapid onset and offset of action and reduced cardiovascular depression (Ikonomidou et al., 1999, Sanders et al., 2003, Hecker et al., 2004, Jordan and Wright, 2010). Despite the extremely high costs of xenon, new technologies have been designed to allow for the recycling of air during anaesthesia to allow less of the gas to be used for each surgery (Mellon et al., 2007). In addition to its benefits as an anaesthetic, xenon has also been proven to have a range of organoprotective effects *in vitro* (Durieux and Davis, 2010), *in vivo* (Esencan et al., 2013, Loepke et al., 2008), and is even now in clinical trials (Jevtovic-Todorovic and Olney, 2008). These trials include CoolXenon3 (ClinicalTrials.gov identifier: NCT02071394) and TobyXe (ClinicalTrials.gov identifier: NCT00934700). However, although the cost of xenon may be overcome using specialised equipment, it still has an extremely high cost of usage which could limit uptake. In addition, an important consideration which could discourage use as a neuroprotective agent is one of the main reasons why xenon is so widely used already; its anaesthetic action. There are a number of risk factors associated with using anaesthetics agent in the context of brain injury, for example due to increased intracranial blood pressure (Brambrink et al., 2012), decreased cerebral blood flow (Creeley et al., 2013) and altered blood glucose levels, which can impact the brain (Creeley et al., 2013). Xenon may also depress the pontine system (Brambrink et al., 2010a), which is associated with respiratory control, and can induce apnea in subjects (Paule et al., 2011). This could be particularly dangerous for patients after hypoxia incidence, and even more so for young infants where there may be different sensitivities. This, when combined with its relatively higher density (Table 2.4) which can increase airway resistance (Sun, 2010), could

indicate that particularly in the case of neonates (Modak, 2013), xenon may have side effects which would only exacerbate a hypoxic insult.

Argon has a number of properties which would recommend its use over xenon in a neuroprotective setting. As mentioned above, xenon has a very high cost of use, with estimates of up to \$90 per hour for clinical use, even using a closed loop system . Considering most standard gaseous and volatile anaesthetics cost in the region of \$10 per hour (Kurjak and Chervenak, 2015), this is a significant cost compared with standard clinical costs. Argon on the other hand, with a cost over 200 times less than xenon (Table 2.4), could reasonably be used for hours on end without the need for a closed loop system and incur a fraction of the cost of xenon. Adding to this the lack of anaesthetic effects at normobaric pressures reduces the inherent risks as discussed above for xenon, and the lower narcotic potency argon overall has less likelihood of adverse side effects on vulnerable patients. As discussed above, density of a gas can affect the airway resistance of gases, but argon has a density very similar to normal air (argon is 1.784g/L while air is 1.19g/L). This would suggest that argon could be breathed without having any effect as compared to normal air. In these physiological and economic considerations, argon would appear to be superior to xenon as a clinical neuroprotectant.

Table 2.4 **A comparison of the properties of xenon versus argon.** There are a number of differences between the two gases that make argon a better choice for clinical use including narcotic potency, cost (Loetscher et al., 2009) and density.

	Xenon	Argon
Anaesthetic?	Yes	At hyperbaric concentrations
Narcotic potency	25.6	2.3
Protection	Brain, kidney, cell culture, spinal cord	Brain, kidney, cell culture, lung
Abundance	0.1ppm	9300ppm
Cost	€20/L	€0.09/L
Density	5.894g/L	1.784g/L

2.5.3. Neuroprotective research

The first scientific data which pointed to the protective benefit of argon were published just ten years ago. The earliest is one of the few argon publications not focusing on the central nervous system; instead studying cell death in the hair cells of the ear (Yarin et al., 2005). This work showed that argon could protect the organ of corti from cytotoxic substances, in some scenarios inducing 100% protection. Two years later, argon was shown to induce cardioprotection, most likely *via* inhibition of the mitochondrial permeability transition pore (Pagel et al., 2007). In 2009, two studies were published; one *in vitro* comparing a range of noble gases in a primary neuronal cell culture model (Jawad et al., 2009), and the second an *in vivo* exploration of argon as a neuroprotective agent against both ischaemia and traumatic brain injury (Loetscher et al., 2009). This marked a renaissance in argon research, where over twenty studies have been published in the last six years regarding its organoprotective benefits. These cover a wide range of models, organ systems and disorders, with the relevant neuroprotective studies outlined in Table 2.5. One striking difference between much of the published research is the high level of variability in concentrations of argon used, ranging from 15-95%. This lack of standardisation does

reduce the comparability between much of the data, as it is difficult to extrapolate results from high concentration argon data to performance at lower concentrations. However, variation between models is unavoidable and it does give reassurance that argon shows protection over the range of concentrations used, although more consistently so at higher concentrations.

Table 2.5 **Published data regarding the neuroprotective effects of argon both *in vivo* and *in vitro*.** The bulk of research into the biological effects of argon has been carried out in models of neuroprotection. This table summarises the major discoveries regarding the neuroprotective abilities of argon, as well as the mechanisms by which it may act. The Timing column refers to when argon was administered relative to the insult. ↑; increased, ↓; decreased, BV-2; a cell line, OGD; oxygen glucose deprivation, ROS; reactive oxygen species, NMDA; NMDA receptor agonists to induce excitotoxicity, TBI; traumatic brain injury, tMCAO; transient middle cerebral artery occlusion.

Year	Author	Model	Insult	Pre/Post	Concentration of argon	Outcome
2005	Yarin et al.	Rat (cochlea)	Cytotoxic drugs, hypoxia	During	74%, 95% (hypoxia)	Argon protective
2009	Jawad et al.	Primary neurons	OGD	During/Post	75%	Argon neuroprotective, not as much as xenon
	Loetscher et al.	Hippocampal slices	OGD, TBI	Post	25%, 50%, 74%,	Argon neuroprotective at all conc.
2011	Ryang et al.	Rat (brain)	tMCAO	Post	50%	Argon neuroprotective
2012	David et al.	Coronal slices Rat (brain)	OGD, NMD MCAO	Post	15%, 25%, 37.5%, 50%, 75% 50%	Ex-vivo - 25% toxic with OGD, 37.5-75% protective In vivo - ↓ infarct but ↑ damage subcortically
	Fahlenkamp et al.	Different cell lines	None	-	50%	↑ pH-ERK1/2 in BV-2, but not in primary cell cultures
	Zhuang et al.	Rat (brain)	Perinatal hypoxia	Post	70%	↓ cell death, ↑ neurological score
	Brucken et al.	Rat (brain)	Cardiac arrest	Post	70%	Argon neuroprotective
2013	Harris et al.	Hippocampal slices	TBI	Post	30%, 50%, 70%	Argon neuroprotective at 50% and 70%, less so than xenon
2014	Alderliesten et al.	Pig (brain)	Perinatal asphyxia	Post	50%, 80%	Argon didn't affect physiology (no measure of protection)
	Fahlenkamp et al.	Rat (brain)	tMCAO	Post	50%	Argon neuroprotective but molecular markers varied
	David et al.	Rat (brain)	Amphetamine	Post	75%	Argon ↓ locomotor sensitisation
2015	David et al.	Rat (brain)	Amphetamine	Post	75%	Argon ↓ locomotor sensitisation
	Ulbrich et al. (b)	SH-SY5Y (cells)	Rotenone	Post	25%, 50%, 75%	Time/dose dependent reduction of apoptosis
2016	Hollig et al.	Rat (brain)	Subarachnoid Hemorrhage	Post	50%	↓ mortality, ↑ weight, ↓ cell death
	Broad et al.	Piglet (brain)	Perinatal asphyxia	Post	45-50%	↓ cell death compared to hypothermia alone
	Zhao et al.	Primary neurons Rat (brain)	OGD Perinatal hypoxia	Post	70%	Protected against OGD ↓ inflammation, apoptosis, ROS
	Zuercher et al.	Rat (brain)	Cardiac arrest	Post	50%	No neuroprotection

2.5.4. Mechanisms of action

While almost all the data are in agreement that argon is protective against a range of injuries, there is little certainty about the mechanisms by which it has its effects. The majority of data published to date investigating molecular pathways point towards a general ability to protect the mitochondria from undergoing a destructive cycle, which leads to the release of pro-apoptotic proteins, thus preventing apoptosis and cell death. As described earlier in this chapter, the intrinsic pathway of apoptosis centres on the health and viability of the mitochondria. The Bcl-2 family of proteins contains both pro- and anti-apoptotic molecules which act on the mitochondria to induce or prevent mitochondrial outer membrane permeabilisation (MOMP). When MOMP occurs, pro-apoptotic proteins such as cytochrome c and apoptosis-inducing factor (AIF) are released from the inner mitochondrial membrane to the cytosol. Argon has been shown to increase levels of anti-apoptotic Bcl-2 family member proteins (Zhuang et al., 2012) and decrease pro-apoptotic members (Ulbrich et al., 2014), although in other studies there has been either no change or a decrease in anti-apoptotic Bcl-2 (Rizvi et al., 2010, Ulbrich et al., 2014). In another study, the levels of cytochrome c release were shown to be reduced by argon (Spaggiari et al., 2013). To relate this to downstream reduction of apoptosis, cleaved caspase-3, the effector caspase, has been shown to decrease after argon exposure (Spaggiari et al., 2013, Ulbrich et al., 2014). The mechanism underlying this mitochondrial protection may also rely on more upstream effectors. These include ERK1/2, a signal kinase which is phosphorylated significantly more after argon exposure (Fahlenkamp et al., 2012, Ulbrich et al., 2015b), and nitric oxide synthase (NOS) which is increased by argon (Fahlenkamp et al., 2014). ERK1/2 is thought to have a protective effect by regulating the opening of MPTP *via* downstream activators such as NOS and p53 (Pagel et al., 2007). As a side note, NOS was described in an earlier chapter

in terms of its damaging effects on the cell, whereas in argon studies its presence is deemed to have neuroprotective benefits. This dual role of NOS is widely reported (Moro et al., 2004, Cardenas et al., 2005, Brune et al., 1998) and suggested reasons range from differences in cellular *versus* vasculature effects (Dalkara et al., 1994), while dose response effects (Hu et al., 1999) appear to mediate this difference. Argon has also been shown to affect the expression of a range of cytokines (IL-1 β (Fahlenkamp et al., 2012)), growth factors (NGF, TGF β (Fahlenkamp et al., 2014)) and inflammatory molecules (CD3, IL-6, iNOS (Fahlenkamp et al., 2014)), as well as molecules such as heat shock proteins, heme-oxygenase 1 and HIF-1 α (Rizvi et al., 2010, Ulbrich et al., 2015b). It has also been shown that argon can increase Akt (Mayer et al., 2015), which is known to reduce apoptosis by phosphorylating Bad (del Peso et al., 1997). All these molecules could play a role in the protective effects of argon in different models of injury and toxicity.

The most intriguing unanswered question regarding argon and its biological effects are which receptors it acts upon. Xenon has been shown to act as an NMDA receptor antagonist, however, it has been proven that this is not the case for argon (Harris et al., 2013). It is currently suggested that argon is an agonist for the γ -amino-butyric acid type A (GABA-A) receptor. This was discovered by applying GABA receptor antagonists to rats using high pressure argon in an attempt to reverse the effects of argon narcosis (Abraini et al., 2003). The GABA-A specific antagonist gabazine reduced argon narcosis where a GABA-B antagonist could not. However, as this study had to be carried out using hyperbaric argon concentrations to achieve narcosis, it may not be a true reflection of the action of argon at atmospheric pressure, as is the case for all the results described above. New results have also identified antagonistic action at the μ -opioid receptor (MOR) as the likely site for the psychological actions argon has shown in reducing locomotor sensitisation to

amphetamines (David et al., 2015, David et al., 2014), an *in vivo* model for the long-lasting effects of drugs of abuse. Interestingly, opioid antagonists may be neuroprotective (Sanders et al., 2010b) and MOR have been implicated in reducing apoptosis *via* the phosphatidylinositol 3-kinase/Akt (PI 3-K/Akt) signal transduction pathway (Clanton, 2007). In addition, MOR activation can lead to disinhibition of GABA release since GABA receptor activation has been shown to attenuate excitotoxicity by counteracting glutamatergic overactivity (Zhang et al., 2007). As neither of these mechanisms has been studied in the aforementioned argon research, there is no way to determine whether this is a viable hypothesis for the protective effects of argon; however it does give some idea of possible directions for future pharmacological studies to take. One issue to bear in mind, however, is that the GABA receptors are excitatory in the perinatal period, as described in Section 2.1.2.1, so if argon does indeed act *via* the GABA receptor, its role during this time may be different.

2.5.5. Limitations and considerations for use

There appears to be little consensus regarding the optimal concentration of argon for clinical use. A recent study *in vitro* determined the concentration of argon in cell culture media at concentrations of 25%, 50% and 75% and found that, within two hours, the gas had equilibrated to 16 μ l/ml, 24.9 μ l/ml and 23.1 μ l/ml, respectively (Ulbrich et al., 2015a). At 4 hours, these concentrations had not significantly changed, showing gas concentrations stabilise early on. It is particularly interesting that there is such a small difference between 50% and 75% argon in air compared to how much is dissolved in media, which may explain some results wherein increasing concentrations of gas do not induce increased protection (Loetscher et al., 2009, David et al., 2012), where these studies utilised tissue slices in media. Similar results were obtained in piglets, where argon concentrations in the blood

were measured, with 50% or 80% inhaled argon giving blood concentrations of 23.6 μ l/ml or 34.5 μ l/ml, respectively (Alderliesten et al., 2014). An older study measured the solubility of argon in blood and found it to be similar between species, meaning that translating between human data and rodent data shouldn't pose any major complications (Hlastala et al., 1980).

One final consideration does not necessarily relate to the studies above, but should be noted when reading other research reports, is that many researchers still consider argon to be an inert gas. While chemically it is inert, making it good for neutral environments, there have been publications where argon has been used as the gas used to induce hypoxia instead of nitrogen, as argon dissolves into water faster than nitrogen, leading to quicker equilibrium to a hypoxic environment (Paquet-Durand and Bicker, 2004). The presence of argon in what should be a toxic insult could easily confound data. This illustrates the importance for researchers of keeping abreast of the latest literature regarding their scientific methods (Brar et al., 1999, Frenzel et al., 2002, Tekkok and Goldberg, 2001, Steinbach et al., 2004).

Chapter 3.

Aims and hypothesis

3.1. Aims

The aims of this work are to elucidate the effects of nitrous oxide in both *in vitro* and clinically relevant *in vivo* settings. In addition to this, the neuroprotective effects of the noble gas argon will be investigated in these same models to expand the knowledge base on this relatively newly described treatment. By utilising models with both no insult, and clinically relevant insults, the effects of these two gases in a range of settings can be discussed. In particular, this work will focus on the mitochondrial pathway of apoptosis as a possible convergent pathway by which these two agents could have their effects, whether this be protective or toxic. This work will use four models to assess different aspects of N₂O and argon actions:

1. *In vitro* primary neuronal culture
2. *in vitro* immortalised cell lines
3. *in vivo* neurotoxicity in naïve pups
4. *in vivo* model of perinatal hypoxia

3.2. Hypothesis

1. Nitrous oxide will be neurotoxic to neurons both *in vitro* and *in vivo*
2. Argon will provide neuroprotective benefits when administered in combination with N₂O
3. Both these agents will have effects on proteins which are important for the health of mitochondria

Chapter 4.

General methods and experimental models

4.1. Cell culture

One primary cell culture and two immortal cell lines were used; the H4 neuroglioma cell line and the SH-SY5Y neuroblastoma cell line. A primary neuronal culture was initially used to give the most relevant information regarding the actions of anaesthetics on the perinatal brain. Standard primary culture involves the use of foetal brain tissue from rodents as, at this age, there is a minimal glial cell population, yielding a more pure neuronal culture. This, in combination with the type of media and growth supplements used ensure a pure neuronal culture can be obtained to more easily focus investigations and interpret results.

4.1.1. Cell lines

H4 cells are often used in models of Alzheimer's disease (Colombo et al., 2009, Kinoshita et al., 2002) as they are easily transfected with the amyloid precursor protein (APP) gene. Here, however, we used them in their naïve form as a model of glial cells in the brain as previously used in similar anaesthetic studies (Sun et al., 2014, Zhang et al., 2014, Zhang et al., 2013). H4 cells are a stable cell line obtained from human neuroglioma. SH-SY5Y, on the other hand, is a human neuroblastoma cell line and offers a widely used neuronal model. While neither cell line is as biologically similar to the *in vivo* work as a primary culture (Gordon et al., 2013), immortalised cell lines do not require the use of animals, reducing the number of dams and pups needed for this study, with the added bonus of much higher cell yield to allow for greater experimental flexibility and scope. For this reason, many of the experiments that required high cell numbers, such as for Western blot analysis, use the immortalised cell lines

4.2. Cell maintenance

4.2.1. Primary cell culture

Primary cells were obtained from the cortices of embryonic day 18 (E18) Sprague-Dawley rat foeti. Gravid Sprague-Dawley dams were killed using cervical dislocation and the abdomen was sprayed down with 70% ethanol to sterilise. From this point onwards a sterile working field was maintained to reduce the risk of infection of the cell culture. Using an abdominal incision, the uterus was exposed, excised from the dam and placed in sterile saline. Pups were individually removed from the uterine sac, decapitated and skulls placed in an ice cold high sucrose, high glucose solution (HSG; 1000ml 1X HBSS [Gibco], 0.35g NaHCO_3 (anhydrous) [Sigma], 7g sucrose (anhydrous) [Sigma], 5g D-glucose (anhydrous) [Sigma]). Once all pups were culled (12 pups per dam on average, approximately 2 minutes between excision and completion of decapitations), brains were removed from the skull and placed in fresh ice-cold HSG. From this point all stages were performed serially to ensure all brains were at the same stage of dissection as close in time as possible. All dissection steps, including removal of brains from the skull were performed using 2 fine forceps. The cerebral cortices were first removed from the midbrain and hindbrain, before all meninges were removed to ensure no blood cells contaminated the culture. Following this, a microscope was used to micro-dissect the hippocampus from the cortices to yield pure cortical cultures.

Once pure cortical tissue was obtained, the tissue was roughly minced using fine forceps and transferred to 2ml Accutase (ThermoFisher Scientific) solution in a 60mm petri dish before passing tissue through a fire-polished pipette tip (Brainbits, UK) 4-5 times to mechanically and chemically reduce the tissue to single cells. This dish was transferred to a 37°C incubator with 5% CO_2 for 10 minutes before another 4-5 passes through the fire-

polished pipette followed by a final 10 minute, 37°C incubation. After the second incubation, media containing 10% foetal calf serum (FCS, Gibco) was added to neutralise the Accutase and the solution was triturated with a fire polished pipette tip until a homogenous solution with no tissue clumps was yielded. This solution was centrifuged at 1400RPM for 10 minutes and the pellet resuspended in supplemented Neurobasal media (Gibco) , filtered through a Falcon 40µm cell strainer (Fisher Scientific) to obtain a single cell suspension, and this solution assessed for cell density. Cell density was measured using a haemocytometer, where 100µl of cell suspension was added to the haemocytometer and cells within the blue 4x4 squares (described in Figure 4.1) were counted and an average of the four squares obtained. As each square contains a volume of 0.1µl, cells per 0.1µl could be assessed and cell density for plating calculated.

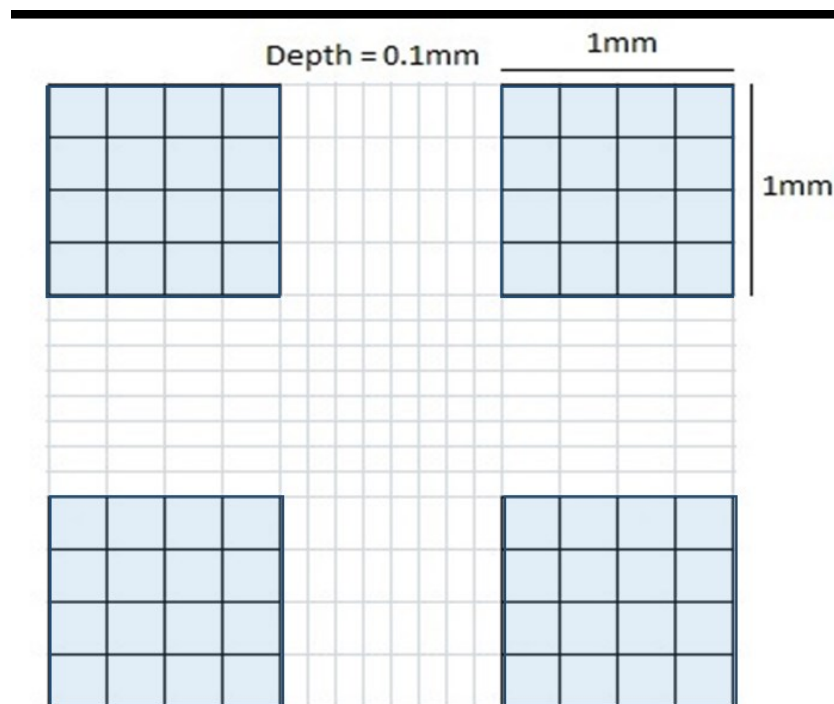


Figure 4.1 **Diagram of a haemocytometer, used to assess cell density of a solution.** Within one blue square is an area of 1mm x 1mm x 0.1mm, i.e 0.1µl. The average of the four blue areas is obtained and this value correlates to cells per 0.1µl. By scaling up, concentrations can be obtained to determine optimal seeding volumes

Cells were diluted as necessary in supplemented Neurobasal media (see Table 4.1) and plated at a density of 12.5×10^4 cells/500 μ l. Cells were plated either on poly-ornathine (Sigma) coated coverslips or 15mm petri dishes which had been pre-coated with poly-L-lysine (Sigma) overnight to ensure cell adhesion for maximal growth. Coverslips in 4 well plates were seeded with 500 μ l of cell suspension and 15mm petri dishes were seeded with 1ml cell suspension. Cells were incubated at 37°C with 5% CO₂ with half of the media changed every 2-3 days to ensure no complete removal of secreted growth factors from the cells. After 7 days *in vitro* (DIV), cells were used for experiments.

4.2.2. Immortalised cell lines

Both H4 and SH-SY5Y cells were treated in the same manner throughout the entire set of experiments, with the exception of the media used for each group, as outlined in Table 4.2. Cells were thawed from liquid nitrogen storage before use by defrosting aliquots of cells in a 37°C water bath before dilution in the relevant media. Once diluted, cells were immediately centrifuged at 1400RPM for 5 minutes to remove remnant of cryopreservative medium which could be toxic due to DMSO. Following this, cells were resuspended in supplemented media; DMEM (Sigma) for H4 cells and DMEM:F12 (Gibco) for SH-SY5Y. After resuspension, cells were plated in 75cm² tissue culture flasks (Greiner Bio-One), incubated at 37°C with 5% CO₂ with media changed every 2-3 days. When cells reached 80% confluence in the flask, cells were subcultured. For subculture, media was removed from the flasks and cells were washed with Dulbecco's Phosphate-buffered Saline (DPBS; ThermoScientific), before the addition of 0.5g/L trypsin EDTA solution (Sigma) to the flask. The flask was incubated for approximately 5 minutes at 37°C until cells rounded up and detached from the flask, with gentle manual force used to ensure all cells were fully detached. Once a fully floating cell suspension was obtained, trypsin was neutralised by addition of an equal volume of media

containing 10% foetal calf serum (Gibco). Cells were centrifuged at 1400RPM for 5 minutes and the resulting pellet resuspended in supplemented media. Cells were subcultured at between 3-8 times dilutions in 75cm² tissue culture flasks as before. For use in experiments, cells were plated onto either poly-ornathine coated coverslips in 4-well plates or 60mm petri dishes for experiments. After plating, cells were maintained at 37°C with 5% CO₂. Cells were used for experiments two days following plating, once cells had returned to their normal morphological shape and reached approximately 80% confluence.

Table 4.1 **Various media types used for different cell lines and the supplements added.** Each media contained phenol red as a pH indicator, with L-glutamine added as an essential amino acid, penicillin/streptomycin to ensure cultures were infection free, and different growth supplements based on the cell type used. While foetal calf serum is the norm for immortalised cell lines, B27 is used for neurons as it is serum free and optimised for this purpose.

	Primary neurons	H4 cells	SH-SY5Y cells	Freezing media
Base media				
L-glutamine				
Penicillin/ Streptomycin				
Growth supplement				
Other				

Table 4.2 **Plasticware used in the culturing of various cell types for experiments.** Cells were seeded either during culturing (primary neurons) or during subculture (immortalised cell lines) for different experimental outcomes. After seeding, primary neurons were left for 7 days in vitro (DIV) before use, while H4 and SH-SY5Y were left only 2 DIV before ready to use. Some cell types were not grown on particular plasticware (not applicable; N/A), while others did not use an adhesion coating (none). P, primary neurons. I, Immortalised cell lines

	Tissue culture flasks	60mm petri dish	35mm petri dish	4-well plate	Glass coverslip in 4-well plate
Area	75cm ²	21.5cm ²	8.8cm ²	1.9 cm ² /well	1.44 cm ²
Adhesion coating	P - N/A	P - N/A	P - Poly-L lysine	P - Poly-L lysine	P - Poly-L lysine
	I - None	I - None	I - None	I - N/A	I - Poly-Ornathine
Seeding density	P - N/A	P - N/A	P - 2.5 x 10 ⁵ cells	P - 1.25 x 10 ⁵ cells	P - 1.25 x 10 ⁵ cells
	I - 2.1 x 10 ⁶	I - 0.8 x 10 ⁶	I - 0.3 x 10 ⁶	I - 0.05 x 10 ⁶	I - N/A
Use	Subculture	Collecting cell lysate for western blot	Collecting whole cells for flow cytometry	Staining live cells for MTT assay	Collecting fixed cells for antibody staining

4.2.3. Cryopreservation and thawing cells

To allow for future use up of immortalised cell lines, cells were periodically frozen down at low passage numbers. This allowed a reliable supply of cells at a low passage number in case of infection, or if cells in use reached too high a passage number. Freezing down cells involved a similar technique to subculturing. Cells were detached from flasks using 0.5g/L trypsin-EDTA. Following trypsinisation, full media was added to neutralise the trypsin. Cells were pelleted in a centrifuge at 1400RPM for 10 minutes at room temperature. This pellet was resuspended in freezing media as described in Table 4.1, which included dimethyl sulfoxide (DMSO) (Sigma) to prevent the formation of ice crystals while freezing, acting as a cryoprotectant. Immediately after resuspension, cells were aliquoted into tubes and placed at -80°C to slowly freeze the cells over the course of a number of hours, usually overnight. After freezing, tubes were transferred to a liquid nitrogen storage container in the vapour phase for long term storage.

4.3. Cell culture gas exposure

4.3.1. Gas exposure

Once cells had been grown for the required time, they were used for gas exposure experiments. Media for these cells was changed 24 hours before gas exposure. Custom made gas exposure chambers were used for experiments. The chambers were 1.5L in capacity with an airtight locking system. All gases were delivered through an anaesthesia machine (Ohmeda International 2, Surrey, UK) via the chamber input, and a recording of the gas from the outlet was made using a capnograph equipped to also measure anaesthetic gas concentrations (Ohmeda Capnomac Ultima, Surrey, UK). Cells treated with either N₂O alone or with argon were removed from the incubator and sealed in the chamber. Gas was set to

flow at 2L/min and circulated through the chamber for at least 3 minutes to ensure all air was flushed before sealing and returning the entire chamber to the incubator. Inside the lid of the chamber a small fan ensured constant circulation of the gas inside the chamber. Control cells were left in the original incubator for the same length of time as treated cells were exposed to the gas. Cells were exposed to either (i) normal incubator air (74% N₂, 21% O₂, 5% CO₂) , (ii) 50% N₂O, 21% O₂, 24% nitrogen and 5% CO₂ or (iii) 50% N₂O, 21% O₂, 24% argon and 5% CO₂, described further in Table 4.3. Naïve cells were not moved from the normal incubator at 5% CO₂. After four hours gas exposure, cells were removed from the chamber.

Table 4.3 **Gas exposure concentrations for *in vitro* cells.** These concentrations were used for all cell types, with gas exposure maintained for four hours in a sealed chamber with no continuous flow of gas. Naïve cells were left in the growth incubator undisturbed until collection.

	Naïve	N ₂ O treatment	N ₂ O + Argon treatment
O ₂	21%	21%	21%
CO ₂	5%	5%	5%
N ₂	74%	24%	-
N ₂ O	-	50%	50%
Argon	-	-	24%

4.3.2. Oxygen Glucose Deprivation

For tumour cell lines, oxygen glucose deprivation (OGD) was carried out to mimic the *in vivo* hypoxic insult. OGD depletes the cell of both the oxygen and glucose needed to maintain cellular energy production. In this model, OGD was performed after the normal 4 hour gas

exposure described above, which in this case was used as a preconditioning element. Following cessation of preconditioning gas exposure, cell culture media was replaced with a balanced salt solution lacking glucose (OGD solution; 6.78g/L NaCl, 0.4g/L KCl, 0.11g/L MgSO₄ hydrate, 0.16g/L NaH₂PO₄ dihydrate, 0.2g/L CaCl₂ dihydrate, 2.18g/L NaHCO₃), which had been deoxygenated by bubbling through with pure nitrogen for 20 minutes in a Dreschel bottle. Once the culture media was replaced by this OGD solution, the cells were placed in a hypoxia chamber (Billups Rothenburg, CA) which was flushed and filled with 95% N₂ and 5% CO₂. This chamber was then placed in a 37°C incubator and left for 75 minutes to allow damage to occur. Once 75 minutes OGD was complete, the OGD solution was removed and fresh media appropriate to the cell line was added, after which the cells returned to the incubator for recovery. A collection timepoint of 24 hours after the start of the preconditioning gas exposure was chosen to allow the effects of the hypoxia to take effect. Cells were collected and analysed as for the normal gas exposure, described below in Section 4.3.3.

4.3.3. Sample collection

Following exposure, cells were collected for a number of different analysis techniques, including MTT assay, propidium iodide staining, western blot, immunocytochemistry, and flow cytometry. For immunocytochemistry, cells on coverslips were washed in phosphate buffered saline (PBS) before fixation using 4% paraformaldehyde (PFA) in PBS for 12 minutes. Once fixed, cells were rinsed and stored in PBS at 4°C until used for staining, as described in Section 4.6.1. For western blot, cells in 60mm plates were washed in PBS while kept on ice to keep cool. Radio-Immunoprecipitation Assay (RIPA) buffer (Sigma) with protease inhibitor (cOmplete Mini, Roche), PMSF (Sigma) and Dithiothreitol (DTT, Sigma) added was used to lyse cells. This supplemented RIPA buffer was added to the washed

dishes which were transferred to a fridge at 4°C for 15 minutes to fully lyse cells. Lysate was collected, centrifuged at 3000G for 5 minutes at 4°C to remove cell debris, and the supernatant was removed and stored at -20°C until used for western blot, as furthered described in Section 4.6.4. Cells used for MTT assay were grown in 4 well plates without coverslips. Following gas exposure, cells were returned to the normal incubator for a further 24 hours to allow for any progression of cell death to occur, before performing the MTT assay, as described in Section 4.6.2. Cells for propidium iodide staining and flow cytometry were used 24 hours after the start time of gas exposure, described in Sections 4.6.3 and 4.6.3.2 respectively.

4.4. Animal experiments

This study was approved by the Home office (UK) and conforms to the United Kingdom Animals (Scientific Procedures) Act of 1986. All efforts were made to minimise any animal suffering and the minimum number of animals necessary to achieve statistical significance were used.

Sprague-Dawley (SD) rats (Harlan UK) were used for *in vivo* experiments. They were housed in a 12-hour-light/12- hour-dark schedule, with temperature (24°C) and humidity (23%) controlled conditions and had access to food and water *ad libitum*.

4.4.1. *In vivo* toxicity

To determine whether the administration of anaesthetic agents without the presence of a hypoxic insult had any effect on molecular pathways within the brain, a protocol was set up to test pups at both postnatal day 3 and postnatal day 7. Pups for these experiments were

born by vaginal delivery, with day of birth designated postnatal day 0, with no intervention until experiments.

4.4.2. Gas exposure

Gas exposure was carried out using a modified chamber, with a tight sealing lid to prevent leakage of gas, and two tightly fitted tubes for inlet and outlet of gases. The chamber base was filled with soda lime pellets and silica gel crystals to absorb excess CO₂ and moisture respectively. The base was then covered to prevent contact of pups with either material. A thermometer was attached to the inner lid of the chamber to monitor temperature within. The entire chamber was placed within a heat box heated to 37°C and left for 30 minutes to equilibrate to 37°C inside the chamber. Pups were removed from the home cage immediately prior to experiments and placed within the chamber. All gases were delivered through an anaesthesia machine (Ohmeda International 2, Surrey, UK) at concentrations described in Table 4.4 via the chamber input, and a recording of the gas from the outlet was made using a capnograph equipped to also measure anaesthetic gas concentrations (Ohmeda Capnomac Ultima, Surrey, UK). The groups consisted of i) Naïve; 21% O₂, 78% N₂ and 0.93% argon, ii) Maternal deprivation; as for naïve but removed from the home cage, iii) N₂ group; 21% O₂, 79% O₂, iv) N₂O group; 21% O₂, 29% N₂, 50% N₂O, and v) N₂O plus argon group; 21% O₂, 29% argon and 50% N₂O. This trolley output was then attached to the chamber input to start the flow of gases to the pups, and the gas flow maintained throughout the experiment to ensure adequate oxygenation and to prevent the build-up of CO₂. The chamber output was monitored for CO₂ output to ensure this did not rise to toxic level. The chamber was of clear plastic so pups could be monitored throughout the experiment. Pups were left for four hours in the chamber before gas flow was stopped and pups removed. As a control, some pups were removed from the mother and placed in the

exposure chamber with the lid off and no gas flow at 37°C to give maternal deprivation. As a pure naive control, pups were left with the mother throughout the experiment

Table 4.4 **Gas exposure concentrations for postnatal day 3 and 7 pups.** These concentrations were maintained for four hours via continuous flow of gas to pups in a clear plastic chamber sealed except for inlet and outlet valves. Maternal deprivation pups were in the chamber but with no lid and no flow of gas. Naïve pups were left with the dam throughout the entire experiment.

	Naive	Maternal deprivation	N ₂ treatment	N ₂ O treatment	N ₂ O + Argon treatment
O ₂	21%	21%	21%	21%	21%
N ₂	78%	78%	79%	29%	-
N ₂ O	-	-	-	50%	50%
Argon	0.93%	0.93%	-	-	29%
Location	Home cage	Exposure chamber	Exposure chamber	Exposure chamber	Exposure chamber

4.4.3. Sample collection

Immediately after cessation of gas exposure, pups were removed from the exposure chamber and kept at 37°C during sample collection. In this experiment, the brains of pups were either fixed for tissue histology or dissected and snap-frozen in liquid nitrogen for Western blotting.

For tissue histology, fixation *via* transcardiac perfusion was carried out. Due to the small size of the pups, a modified procedure was carried out. Pups were deeply anaesthetised using sodium pentobarbital (Central Biological Services, Imperial College London) at a concentration of 200mg/kg, administered intraperitoneally. Once anaesthesia had been verified by a loss of hindpaw pinch reflex, an incision along the sternum was made to expose the rib cage. The rib cage was cut and clamped open to expose the heart. Using a blunted

needle to reduce the risk of puncturing too deep through the heart, the left ventricle was pierced and the needle held in place by hand. A small cut was made in the right atrium to allow the drainage of blood. The needle was connected to two syringes by way of a 3-way valve. Before placement in the heart, the tubing and needle were flushed through with phosphate-buffered saline (PBS). Once placed in the heart, PBS was flushed through the heart and circulatory system by slowly depressing the syringe at a rate of approximately 1.5ml/min. Once the body was drained of blood, as determined by clearance of the liver from a deep brown/red to a pale yellow, the valve was switched to allow the flow of PFA. 5ml of PFA was flushed through the body at a rate of 1.5ml/min until the body had become rigid throughout. Following fixation, the whole brain was immediately dissected from the skull and postfixed in 4% PFA, stored in the fridge at 4°C.

For Western blot, microdissection of the brain was carried out to separate the hippocampus and cortex, or in some cases the whole brain was frozen. Pups were decapitated using sharp scissors to cull, and the brain exposed by cutting through the skull along the midline. The brain was removed and placed in a bath of ice cold PBS for microdissection. In the case of brains dissected, the cortices were removed and the hippocampus dissected out from the cortex. Whole brains or cortices were wrapped in tin foil while hippocampi were placed in 1.5ml Eppendorf tubes before being placed in liquid nitrogen to snap freeze. These samples were then stored at -80°C before use.

4.5. *In vivo* hypoxia

There are a number of different perinatal hypoxia models which are currently used, and each has its distinct advantages and disadvantages. The model used here, wherein pups are not removed from the uterus but instead the whole uterine horn placed in a water bath

without maternal blood supply, is one the most clinically reflective of the distress and hypoxia most commonly encountered during labour and birth (Weitzdoerfer et al., 2004). However the disadvantage of such a model is the relative immaturity of the pups at this age. This surgery is carried out on the last gestational day for the dam, embryonic day 22 (E22) for the pup. The brain of pups at this stage is still quite immature compared with a term human infant (Clancy et al., 2007), so comparisons need to take this into consideration.

4.5.1. Preconditioning gas exposure

To expose the pups to preconditioning gases while still in the uterus, the dams in the final 24 hours of gestation were exposed to the gas mixtures of interest, shown in Table 4.5 as these gases are known to cross the placenta and enter the blood stream of the foetus (Karasawa et al., 2003). A plexiglass chamber was used for gas exposure which had soda lime pellets and silica gel crystals in the bottom below a mesh floor, to absorb excess CO₂ and moisture respectively. The mesh floor was covered over with tissue and bedding to provide a more comfortable base for the dams. All gases were delivered through an anaesthesia machine (Ohmeda International 2, Surrey, UK) via the chamber input, and a recording of the gas from the outlet was made using a capnograph equipped to also measure anaesthetic gas concentrations (Ohmeda Capnomac Ultima, Surrey, UK). The dams were placed inside the chamber and gas flow immediately started. Groups consisted of i) Naïve group; dams were left in the home cage, ii) N₂O group; 30% O₂, 20% N₂ and 50% N₂O, and iii) N₂O plus argon group, 30% O₂, 20% argon, 50% N₂O. The chamber was covered over with paper to keep the dam in a darkened environment and to reduce stress. The dam was regularly monitored throughout the exposure. At the end of four hours exposure, the dam was removed for surgery.

Table 4.5 **Gas exposure concentrations for pregnant dams.** These concentrations were maintained for four hours via continuous flow of gas to dams in a clear plexiglass chamber sealed except for inlet and outlet valves. Naïve dams were left undisturbed in their home cage until surgery.

	Naïve	N ₂ O treatment	N ₂ O + Argon treatment
O ₂	21%	30%	30%
N ₂	78%	20%	-
N ₂ O	-	50%	50%
Argon	0.93%	-	20%

4.5.2. Hypoxia surgery

After removing the dam from the chamber, she was immediately culled via cervical dislocation. In the case of dams not exposed to anaesthetic gas mixtures, they were removed from the home cage and immediately culled by cervical dislocation. After death had been confirmed, a laparotomy was performed and the uterus exposed. As the uterus in a rat is comprised of two horns, one either side of the body and both leading to the vagina, this allowed the use of both uterine horns for different surgical interventions. One horn was clamped at either end to create a sealed uterine environment with pups still inside. This horn was removed and placed inside a water bath filled with sterile saline at 37°C. While these pups were in the water bath, the second uterine horn was removed; pups were excised from the uterus and removed from their individual amniotic sacs. After quickly stimulating all pups to breathe using cotton buds to remove moisture and prevent excess force, the umbilical cord was cut and pups were placed in a 37°C heat box for close monitoring. After the desired hypoxia time, the uterine horn in the water bath was removed and pups taken out and stimulated to breathe as for the caesarean section controls.

4.5.3. Surgical aftercare

Pups were monitored closely for one hour following surgery, regularly stimulated with warm, saline soaked cotton swabs to ensure movement and regular breathing was maintained. At the end of one hour, pups were marked according to treatment group using tattoo ink in a 0.3ml insulin syringe (BD Micro-fine+ U-100) injected into either the paw pad of front or hind paws, or the base of the tail. The location of tattoo was randomised between litters. After tattoo identification, pups were placed back to the heat box while the surrogate dam was prepared. The surrogate dam was a dam that had given birth to a litter within the preceding 24 hours. Her pups, which were used as naïve controls due to their natural vaginal delivery, were removed and counted. A small amount of faeces was removed from the cage and dissolved in warm saline. This solution was then rubbed on the backs of surgery pups to scent them and reduce the chances of rejection by the surrogate dam. A certain number of naïve pups were also tattooed and returned to surrogate dam if the total number of pups did not exceed 15, to avoid overburdening the dam. Pups were weighed on postnatal days 1, 3 and 7 to monitor growth, and samples were taken on postnatal days 1, 3 and 7. Postnatal day 1 and 3 samples were taken and used for Western blot, while postnatal day 7 samples were used for tissue histology.

4.5.4. Sample collection

Sample collection for both perfused tissue for histological analysis and frozen tissue for Western blot were collected in the same manner as Section 4.4.3, except on postnatal days 1, 3 and 7.

4.6. Analysis methods

4.6.1. Immunofluorescence staining

Immunofluorescence staining relies on antibody binding, with a primary antibody binding to the protein of interest, followed by binding of a secondary antibody conjugated to a fluorescent probe, which can then be visualised using a fluorescent microscope with lasers equipped to read the specific wavelength of the fluorescent probe used. While primary antibodies bound to fluorophores are commercially available, the two step primary-secondary is a more widely used method as it allows for signal amplification, as more than one secondary antibody molecule can bind to each primary antibody molecule. To prevent background staining, cells are initially blocked in a serum from the same animal as the secondary antibody was raised in, for example if a donkey anti-rabbit secondary antibody is used, donkey serum is used as a blocking agent. This means that once the secondary antibody is applied it should only bind to primary antibody sites, reducing background. To ensure there is no non-specific binding, control samples are used where no primary antibody is used, secondary antibody is applied and non-specific fluorescence is assessed.

Cells which had been previously fixed using 4% paraformaldehyde as described above were used for immunofluorescence staining. Initially, cells were blocked to prevent non-specific antibody binding which may have introduced high background staining. For all experiments, a 10% normal donkey serum solution in phosphate-buffered saline (PBS) with 0.5% Triton X-100 (PBS-T) was added to coverslips for 1 hour at room temperature. Immediately following blocking, cells were incubated overnight at 4°C in the desired primary antibody diluted in PBS-T with 5% normal donkey serum (see Table 4.8 for full list of antibody dilutions). Following primary antibody incubation, cells were washed three times with PBS before the

addition of fluorescent labelled secondary antibodies raised against the host animal of each primary antibody. Secondary antibodies were conjugated to either a rhodamine (red) or FITC (green) fluorescent molecule. All stages from this point onwards were performed in lower light settings so as to prevent photobleaching of the fluorescent probes. Secondary antibodies, diluted in PBS, were incubated with the cells for 1 hour at room temperature before washing three times with PBS. Cells were then mounted using a mounting media containing DAPI (Vector Laboratories, Peterborough, UK), a dye which specifically binds and stains nuclear material giving a blue fluorescence. Cells were mounted on glass microscope slides (VWR International, Lutterworth, UK) using DAPI mount and the edges of the coverslip sealed with clear nail varnish to ensure coverslips could not move or dry out, preventing damage to the cells. Cells were stored at 4°C in the dark before imaged using an Axiocam light microscope (Zeiss Gottingen, Germany).

4.6.2. MTT assay

The MTT assay works on a principal of live cells with healthy mitochondria have the ability to reduce the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a yellow colour, to formazan, a purple colour (Lobner, 2000). This test requires accurate cell seeding densities so that any difference in reduction is due to cellular changes as opposed to greater or lesser cell numbers resulting in greater or lesser reduction. The formation of purple formazan crystals within the cells is a result of this reduction. By dissolving these crystals in DMSO to create a solution, the depth of purple colour can be used as a surrogate marker of mitochondrial health and cell viability. Cells for MTT assay were grown in four well plates without coverslips. After gas exposure, cells were returned to the incubator for 24 hours to allow injury to develop. To carry out MTT assay, cells were first rinsed in PBS before the addition of a 0.5mg/ml MTT solution (Calbiochem) diluted in PBS. The cells were

returned to the incubator at 37°C with 5% CO₂ for 2 hours to allow reducing of the dye. The dye was then removed and 300µl DMSO (Sigma) was added to each well to lyse the cells and obtain a precipitate of the cell contents and dye. This solution was added to a 96 well plate in a 5x dilution in DMSO and read on a MRX II Microplate Reader (Dynex Technologies, Worthing, UK) plate reader at 595nm.

4.6.3. Propidium iodide staining

Propidium iodide (PI) is a fluorescent molecule, which cannot permeate the membrane of live cells. Once inside a cell, PI binds to DNA and fluoresces red (Zamai et al., 2001). For this reason, PI is often used as a nuclear stain for counterstaining different fluorescent protocols. However this also means that if the membrane is damaged, then the addition of PI and subsequent imaging will give an indication of which cells are alive and which are dead. In this way, a quantifiable measurement of the population of live versus dead cells can be obtained.

4.6.3.1. Microscopy

24 hours following gas exposure, primary neurons grown on coverslips for propidium iodide staining were removed from the incubator and the media removed. This was replaced by 5µm propidium iodide solution in PBS (Sigma). This solution was left on the cells for 5 minutes at room temperature away from light which could bleach the fluorescence. This PI solution was then removed and cells were washed three times with PBS to ensure there was no remaining PI solution before fixation. 4% PFA was added to the cells for 15 minutes at room temperature to fix the cells, which was then washed off. Cells were washed three times with PBS before mounting with a mounting media containing DAPI (Vector) and the edges of the coverslip sealed with clear nail varnish to ensure coverslips could not move or

dry out, preventing damage to the cells. Cells were stored at 4°C in the dark before imaged using an Axiocam light microscope (Zeiss).

4.6.3.2. Flow cytometry

Flow cytometry is a technique which allows for the quantitative measurement of fluorescently labelled probes or fluorescent molecules at a single cellular level (Riccardi and Nicoletti, 2006). Cells are stained and suspended in a single cell solution. This solution is then run in a stream of single cells through a laser beam where cells are analysed individually for the scatter of light in forward and side dimensions as measures of size and granularity respectively. This can be used to sort different cell types in a mixed cell population, or determine different sized particles in a single cell type solution, for example, to determine which populations are whole cells or debris. The fluorescence emitted by any fluorescent probes is also measured via specific sensors. For tumour cell lines, PI staining was assessed using flow cytometry as this allowed for more accurate staining and a higher throughput. This was attempted with primary neurons but the correct technique could not be determined which did not damage the cells and result in cell permeability and excess staining regardless of treatment. Following gas exposure or OGD, cells in petri dishes were placed back in the incubator for 24 hours to allow for the development of cell death. To prepare cells for flow cytometry, they need to be removed from the plate without damaging the outer membrane. For this, trypsin was applied for a short period to encourage detachment. Trypsin was added to plates for 7 minutes at room temperature before being neutralised by an equal or greater volume of media containing 10% FCS. Cells were agitated by gentle pipetting with a wide 1ml pipette tip, swirling the solution to gently lift the cells without breaking. This cell suspension was centrifuged at 1200RPM for 5 minutes and the supernatant discarded. The cell pellet was resuspended in PBS. This wash procedure was

repeated twice before the addition of a ready-made PI solution (eBioscience Inc.) for 5 minutes incubation. This cell suspension/dye combination was then run, without washing off the dye, on a Cyan ADP flow cytometry analyser (Dako) with an event limit of 10'000 events.

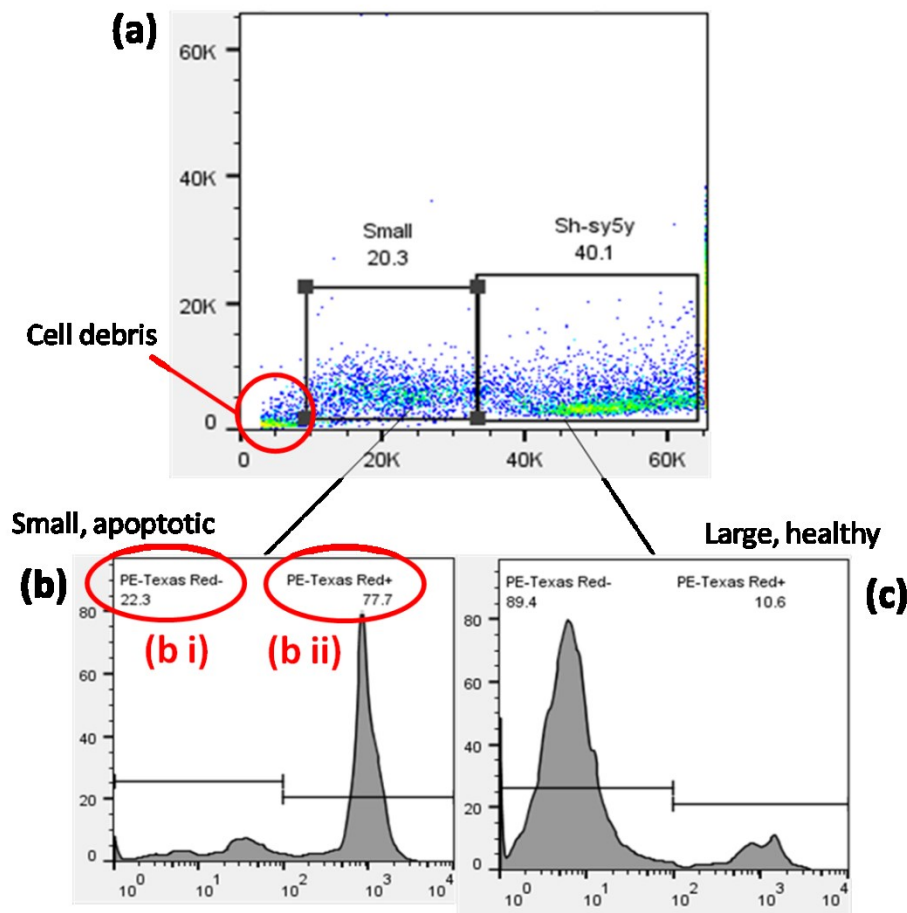


Figure 4.2 **A representative cell count from flow cytometric analysis of propidium iodide staining.** Cells counted were first gated according to size (a, x axis = forward scatter). Two sub-populations were gated, one of larger cells (designated SH-SY5Y) and one smaller (designated small). Propidium iodide fluorescence was analysed as either positive or negative in the small apoptotic group (b) or the large healthy group (c). Within each group, there is a PI negative set of cells (b i) and a set of PI positive cells (b ii).

4.6.4. Western Blotting

4.6.4.1. Tissue homogenisation

All tissue from *in vivo* samples had to be homogenised before use for Western blot. This involved using a mixture of lysis buffer and mechanical agitation to dissociate cells and

release intracellular contents. Two mechanical techniques were used, sonication for smaller tissues such as hippocampus, and a blade tissue homogeniser for larger pieces such as whole brain or cortex. Lysis buffer was prepared using a 10x lysis buffer stock (Cell signalling), diluted 1:9 with deionised water (dH₂O). To this solution, 5mM DTT (Sigma) was added to prevent oxidation, protease inhibitor (cOmplete Mini tablets, Roche, 1 tablet/10ml lysis buffer) and PMSF (Sigma, 1mM) were added to prevent protease activity during homogenisation and lysis. Samples were removed from -80°C storage and kept in liquid nitrogen short term before homogenisation. Working on a dilution factor of approximately 5ml/g of tissue, two hippocampi in an Eppendorf were diluted in lysis buffer. This tube was placed in the holder of the sonicator, the sonicator tip placed in the solution and pulsed on and off for approximately 10 seconds at an amplitude power of 15 microns. The sample was not sonicated for long periods and moved immediately to ice to prevent excess heat formation which could damage cellular elements. For larger tissue pieces, the same dilution factor was used. The blade homogeniser had similar limitations as sonication in that excess pulsing could lead to high heat levels, causing damage to tissue. For this reason tissue was homogenised in pulses for approximately 20 seconds and placed immediately on ice. From this point, samples were centrifuged at 20,000xG for 15 minutes to remove debris.

4.6.4.2. Protein quantification

The concentration of protein in each sample was determined using the Bradford assay (Bio-Rad). In this simple colorimetric assay, the dye Coomassie Brilliant Blue is changed from an initial reddish-brown colour to blue dependent on an increasing concentration of protein (Bradford, 1976). A standard concentration curve was obtained using bovine serum albumin (BSA) diluted in deionised water (dH₂O) at serial dilutions of 1000µg/ml, 500ug/ml,

250ug/ml, 125ug/ml, 12.5ug/ml and 1.25ug/ml. Each sample was diluted x25 in dH₂O, and 10µl of each sample and standard was plated in duplicate in a 96 well plate. The assay dye was diluted in dH₂O in a 1:4 ratio, and 200µl of dye added to each sample and standard. The standards and samples were read on an MRXII microplate reader (Dynex Technologies, Worthing, UK).

4.6.4.3. Gel electrophoresis

Gel electrophoresis is dependent on the separation of different proteins based on size and charge, allowing for the individual identification and quantitative measurement of individual proteins based on molecular weight. Following protein quantification, samples were prepared to run by gel electrophoresis. A vital step of gel electrophoresis is the denaturation of proteins from their folded quaternary, tertiary and secondary structures to a more basic unfolded form, which can then run through the gel. This is facilitated by each step in the process; the addition of SDS, DTT and heat denaturation. When samples were prepared, protein loading buffer containing SDS and DTT were added, to alter the charges of the proteins and break disulphide bonds respectively. Finally, heat was applied to ensure both SDS and DTT have the full desired effect and render the proteins straight and ready to run. To prepare samples, a 4X loading buffer solution (NuPage Loading Buffer, ThermoFisher) was used, alongside 200mM DTT and enough sample to make up to a certain concentration, as determined by quantification. dH₂O was added to bring the solution up to an even volume. Depending on the size of well used for gel electrophoresis, the loading volume changed, as outlined in Table 4.6. Final loading samples were heated in a shaking heat plate at 95°C for 5 minutes and rapidly transferred to ice to prevent refolding of the proteins before loading into gels. Two protein standard ladders were used, one coloured ladder (Novex Sharp protein standard, Invitrogen, UK) to measure the running of the gel and to

allow for accurate cutting of the membrane in the next step. A fluorescent protein standard (MagicMark XP protein standard, Invitrogen, UK) was also used to allow for accurate identification of bands during imaging, again described in further detail below. Gels used were precast bis/tris 12% gels (Invitrogen). Gels were mounted in a XCell SureLock box (Invitrogen) which was filled with NuPage MES SDS running buffer (Invitrogen). The gels were run at 200 volts for 35 mins using a PowerEase 500 powerpack (Invitrogen) until the 4kDa band was almost run off the gel, to ensure an even spread of all proteins.

Table 4.6 **Determining the loading volumes for gel electrophoresis.** The loading volumes for gel electrophoresis primarily depends on the size of the well being used. The sample volume stays the same regardless of the well size, but changes between samples as they vary in concentration, so this volume is not fixed.

Gel well size	Max loading volume	Sample volume	Loading buffer	200mM DTT	dH ₂ O	Final loading volume
12 well, 1.5mm	18µl	Xµl	5µl	1.8µl	11.2 - Xµl	18µl
15 well, 1.5mm	15µl	Xµl	3.5µl	0.75µl	10.57 - Xµl	15µl

4.6.4.4. Membrane transfer and staining

To stain the proteins which have been separated by gel electrophoresis, it is standard practice to transfer the proteins to a membrane. In these experiments, a specialised dry-blot system was used, which is faster than the traditional wet set-up. PVDF membranes were used for all experiments as generally PVDF is more compatible with the proteins of interest used here and is not as delicate as nitrocellulose, the other commonly used membrane type. After running the gel to completion, the gels were removed from the plastic casing and

placed on a PVDF membrane (iBlot Transfer Stack, Invitrogen, UK). This gel is then overlaid with wet filter paper, a conduction layer and finally a sponge on top. This whole sandwich is then run through with 20 volts current for 7 minutes, resulting in a membrane bound with proteins. This membrane was briefly rinsed with methanol to activate the normally hydrophobic PVDF, before rinsing in Tris-buffered saline with Tween-20 (TBS-T). A blocking solution of 5% dried skimmed milk powder (Cell Signalling) diluted in TBS-T was used for one hour at room temperature to block non-specific antibody binding. Following blocking, the membrane was incubated with antibodies as listed in Table 4.8, all diluted in 5% milk in TBS-T. Membranes were cut into 2-3 portions to allow the incubation of different proteins of various molecular weights. For example, using the coloured ladder with bands at different points, as shown in Figure 4.3, by cutting the gel at 30kDa and 65kDa, three pieces could be obtained to stain for PARP, phospho-Erk 1/2 and Bcl-2.

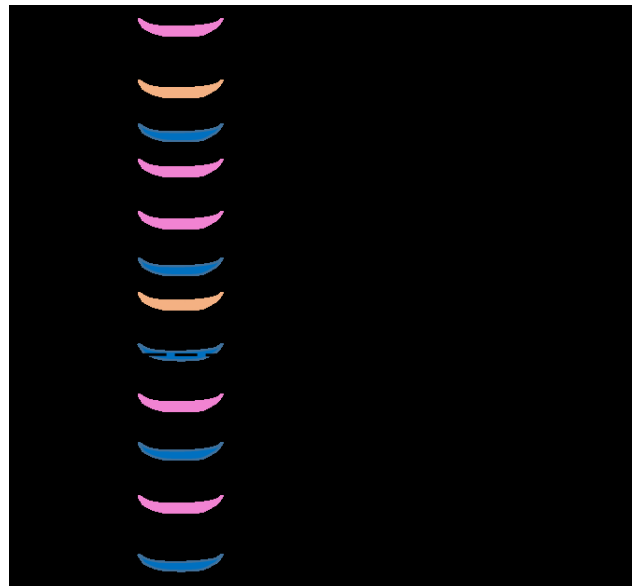


Figure 4.3 **Diagram of protein standard used during Western blot procedure.** The protein standard allowed the PVDF membrane to be cut into different pieces to allow for probing multiple proteins from one membrane. The topmost section was stained for PARP, the middle section stained for phospho-ERK 1/2 followed by GAPDH as an internal control, and the bottom section stained for two of four possible proteins. Bcl-2 was always stained on the same piece as Bax, while Puma and Cleaved caspase-8 were always probed on the same piece to avoid overlap of proteins if electrophoresis had not separated the proteins enough.

After incubating in primary antibody overnight at 4°C, the membrane was washed in TBS-T before incubation in the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody in 5% milk in TBS-T, as outlined in Table 4.8. After one hour room temperature incubation, the membrane was rinsed 3x in TBS-T and once in Tris-buffered saline (TBS). The housekeeping protein, GAPDH, was applied to the membrane in combination with the secondary antibody for one hour at room temperature due to the highly sensitive nature of the antibody. The housekeeping protein is one that is present in similar quantities in all cells, and can be used as a way to check the total amount of protein loaded, and allow for normalisation of samples. For antibody detection, a luminol detection (Santa Cruz Biotech) method was used, wherein a solution is applied to the membrane and the HRP in the secondary antibody mediates oxidation of the solution, resulting in light emission. Using a dark box and camera, the emitted light can be captured in an image. Luminol detection of bands was carried out with varying exposure times dependent on the antibody used, with approximately 2-5 minutes used for all antibodies, with the exception of the housekeeping protein which had approximately 30 seconds exposure. For the smaller molecular weight proteins, since many were in close proximity on the membrane, the membrane was stripped and re-probed with a different primary antibody. This was carried out using Restore Stripping buffer (Thermo Scientific) for 15 minutes, before rinsing 3x in TBS-T. From this point, blocking, primary and secondary antibody steps were carried out as before.

4.6.5. Tissue histology

Tissue histology was used on whole brain samples which had been previously fixed and stored in 4% PFA at 4°C. There are a number of different staining protocols which can be

used on fixed, sectioned tissue, with the biggest advantage being the ease of analysis in discrete brain areas which is more difficult with techniques such as Western blot where different areas need to be dissected out.

4.6.5.1. Tissue processing and sectioning

Tissue processing is the name by which fixed tissue is prepared for sectioning. For these experiments, tissue was embedded in paraffin and sectioned on a microtome, as opposed to the other commonly used technique of frozen sectioning in a cold mounting solution. The advantages of paraffin sectioning are the much smaller section thickness which can be achieved, as little as 3-4µm thick. However, the tissue processing for paraffin sectioning results in substantial shrinkage of the tissue so is not suitable for smaller sample sizes.

Tissue processing was carried out in an STP 120 automatic spin tissue processor (Thermo Scientific). Samples were placed in PFA and run through increasing concentrations of alcohol solutions to dehydrate the tissue. This is followed by 3 cycles in xylene to replace the alcohol, before placing samples in warm, liquid paraffin. This gives a sequential replacement of water with alcohol, then xylene, then paraffin to give a final sample which is completely infiltrated through with wax. The solutions and timings for different samples are shown in Table 4.7. Following tissue processing, the samples were then embedded in wax blocks to facilitate cutting on the microtome.

Table 4.7 **Steps to prepare paraffin sections for histological staining.** Samples were run through solutions first to remove the paraffin wax (xylene), then rehydrated over decreasing concentrations of alcohol with water, before a final rinse in either water or PBS, depending on the subsequent staining procedure.

Step	Solution	Duration
1	Xylene	2 min
2	Xylene	2 min
3	100% Alcohol	2 min
4	100% Alcohol	2 min
5	95% Alcohol	2 min
6	70% Alcohol	2 min
7	dH ₂ O/ PBS	As necessary

Sections were cut at 8µm thickness using a microtome for all samples and floated onto a warm water bath to smooth out any wrinkles. These sections were then mounted onto glass slides (Superfrost, VWR) which had been coated in a gelatinous subbing solution to promote tissue adhesion and prevent samples from being washed off during staining. Samples were left overnight to dry completely before staining. For all staining procedures, the same initial steps were carried out to deparaffinise and rehydrate the sections.

4.6.5.2. Cresyl violet staining

Cresyl violet, also known as Nissl stain, is used to stain the Nissl bodies, a type of large granular organelle specific to neurons (Pilati et al., 2008). This gives a cellular stain of the cytoplasm of neurons in the brain, giving a clear histological staining of the tissue. A 0.1% cresyl violet solution was prepared from a powder, with cresyl violet acetate (Sigma) dissolved in dH₂O, then adjusted to pH 3.5 using glacial acetic acid. Following rehydration, samples were placed in dH₂O for rinsing. Cells were immersed in cresyl violet for

approximately 5 minutes before rinsing again in dH₂O. Acid alcohol, comprised of 95% alcohol with 2.5% glacial acetic acid, was used as the differentiation solution. Slides were very briefly dipped in the acid alcohol to remove any excess dye and obtain clear staining. Slides were then brought through a dehydration ladder of; 1) 95% alcohol, 2) 100% alcohol, 3) 100% alcohol, 4) xylene, 5) xylene before mounting with DPX (BDH Laboratory Supplies), a xylene based mounting media.

4.6.5.3. Immunohistochemistry

Immunohistochemistry refers to the staining of proteins in sectioned tissue using antibodies. In this study, DAB visualisation was used to display different proteins of interest in the brain. DAB staining is a multi-step process where different conjugated molecules form a final complex which allows the enzymatic breakdown of 3,3'-Diaminobenzidine (DAB) to a brown precipitate visible to the naked eye. As sections were cut from tissue which had been fixed with paraformaldehyde, the first step in staining was to unmask the antigens to allow antibody binding, known as antigen retrieval. PFA fixation creates cross-links between proteins, so the breakdown of these cross-links exposes antigen sites. The method of antigen retrieval was dependent on the antibody used, with the two most commonly used antigen retrieval buffers used being citrate buffer and sodium acetate buffer. Following rehydration, slides were placed into boiling retrieval buffer for 20 minutes, followed by resting in the still-hot buffer away from the heat source, i.e. no longer boiling but gradually cooling down. Endogenous peroxidases which can interfere with DAB staining were quenched by incubation for 5 minutes in 3% H₂O₂. Following this step, sections were blocked in 5% donkey serum in PBS-T for one hour at room temperature. Primary antibodies were made in PBST with 1% donkey serum to the concentrations shown in Table 4.8. Samples were incubated overnight at 4°C in primary antibody. The following day, samples

were rinsed in PBS before incubation in biotinylated secondary antibody at room temperature for 2 hours. Using the Vectastain ABC detection kit (Vector), a working solution of avidin-biotin complex (ABC) was prepared and the slides were incubated in this solution for 30 minutes. Avidin has a high affinity for biotin, so a complex is formed of avidin with HRP-conjugated biotin. This complex uses less biotin than avidin, to leave some binding sites free. Once this solution is added to the slides, the remaining binding sites bind to the biotin on the secondary antibody, creating a large complex of avidin, biotin and HRP enzyme, creating more than 3-fold amplification of signal. Finally, the DAB peroxidase solution was applied to the slides (Vector). The HRP enzyme reacts with the DAB to form a brown precipitate. This reaction takes no longer than 10 minutes to react, but different antibodies were optimised for best staining. The reaction was stopped after the time optimised in early experiments by rinsing the solution with dH₂O. Cells were then dehydrated as above before mounting in a xylene based mounting media, DPX. Counting was performed manually under the microscope by the experimenter blinded to treatment groups using an AxioCam microscope (Zeiss).

4.6.5.4. TUNEL staining

Terminal deoxynucleotidyl transferase dUTP end-nick labelling (TUNEL) staining is a protocol by which damaged and fragmented DNA is visualised by staining (Van de Berg et al., 2002). It relies on an enzyme called terminal deoxynucleotidyl transferase (TdT) which adds nucleotides to the 3' ends of DNA. By conjugating these nucleotides with molecules which can be visualised, any free ends of DNA caused by fragmentation can be identified. In this protocol, a 3,3'-diaminobenzidine (DAB) conjugation protocol was used, although fluorescent protocols are available.

TUNEL staining was carried out using the ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Millipore) according to the manufacturer's instructions. Briefly, after deparaffinising and rehydrating the samples, the tissue was treated with proteinase K to expose the DNA for staining. Endogenous peroxidases were then quenched using 3% H₂O₂ for 5 minutes, before the application of the TdT enzyme. The TdT enzyme, which binds nucleotides to the 3' end of DNA, was diluted in a reaction buffer which contains free nucleotides which have been conjugated to digoxigenin. This results in the free 3' ends of the DNA being labelled with digoxigenin. After removing the TdT enzyme and nucleotides, an anti-digoxigenin antibody conjugated to peroxidase was applied. In the final step, a peroxidase substrate was applied, with the bound peroxidase enzymatically creating a brown precipitate which can then be imaged under the microscope. The TUNEL process is similar to DAB staining (Figure 4.4) where HRP is utilised to create a brown precipitate for staining, but instead of an avidin/biotin intermediary binding step, a digoxigenin/anti-digoxigenin complex is used. Similarly to DAB, optimal reaction times are determined and used throughout experiments. Once staining is complete, the DAB solution is washed off and cells are dehydrated and mounted in DPX, a xylene based mounting medium.

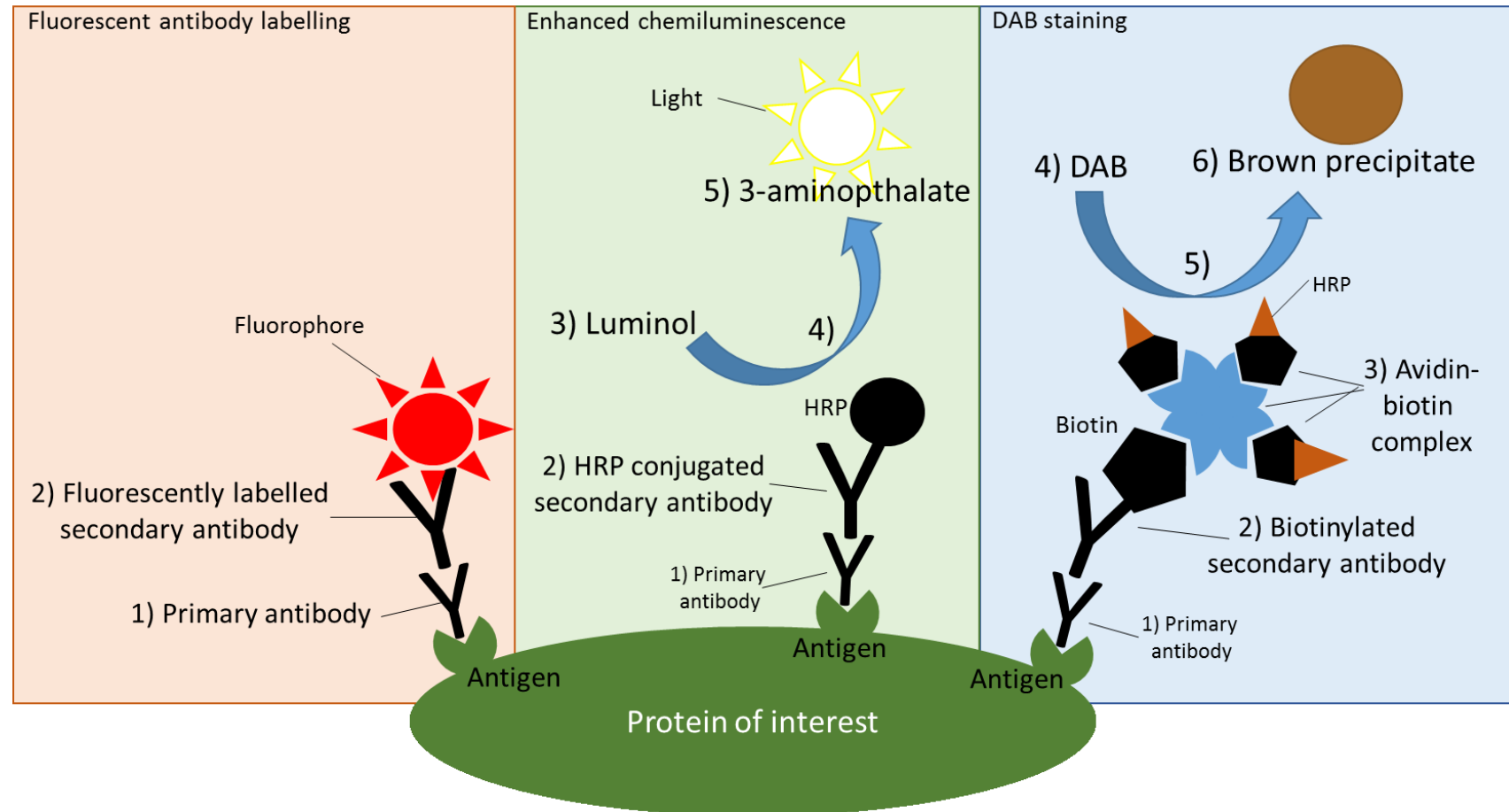


Figure 4.4 **A diagram of the different methods of antibody detection used** . (A) Fluorescent antibody staining is the most simple of the methods used , where a primary antibody raised against the protein of interest is bound, followed by a fluorophore conjugated secondary antibody. This allows for simple measurement of fluorescent intensity to determine protein expression. (B) Enhanced chemiluminescence is used during Western blotting, where instead of a fluorophore conjugated secondary antibody, a HRP molecule is bound. A solution is then added which reacts with the HRP to form a light emitting end product, which can be detected using a simple camera in a dark box. (C) DAB staining is a more complex staining protocol which utilises the strong natural affinity for the protein avidin to biotin. As avidin has multiple biotin binding sites, large avidin biotin complexes can be obtained which result in signal amplification. By adding DAB substrate which, like the chemiluminescence protocol, relies on bound HRP to drive a reaction, a brown precipitate is formed which can be used to analyse samples under a brightfield microscope

Table 4.8 **Antibodies and their concentrations for this thesis.** Antibody concentrations were determined first by recommendation from data sheets supplied with each antibody, and further optimised where necessary to improve the quality of staining

Primary antibody concentrations					
	Specificity	Source	IF	WB	IHC
Cleaved caspase-3	Rabbit	Cell Signaling	1:200		1:300
Bcl-2	Rabbit	Abcam	1:200	1:800	1:200
Phospho-ERK1/2	Rabbit	Cell Signaling		1:1000	
Puma	Rabbit	Abcam		1:1000	1:100
Caspase-8	Rabbit	Cell Signaling		1:1000	
Bax	Rabbit	Cell Signaling	1:200	1:1000	
Bcl-xL	Rabbit	Cell Signaling	1:200		
GAPDH	Mouse	Millipore		1:20,000	
β-Tubulin	Rabbit	Cell Signaling	1:500		
Secondary antibody concentrations					
	Specificity	Source	IF	WB	IHC
Anti-rabbit FITC	Donkey	Abcam	1:200		
Anti-rabbit Rhodamine	Donkey	Millipore	1:200		
Anti-rabbit HRP conjugated	Donkey	Cell Signaling		1:1000	
Anti-mouse HRP conjugated	Donkey	Cell Signaling		1:1000	
Anti-rabbit biotinylated	Donkey	Millipore			1:200

4.6.6. Statistical analysis

All data were input to Microsoft Excel for data normalisation, and this data was further input to GraphPad Prism to create data graphs and carry out statistical analysis. Where data sets had sufficient n numbers, normality tests were carried out to determine whether data were normally distributed. Where data met the criteria for ANOVA analysis, One way ANOVA with Bonferroni's comparisons were made. Where data sets were not normally distributed, Kruskal-Wallis testing was carried out with Dunn's multiple comparisons.

Data are presented as scatter dot plots in chapters 5-7, and as bar graphs in chapter 8, with corresponding n numbers for each group presented in a table in the supplementary data. All data are presented as mean $\pm$ standard deviation.

Chapter 5.

Investigation of the neurotoxic potential of N₂O in a primary neuronal cell culture model

5.1. Introduction

Neuroscience research occurs on a scale of increasing complexity, with the highest level being the human brain. However, the human brain is a complex organ which is affected by constant input, ever-changing physiological status and other confounding factors. For this reason, as well as due to the practical and ethical considerations of studying the human brain, most neuroscience research is carried out on systems which are levels below in complexity. These range from simple cell cultures and *ex vivo* tissue samples, through small animals such as the nematode, *Drosophila melanogaster* and the zebrafish (Sattelle and Buckingham, 2006, Stewart et al., 2014), up to more complex whole animal systems in rodents, cats and non-human primate models (Capitanio and Emborg, 2008). Researchers often work with simpler model systems to lay a foundation for future work (Koslow, 2002) before moving on to more complex models where data may be more meaningful but a wider range of variables may influence the outcomes.

Primary neuronal cultures are an extremely useful biological tool which can help us to understand the fundamental interactions of the brain in an easy to control environment. Primary cultures can be made of almost any cell type, given sufficiently precise dissection of discrete brain areas for culturing (Giordano and Costa, 2011). Generally, cortical and hippocampal cultures are the most commonly used, but a wide range of different cell types can be obtained for investigation. There are many options available for culturing neurons, including set-ups which allow the spatial separation of soma from processes, co-culturing of multiple cell types, inducing neurons from stem cells (Bai et al., 2013) and culturing of slices of whole brain to study intact neuronal networks (Millet and Gillette, 2012). These different options allow the study of various physiological factors such as cellular interactions,

development and the effects of extracellular stimuli on neuronal growth. However, the simple primary neuronal culture is still a widely used model which allows the freedom to culture cells for a number of different outcomes measures, such as on coverslips, in electrophysiology chambers, or in chambers of varying sizes for different applications.

Primary neuronal cultures have previously been used to study the effects of a range of anaesthetics, including ketamine (Bai et al., 2013, Bosnjak et al., 2012), propofol (Pearn et al., 2012, Twaroski et al., 2014), and volatile anaesthetics (Yan et al., 2013, Qiu et al., 2015, Wei et al., 2005). However there are currently very few studies of the *in vitro* effects of nitrous oxide on a primary neuronal culture. Most of these studies use N₂O in combination with different anaesthetics (Campbell et al., 2011, Zhen et al., 2009), and no studies thus far have studied the neurotoxicity of N₂O alone in cell culture. One of the first studies into the neurotoxic effects of N₂O was published in 1998, which investigated whether N₂O acted upon the NMDA receptor, and if it exhibited similar neurotoxic effects as other NMDA antagonists such as ketamine and MK-801 (Jevtovic-Todorovic et al., 1998). This study, which found that administering high concentration N₂O gave neurotoxicity in a pattern similar to MK801 and ketamine, used a primary hippocampal neuronal culture to take electrophysiological recordings which were used to determine whether N₂O was an NMDA antagonist, but the toxicity work was carried out in an *in vivo* rat model. This shows the flexibility of primary neurons to support *in vivo* research, but leaves room to investigate N₂O in a primary neuronal culture to add new data to the field.

Argon has been subjected to slightly more analysis in cell culture models, of the few papers studying argon protection; fewer still have used cell cultures to do so. Of the neuronal models, two used primary neurons (Jawad et al., 2009, Zhao et al., 2016), two used a cell

line (Spaggiari et al., 2013, Ulbrich et al., 2015a) and another used both primary neurons and a glial cell line (Fahlenkamp et al., 2012). Interestingly, one of the studies also investigates N₂O but does not discuss the results of N₂O exposure (Spaggiari et al., 2013). For the three studies, concentrations of argon used ranged between 25-75%, either in combination with an insult such as OGD (Jawad et al., 2009), mitochondrial toxins (Spaggiari et al., 2013), or alone (Fahlenkamp et al., 2012). Argon was neuroprotective in studies containing insult, and the Erk 1/2 pathway was highlighted as a likely mechanism for this neuroprotection by Fahlenkamp et al. (2012). These studies give reasonable confidence that argon can have an effect in an *in vitro* model; however the test in these experiments will be the performance of argon at a much lower concentration than previously investigated.

The pathway which will be investigated in this study focuses primarily on mitochondrial control of apoptosis. Using three Bcl-2 family proteins and the executor caspase, cleaved caspase-3, the effects of these gases on the mitochondria and downstream caspase activation can be investigated. By then investigating cell viability, the effects of any changes to these proteins can be correlated with cell survival. Bax is a Bcl-2 family pro-apoptotic family which acts upon the mitochondrial outer membrane and can induce the permeabilisation of this membrane via pore formation (Raemy and Martinou, 2014). The anti-apoptotic proteins Bcl-2 and Bcl-xL both act upon the mitochondria to protect the outer membrane from the formation of these pores. As described above, cleaved caspase-3 expression signals the cells have reached the final stages of apoptosis and can be used as a marker of cells fated to die. Using both propidium iodide and the MTT assay, cell viability can be measured and give a final outcome 24 hours after the termination of gas exposure, whilst the above proteins are measured at earlier timepoints.

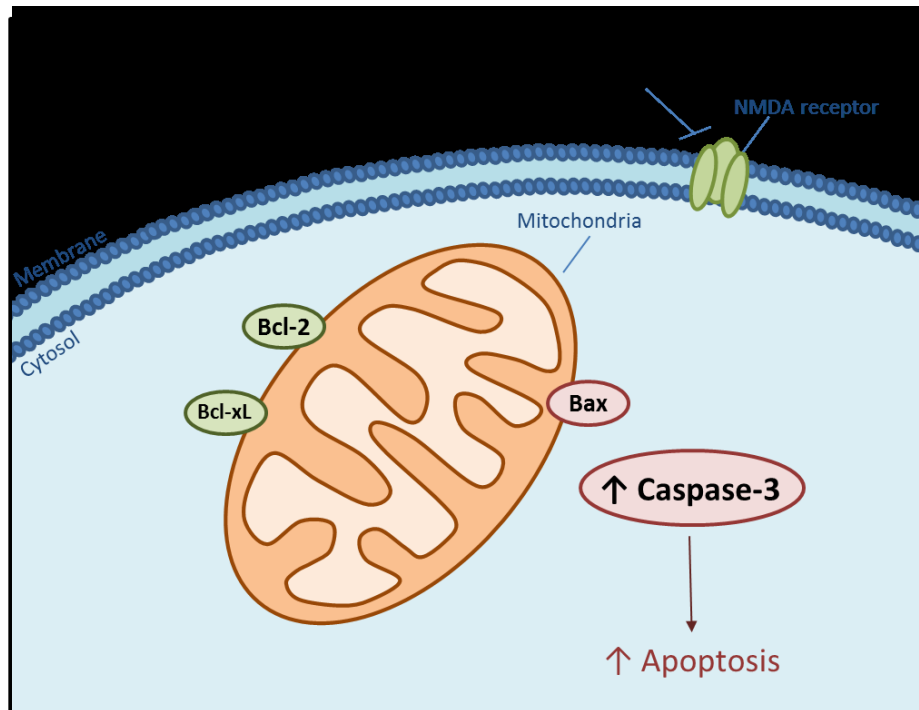


Figure 5.1. **Proteins to be measured in this chapter.** Four proteins will be examined in this chapter, two pro-apoptotic (cleaved caspase-3 and Bax) and two anti-apoptotic (Bcl-2 and Bcl-xL). Bax acts upon the mitochondria to induce pore formation, leading to the release of proteins which induce caspase-3 cleavage, ultimately causing apoptosis. Bcl-2 and Bcl-xL both act to protect the mitochondria from such pore formation, and ultimately protect the cell from death.

5.2. Hypothesis and aims

The aim of this chapter was to determine in the simplest terms what the effects of nitrous oxide are on neurons. In addition to this, the combination of N_2O and argon was investigated to determine whether argon can have a beneficial additive effect.

5.3. Methods

Primary neuronal culture is a relatively straightforward type of culturing, where neuronal tissue is removed from an animal and immediately trypsinised into a single cell suspension for culturing. Unlike cell lines, primary cultures do not expand or multiply, instead maturing over time until the desired morphology is reached. A number of precautions need to be

taken to ensure a pure culture is obtained. In this study, embryonic day 18 rat pups were used, as is standard for most primary neuronal cultures. Due to the relative immaturity of the brain at this stage of development there are few astrocytes in the brain, which, in combination with the media and supplements used, ensures a clean neuronal culture devoid of glial cells. Microdissection techniques were also used to ensure a culture of a specific subset of neurons. By removing all meninges and peripheral brain regions such as olfactory bulbs, brainstem and midbrain, as well as careful dissection of the hippocampus, pure cortical cultures were obtained. Cells were grown to 7 days *in vitro* (DIV) to allow for maturation of cells, shown by the density of cellular arborisation (see Figure 5.2 for examples).

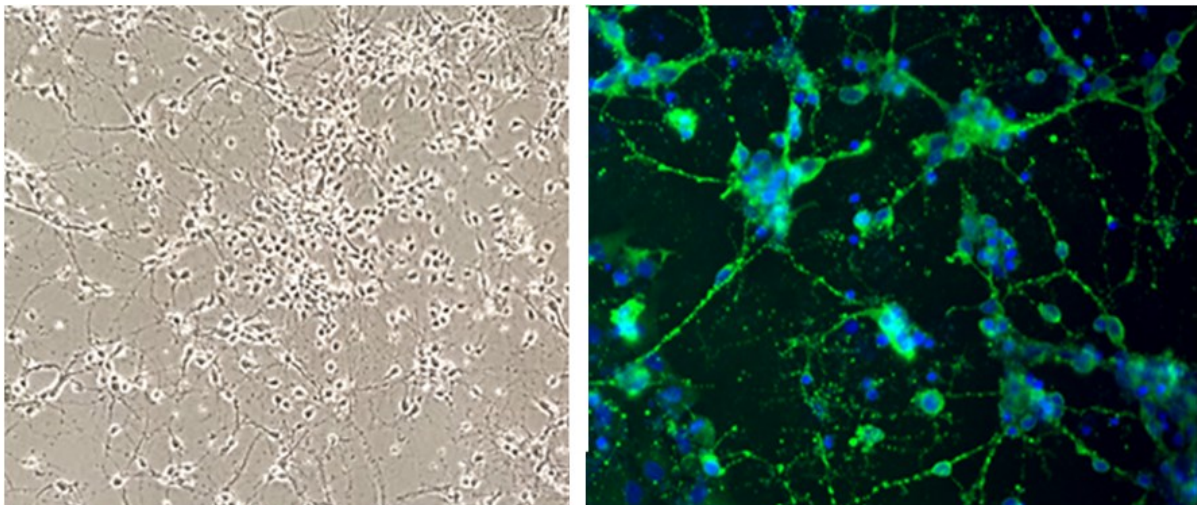


Figure 5.2 **Neuronal structure at 7 days in vitro.** Images were taken using either brightfield microscope without staining (a) or stained with an antibody against β -tubulin (green) and counterstained with DAPI (blue) to show the cellular processes and nucleus respectively (b). Both images show the presence of the dendritic arborisation which occurs between neurons grown in culture

On 7 DIV, cells for gas exposure were removed from the incubator and treated with the correct gas for four hours. A simple time course analysis was performed to determine what

the optimal duration of anaesthetic exposure would be. Four hours was decided as the optimal duration as it resulted in peak expression of both caspase-3 and Bcl-2 immunofluorescence, and was in line with the timings used in the *in vivo* work. Cells were exposed to the gas of interest for 4 hours in airtight exposure chambers. Immediately following gas exposure, cells for immunofluorescence were fixed using 4% paraformaldehyde. Cells for propidium iodide staining were returned to the incubator for 24 hours to allow damage to develop before staining. Naïve control cells were left in the incubator throughout experiments.

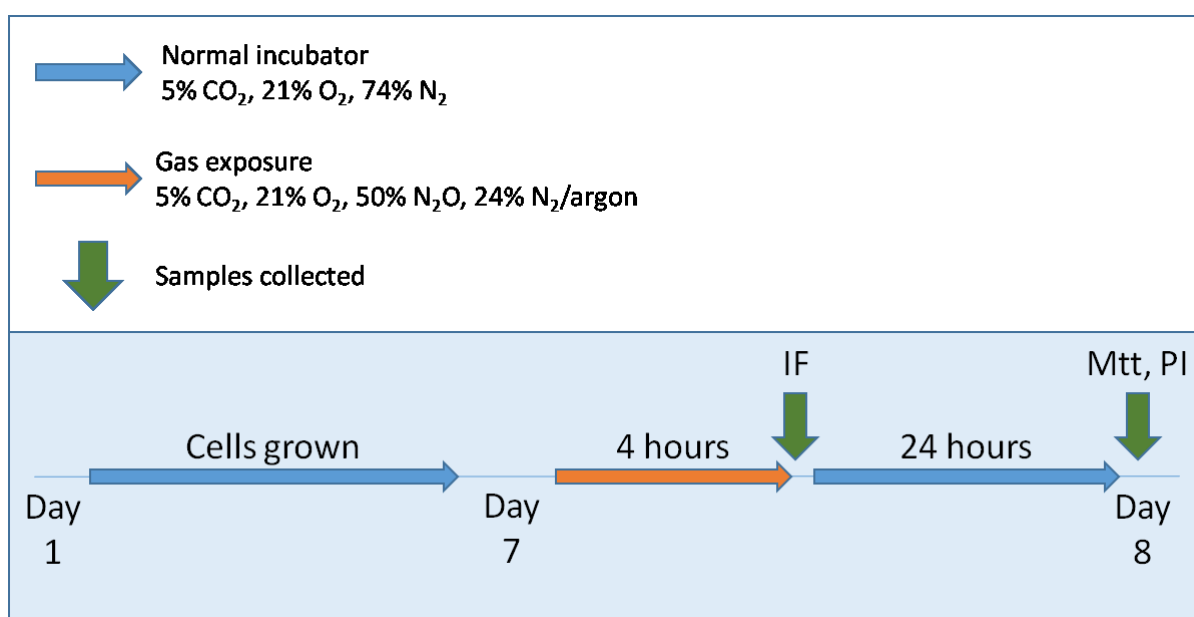


Figure 5.3 **Timeline of experimental protocol.** Cells were cultured for 7 days before experiments began. Treated cells were exposed to four hours of gas exposure on 7 DIV in a sealed exposure chamber kept at 37°C. Naïve control cells were left in the original incubator. Following gas exposure, cells were either immediately fixed for immunofluorescence (IF) or maintained for 24 hours for propidium iodide (PI)/MTT viability assay.

Cells were exposed to the gas of interest for 4 hours in airtight exposure chambers, using concentrations outlined in Table 5.1. For each cell line, 21% O₂ was used to mimic normal oxygenation in the incubators, and 5% CO₂ used as this is standard procedure for cell

cultures, and again what cells would have been exposed to in the incubators. The remainder of the gas mixture could then be altered, and a concentration of 50% N₂O was fixed upon, as this was the same concentration used for the *in vivo* work, being the concentration administered to labouring mothers for pain relief. The remainder of the gas concentration was then set as argon or nitrogen depending on the treatment group. These chambers were custom made, comprising of a small interior chamber which held a metal tray. This tray was loaded with samples, usually cells in petri dishes or 4 well plates, and the lid of the chamber closed. This lid was held in place using two large clamps to ensure an airtight seal. The inside of the lid contained a small fan which ensured even distribution of the gas throughout the chamber, which was powered by a mains connection on the outside of the lid. Once cells were sealed inside the chamber, the chamber was connected to the output of the anaesthesia trolley and the flow of gas was started. This remained flowing through the chamber for approximately 4 minutes until the chamber was flushed of air and filled only with the gas mixture of interest. The chamber was then sealed, the fan switched on and the entire system placed inside an incubator to ensure the cells remained at 37°C. Naïve control cells were left in the incubator throughout experiments. Following the gas exposure, cells for immunofluorescence were fixed in 4% paraformaldehyde. Cells for propidium iodide staining were returned to the incubator for 24 hours as apoptosis is not an immediately visible outcome, with time needed for cellular mechanisms to instigate and carry out apoptosis.

Table 5.1 **Gas exposure concentrations for *in vitro* primary cortical neurons.** These concentrations were used for all cell types, with gas exposure maintained for four hours in a sealed chamber with no continuous flow of gas. Naïve cells were left in the growth incubator undisturbed until collection.

	Naive	N ₂ O treatment	N ₂ O + Argon treatment
O ₂	21%	21%	21%
CO ₂	5%	5%	5%
N ₂	74%	24%	-
N ₂ O	-	50%	50%
Argon	-	-	24%

5.4. Analysis and Statistics

MTT assay was analysed by reading the colour of different samples using an MRX II Microplate Reader (Dynex Technologies) at 595nm. For each experiment, 4 repeats were measured to reduce the variability associated with differences in cell density. Raw absorbance values were obtained for each sample and the average of the four repeats was calculated. These raw absorbance values were then plotted using GraphPad Prism 5. Propidium iodide staining was analysed by taking images of both the red and blue fluorescence channels to capture PI and DAPI fluorescence respectively. Ten images were taken of each coverslip. The number of red cells and blue cells were counted from each image taken and the percentage of propidium iodide positive cells was calculated.

For immunofluorescence, cells were stained for cleaved caspase-3, Bax, Bcl-xL and Bcl-2. Images were captured using an Olympus BX60 microscope fitted with a Zeiss Colour AxioCam camera. For each coverslip, 10 pictures were taken randomly around the coverslip. In each image, 5 cells were measured for mean fluorescent intensity (MFI) of the protein of

interest. 5 measurements of the background were taken and the background MFI was subtracted from the protein MFI to get a measurement of fluorescence of the protein of interest. The average fluorescence of the 10 images was then obtained to give the MFI for one sample. To normalise samples, all naïve values were averaged and this average naïve value was used as the normalising value, described in more detail in Figure 5.4.

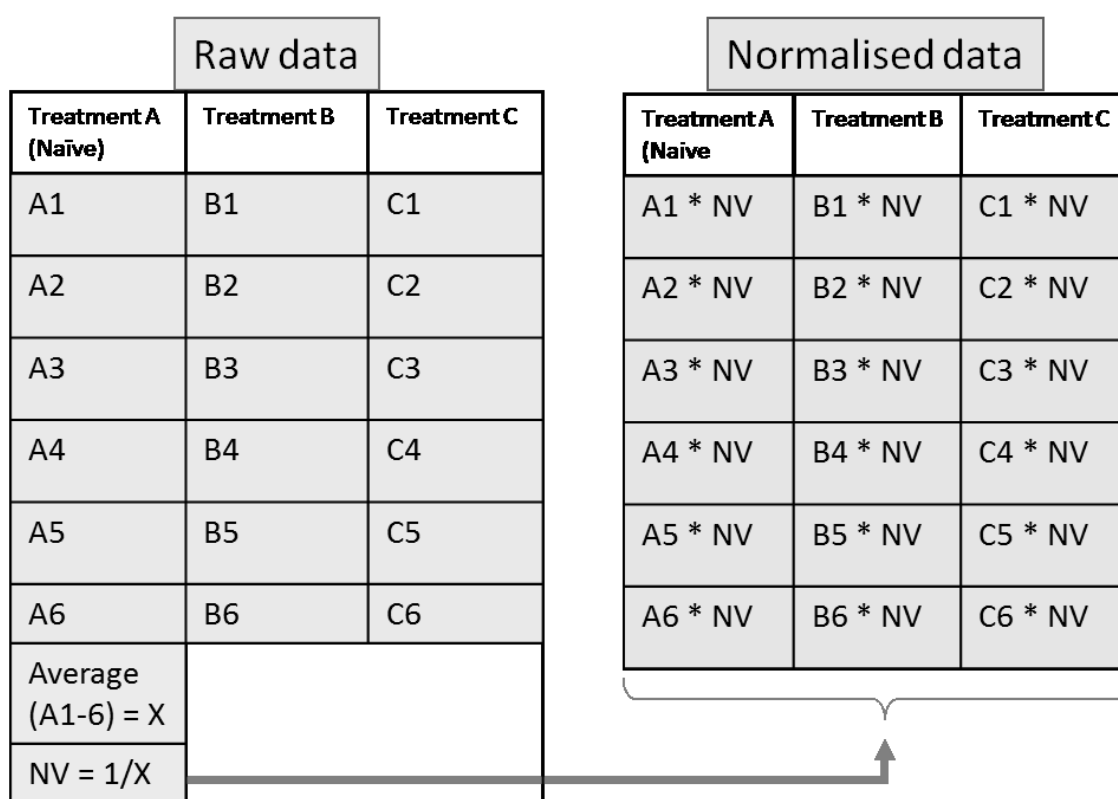


Figure 5.4 **Method for normalising data.** The average of all naïve samples was calculated, giving value X. A normalised value (NV) was obtained by dividing 1 by X. This NV was then applied to all original values to obtain a normalised data set. This can be confirmed by averaging the normalised naïve values which should equal 1.

Statistical analysis was carried out using GraphPad Prism software. When $n > 3$, Kolmogorov-Smirnov analysis was used to assess normality of distribution. Where samples were normally distributed, samples were analysed using One-Way ANOVA with Bonferroni's post-hoc analysis. For non-parametric data or sample sizes of $n \leq 3$, Kruskal-Wallis analysis with

Dunn's multiple comparison for post-hoc analysis was used. Data are presented as mean $\pm$ SEM.

5.5. Results

5.5.1.1. Cell viability

Two measures of cell viability were used for primary neuronal cell culture analysis, the MTT assay and propidium iodide staining. While the MTT assay showed no significant difference between groups and no variability (One-way ANOVA; $p = 0.9291$, $n = 17$, Figure 5.5a), propidium iodide staining showed a significant, albeit small, increase in cell death after treatment with N_2O when compared with naïve (Kruskal Wallis; $p = 0.0294$, Dunn's multiple comparison; Naïve vs. $N_2O < 0.05$, $n = 3-4$, Figure 5.5b). The addition of argon to the N_2O exposure was not significantly different from naïve cells, indicating the argon had a neuroprotective role.

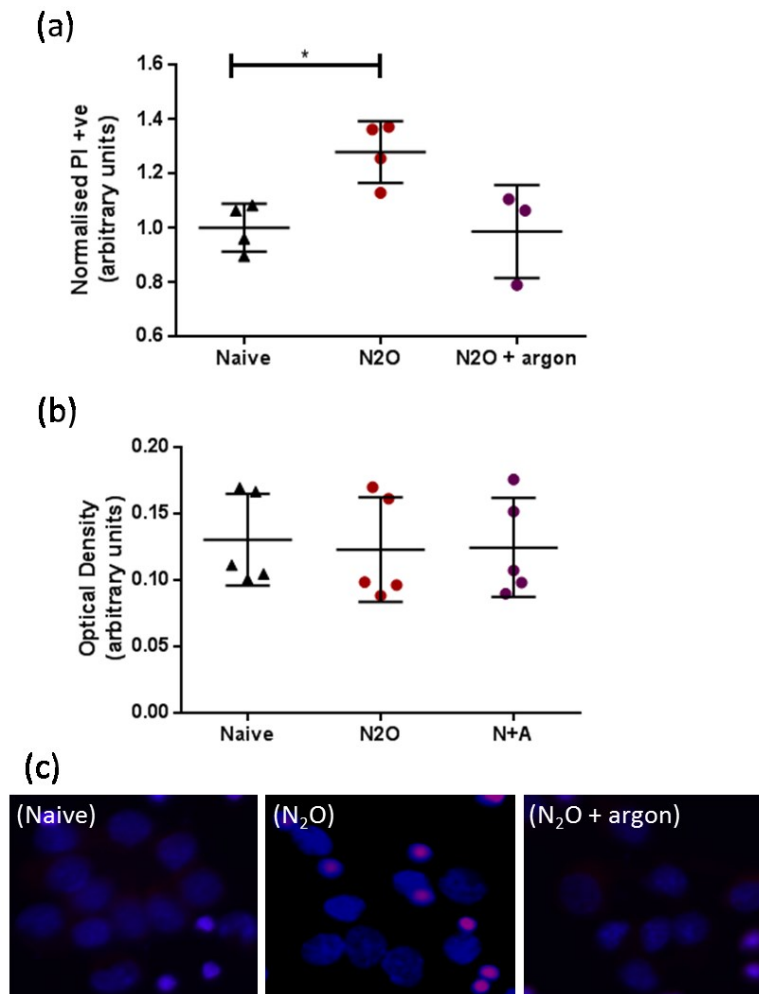


Figure 5.5 **Cell viability assays on primary neuronal cultures exposed to N₂O alone or in combination with argon.** Primary neuronal cultures were exposed to cells treated with 50% N₂O $\pm$ 25% argon for 4 hours. Control cells had no treatment. (a) Propidium iodide staining as a measure of cell viability showed a significant increase in PI staining following exposure of the cells to N₂O when compared with naïve control. While the addition of argon clearly reverses the increased PI staining, no significance was found, most likely due to a small number of repeats (n=3). n=3-4, Kruskal-Wallis; $p < 0.05$, Dunn's multiple comparison test; Naive vs N₂O $p < 0.05$. (b) MTT assay showed no significant difference between control neurons or either treatment group. n=17, One-way ANOVA; no significance ($p = 0.9291$) (c) Representative images of propidium iodide staining showing dead cells stained both blue and red, with healthy cells stained only blue. Red; PI dye, Blue; DAPI

5.5.1.2. Pro-apoptotic proteins

The apoptotic cascade contains a large number of receptors, proteins and activation pathways. Involved in all this are a number of proteins which can swing the balance either towards or away from cells reaching the final stages of apoptosis. Pro-apoptotic proteins are involved in causing apoptosis within the cell, with caspase 3 being the executor caspase, one

of the final stages in the pathway, and Bax playing an integral role in mitochondrial stability. These proteins were measured in cells by immunofluorescent cell staining.

Cleaved caspase-3 showed a trend to increasing expression following N₂O gas exposure compared to either naïve or N₂O plus argon (Figure 5.6a). This difference was significant by One-way ANOVA, however Dunn's multiple comparison test did not pick out any groups as being significantly different from each other (One-way ANOVA; $p = 0.0492$ (*), Dunn's multiple comparison = NS, $n = 7$). Bax expression, on the other hand, showed an increased expression only in the N₂O plus argon group, although this did not reach significance (Kruskal Wallis; $p = 0.4313$, $n = 3-4$, Figure 5.6b).

5.5.1.1. Anti-apoptotic proteins

On the other side of the apoptotic pathway are the anti-apoptotic proteins which include Bcl-2 and Bcl-xL, which act to protect the mitochondria and thus prevent cell death. Again, these proteins were measured in cells by immunofluorescent cell staining.

Bcl-2 showed a trend towards decreasing expression directly after N₂O exposure, while the mixture of N₂O plus argon gave a significantly increased expression of Bcl-2 compared to N₂O alone (One-way ANOVA; $p = 0.0168$, Bonferroni's multiple comparison; N₂O vs N₂O + argon < 0.05 , $n = 6$, Figure 5.7a). Bcl-xL showed a similar trend, in that N₂O plus argon had a higher expression level than either naïve or N₂O alone, however this did not reach significance (Kruskal Wallis; $p = 0.4457$, $n = 4$, Figure 5.7b).

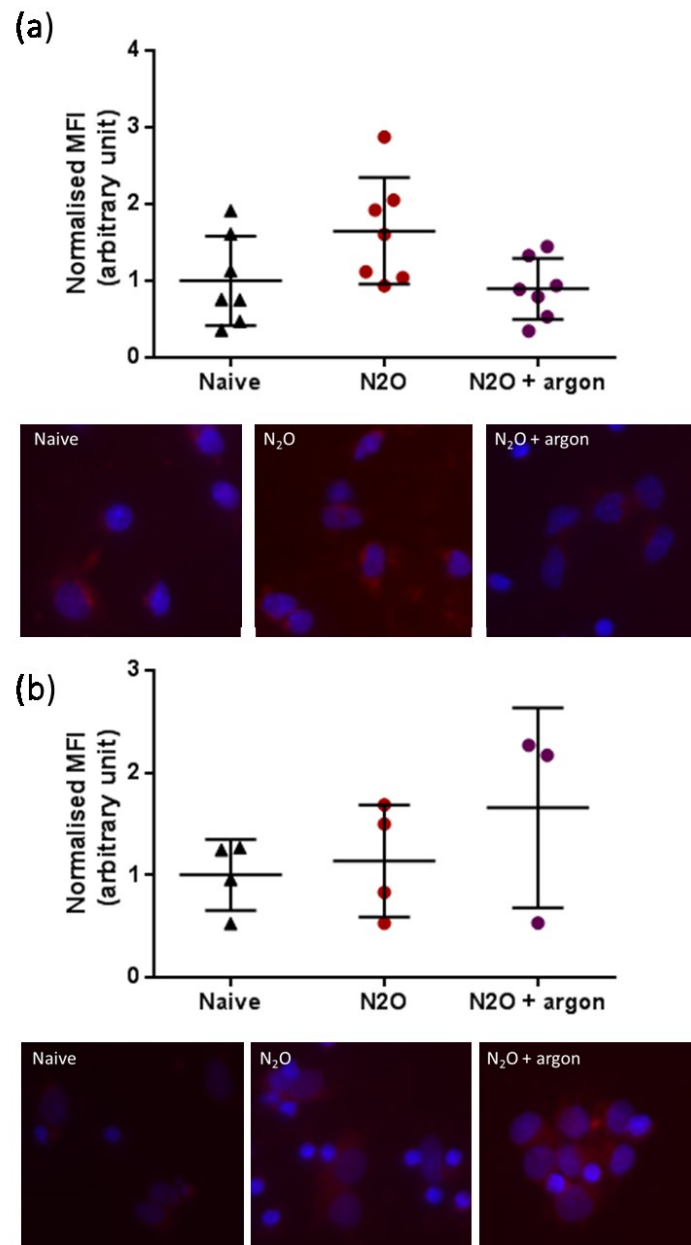


Figure 5.6 **Pro-apoptotic proteins expressed in cells after exposure to anaesthetics.** Primary neuronal cultures were exposed to cells treated with 50% N₂O $\pm$ 25% argon for 4 hours. Control cells had no treatment. Levels of pro-apoptotic proteins were measured using immunohistochemistry. (a) Levels of cleaved caspase-3, a late stage marker of apoptosis, were assessed using immunofluorescent staining. A clear trend of increased cleaved caspase-3 expression can be seen, and there was a significant difference between the three treatment groups. $n=7$, One-way ANOVA; $p=0.049$, however Bonferroni's multiple comparison test showed no significant difference between groups. (b) The pro-apoptotic protein Bax was not found to be significantly difference between any of the groups tested, although the addition of argon appears to paradoxically trend towards an increase in expression, albeit with a smaller n number. $n= 3-4$, Kruskal-Wallis; no significance ($p = 0.43$)

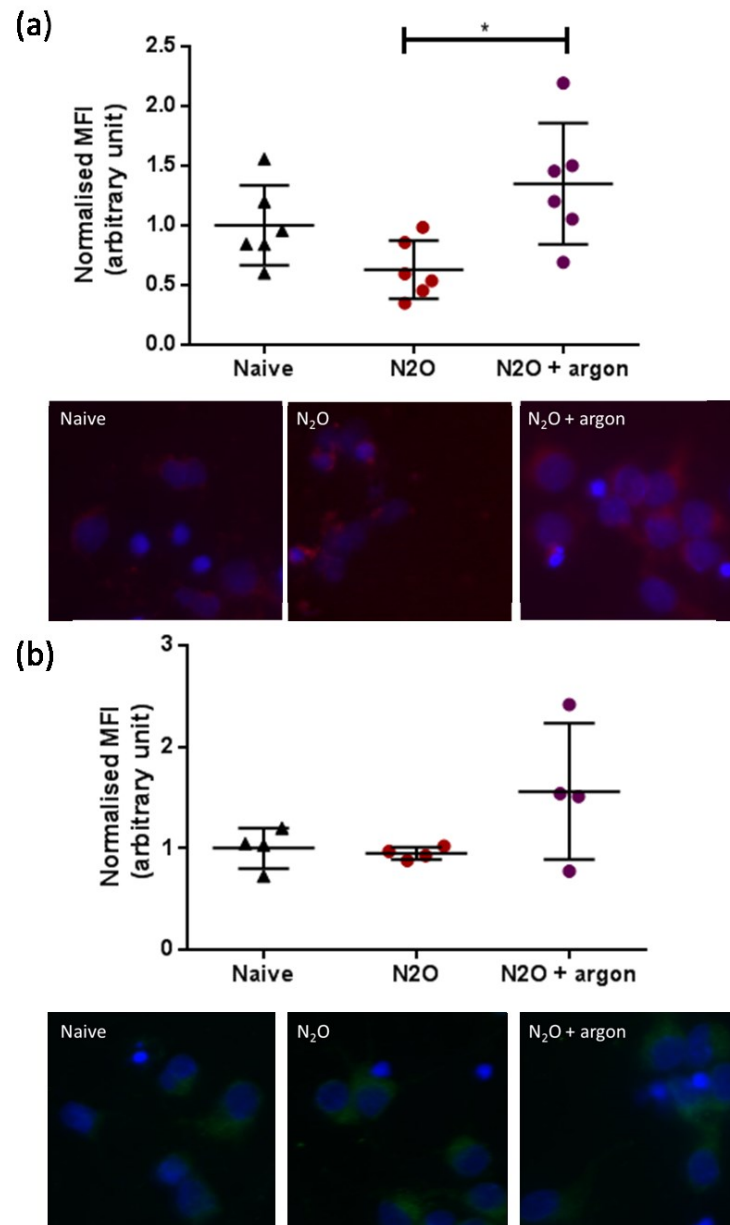


Figure 5.7 **Anti-apoptotic proteins expressed after four hours gas exposure.** Primary neuronal cultures were exposed to cells treated with 50% N₂O ± 25% argon for 4 hours. Control cells had no treatment. Levels of anti-apoptotic proteins were measured using immunofluorescence. (a) Bcl-2 expression was significantly increased after N₂O + argon exposure when compared with N₂O exposure alone. n=6, One-way ANOVA; p=0.017, Bonferroni's multiple comparison test; N₂O vs N₂O + argon < 0.05. (b) Bcl-xL expression was not significantly affected by any treatment. However, the addition of argon did show a trend towards increased Bcl-xL expression. n=4, Kruskal-Wallis; no significance (p=0.245)

5.6. Discussion

Primary neuronal cultures are a widely used model to assess neurotoxicity of drugs and interventions which allow for in-depth molecular investigations. While there are a number

of immortalised cell line models for neuronal investigation, some of which are used in later chapters of this thesis, it is important to verify results in a primary culture to ensure data is as close to the *in vivo* models as possible. For this reason, we investigated some of the simpler molecular pathways to determine the effect N₂O had on these cells, as well as to look closely into possible mechanisms of neurotoxicity. In combination with this, we also looked at the possible neuroprotective potential of argon in this model to relate this work to the *in vivo* investigations.

These data show that in primary neurons, N₂O can have a toxic effect, while the addition of argon to N₂O can reverse this toxicity. Further analysis of apoptotic proteins indicate that this is likely due to the balance between pro and anti-apoptotic proteins in the cell, with N₂O increasing cleaved caspase-3 levels, while N₂O plus argon had higher levels of Bcl-2 and Bcl-xL, both known to stop apoptosis. These data strongly support the theory that N₂O is neurotoxic, even with just a four hour exposure at a concentration of 50%. Even more novel is the finding of positive effects of argon at a very low concentration of 25%. Previously, the lowest concentration of argon to show any physiological effect in cells was 50%, so these data indicate a significantly wide variation in the concentrations at which argon can have an effect.

Figure 5.8 shows the effects of both N₂O and argon on the proteins measured in this study. N₂O increased the concentration of cleaved caspase-3 in the cell, while the addition of argon increased Bcl-2 and Bcl-xL, although the latter not to a significant degree. Neither gas appeared to affect the expression of Bax, although with higher repeats some difference between N₂O versus N₂O + argon may have been seen. The most widely recognised likely mechanism for the increase in caspase-3 cleavage is also outlined in Figure 5.8. As described

in Section 2.2, permeabilisation of the mitochondrial membrane leads to the release of pro-apoptotic factors such as cytochrome c and apoptosis inducing factor (AIF). Cytochrome c can bind to APAF1 to form the apoptosome, which in turn cleaves caspase-3. This is the likely mechanism by which damage to the mitochondria can lead to apoptosis. One interesting analysis which can be done to support this idea is to create a Bcl-2/Bax ratio. This is a widely reported measurement of two of the major proteins involved in the balance of mitochondrial health and stability. It is often used in clinical measurements, for example to predict the success of chemotherapy (Matsumoto et al., 2004, Salakou et al., 2007, Scopa et al., 2001). The Bcl-2/Bax ratio is a single number which describes the shift in balance either towards (<1) or away (>1) from apoptosis. In this study, the administration of N_2O alone gave a Bcl-2/Bax ratio of 0.55, whilst for N_2O + argon this ratio was 0.81. This reflects the cell viability findings, where N_2O induced apoptosis, while the addition of argon reversed this. Noticeably, argon combined with N_2O does not bring the ratio back to 1, which would indicate healthy mitochondria, but the Bcl-2/Bax ratio does not take into consideration other proteins, including Bcl-xL and similar protective and apoptotic proteins, so cannot give a complete picture of intracellular homeostasis. Nevertheless, it is a useful measure of mitochondrial stability, particularly when supported by viability data, as shown above.

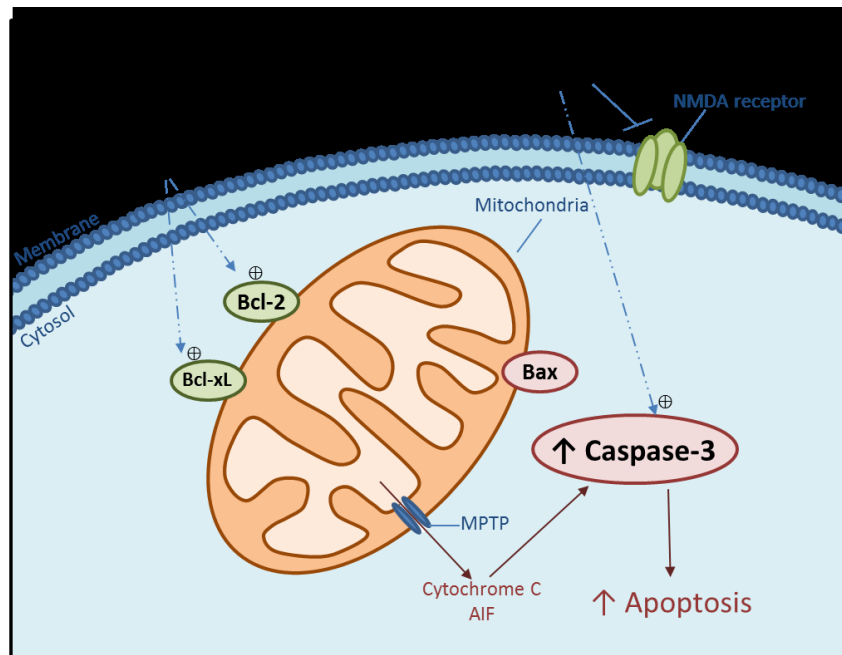


Figure 5.8. **Pathways suggested by this data.** From this *in vitro* data, argon has been shown to increase the anti-apoptotic protein Bcl-2, while N₂O increases the pro-apoptotic protein cleaved caspase-3. This is reflected in the cell viability data which shows that N₂O increases cell death, while the addition of argon could reduce cell death to naive levels. This is in line with the mitochondrial pathway of apoptosis, where Bcl-2 can prevent the apoptotic cascade.

In this study, there were a number of limitations to the findings. A set time point of four hours gas exposure was chosen to reflect the *in vivo* experiments, where four hours gas exposure was given to parturient dams before insult to the pups. As well as this, a secondary method to measure protein concentrations was not used to validate the immunofluorescent staining. Samples were collected for Western blot analysis in the early stages of the project, but due to the nature of primary cell culture, where low cell yields are common, sufficient protein for analysis was not obtained. As well as this, flow cytometry was attempted on the cells but a method of removing the cells from growth plates without damaging them could not be found, and the majority of cells were too damaged and fragmented for meaningful analysis. Similarly, the effects of argon alone were not studied here. This was primarily a logistical issue, based around the instruments used to measure gas concentrations. The anaesthesia monitor used in this study could measure O₂, CO₂ and N₂O, as well as volatile

anaesthetics which we do not consider here. However, it could not measure either argon or N₂. In the groups used above, described in Table 5.1, argon and N₂ were never administered together as argon replaced the N₂ component in the combined N₂O + argon treatment. However, to have argon alone group, these two gases would have had to have been combined together. It is possible to work out the relative ratios of these gases based on flow rate analysis, but this can be imprecise and requires calibration. It was felt that since no argon alone group was to be used in the *in vivo* work, and previous studies on argon alone have been carried out, this group was not of high importance so was not included.

In summary, this chapter gives encouraging evidence that nitrous oxide can indeed have neurotoxic effects in a primary cell culture. In addition to this, the benefits of argon at very low concentrations give evidence that argon can be neuroprotective at concentrations below 50%. If these trends continue in the *in vivo* models, where low concentrations of argon are needed, this work could have significant implications for future clinical administration.

Chapter 6.

Effects of N₂O alone or in combination with argon
on the mitochondrial apoptotic pathway in two cell
lines

6.1. Introduction

Immortalised cell lines are a widely used research tool in many different research fields. In neuroscience, there are a number of different cell lines which can help to answer scientific questions without the need to use large quantities of animal or human samples, such as SH-SY5Y or PC-12 (Hopkins et al., 2015). Immortalised cell lines, unlike neurons, have the ability to replicate in culture (Giordano and Costa, 2011). They are often obtained from tumours, such as neuroblastomas or neurogliomas (Shastry et al., 2001). These cell lines therefore have the many of the cellular properties of the cell type of the tumour, such as neurons for neuroblastomas and glial cells from neurogliomas. However due the obviously aberrant phenotype of tumour cells, they are not an ideal match for primary cells, and have some drawbacks despite their ease of use. For instance, immortalised cell lines tend to shift away from the initial cellular phenotype over repeated generations (Hughes et al., 2007). However despite this, using cell lines has a number of advantages; much greater cell yields can be obtained for different analysis types, cells are readily available at all times for experimentation without the need for surgery, culturing and waiting for maturity, and cell lines help reduce the number of animals used in research (Blakemore et al., 2012). Importantly, tumour cells can also be easily genetically manipulated by transfection to induce genetic manipulation (Farah et al., 2000).

The two cell types used in this chapter are H4 neuroglioma cells and SH-SY5Y neuroblastoma cells. H4 cells have been previously shown to undergo apoptosis after exposure to anaesthetics such as sevoflurane (Dong et al., 2009), isoflurane (Zhang et al., 2011) and nitrous oxide in combination with isoflurane (Zhen et al., 2009). SH-SY5Y cells have also been shown to be sensitive to anaesthetic toxicity in some instances (Mak et al., 2010), but

on the whole show less vulnerability to anaesthetics than H4 cells (Lin et al., 2011a), although there are significantly less studies regarding anaesthetic toxicity in SH-SY5Y cells compared with H4 cells. SH-SY5Y cells are a widely used model of neuronal cells as they can be differentiated from their neuroblastoma original phenotype to a more primary neuron-like phenotype (Kovalevich and Langford, 2013a). This is achieved by the addition of substances to inhibit growth and induce differentiation, such as retinoic acid. Undifferentiated SH-SY5Y cells show a phenotype more similar to immature neurons, and proliferate at a high rate, whilst differentiated cells show mature neuronal phenotype, axonal branching and a decreased proliferation (Constantinescu et al., 2007, Kovalevich and Langford, 2013b). However this differentiation process takes up to 5 days and once differentiated cells cannot be split readily due to poor growth, meaning continuing both undifferentiated and differentiated cell lines can take quite some time, space and resources. This chapter will expand upon Chapter 5 by using the same gas exposure parameters in cell types which are more amenable to different analysis methods. The biggest addition of this chapter to the primary neuronal data will be to attempt to validate the findings using a wider range of outcome measures. With primary cultures, due to low seeding densities and physiological constraints, it was not possible to perform either Western blot or flow cytometry analysis, so by using tumour derived cell lines which proliferate and are more robust, hopefully a clearer picture of the underlying molecular mechanisms surrounding anaesthetic neurotoxicity and argon neuroprotection can be gained. As well as this, due to the availability of greater amounts of cells, it will be possible to include a hypoxia model to mimic the *in vivo* work to be discussed later. This was not possible in the primary cells as it introduced too many groups and sufficient repeats were not achievable. A slightly different cohort of proteins will be analysed in these cell lines as were analysed in the primary

neurons, due in large part because of the different analysis methods used, with Western blot allowing for a greater range of proteins analysed. As before, cleaved caspase-3 will be analysed as an endpoint measurement for apoptosis. Puma and Bax, both pro-apoptotic Bcl-2 family members will also be measured to assess the impact on the mitochondria. Cleaved caspase-8 will also be measured as a different mechanism of cell death, acting via the so-called “death receptors”, which ultimately leads to apoptosis. Finally, ERK 1/2 will be analysed as a neuroprotective mechanism by which argon may have its effects, as shown in previous publications

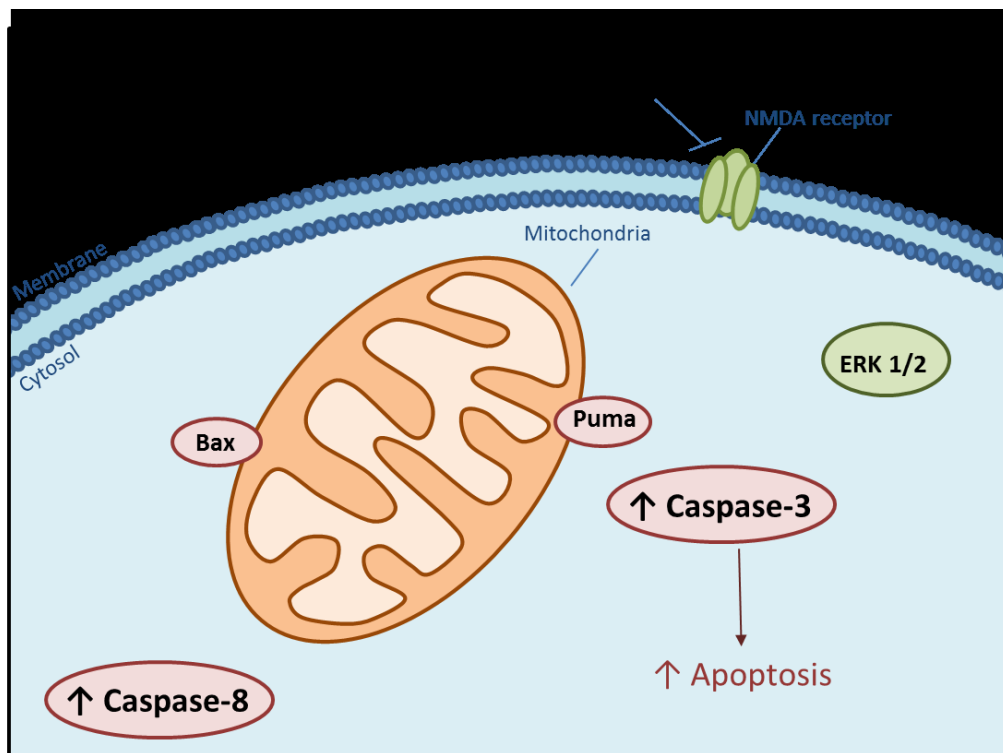


Figure 6.1 **Proposed proteins to be investigated in this protein.** To expand on the apoptotic mechanisms by which N_2O could have its effect, and the protective mechanisms of argon, proteins from both the intrinsic pathway and other molecular pathways will be investigated. Bax and Puma both act upon the mitochondria to produce membrane pore formation to trigger the apoptotic cascade, while caspase-8 is directly upregulated after activation of the death receptor. Erk 1/2 is a protein with a complex activation process, but which is strongly implicated in neuronal survival. The cleavage of caspase-3 is one of the final steps in the apoptotic cascade.

6.2. Hypothesis and aims

The aim of this chapter is to examine the effects of both N_2O alone and N_2O with argon on two different immortalised cell lines. There is also a secondary aim to examine the effects of preconditioning with these gases on a model of oxygen glucose deprivation.

6.3. Methods

Cells from this study were obtained either from stock cultures within the lab, frozen down by previous experimenters at passage number 3 and brought up as necessary throughout experiments, or generously donated from the group of Prof. Chris Dobson at the Centre for Misfolding Disease, Cambridge University, UK at passage number 2. Passage number denotes the number of times a flask of cells was grown to reach confluence and split, either into separate flasks or for storage (see Section 4.1.1 for further details). As discussed above, the genotype of the cells can shift over generations, so this must be closely monitored. When being used in experiments, cells can easily go through 3 or more generations in one week with continuous splitting and subculturing. For this reason, most experimenters don't use cells which have gone past a certain levels of generations. In these experiments, cells above passage 15 were not used for experiments.

Once removed from long term liquid nitrogen storage, cells were quickly thawed, rinsed of the freezing medium and resuspended in the appropriate media for the cell type (see Table 4.1). Cells were grown until approximately 80% confluence before splitting into appropriate culture vessels for experimentation. Seeding density was determined by the time at which experiments were to take place; with a higher seeding density used the earlier cells were needed for experiments. In general, for the following experiments, cells were seeded at

approximately 50% confluence and used for experiments approximately 40 hours after seeding. The plasticware used for different outcome measures are listed in Table 4.2.

6.3.1. Gas exposure

On the day of experimentation, cells were removed from the incubator and placed in gas exposure chambers which were then sealed. Media was not changed before experiments commenced. These chambers were custom made, comprising of a small interior chamber which held a metal tray. This tray was loaded with samples, usually cells in petri dishes or 4 well plates, and the lid of the chamber closed. This lid was held in place using two large clamps to ensure an airtight seal. The inside of the lid contained a small fan which ensured even distribution of the gas throughout the chamber, which was powered by a mains connection on the outside of the lid. Once cells were sealed inside the chamber, the chamber was connected to the output of the anaesthesia trolley and the flow of gas was started. This remained flowing through the chamber for approximately 4 minutes until the chamber was flushed of air and filled only with the gas mixture of interest. The chamber was then sealed, the fan switched on and the entire system placed inside an incubator to ensure the cells remained at 37°C. Cells were exposed for four hours to (a) naïve, (b) N₂O or (c) N₂O + argon treatments, the exact gas mixtures of which are outlined in Table 6.1. For the standard gas exposure protocol, cells were removed from the incubator at this point and either collected for immunofluorescence staining or Western blot, or returned to the growth incubator for 20 hours for propidium iodide analysis. The top branch of the experimental timeline (Figure 6.2) shows this process.

Table 6.1 **Gas exposure concentrations for *in vitro* immortalised cell lines.** These concentrations were used for all cell types, with gas exposure maintained for four hours in a sealed chamber with no continuous flow of gas. Naïve cells were left in the growth incubator undisturbed until collection.

	Naive	N ₂ O treatment	N ₂ O + Argon treatment
O ₂	21%	21%	21%
CO ₂	5%	5%	5%
N ₂	74%	24%	-
N ₂ O	-	50%	50%
Argon	-	-	24%

6.3.2. Oxygen glucose deprivation

Oxygen glucose deprivation is a commonly used *in vitro* model of a cessation of blood flow, leading to reduced oxygen and blood glucose. As neurons have an extremely high demand of both oxygen and glucose, this provides quite an extreme insult to these cells. A protocol previously used within the group was used for these experiments. In this model, the four hour gas exposure was still carried out, becoming a preconditioning model to mimic the *in vivo* experiments. Briefly, after cessation of 4 hour gas exposure as described above, cell media was changed to an OGD solution. This consisted of a balanced salt solution lacking glucose, prepared in advance and bubbled through with pure N₂ for 20 minutes to create an oxygen free solution. For naïve cells, the media was exchanged for fresh media to keep manipulations of the cell consistent between groups. Once the media had been changed, cells were placed in a clear hypoxia chamber which was sealed and flushed with 95% N₂ and 5% CO₂. This chamber was placed in an incubator for 75 minutes to give the OGD insult. After 75 minutes, cells were removed and the media exchanged for fresh supplemented media and cells returned to the incubator for recovery. Cells were left until 24 hours after

the end of the initial preconditioning gas exposure. This was due to the fact that often the time between ceasing preconditioning and starting OGD could vary between samples depending on other sample collection, the quantity of samples being handled that day etc., so to standardise as much as possible, a set endpoint based on the initial startpoint was utilised. Following the recovery period, cells were then collected either for immunofluorescence, Western blot analysis or propidium iodide staining.

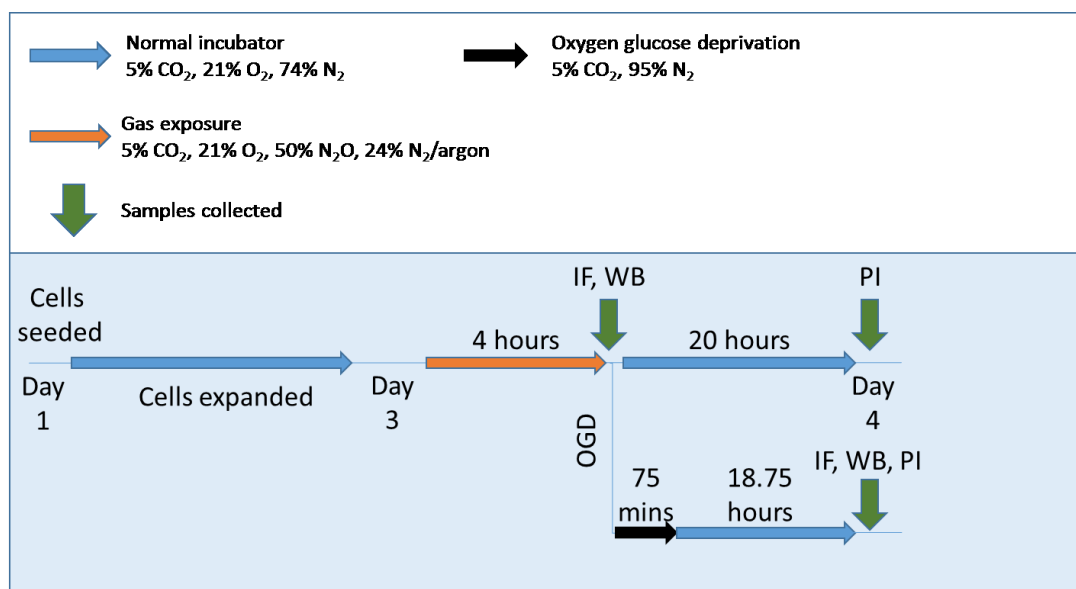


Figure 6.2 **Timeline of experimental protocol.** Cells were cultured for 3 days before experiments began. Treated cells were exposed to four hours of gas exposure on 3DIV in a sealed exposure chamber kept at 37°C. Naïve control cells were left in the original incubator. Following gas exposure, cells were either immediately fixed for western blot (WB) or immunofluorescence (IF), maintained for 20 hours for propidium iodide (PI) viability assay or immediately exposed to an oxygen glucose deprivation (OGD) insult. OGD was given for 75 minutes in a glucose free solution, in an environment of 95% N₂ and 5% CO₂. After 75 minutes, the cells were removed and returned to the original incubator for 18.75 hours, before collection for western blot, Immunofluorescence or PI staining.

6.4. Analysis and Statistics

For immunofluorescence, cells were stained for cleaved caspase-3 or Puma. Images were captured using an Olympus BX60 microscope fitted with a Zeiss Colour AxioCam camera. For each coverslip, 10 pictures were taken randomly around the coverslip. In each image, 5

cells were measured for mean fluorescent intensity (MFI) of the protein of interest. 5 measurements of the background were taken and the background MFI was subtracted from the protein MFI to get a measurement of fluorescence of the protein of interest. The average fluorescence of the 10 images was then obtained to give the MFI for one sample. To normalise samples, all naïve values were averaged and this average naïve value was used as the normalising value, described in more detail in Figure 5.4.

For Western blot, samples were stained for cleaved caspase-8, Bax, Puma, and Erk 1/2. ImageJ software was used to analyse samples. The optical density of bands was obtained using ImageJ for all proteins of interest and the housekeeping protein GAPDH. Each value of the protein of interest was then divided by the corresponding value of GAPDH to obtain the normalised optical density for each sample. As there was often variability in protein staining intensity between membranes, each membrane was normalised individually to the naïve samples on the membrane, instead of normalising all naïve samples across all gels.

For flow cytometry, the first stage involved gating the cells to select the appropriate population of cells. As can be seen in Figure 4.2, there were two distinct populations in the cells, a large population which was primarily PI negative, and a smaller population, both in number and size, which showed very high PI staining. It is likely that the smaller population are shrunken apoptotic cells, hence their small size and PI staining, so both populations were combined together to get the complete profile of the cell population. The channel for PE Texas Red was analysed, corresponding to the emission spectrum of propidium iodide.

6.5. Results

6.5.1. Cell viability

For both cell types, there was no significant effect on cell viability with either N₂O or N₂O + argon treatment. With the addition of OGD, neither H4 nor SH-SY5Y cells had any significant increase in propidium iodide staining, regardless of the preconditioning group (SH-SY5Y; One way ANOVA, $p = 0.7180$, $n=4-5$, Figure 6.3b). It is worth noting that for H4 cells, only 2 repeats were achieved so no statistical analysis could be carried out.

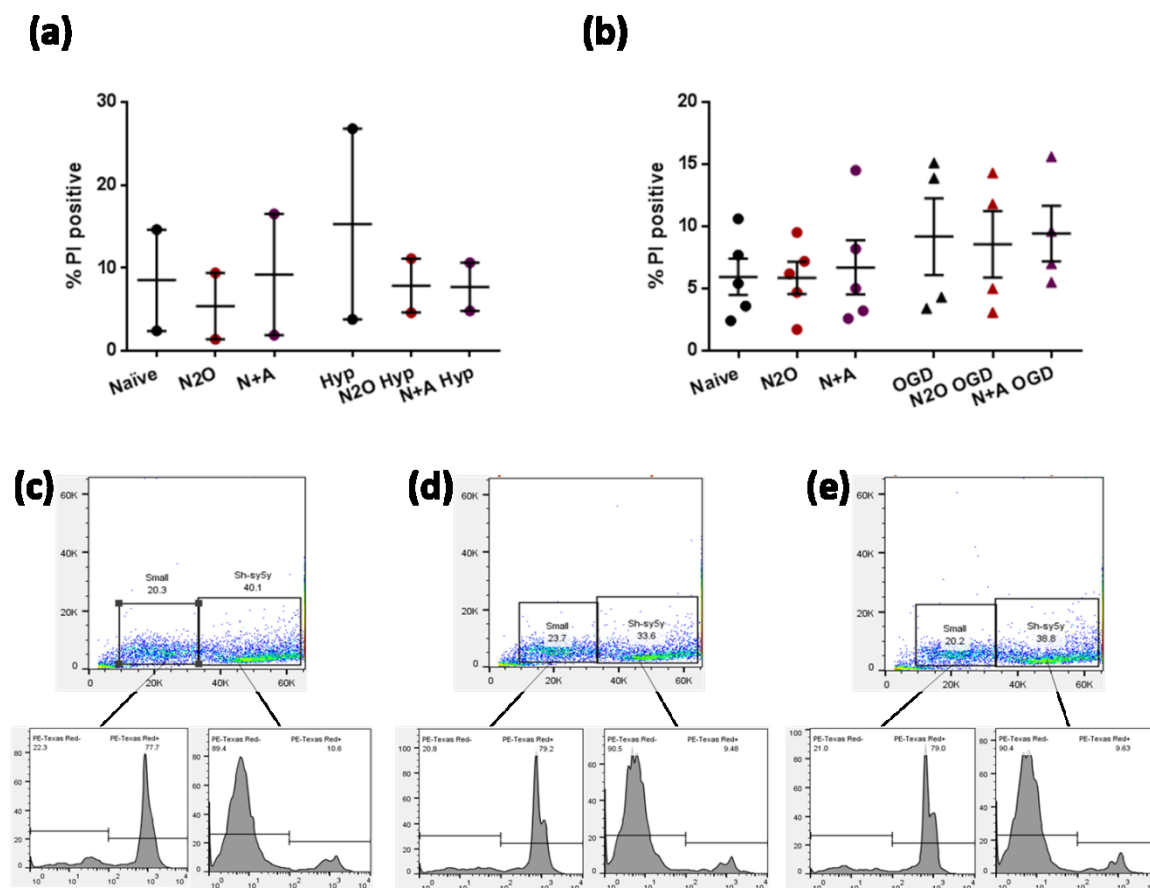


Figure 6.3 Cell viability results. Cell viability analysis was carried out by propidium iodide (PI) staining and analysed by flow cytometry. (a) H4 cells did not reach sufficient repeats for statistical analysis. (b) SH-SY5Y cells showed no significant difference in PI staining, indicating no changes following anaesthetic exposure either with or without oxygen glucose deprivation (OGD). Representative cell counts for propidium iodide fluorescence are shown at the bottom for naive (a), N₂O (b) or N₂O plus argon (c) treated SH-SY5Y cells. Cells showed two populations, one larger primarily PI negative subset and one smaller, primarily PI positive subset. One way ANOVA, $p = 0.7180$, $n=4-5$.

6.5.2. Pro-apoptotic proteins

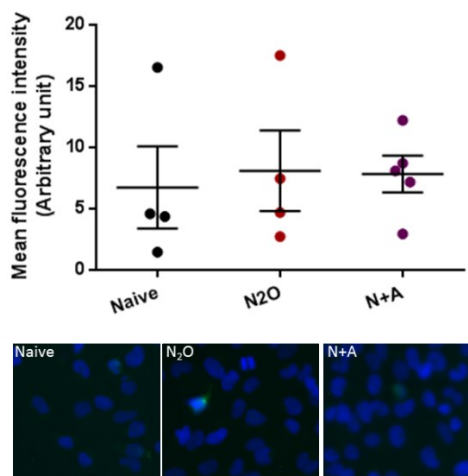
Cleaved caspase-3 expression was measured using immunofluorescence staining for gas exposure alone. For both H4 and SH-SY5Y cells, there was no significant difference in cleaved-caspase-3 expression, although SH-SY5Y was not high enough n number for statistical analysis (H4; Kruskal-Wallis, $p = 0.6251$, $n=4-5$,

Figure 6.4a). Puma and cleaved caspase 8 (18kDa fragment) were analysed by Western blot, again with no significant difference between any of the treatment groups. For H4 cells, data for the OGD insult was not obtained. (H4 Puma; Kruskal Wallis, $p = 0.8741$, $n=4$,

Figure 6.5a, SH-SY5Y Puma; Kruskal Wallis, $p = 0.4298$, $n=3$,

Figure 6.5b; H4 Casp8 p18, One Way ANOVA, $p = 0.6346$, $n=6-7$, Figure 6.6a, SH-SY5Y Casp8 p18, Kruskal Wallis, $p = 0.3487$, $n=3$, Figure 6.6b). For SH-SY5Y cells, Bax was also analysed using Western blot, with no significant difference between any groups (SH-SY5Y Bax, Kruskal Wallis, $p = 0.4298$, $n=3$, Figure 6.7).

(a)



(b)

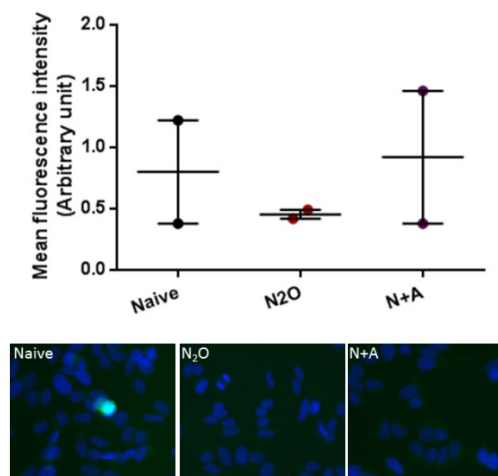


Figure 6.4 **Cleaved caspase-3 expression.** Expression of the proapoptotic protein cleaved caspase-3 in two cell lines following four hours exposure to either N₂O alone (N₂O) or N₂O plus argon (N+A). (a) Exposure in H4 cells showed no significant difference between treatment groups. (b) SH-SY5Y cells also showed no difference between treatment groups

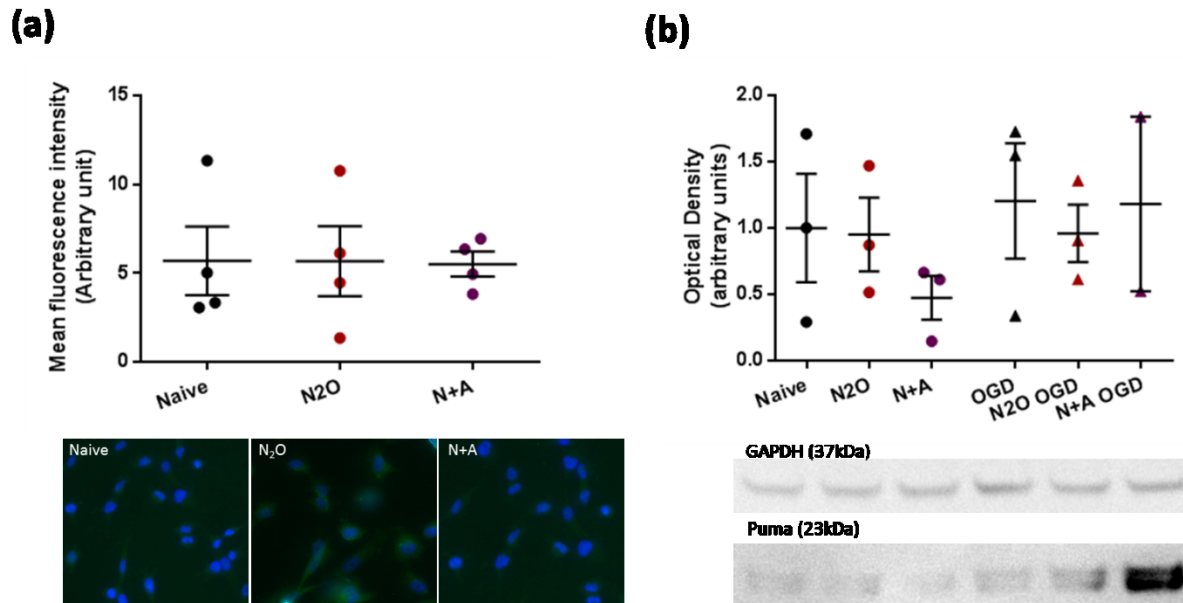


Figure 6.5 **Puma expression.** Expression of the pro-apoptotic protein Puma in two cell lines following four hours exposure to either N₂O alone (N₂O) or N₂O plus argon (N+A). (a) Exposure to H4 cells showed no significant difference between treatment groups. (b) Sy-shy5y cells were also exposed to 75 minutes oxygen glucose deprivation (OGD) with or without pre-treatment with N₂O or N+A. Again, none of the groups showed a significant difference.

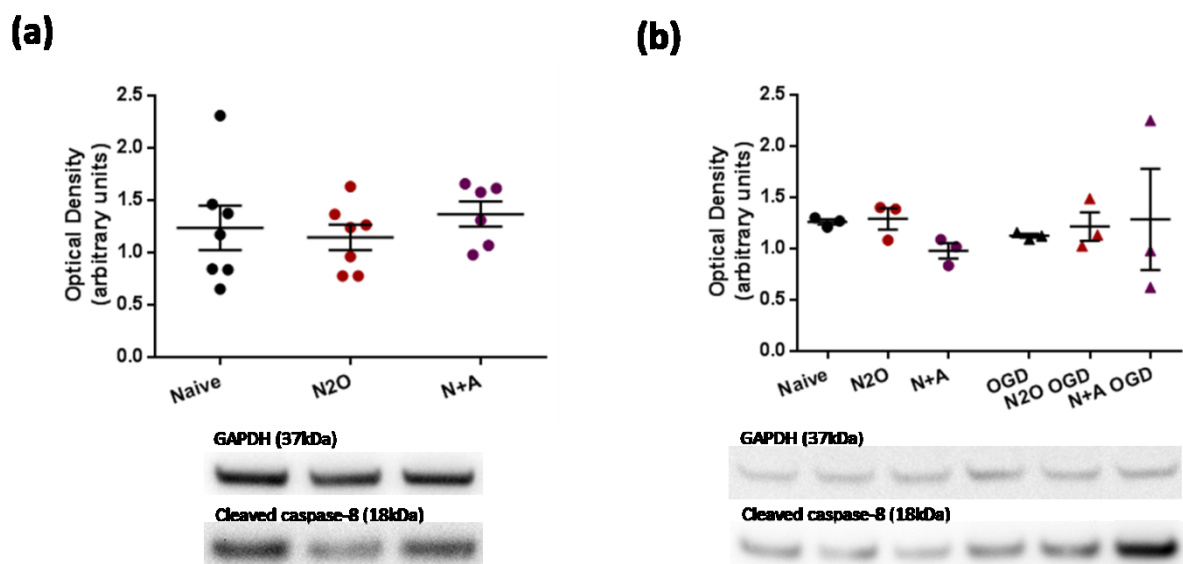


Figure 6.6 **Cleaved caspase-8 expression.** Expression of the 18 kDa cleavage product of the pro-apoptotic protein cleaved caspase-8 in two cell lines following four hours exposure to either N₂O alone (N₂O) or N₂O plus argon (N+A). (a) Exposure to H4 cells showed no significant difference between treatment groups. (b) Sy-shy5y cells were also exposed to 75 minutes oxygen glucose deprivation (OGD) with or without pre-treatment with N₂O or N+A. Again, none of the groups showed a significant difference.

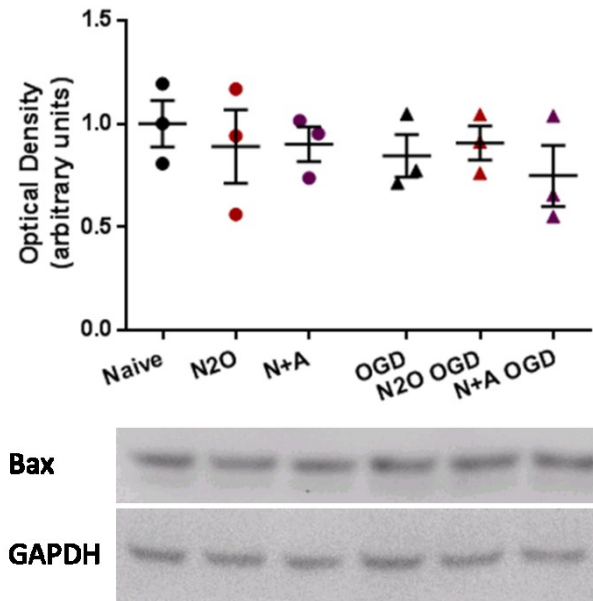


Figure 6.7 **Bax expression.** Expression of the pro-apoptotic protein Bax in SH-SY5Y cells following four hours exposure to either N₂O alone (N₂O) or N₂O plus argon (N+A). Cells were also exposed to 75 minutes oxygen glucose deprivation (OGD) with or without pre-treatment with N₂O or N+A. None of the groups showed a significant difference.

6.5.3. Anti-apoptotic proteins

For both H4 and SH-SY5Y cells, pErk 1/2 expression was analysed using Western blot, but showed no significant difference between groups. Again, H4 cells exposed to OGD only reached n=2 so could not be included for statistical analysis (H4 pErk1/2; Kruskal Wallis, p = 0.697, n=4-6, Figure 6.8a, SH-SY5Y pErk1/2, Kruskal Wallis, p = 0.8587, n=5-6, Figure 6.8b).

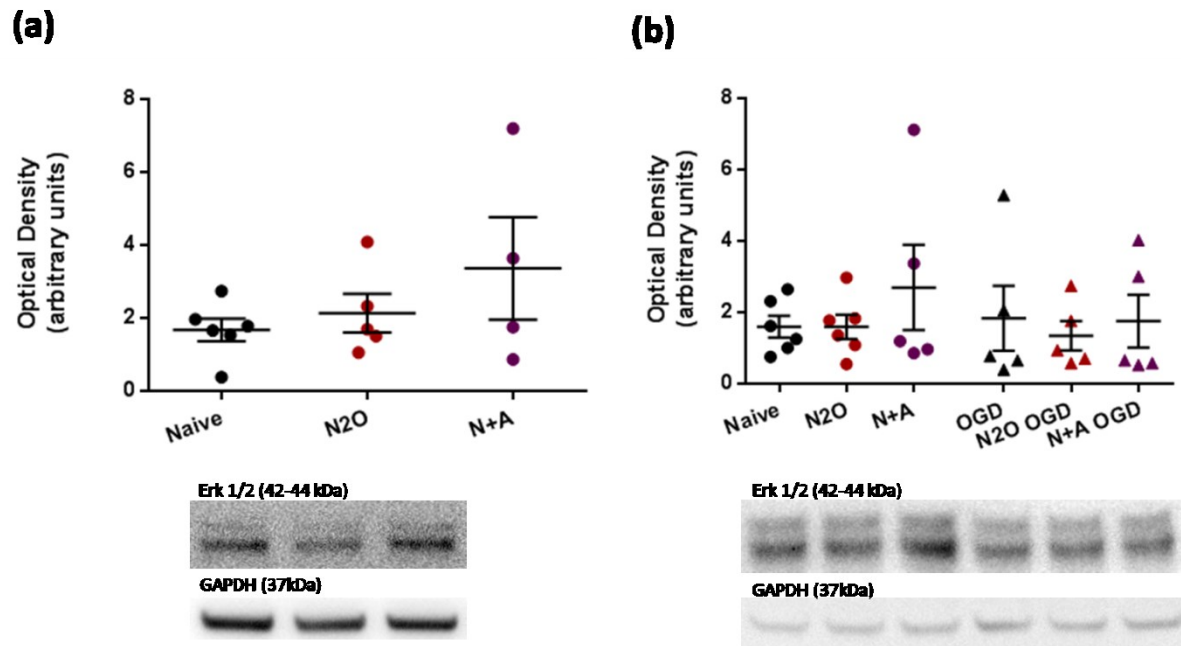


Figure 6.8 **Erk 1/2 expression.** Expression of the anti-apoptotic protein Erk 1/2 in two cell lines following four hours exposure to either N₂O alone (N₂O) or N₂O plus argon (N+A). (a) Exposure to H4 cells showed no significant difference between treatment groups. (b) Sy-shy5y cells were also exposed to 75 minutes oxygen glucose deprivation (OGD) with or without pre-treatment with N₂O or N+A. Again, none of the groups showed a significant difference.

6.6. Discussion

The data from flow cytometry, Western blot and immunofluorescence together clearly suggests there is no significant effect of either N₂O alone or in combination with argon on the viability or protein expression of H4 and SH-SY5Y cells. This is in contrast to what was shown in primary neuronal cells. This data also shows that OGD had no effect on either viability or protein expression on either cell type, although for H4 cells this data cannot be reliably discussed due to small repeats and fewer proteins measured. Neither cell type appeared to show any differences in terms of vulnerability to different treatments or insults.

There are a number of reasons why no response was seen in either cell type to any insult or treatment given. The most likely is that in general, tumour cell lines are more robust to insult than primary neurons. The harsh environment of a tumour, with hypoxia often

present, and the cells undergoing rapid growth, could create cellular mechanisms which readily adjust to insult (Shannon et al., 2003). Tumour cells often have aberrant apoptotic pathways which confer the characteristics necessary to cancer cells, namely resistance to apoptosis (Igney and Krammer, 2002). Considering also that the changes seen in primary cells in Chapter 5 were relatively small, it is probable that the cancer cell lines used here were more readily able to resist the toxic effects of N_2O . In terms of the OGD insult, it is quite possible that the level of insult was again just not great enough for these rather robust cells. The protocol used here was optimised in this group for primary cell lines (Rajakumaraswamy et al., 2006, Ma et al., 2003), and had not been tried in tumour cells before. It is apparent from reading the literature that up to 24 hours OGD can be used (Fordel et al., 2007). On the other hand, for SH-SY5Y cells, OGD times as short as between 60 minutes (Pei and Cheung, 2003) and 90 minutes (Lee et al., 2010) have been used, so 75 minutes may have been expected to show a minor change. Indeed, although the data did not reach significance, there does appear to be a trend towards increased PI staining in SH-SY5Y cells which underwent OGD, indicating at least some level of damage was occurring. Perhaps with longer time points this may have become more pronounced.

One important consideration when comparing the effects of treatments on these two cell lines is their different source cell type. H4 cells are of neuroglioma origin, a tumour of glial cells (Sun et al., 2014). SH-SY5Y on the other hand were obtained from a human neuroblastoma, stemming from a tumour of neuroblast cells, an undifferentiated neuronal precursor cell type (Kovalevich and Langford, 2013a). This gives two quite different cell types, glial versus undifferentiated neurons. It is becoming widely recognised that glial cells have an extremely important role in the protection and normal maintenance of the brain, but these cells are physiologically and biochemically quite different from neurons. Therefore

it would not be expected that these two cell types should react in a similar manner to insult. Unfortunately in this study, neither cell line showed any particularly reaction to either the anaesthetic gases or the OGD insult, which makes a comparison of the two cell types difficult in this instance. One thing that is noticeable when all the data of gas exposure is compared together, disregarding the OGD insult, is that the H4 cells appear to have almost no reaction to any treatment, with virtually no hint of change between groups, while SH-SY5Y cells, although not reaching significance, do show some more clear differences between N₂O and N₂O + argon groups, particularly for Puma, phosphorylated Erk 1/2 (pERK 1/2) and even cleaved caspase-8. Perhaps then, the more neuronal like cell line is more susceptible to different treatments as opposed to glial cells, as has been shown before with different toxins (Iwata-Ichikawa et al., 1999, Silva et al., 2002).

This study did have a number of limitations, not least of which was small n numbers for some of the groups, particularly for H4 cells. It was hoped that larger n numbers could have been reached but unfortunately with changes to protocol through the experiment which made older data unusable and infections wiping out cell lines, the experiments could not reach optimal repeats. However the data was included here to give a more rounded picture of the effects of these anaesthetics. As there was such a difference between the reactions of the primary neurons and the cell lines to the treatments, it was important to include all data to allow a full consideration of the actions of N₂O and argon on the brain. Many studies use either primary neurons or cell lines, but few use both, so it is important to ensure the data between the two can be reliably discussed as representing the same thing. In this data, it is clear that the two different models do not indeed have the same reactions to this relatively mild insult of N₂O toxicity.

This data, in combination with the results from the primary neuronal culture, still gives reason to believe that these anaesthetics require further investigation, particularly in the presence of a secondary insult such as hypoxia. Whilst tumour derived cells did not show vulnerability to anaesthetic toxicity, the presence of toxicity in primary neurons, which are a truer model of *in vivo* cells would encourage the continuation of investigations in higher model systems.

Chapter 7.

Investigation of anaesthetic neurotoxicity in a perinatal pup model

7.1. Introduction

The majority of research regarding the neurotoxicity of anaesthetic agents is carried out in rodent models. While *in vitro* and *ex vivo* research can help to answer fundamental questions regarding mechanisms, eventually an *in vivo* model is necessary, with whole organ and whole systems needed to understand the full effects of these anaesthetics before considerations of clinical effects can be started. Thus far, only one investigation into the actions of N₂O alone on the young brain has been carried out. This study, which measured apoptosis and protein expression in pups exposed to N₂O either alone or in combination with isoflurane and midazolam, found that N₂O alone at 50%, 75% or 150% showed no signs of neurotoxicity (Yon et al., 2005). A number of studies have examined N₂O in combination with volatile anaesthetics in neonatal pups (Lu et al., 2006, Shu et al., 2010, Yon et al., 2006), and investigations into N₂O alone have been carried out in adult rats (Jevtovic-Todorovic et al., 2003a, Jevtovic-Todorovic et al., 1998), although at hyperbaric concentrations. As has been shown in Chapter 5, exposure to just 50% N₂O can have neurotoxic effects on a primary cell culture, so it is important to try to further investigate these findings in an *in vivo* setting. The effects of N₂O in the immature brain are somewhat difficult to hypothesise, as the neonatal brain has a number of differences from the mature adult brain which may alter the vulnerability, described in greater detail in Section 2.1.2. These include immature systems to handle reactive oxygen species and an altered function of GABAergic neurons, which are excitatory instead of inhibitory for the first week of life in both rodents and humans (Tyzio et al., 2007, Leinekugel et al., 1999).

As this project seeks to understand the actions of N₂O and argon on the perinatal brain, young pups were used for *in vivo* toxicity investigation. Both postnatal day (P) 3 and P7 pups

were used as these are said to correlate with the preterm and term human brain development (Workman et al., 2013). P7 is important as this is the likely brain development stage of infants exposed to anaesthetics in the postnatal period. P3 pups were also used to determine differences in vulnerability at different ages and developmental stages. These timepoints are widely reported and used, and are based on a number of different neurodevelopmental stages which can be correlated between humans and rats. This estimate of comparative developmental stages was initially determined by a study comparing cell numbers, myelination and brain weights (Clancy et al., 2007, Dobbing and Sands, 1979), and it is from these first studies that the majority of papers base their timescales, with these studies from the 1970's still cited today (Tchekalarova et al., 2013, Sanders et al., 2009, Souza et al., 2013). One consideration when using pups this young is their ability to remain away from the nest without experiencing adverse effects associated with lack of feeding or other effects being separated from their mother could cause. Studies investigating maternal deprivation have looked at varying ages and durations of deprivation, and the resulting stress and cell death responses which occurred. However the majority of these studies use timescales of 24 hours and above (Zhang et al., 2002, Rentesi et al., 2013), and it has been found that until approximately 8 hours, there was little stress response in pups at P3 or P7 (Levine et al., 1991). For this reason, a 4 hour duration of anaesthesia away from the dam was deemed unlikely to interfere with the experiment. To ensure no effects however, a control group of maternal deprivation, separated from the dam and kept in the anaesthesia chamber without the lid sealed and with no gas flowing, were used for comparison.

As was investigated for the *in vitro* work, this study examined proteins which act on the mitochondria to damage or protect them, or otherwise act upon the apoptosis pathway to

alter cell viability in the face of injury were measured. The pro-apoptotic proteins Bax, Bcl-2, Puma, cleaved caspase-8 and cleaved caspase-3 were analysed either in whole brain, hippocampus or both. Anti-apoptotic proteins phosphorylated Erk 1/2 and Bcl-2 were also measured in different sample types.

As well as studying a particular mechanism of cell death following anaesthetic administration, this study will also investigate the effects in a particular brain region. While it is of interest to see global protein changes, there are variable patterns of vulnerability in the brain, and any damage in particular brain structures can be lost in the whole brain study. For this reason, the hippocampus will also be investigated as a specific substructure which is known to be vulnerable to neurotoxicity (Gozal et al., 2001), and which is easily dissected to ensure this specific brain area is reliably analysed between animals. A number of studies have shown anaesthetics such as propofol, sevoflurane and isoflurane, can increase apoptosis and induce changes in gene expression in the hippocampus (Han et al., 2015, Feng et al., 2012a, Pekny et al., 2014, Matsuoka et al., 1999). The hippocampus is a brain structure well associated with memory, and again anaesthetics have been shown to impair memory in both young and old rats (Shih et al., 2012, Wang et al., 2013, Culley et al., 2004). While this study will not be carrying out behavioural analysis on these animals following anaesthetic exposure, the hippocampus will be studied directly to determine any changes in this region.

7.2. Hypothesis and aims

The aim of these experiments was to investigate the translatability of pathways found to play a role in an *in vitro* model of anaesthetic exposure. Data from naïve pups at either postnatal day 3 or postnatal day 7 N₂O alone or in combination with argon was used to explore:

1. Is N₂O neurotoxic to naïve neonatal rat pups?
2. Can argon protect against any N₂O neurotoxicity?
3. Does the postnatal age of the animal alter the biological response?

7.3. Methods

Pups were born via natural vaginal delivery with no intervention from experimenter. Pups were left undisturbed in the home cage until the day of experiment. On either P3 or P7 pups were used for experiments.

7.3.1. Gas exposure

Gas exposure was carried out using a modified chamber. The chamber base was filled with soda lime pellets and silica gel crystals to absorb excess CO₂ and moisture respectively. The base was then covered to prevent contact of pups with either material. A thermometer was attached to the inner lid of the chamber to monitor temperature within. The entire chamber was placed within a heat box heated to 37°C and allowed to equilibrate to 37°C inside the chamber. Pups were removed from the home cage immediately prior to experiments and placed within the chamber. Anaesthetic gases were mixed using an anaesthesia trolley, and the output was measured using a monitor capable of measuring the concentration of gaseous or volatile anaesthetic agents and other gases. This trolley output was then

attached to the chamber input to start the flow of gases to the pups, and this gas flow was run continuously throughout the experiment to keep the chamber flushed of CO₂. The chamber output was monitored for CO₂ output to ensure this did not rise to toxic level. The chamber was of clear plastic so pups could be monitored throughout the experiment. Pups were left for four hours in the chamber before gas flow was stopped and pups removed. As a control, some pups were removed from the mother and placed in the heat box at 37°C to control for any toxicity which may have been associated with the disruption in feeding due to removal from the home cage. Besides naïve control and maternal deprivation (only for P7 hippocampal samples), there were three gas exposure groups, 50% N₂O either with or without 29% argon, and the third control of N₂ with 21% O₂ to ensure the gas exposure itself had no adverse effects. The gas concentrations and where the pups were for each group are outlined in Table 7.1.

Table 7.1 Gas exposure concentrations for postnatal day 3 and 7 pups. These concentrations were maintained for four hours via continuous flow of gas to pups in a clear plastic chamber sealed except for inlet and outlet valves. Maternal deprivation pups were in the chamber but with no lid and no flow of gas. Naïve pups were left with the dam throughout the entire experiment.

	Naïve	Maternal deprivation	N ₂ treatment	N ₂ O treatment	N ₂ O + Argon treatment
O ₂	21%	21%	21%	21%	21%
N ₂	78%	78%	79%	29%	-
N ₂ O	-	-	-	50%	50%
Argon	0.93%	0.93%	-	-	29%
Location	Home cage	Exposure chamber	Exposure chamber	Exposure chamber	Exposure chamber

7.3.2. Sample collection

Immediately following gas exposure, samples were taken for either histology or Western blot. For immunohistochemistry, pups were perfused transcardially with saline, followed by 4% paraformaldehyde (PFA) to fix the tissue. The whole brain was removed and stored in 4% PFA until paraffin embedded and sectioned at 8µm. Sections containing the hippocampus at the same size for all animals, approximately Bregma -2.20 of P7 rat brain (Khazipov et al., 2015b, Khazipov et al., 2015a), were then stained for cleaved caspase-3, Bcl-2 and Puma using DAB immunostaining. For Western blot, samples were either snap frozen in liquid nitrogen as whole brain samples, or the cortex and hippocampi were dissected and frozen separately for each animal.

7.4. Analysis and statistics

For immunohistochemistry, cell counting was performed using a light microscope. Positive cells, as determined by a deep red/brown stain of a rounded shape to denote a cell, were counted by an experimenter blinded to the treatment of each sample. The CA1 and CA3 regions of the hippocampus were counted separately and representative images taken.

For Western blot analysis, the proteins measured were Puma, cleaved caspase-8, Bax, Bcl-2 and Erk 1/2. ImageJ software was used to analyse samples. The optical density of bands was obtained using ImageJ for all proteins of interest and the housekeeping protein GAPDH.

Each value of the protein of interest was then divided by the corresponding value of GAPDH to obtain the normalised optical density for each sample. As there was often variability in protein staining intensity between membranes, each membrane was normalised individually to the naïve samples on the membrane, instead of normalising all naïve samples across all gels.

Statistical analysis was performed using GraphPad Prism. A normality test was carried out to determine if the data were normally distributed, followed by either a One-Way ANOVA with Bonferroni's multiple comparison or Kruskal Wallis with Dunn's multiple comparison test.

Data are presented as mean $\pm$ standard deviation (SD).

7.5. Results

7.5.1. Whole brain samples

7.5.1.1. Pro-apoptotic proteins

For whole brain samples at postnatal day 3 and 7, Bax was analysed via Western blot. For both P3 and P7 pups, there was no significant difference between any of the treatment groups (P3 Bax; Kruskal Wallis, $p = 0.5360$, $n=3$, Figure 7.1a, P7 Bax; Kruskal Wallis, $p = 0.2001$, $n=3$, Figure 7.1b).

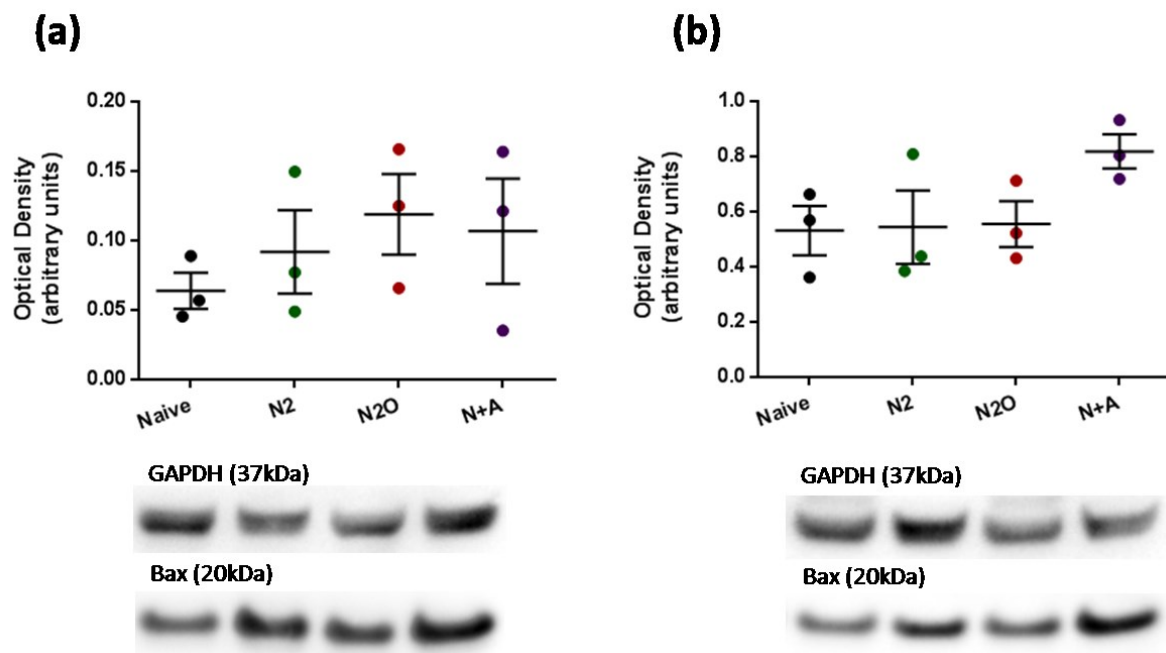


Figure 7.1 **Bax expression.** Bax expression was measured by Western blot analysis in the whole brain of both P3 (a) and P7 (b) pups exposed to 4 hours continuous gas. There were no significant differences between any of the treatment groups immediately after gas exposure.

7.5.1.2. Anti-apoptotic proteins

For both P3 and P7 samples, pErk 1/2 was analysed by Western blot. There were no significant differences for either timepoint between any treatment group (P3 pErk 1/2; Kruskal Wallis, $p = 0.1808$, $n=3$, P7 pErk 1/2; Kruskal Wallis, $p = 0.5566$, $n=3$). Bcl-2 was also measured for P3 samples; however bands were not obtained for P7 samples. Again, there was no significant difference between any treatment groups (P3 Bcl-2; Kruskal Wallis, $p = 0.1104$, $n=3$).

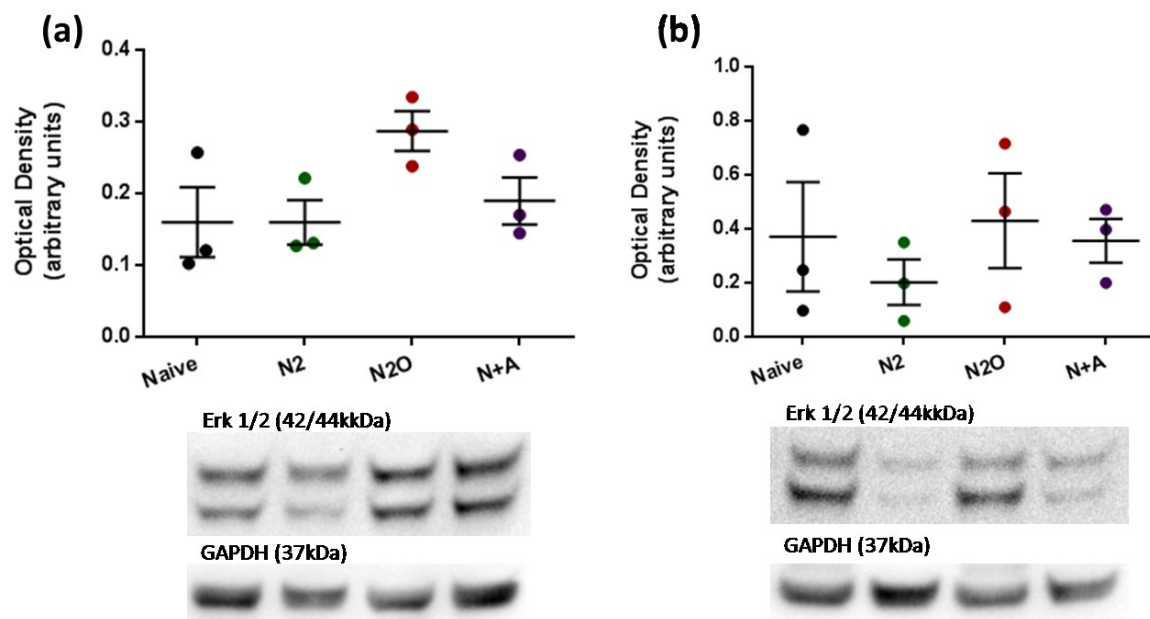


Figure 7.2 **Erk 1/2 expression.** Erk 1/2 expression was measured by Western blot analysis in the whole brain of both P3 (a) and P7 (b) pups exposed to 4 hours continuous gas. There were no significant differences between any of the treatment groups immediately after gas exposure.

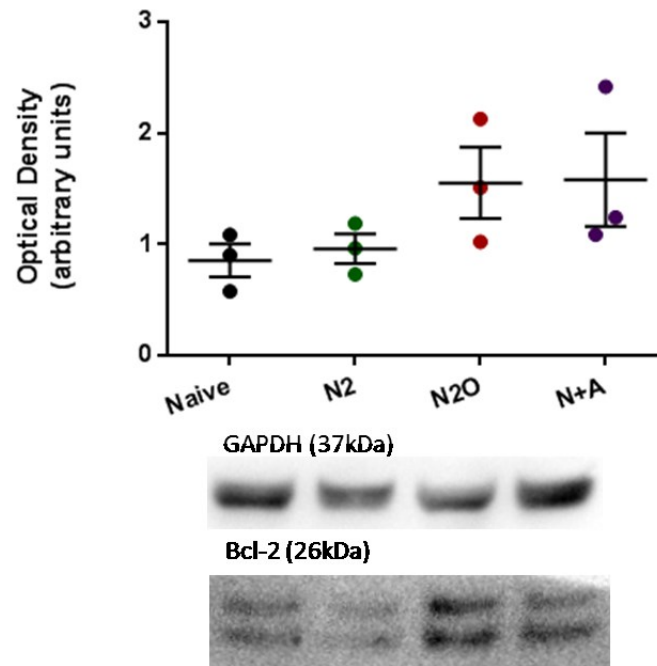


Figure 7.3 **Bcl2 expression.** Bcl-2 expression was measured by Western blot analysis in the whole brain of both P3 (a) and P7 (b) pups exposed to 4 hours continuous gas. There were no significant differences between any of the treatment groups immediately after gas exposure.

7.5.2. P7 hippocampus

For hippocampal samples, only postnatal day 7 animals were used. A new control group of maternal deprivation was also included for these samples.

7.5.2.1. Pro-apoptotic proteins

For P7 pups, the hippocampal expression of cleaved caspase-8 (18kDa fragment) was analysed by Western blot for all treatment groups. There was no significant difference between any of the treatment groups (P7 cleaved caspase-8; Kruskal Wallis, $p = 0.3142$, $n=3-6$). Puma was also analysed via Western blot, but there was no significant difference between any group for this protein either (P7 Puma; Kruskal Wallis, $p = 0.5965$, $n=4-6$). Cleaved caspase-3 was also analysed by tissue immunohistological staining, with data presented as total positive cells in either the CA1 or CA3 region of the hippocampus. There

was no significant difference between any of the groups for either region (P7 cleaved caspase-3; CA1, Kruskal Wallis, $p = 0.5519$, $n=2-3$, CA3, Kruskal Wallis, $p = 0.5483$, $n=2-3$).

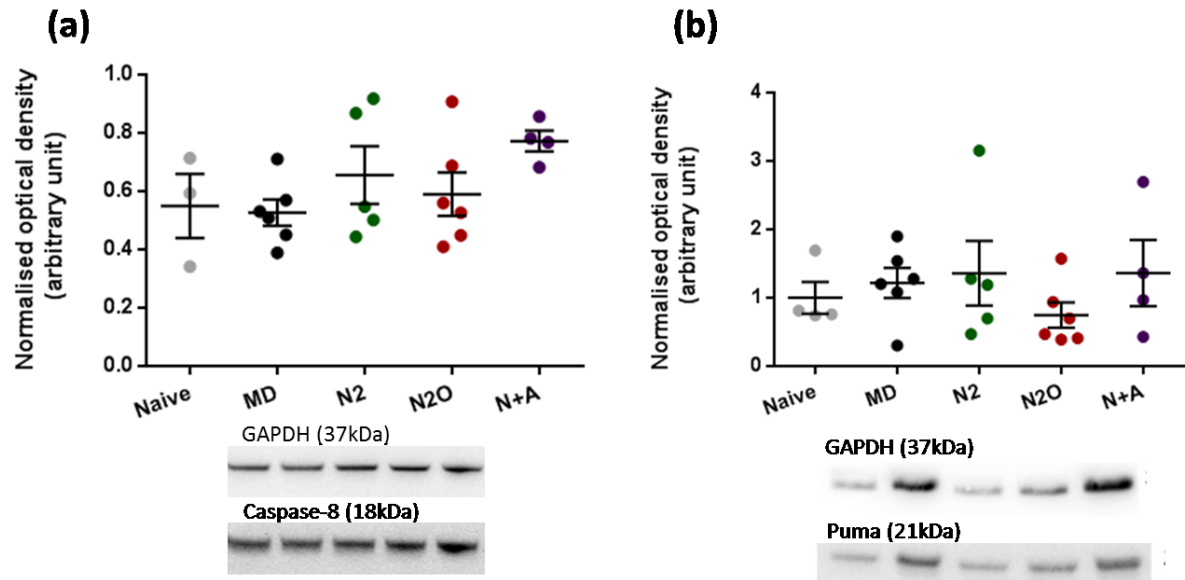


Figure 7.4 **Casp8 and Puma expression.** Following four hour gas exposure, Western blot analysis was performed to measure the expression of cleaved caspase-8 (a) or Puma (b) in the hippocampus of postnatal day 7 rat pups. There were no significant changes in protein expression in any of the groups.

7.5.2.2. Anti-apoptotic proteins

Phosphorylated Erk 1/2 was analysed by Western blot for all treatment groups, however there was no significant difference between any group (P7 pErk 1/2; Kruskal Wallis, $p = 0.4379$, $n=4-6$).

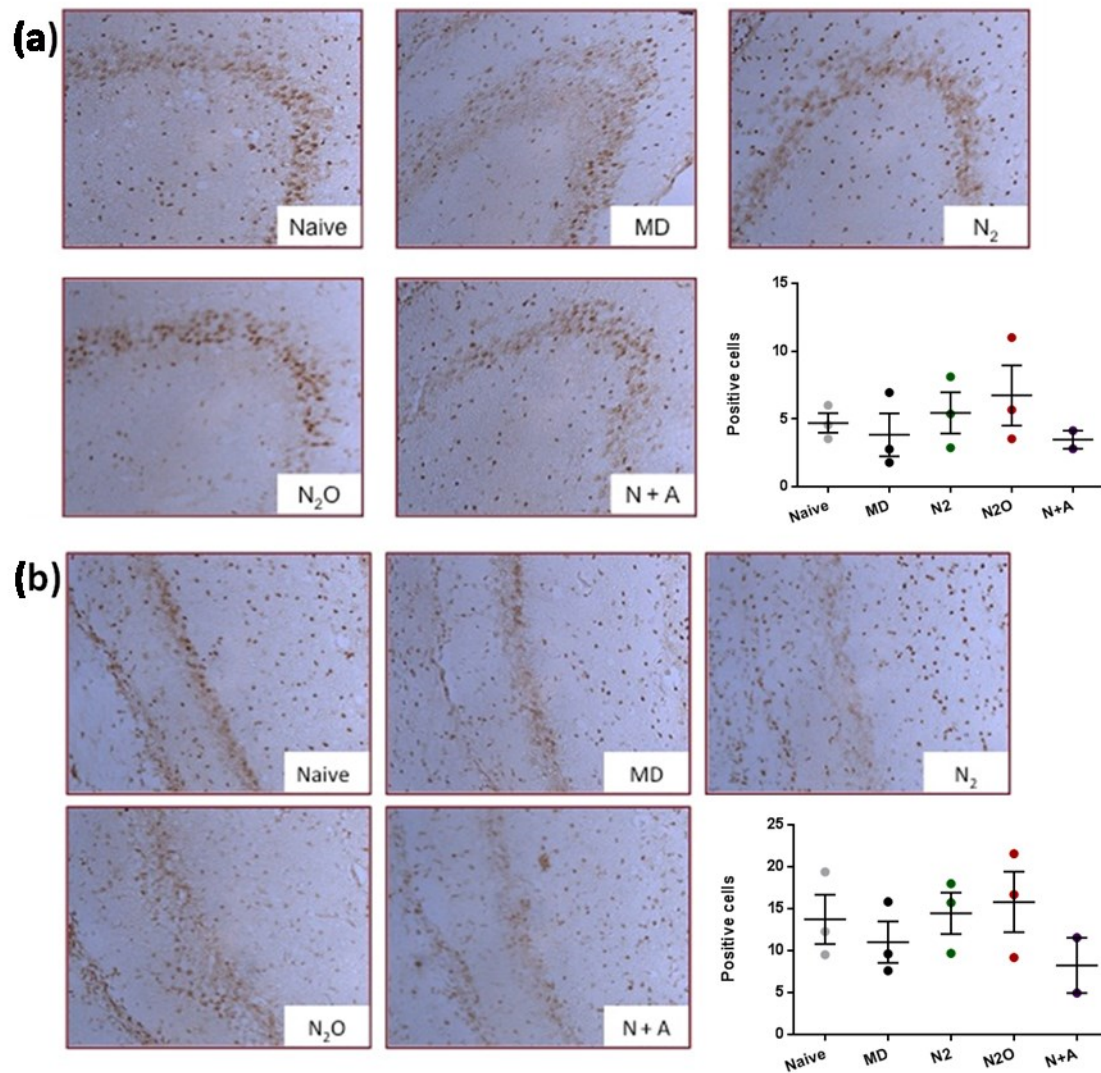


Figure 7.5 **Caspase-3 expression.** Immunohistochemistry was performed to measure the expression of cleaved caspase-3 in the hippocampus of postnatal day 7 rat pups. Positive cells were counted in both the CA3 (a) and CA1 region of the hippocampus. There were no significant changes in expression between any of the groups.

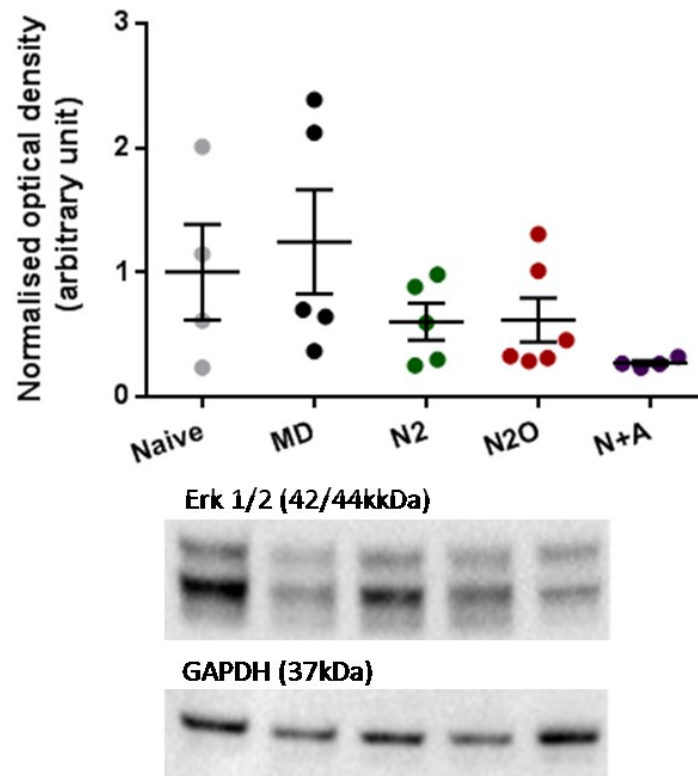


Figure 7.6 **Erk 1/2 expression.** Western blot analysis was performed after four hour gas exposure, to measure the expression of Erk 1/2 in the hippocampus of postnatal day 7 rat pups. There was no significant difference in protein expression between any of the groups.

7.6. Discussion

In these experiments, pups at either postnatal day 3 or 7 were removed from the home cage and exposed to four hours of one of four treatments, or left in the home cage as naïve controls. For all groups involved, there were no significant differences in any of the proteins analysed, either in whole brain or hippocampal samples. While we would not have expected to see any differences between naïve controls and either maternal deprivation or nitrogen treated animals, exposure to N₂O alone or in combination with argon may have had an effect based on primary cell culture data. However, based on mixed *in vivo* data from the past (Rizzi et al., 2010), there was an expectation that N₂O at this concentration for such short durations would not have an adverse effect.

There are a number of reasons why no neurotoxicity was seen after 4 hours N₂O at either age. There is the obvious possibility that N₂O is not neurotoxic under these circumstances, but there is also the possibility that the experimental design did not allow for the capture of any neurotoxicity. Pups were exposed for four hours and samples were taken immediately afterwards, reflecting the *in vitro* protocol. However, perhaps *in vivo*, a longer time is needed to see any changes occur. In future experiments, it would be prudent to carry out a timecourse analysis for time post-exposure, to determine how long after exposure any changes are seen, perhaps timepoints of 6, 12 and 24 hours post exposure to capture any changes. However, in previously published experiments, samples have been collected in short timeframes following cessation of gas exposure to measure cleaved caspase-3 as a marker of apoptosis, so the protocol is comparative to other published data (Yon et al., 2005).

The age used in perinatal investigations is a complex factor which can never be agreed upon. As mentioned above, the standard timepoints used are postnatal day 3 for preterm brain development, and postnatal day 7 for term infant development. These timepoints are based on older investigations, however, and it is perhaps worthwhile questioning the relevance of these to ensure data obtained is as relevant as possible to clinical scenarios. Newer resources, taking into consideration a range of different technologies and methods of statistical extrapolation, have been compiled into a website, <http://www.translatingtime.net> (Workman et al., 2013), where researchers can input the neurodevelopmental system and species required, and get a correlating age. Using this resource, 270 days post-conception (approximately term birth for a normal length pregnancy), is equivalent to 31 days post-conception for a rat, closer to postnatal day 9 than P7. Creating even more confusion is the conflicting data of whether the immature rat brain

is more or less susceptible to toxic insult. While much of the data agrees that approximately postnatal day 7-10 are generally quite vulnerable to a range of insults, younger ages are generally more robust in comparison. In many studies where insults were given over a range of ages from P0 upwards, peak vulnerability appears to be during the second week of life for rat pups (Yon et al., 2005, Levine et al., 1991, Ikonomidou et al., 1989). This means that although the human preterm brain is more vulnerable than the term infant, the same may not be true in rat studies, casting doubt on the translatability of this data (Ment and Vohr, 2008, Soria-Pastor et al., 2009).

Another important consideration for these neurotoxicity studies is the relevance of the anaesthetics and concentrations being studied. As mentioned previously, much of the original N₂O neurotoxicity data was performed using hyperbaric concentrations or excessively long durations which would never be used clinically (Jevtovic-Todorovic et al., 2003a, Jevtovic-Todorovic et al., 1998). While these studies were important to understand the actions of N₂O, they have limited use in the current context of data which does not seem to be able to replicate this N₂O toxicity in relevant concentrations (Zou et al., 2011, Yon et al., 2005). On the other hand, it is not wise to discount the toxicity of N₂O based solely on its lack of effect alone in naïve animals. Much evidence does show that in combination with other anaesthetics, particularly volatile anaesthetics with which it is often combined in clinical use; it can induce apoptosis in the brain of rodents and non-human primates (Zou et al., 2011, Beals et al., 2003, Culley et al., 2004, Yon et al., 2005, Yon et al., 2006, Culley et al., 2007, Shu et al., 2012). In reality however, it is unlikely that young infants will receive prolonged exposure to 50% N₂O alone. Currently, N₂O is being investigated and used in a 50:50% mixture with O₂, known in the UK as Entonox as an analgesic during dental surgeries and painful short procedures such as sutures, fixing broken bones, taking blood etc

(Reinoso-Barbero et al., 2011, Onody et al., 2006, Annequin et al., 2000). However in France at least, guidelines state that this 50% N₂O, 50% O₂ mixture, known there as EMONO (Equimolar Mixture of Oxygen and Nitrous Oxide), should not be used on children younger than four years and for a duration of over 30 minutes (Tepeneu and Chiru, 2013).

Overall, it can hardly be considered bad news if N₂O doesn't show neurotoxicity at clinically relevant concentrations. It is an extremely versatile drug, with a high safety profile in general terms (no respiratory depression etc.), and can be used in a wide range of scenarios, making it an extremely important drug to have available. However, to be sure of the total safety of N₂O, its effects alongside other insults have not been fully investigated, which is what the final part of this thesis will consider. Like many anaesthetics, N₂O is often given alongside either painful stimuli or in the period before or after events such as cardiac damage or hypoxia. If N₂O has the ability to exacerbate these insults, then its use must be considered and contraindicated wherever necessary.

Chapter 8.

Argon pre-treatment in combination with N₂O in a clinically relevant *in vivo* model of perinatal hypoxia

8.1. Introduction

Perinatal hypoxic ischaemic encephalopathy (HIE) is a widespread disorder, with an incidence of 8.5 per 1000 live births on average (Lee et al., 2013a), with an estimated 96% of infants affected by HIE born in low- and middle-income countries. Infants exposed to HIE at birth are at risk of developing a range of life-altering disorders, including cerebral palsy and cognitive impairment (Ellenberg and Nelson, 2013, Perlman, 2006). Up to 1 million infants per year worldwide are thought to develop cerebral palsy due to hypoxia at birth (World Health Organisation, 2005), and it has been calculated that 6132 years lived with disability per 100,000 people were caused by perinatal asphyxia in 2010 (Vos et al., 2012). Therefore the burden of this disorder can be seen to be great, and new treatment options are urgently required. Currently, there is only one approved treatment for birth asphyxia, which is hypothermia, cooling the brain to 33°C for long periods in an attempt to slow or prevent cell death and brain damage (Tagin et al., 2012). A number of preclinical studies have determined benefits of xenon in combination with hypothermia for perinatal hypoxia, and safety trials in human infants have been carried out (Dingley et al., 2014). Xenon plus hypothermia is now undergoing clinical trials in the UK to determine if the benefits found *in vivo* can be translated to humans, in both the Toby Xe trial (International Standard Randomised Controlled Trial Number ISRCTN08886155) and the Cool Xenon trial, ISRCTN75602528 (Dingley et al., 2014). However, xenon has a number of drawbacks which could preclude it from widespread feasibility, including extremely high cost, the need to use a closed loop system, and the anaesthetic effects shown above 60% (Sanders et al., 2003). Therefore interest has been piqued regarding the use of argon, which shows similar neuroprotective benefits but without the side effects listed above.

Argon, like xenon, is a noble gas, meaning it has no chemical reactivity. However, despite the chemical unreactivity leading to its name, derived from the Greek word for lazy, it has proven to be quite an active molecule biologically. This is likely due to its interaction at receptors via van der Waals interactions, although the exact receptor is not yet known (Harris et al., 2013). In particular, it has been investigated for its neuroprotective benefits in a number of *in vivo* models (Brucken et al., 2013, David et al., 2012, Ryang et al., 2011, Zhuang et al., 2012). It appears to have a lower level of neuroprotection compared with xenon (Harris et al., 2013), however in general the reduced costs, ease of use and lack of anaesthetic action may counteract these to make it an attractive option nonetheless (Ezzeddine, 2011). In this study, the neuroprotective benefits of argon alone will not be investigated; instead the combination treatment of N₂O and argon will be given and compared with N₂O alone. This is to mimic the clinical scenario of labour, described in more detail below.

Perinatal hypoxia, in general, cannot be predicted, making prophylactic treatment options difficult to administer. For this reason, the use of N₂O as a carrier agent for a neuroprotective strategy was considered. In the UK, between 60-70% of woman use N₂O during labour as an analgesic (Rosen, 2002, Rooks, 2007), making it one of the most widely used drugs during the period immediately before birth. This, therefore, could be a useful avenue to introduce a preconditioning treatment to women before birth. A pre-treatment may generally be better for perinatal hypoxia as often, by the time the infant is recognised as having undergone a hypoxic incident, cared for and assessed, and treatment begun, many hours may have passed allowing the initial injury to spread. A preconditioning treatment which worked would allow this onset of injury to be reduced, overall giving better outcomes for the infant. As well as the use of N₂O labour analgesia as a method of delivering a

neuroprotective strategy, this study will analyse the effects of N₂O alone in combination with perinatal hypoxia. This will provide information regarding any potential for N₂O to exacerbate or reduce brain damage. This is particularly relevant in the case of perinatal hypoxia as N₂O acts on the NMDA receptor, an important mediator of the excitotoxicity seen after hypoxic brain injury. This NMDA antagonism could help to reduce excitotoxic damage, however N₂O is known to increase levels of homocysteine in the body, a known neurotoxin. Therefore the overall effects of N₂O in a model of hypoxia need to be investigated. Previous work has found that N₂O can exacerbate transient hypoxic injury in the gerbil (Taninishi et al., 2008) and rat (Baughman et al., 1988), and alter electrophysiology in the hippocampus of rats following ischaemia (Amorim et al., 1997).

In this study, a widely used model of perinatal hypoxia was utilised, where the entire uterus was excised, one half clamped and placed in a water bath at 37°C to induce hypoxia, and the other half birthed immediately as caesarean section control pups. For the preconditioning model, dams were exposed to four hours of gas immediately before caesarean section. This model hopes to mimic the effects of N₂O during labour, combined with hypoxia at the time of birth, to give a clinically relevant model. As well as this, by using both uterine horns for different insults, caesarean section pups can act as a control for hypoxia treated animals, undergoing the same maternal effects. Samples were collected for Western blot or histological analysis of protein expression or cell death. As with the previous chapters, proteins which act upon the mitochondria to swing the balance of apoptosis were measured, along with cleaved caspase-8, part of the death receptor apoptotic pathway, and phosphorylated Erk 1/2, a protein shown to be upregulated by argon in previous studies (Fahlenkamp et al., 2012, Ulbrich et al., 2015b).

8.2. Hypothesis and aims

The aim of these experiments was to investigate the effects of N₂O and argon in a clinically relevant model of perinatal hypoxia. Samples from pups with or without the hypoxic insult were analysed at postnatal day 1, 3 or postnatal day 7 to examine the effects of pre-treatment with N₂O alone or in combination with argon to determine:

1. Does exposure to N₂O before perinatal hypoxia affect outcomes in pups
2. Can argon protect against any hypoxic or N₂O neurotoxicity?
3. Is the mitochondrial apoptotic pathway involved in any neurotoxicity seen?

8.3. Methods

Dams were delivered to the facility approximately one week before delivery to allow adjustment to the facility to prevent birth, and to reduce the risk of premature delivery and lessen the effects of stress which can affect pups (Philpot et al., 2012, Lordi et al., 1997). They were kept in a 12hr light:dark cycle, in standard plastic cages with woodchip bedding and cardboard tube. Dams had access to water and standard lab chow *ad libitum*. 2-3 days before due to give birth, extra shredded paper bedding was added to allow nest building. Experiments were performed on dams on the 21st day of gestation.

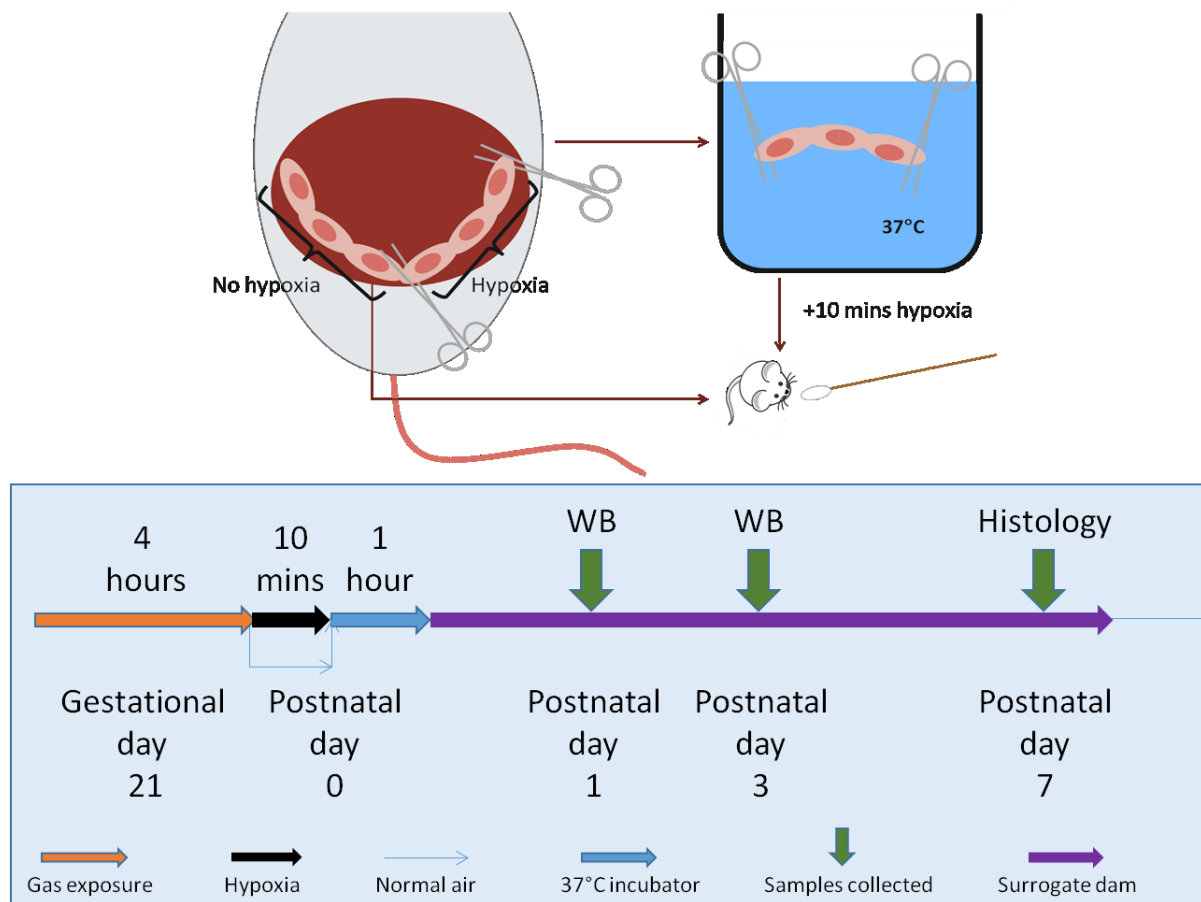


Figure 8.1 **Timeline of experiment for perinatal hypoxia and sample collection.** Dams were exposed to the pretreatment gas of interest for four hours on gestational day 21. Immediately after cessation of gas exposure, dams were culled and the uterus removed. One uterine horn was given 10 minutes hypoxia, while the second horn had the pups removed and stimulated to breathe immediately. Following one hour recovery, pups were given to a surrogate dam and samples collected for either Western blot or immunohistochemical analysis on postnatal day 1, 3 or 7.

8.3.1. Gas exposure

Gas exposure was carried out using a sealed anaesthesia chamber made of clear plexiglass. The chamber base was filled with soda lime pellets and silica gel crystals to absorb excess CO_2 and moisture respectively. A metal base was placed over to prevent contact of dams with either material and to prevent access to the gas inlet valve. This metal base was then covered with tissue material and some bedding for comfort. Once the dam was inside, the chamber was covered with paper to minimise stress and exposure to bright lights for the

dam, but a gap was left to maintain observation throughout gas exposure. Anaesthetic gases were mixed using an anaesthesia trolley, and the output was measured using a monitor capable of measuring the concentration of gaseous or volatile anaesthetic agents and other gases. This trolley output was then attached to the chamber input to start the flow of gas. Each dam was left for four hours in the chamber before gas flow was stopped. The gas concentrations for each group are outlined in Table 8.1.

Table 8.1 **Gas exposure concentrations for pregnant dams on gestational day 21.** Treatment dams were exposed to gases for four hours immediately prior to caesarean section birth. Naïve dams were left in the home cage until surgery.

	Naïve	N ₂ O treatment	N ₂ O + Argon treatment
O ₂	21%	30%	30%
N ₂	78%	20%	-
N ₂ O	-	50%	50%
Argon	0.93%	-	20%

8.3.2. Hypoxia induction

8.3.2.1. Surgery

Immediately following cessation of gas exposure, dams were culled by cervical dislocation. Dams that did not undergo gas exposure were culled by cervical dislocation immediately from the home cage. After culling, the dam was placed on a heat mat to keep pups warm, an abdominal incision was made and the uterus examined to determine how many pups were present. As the rat uterus is split into two horns, one on each side of the body, each horn was clamped separately. One horn was removed by cutting the blood vessels and uterus, as shown in Figure 8.2. In the figure below, there is an obvious discrepancy between

pup numbers in each horn. Where this was the case, the pup number was counted and approximately half way along the uterus was clamped and cut from there. While clamping at either end was quite simple, as can be seen in the diagram there is a clear area with no pups, clamping in the centre often involved manual manipulation of the pups inside the uterus to ensure no limbs were clamped. Once excised, the entire uterine horn was placed in a beaker of pre-warmed sterile saline in a water bath, at a temperature of 37°C and a timer started. After placing one horn in the water bath, the second horn was quickly cut out; placed onto the heat mat, cut open and the pups removed. Each pup had to be cut from its individual amniotic sac, the amniotic fluid cleared from the mouth with a dry cotton swab, and stimulated by gentle manipulation with the cotton swab until breathing commenced for each pup. Once all pups were breathing, the umbilical cord was cut to separate the pup from the placenta, and the pups were transferred to a heat chamber kept at 37°C. After 10, 15, 17 or 19 minutes hypoxia, this procedure was then repeated for the hypoxic pups.

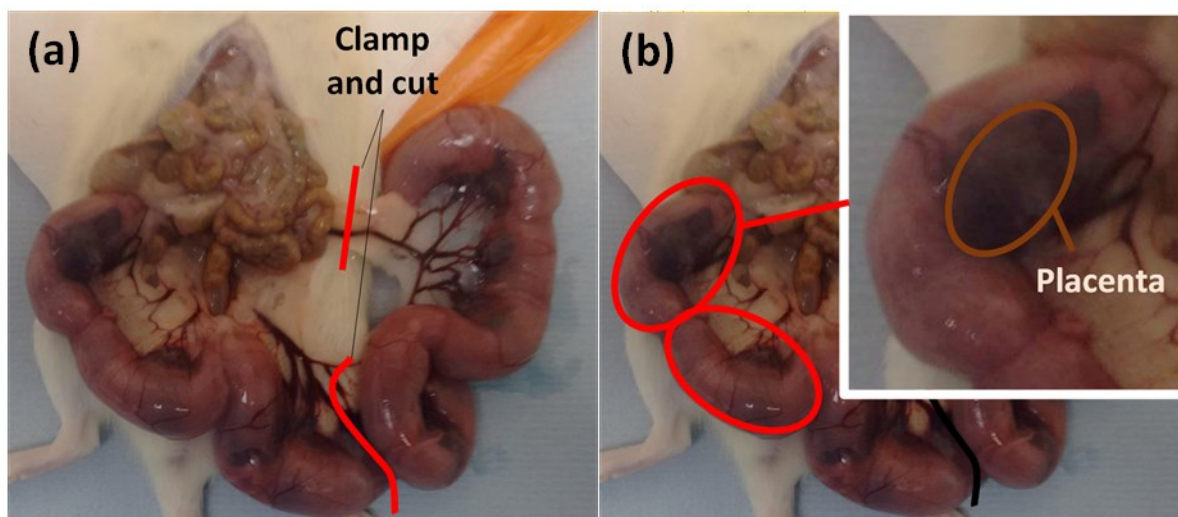


Figure 8.2 **Clamping the uterus to create two groups for different insults.** (a) Clamps were placed along the red lines shown to create two separate uterine horns, each still sealed from the extra-uterine environment. After removing the clamped horn by cutting between the clamp and the maternal blood supply, the pups were in a hypoxic environment. (b) Red circles outline individual pups within the uterus. In the insert, the placenta is outlined.

8.3.2.2. Pup aftercare

Once in the heat chamber, pups were closely monitored for one hour to ensure they continued to breathe and did not bleed from the umbilical cord. If bleeding began, pressure was applied to the cord with a cotton swab until it stopped. After one hour, pups were removed and placed onto a heat mat to keep warm, and using an insulin syringe filled with tattoo ink, marks were made on paws, either front or back, or the base of the tail for future identification. All pups of one treatment were given the same tattoo. A surrogate dam was used, who had given birth in the preceding 24 hours. Her pups were removed, counted and, where necessary, marked with tattoo and returned to the home cage. Each dam was given a maximum of 15 pups, so in combination with the surgery pups, enough naïve pups were kept to make the litter up to this number. Remaining naïve pups were culled at this time and used as P1 samples. Before placing surgery pups with the dam, faeces from the surrogate was dissolved in water and rubbed on the pup's backs to mark them with her scent to reduce the chance of rejection.

8.3.3. Sample collection

Samples were collected on either postnatal day (P) 1, P3 or P7. P1 and P3 samples were used for Western blot and P7 samples for tissue histology, both using brain samples only. Western blot samples were obtained by culling the pups using decapitation, removing the brain and dissecting out the cortices and hippocampi. Both cortices and hippocampi were snap frozen, with the two cortices of each animal frozen separate from the two hippocampi. Hippocampal dissection is outlined in Figure 8.3. P7 samples for tissue histology were collected after transcardiac perfusion with saline followed by 4% paraformaldehyde. The whole brain was then removed and stored at 4°C until paraffin embedding and sectioning.

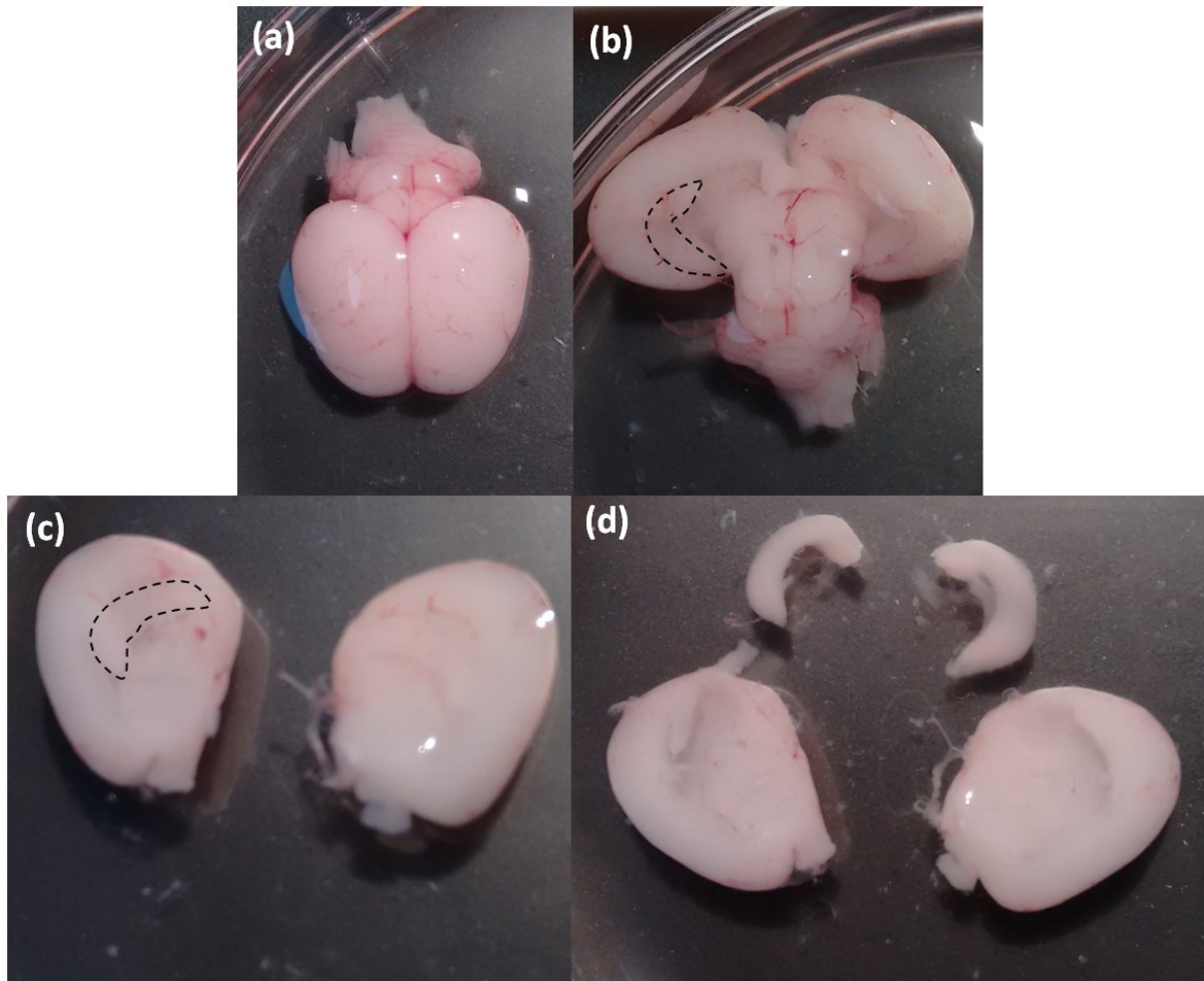


Figure 8.3 **Dissection of the cortex and hippocampus.** Dissection of the brain to obtain cortices and hippocampi for subsequent analysis. Dissection took place in cold saline (a), beginning with separating the two cortices from the midbrain structures (b). Within the cortex, the hippocampus is outlined (c), which could easily be dissected (d).

8.4. Analysis and statistics

For Western blot analysis, samples were analysed for the proteins Puma, Bax, cleaved caspase-8, Erk 1/2, and Bcl-2. ImageJ software was used to analyse samples. The optical density of bands was obtained using ImageJ for all proteins of interest and the housekeeping protein GAPDH. Each value of the protein of interest was then divided by the corresponding value of GAPDH to obtain the normalised optical density for each sample. As there was often variability in protein staining intensity between membranes, each

membrane was normalised individually to the naïve samples on the membrane, instead of normalising all naïve samples across all gels.

For cell counting, cell numbers were counted manually by an experimenter blinded to the treatment groups, and data is expressed either as total positive cells per area for TUNEL staining, or a percentage of damaged cells in a certain area for cresyl violet staining. For the cortex, the area analysed encompassed the entire depth of the cortex, as outlined in Figure 8.4d. For hippocampal TUNEL staining, the entire pyramidal cell layer of CA1, CA2 and CA3 was counted. For cresyl violet CA1 staining, an area the depth of the entire CA1 pyramidal cell layer was analysed, as outlined in Figure 8.4b

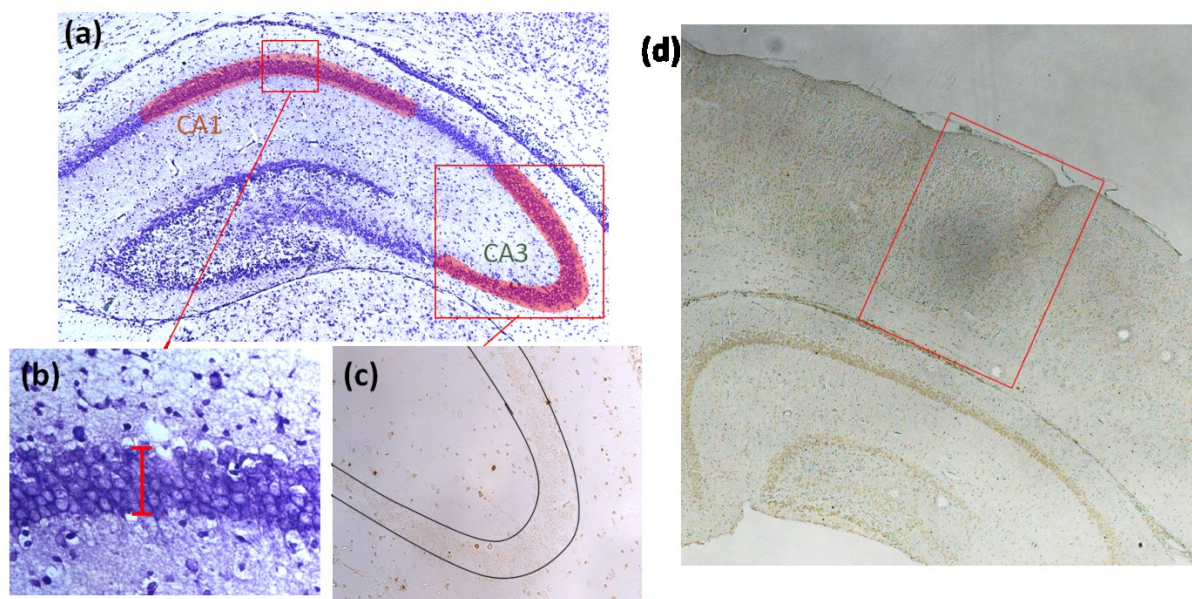


Figure 8.4 Histological analysis of the hippocampus and cortex at postnatal day 7. In the hippocampus, two areas were examined: the CA1 region and the CA3 region (a). For both regions, only the pyramidal cell layer was counted, marked with a red bar in (b) for cresyl violet staining, and outlined in (c) for TUNEL staining. In the cortex, the entire thickness of the cortex directly above the CA1 region of the hippocampus was analysed for TUNEL positive cells

Statistical analysis was performed using GraphPad Prism. A normality test was carried out to determine if the data were normally distributed, followed by either a One-Way ANOVA with

Bonferroni's multiple comparison if normally distributed, or Kruskal Wallis with Dunn's multiple comparison test if not. Data were analysed via six selected sample comparisons; i) Naïve vs CS, ii) CS vs N₂O CS, iii) CS vs N+A CS, iv) CS vs HI10, v) HI10 vs N₂O HI, vi) HI10 vs N+A HI. Data sets of less than 3 samples were not included for statistical analysis. Data are presented as mean $\pm$ standard deviation (SD).

8.5. Results

8.5.1. Determination of optimal hypoxia duration

Four hypoxic time points were initially tested to determine the one most likely to give damage, while still allowing for sufficient sample collection to meet all the aims of the project. From initial data, the 24 hour survival rates for the 10, 15, 17 and 19 minutes were 97.5%, 89%, 58% and 13.8% respectively, as shown in Figure 8.5. Due to the low survival rates at 17 and 19 minutes, they were not analysed further as it was felt that to achieve sufficient sample numbers for the various measurements would result in too high a mortality rate which could not be justified.

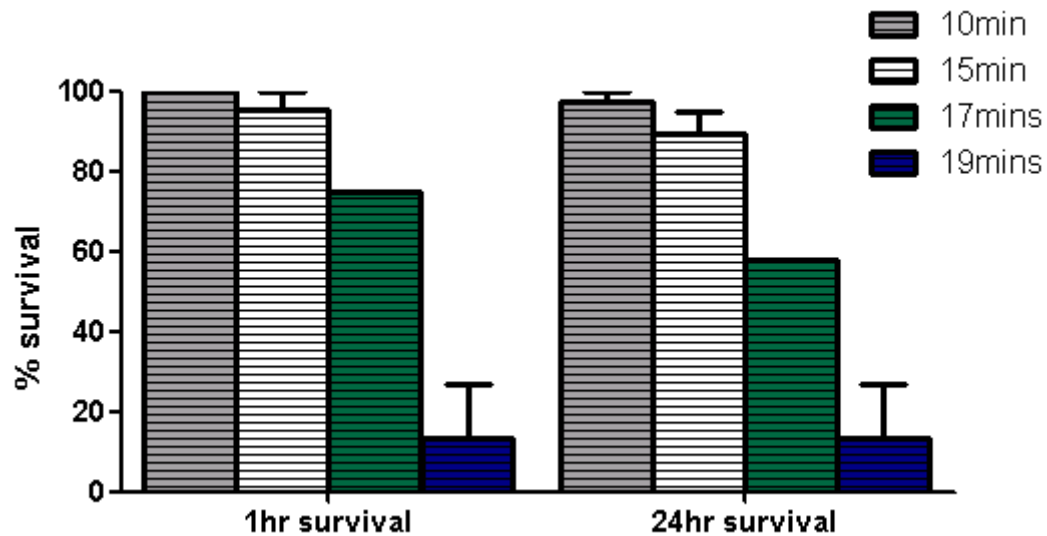


Figure 8.5 **Survival rates following four experimental hypoxic time points.** While 10 and 15 minutes hypoxia showed no dramatic decrease in survival rates (97.5% and 89% respectively), both 17 and 19 minutes showed quite noticeable decreases in mortality rates, with only 58% and 13.8% of pups surviving respectively at 24 hours following insult.

Samples from both 10 and 15 minutes hypoxia were analysed for some measures of neuronal damage to determine optimal time point. TUNEL staining to analyse DNA damage, indicative of neuronal apoptosis, was analysed in both the cortex and hippocampus. In neither the cortex nor the hippocampus was there significant differences in TUNEL positive cells between any of the hypoxic time points or controls (P7 Cortex TUNEL; Kruskal Wallis, $p = 0.1898$, $n=3-4$, P7 Hipp TUNEL; Kruskal Wallis, $p = 0.1168$, $n=3-4$, Figure 8.6).

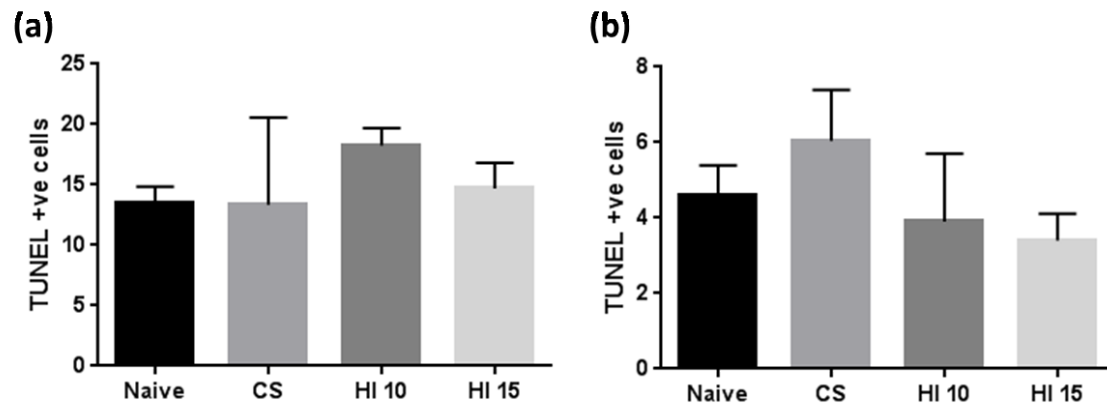


Figure 8.6 **Number of TUNEL positive neurons in the brain following different hypoxic times.** Number of TUNEL positive cells following either no insult, caesarean section alone (CS), 10 minutes hypoxia with no pre-treatment (HI 10) or 15 minutes hypoxia with no pre-treatment (HI 15) in either the cortex (a), or hippocampus (b)

Similarly, there was no significant difference between total damaged cells in the CA1 region of the hippocampus at either 10 or 15 minutes hypoxia when compared with CS control (P7 Cortex TUNEL; Kruskal Wallis, $p = 0.4418$, $n=3-4$, Figure 8.7).

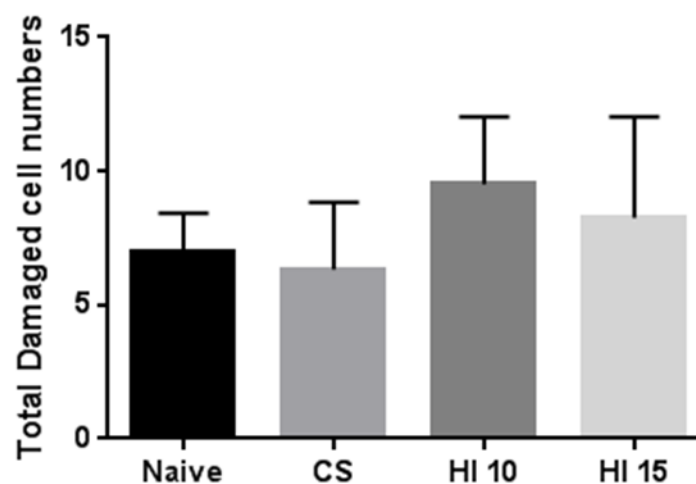


Figure 8.7 **Damaged neurons in the CA1 region of the hippocampus following different hypoxic times.** Percent of damaged neurons from total cells in the CA1 region of the hippocampus as determined by cresyl violet staining following either no insult, caesarean section alone (CS), 10 minutes hypoxia with no pre-treatment (HI 10) or 15 minutes hypoxia with no pre-treatment (HI 15).

From this early data, it was clear that there was no significant difference between 10 and 15 minutes hypoxia. However, there appeared to be a trend that 10 minutes hypoxia was

capable of inducing increased TUNEL staining in the cortex and neuronal damage in the hippocampus. Since earlier work published from this group was carried out using a 10 minute hypoxia model (Yang et al., 2012) the remainder of the work was carried out using 10 minutes hypoxia.

8.5.2. Post-hypoxia observations and survival rates

After hypoxia, pups took longer to resuscitate and generally needed more constant observation for the initial 15-20 minutes following resuscitation. While caesarean section (CS) pups began to vocalize within 15 minutes on average, hypoxia (HI) pups were silent for much longer periods, 30-45 minutes. As can be seen in Figure 8.8, HI pups were paler, showed signs of muscle rigidity and held a straight-body posture, as opposed to CS pups which were pinker in colour, and maintained a curled posture. As well as this, there was a tendency of the umbilical cord of HI animals to bleed for longer than CS pups. This appeared consistent regardless of the pre-treatment group. These data are purely observational, and no attempt to quantify these values was made, however the postural and colour differences seen in Figure 8.8 were highly representative of all pups.



Figure 8.8 **Pups 30 minutes following birth by caesarean section, either with or without hypoxic insult.** (a) Pups with a 10 minute hypoxic insult were visibly paler than caesarean section alone pups (b). As well as this, hypoxic pups showed a clear stiff-bodied posture. This was maintained for at least an hour following insult, but was not present 24 hours later.

Survival rates were measured at 24 hours post hypoxia, when pups were weighed and P1 samples taken. As can be seen in Figure 8.9, survival rates did not fall below 90% for any treatment group. These survival rates did not reach statistical significance (P1 survival, Kruskal Wallis, $p = 0.5028$, $n=4-16$ surgeries). The survival rates at 24hours for each treatment group are: Naïve; 18 out of 19 pups from 5 surgeries, CS; 48 out of 53 pups from 9 surgeries, N_2O CS; 40 out of 41 pups from 7 surgeries, N+A CS; 31 out of 32 pups from 6 surgeries, HI10; 62 out of 65 pups from 11 surgeries, N_2O HI; 30 out of 32 pups from 5 surgeries, and N+A HI; 24 out of 24 pups from 4 surgeries. As a note, for each surgery, the

two horns were sometimes used for different hypoxic insults, so the number of surgeries for 10 minutes hypoxia will not always match the number for caesarean section for each given treatment. This was due to use of both horns for different hypoxic timepoints during the initial stages of determining optimal hypoxia. Also, while the number of surgeries for naïve samples may seem low, these include only cohorts in which the number of pups could be assessed directly after birth, to avoid the loss of pups never counted and therefore lose data. As most dams would spontaneously give birth during the night, it was difficult to obtain sufficient numbers so these data sets come from naïve pups who were born during the day whose dams went on to be used as no gas exposure surgery dams, hence the small numbers.

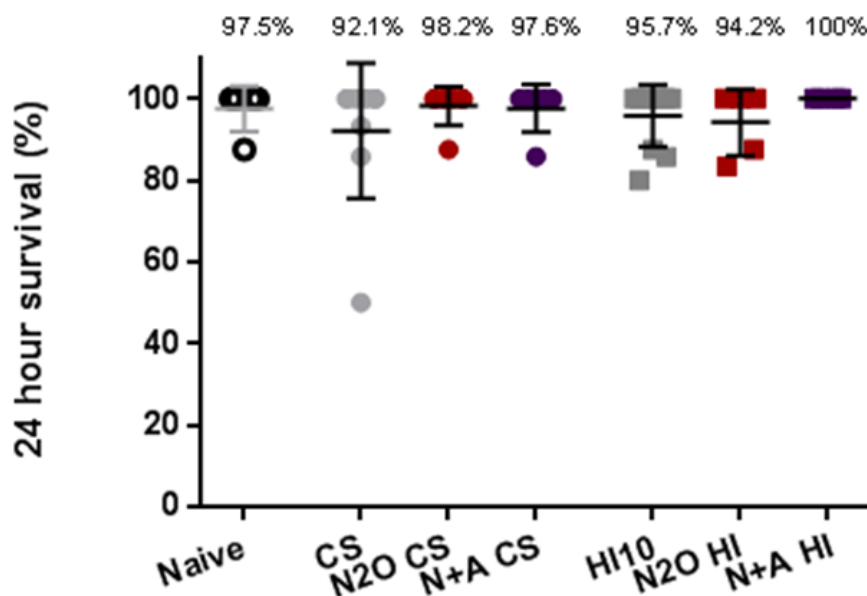


Figure 8.9 **Pup survival rates 24 hours following birth.** Survival was assessed 24 hours after insult to determine any impact of insult or pre-treatment on pup viability. Survival rates stayed high for all groups; however hypoxic insult with N₂O alone pre-treatment did give the worst outcomes, while the addition of argon to this resulted in the highest survival rate of 100%. There were no significant differences between any groups. (P1 survival, Kruskal Wallis, $p = 0.5028$, $n=4-16$ surgeries)

8.5.3. Postnatal weight

Pup weight was measured on P1, P3 and P7 before samples were taken. As seen in Figure 8.10, on P1, there was a significant difference in pup weights (P1 weight, Kruskal Wallis, $p < 0.0001$, $n=24-97$). With post-hoc comparison tests, there was a significant difference between naive control pups and CS pups (naive vs CS, Dunn's multiple comparison = **, $n=44-97$). There was also a significant difference between 10 minute HI pups without any treatment versus treatment with N_2O + argon (HI10 vs N+A HI, Dunn's multiple comparison = **, $n=24-66$). On P3, there was a significant difference in pup weight, but post hoc multiple comparisons did not identify any particular pairs as being significantly different (Kruskal Wallis, $p = 0.0022^{**}$, $n=15-31$). The same was found for P7 weight (Kruskal Wallis, $p = 0.0487^*$, $n=8-24$).

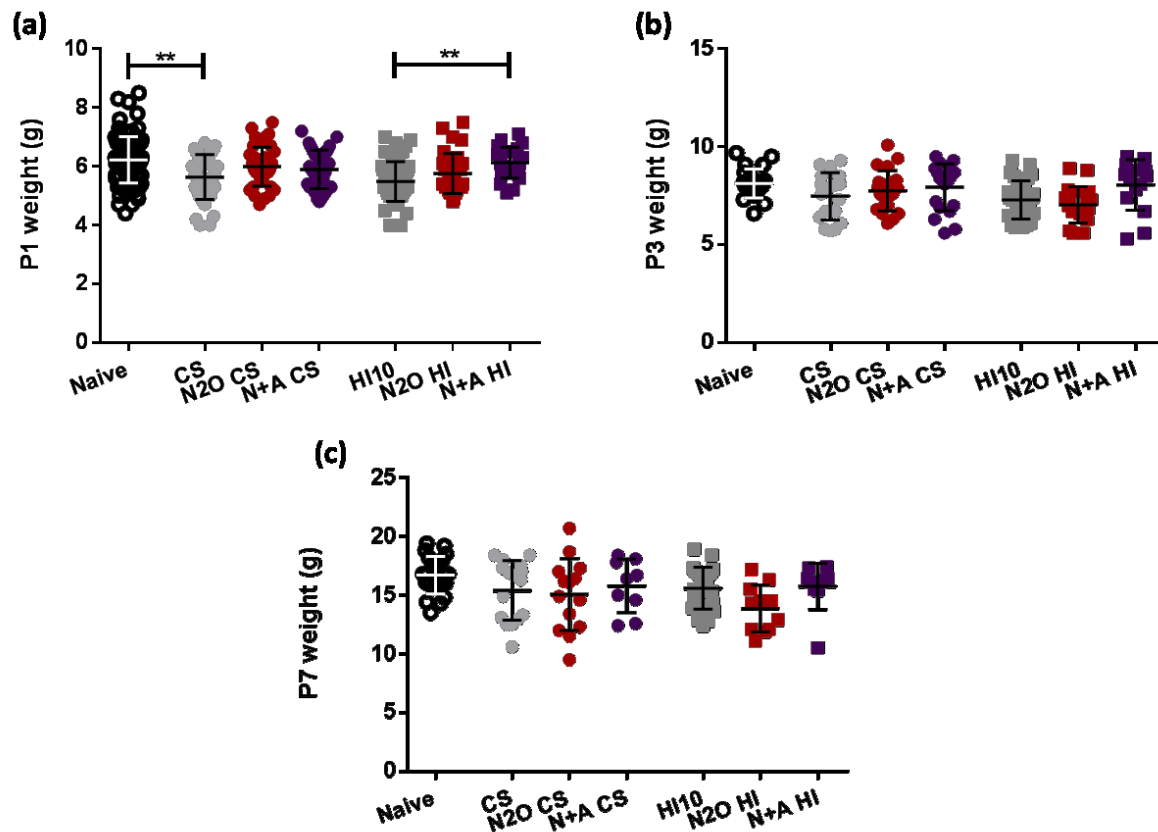


Figure 8.10 **Pup weight 1, 3 and 7 days after birth.** Pup weight 24 hours following birth by caesarean section was significantly different from naive pup weight, but not significantly different from 10 minutes hypoxic incident. Pre-treatment with N₂O + argon before hypoxic insult could return pup weight to naive levels on postnatal day 1. On postnatal days 3 and 7 there were no significant differences between any of the groups, however there is a trend of hypoxic pups pre-treated with N₂O showed a consistent decline in weight compared with all other treatment groups.

8.5.4. Postnatal day 1 protein expression

Protein was measured on P1 by Western blot analysis of both the hippocampal and cortical fractions.

8.5.4.1. Pro-apoptotic proteins

The pro-apoptotic proteins measured were Puma, Bax and the 18kDa fragment of cleaved caspase-8. As can be seen in Figure 8.11, Figure 8.12, and Figure 8.13, neither Puma (P1 Cortex Puma; Kruskal Wallis, $p = 0.9338$, $n=4$, P1 Hipp Puma; Kruskal Wallis, $p = 0.4366$, $n=4$, Figure 8.11), Bax (P1 Cortex Bax; Kruskal Wallis, $p = 0.8574$, $n=5-6$, P1 Hipp Bax; One Way ANOVA, $p = 0.8624$, $n=6$, Figure 8.12) nor cleaved caspase-8 (P1 Cortex cleaved Caspase-8;

One Way ANOVA, $p = 0.5270$, $n=5-6$, P1 Hipp cleaved Caspase-8; Kruskal Wallis, $p = 0.779$, $n=6$, Figure 8.13) showed any significant differences between treatment groups in either brain area.

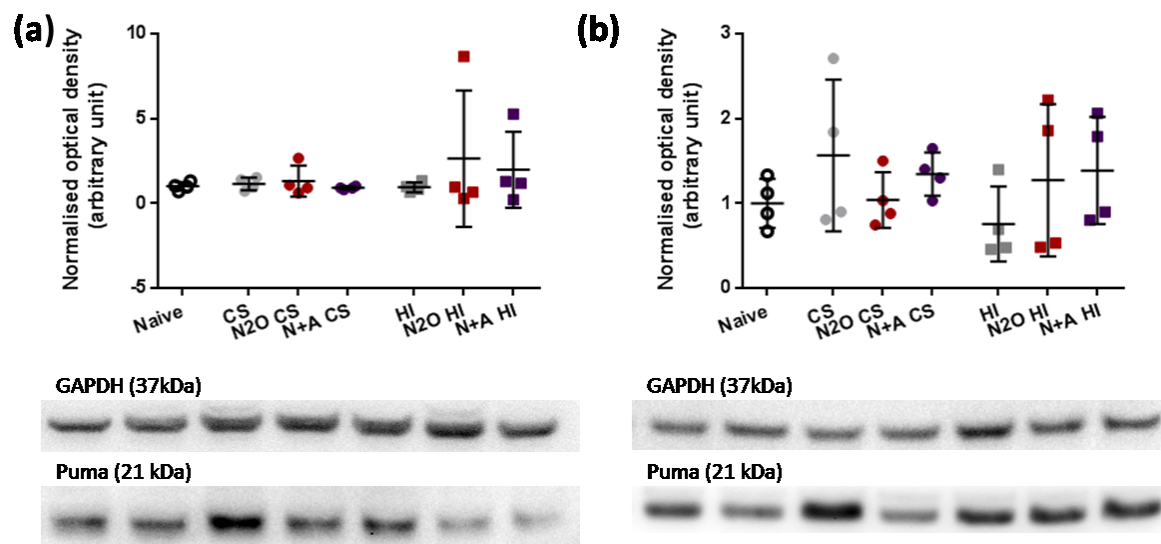


Figure 8.11 **Expression of Puma on postnatal day 1.** The relative expression of the pro-apoptotic protein Puma was analysed by Western blot 24 hours after birth. Both the cortex (a) and hippocampus (b) were analysed, but there were no significant differences between any groups.

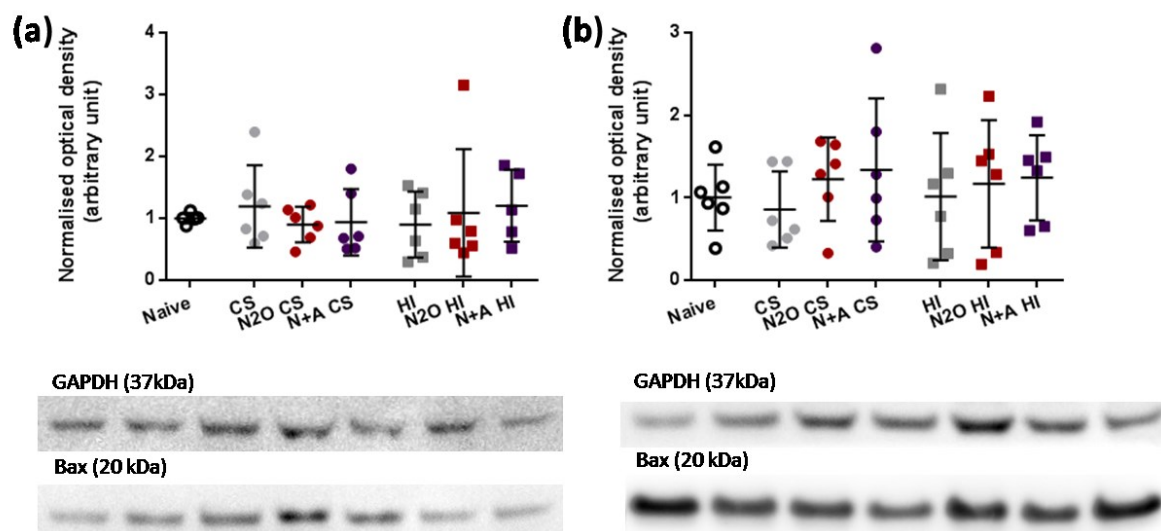


Figure 8.12 **Expression of Bax on postnatal day 1.** The relative expression of the pro-apoptotic protein Bax was analysed by Western blot 24 hours after birth. Both the cortex (a) and hippocampus (b) were analysed, but there were no significant difference

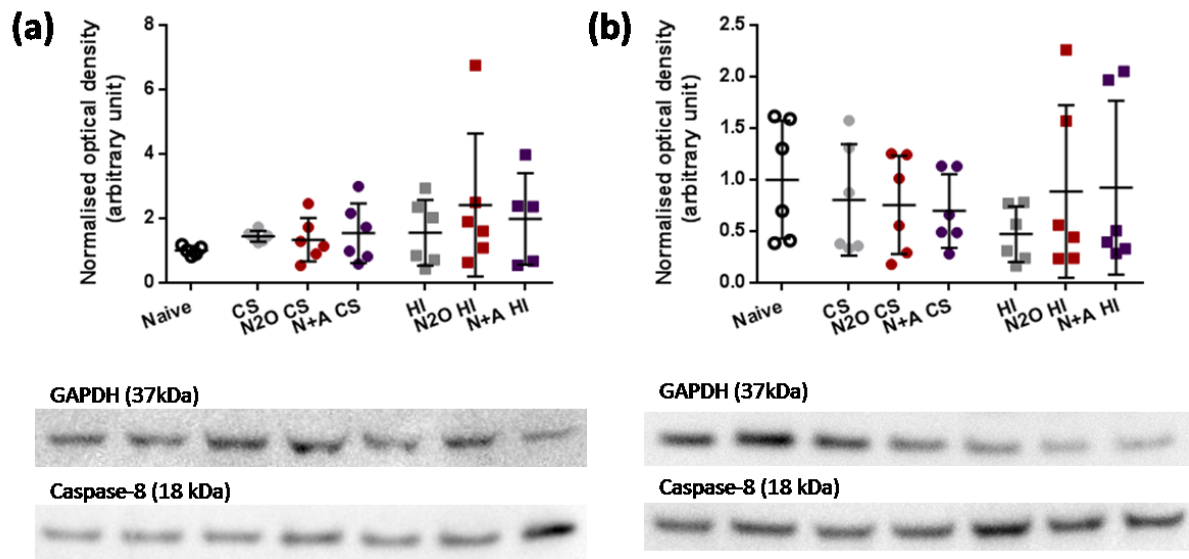


Figure 8.13 **Expression of cleaved caspase-8 on postnatal day 1.** The relative expression of the 18 kDa fragment of the pro-apoptotic protein cleaved caspase-8 was analysed by Western blot 24 hours after birth. Both the cortex (a) and hippocampus (b) were analysed, but there were no significant differences between any groups.

8.5.4.2. Anti-apoptotic proteins

The anti-apoptotic proteins measured were Bcl-2 and phosphorylated Erk 1/2. As with the pro-apoptotic proteins, there was no significant difference between any treatment group for either Bcl-2 (P1 Cortex Bcl-2; Kruskal Wallis, $p = 0.8722$, $n=5-6$, P1 Hipp Bcl-2; Kruskal Wallis, $p = 0.9925$, $n=4$, Figure 8.14) or phosphorylated Erk 1/2 (P1 Cortex pErk 1/2; Kruskal Wallis, $p = 0.9018$, $n=6$, P1 Hipp pErk 1/2; Kruskal Wallis, $p = 0.9408$, $n=2-5$, Figure 8.15). However, for pErk 1/2, protein expression in the hippocampus between 10 minute HI and either N₂O alone HI or N₂O with argon HI could not be analysed as there were not enough samples for statistical analysis.

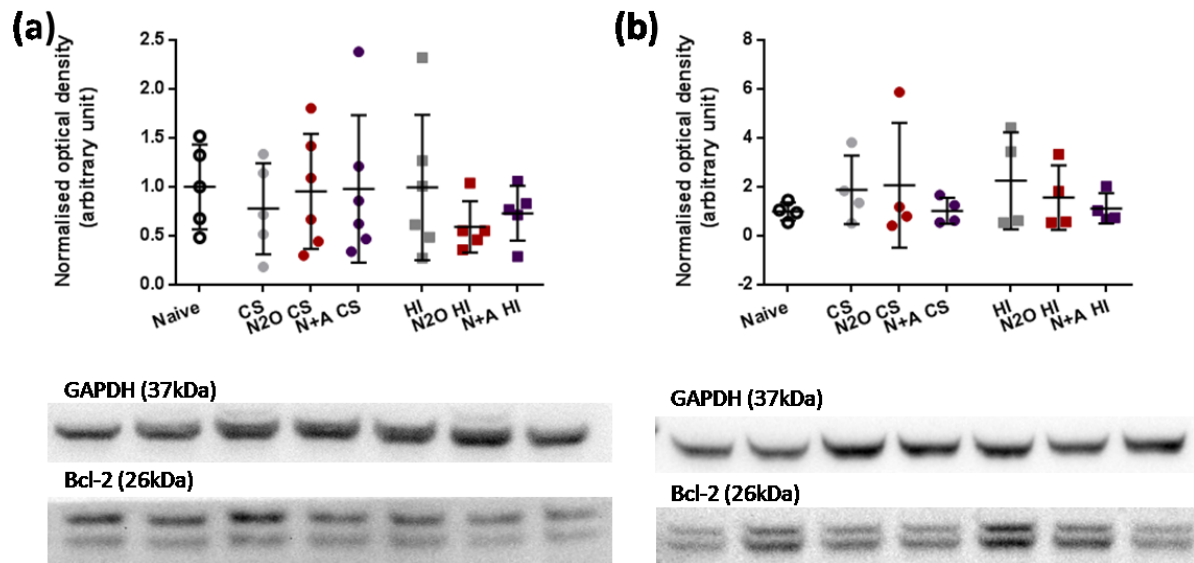


Figure 8.14 **Expression of Bcl-2 on postnatal day 1.** The relative expression of the anti-apoptotic protein Bcl2 was analysed by Western blot 24 hours after birth. Both the cortex (a) and hippocampus (b) were analysed, but there were no significant differences between any groups.

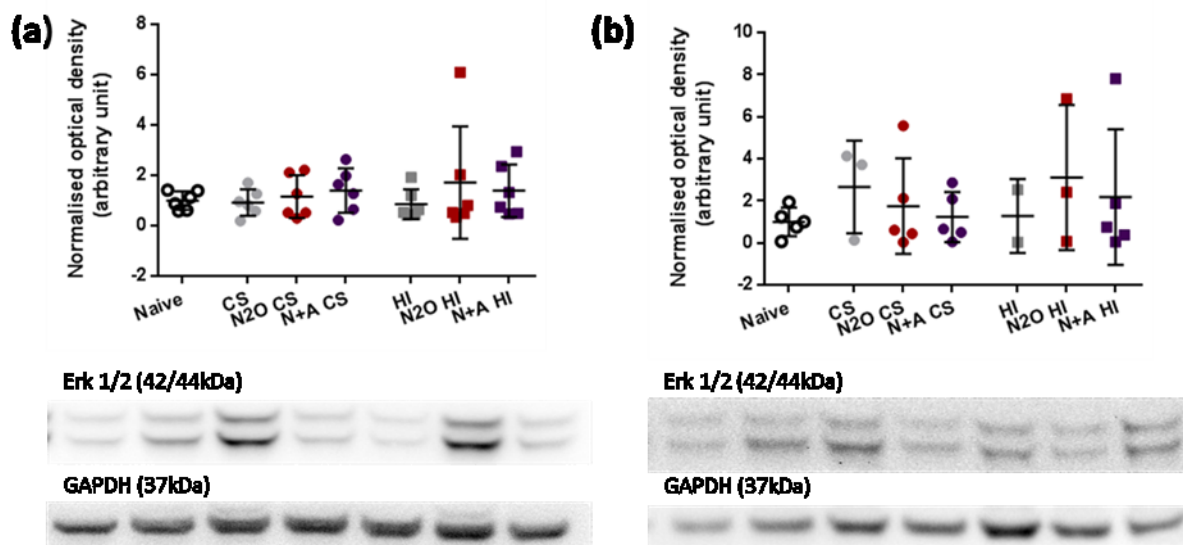


Figure 8.15 **Expression of Erk 1/2 on postnatal day 1.** The relative expression of the neuroprotective protein Erk 1/2 was analysed by Western blot 24 hours after birth. Both the cortex (a) and hippocampus (b) were analysed, but there were no significant differences between any groups.

8.5.5. Postnatal day 3 protein expression

Samples from pups taken 3 days post-insult were analysed for protein expression in both the cortex and hippocampus.

8.5.5.1. Pro-apoptotic proteins

The pro-apoptotic proteins measured did not show statistically significant differences for any treatment group in either brain area. This was true for Puma (P3 Cortex Puma; Kruskal Wallis, $p = 0.6906$, $n=3-4$, P3 Hipp Puma; Kruskal Wallis, $p = 0.0981$, $n=4$, Figure 8.16), Bax (P3 Cortex Bax; Kruskal Wallis, $p = 0.8008$, $n=3-4$, P3 Hipp Bax; Kruskal Wallis, $p = 0.3762$, $n=4$, Figure 8.17) or cleaved caspase-8 (P3 Cortex cleaved Caspase-8; Kruskal Wallis, $p = 0.8017$, $n=3-4$, P3 Hipp cleaved Caspase-8; $p = 0.9417$, $n=4$, Figure 8.18)

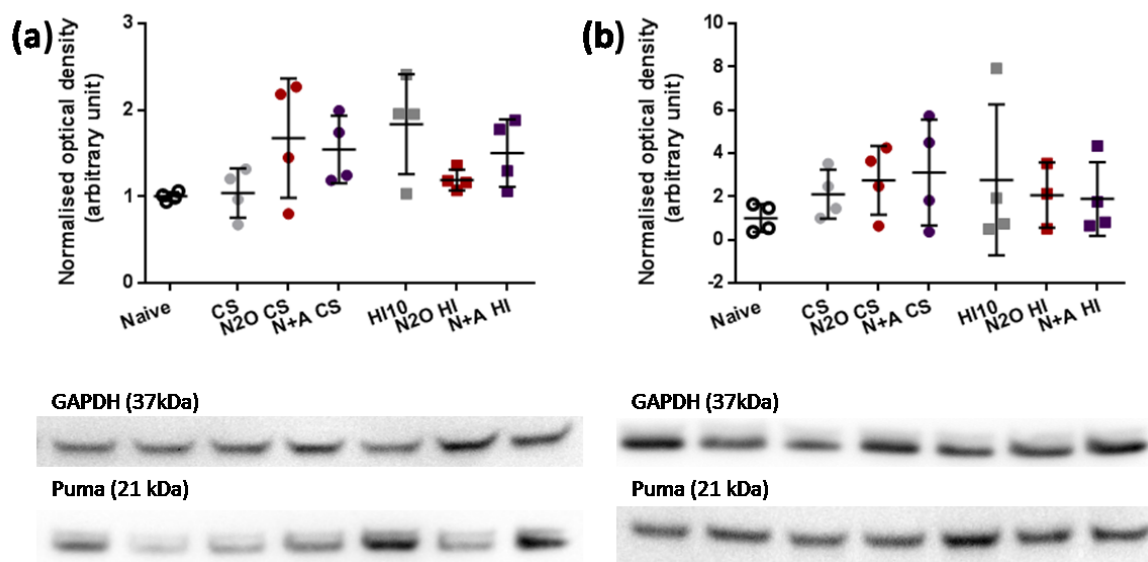


Figure 8.16 **Expression of Puma on postnatal day 3.** The relative expression of the pro-apoptotic protein Puma was analysed by Western blot 3 days after birth. Both the cortex (a) and hippocampus (b) were analysed, but there were no significant differences between any groups.

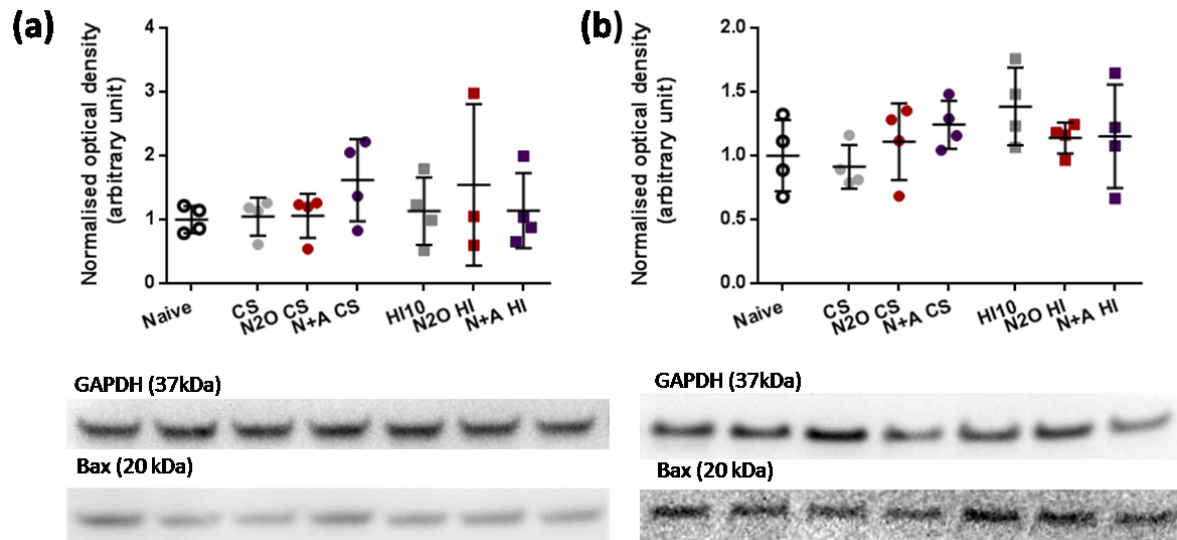


Figure 8.17 **Expression of Bax on postnatal day 3.** The relative expression of the pro-apoptotic protein Bax was analysed by Western blot 3 days after birth. Both the cortex (a) and hippocampus (b) were analysed, but there were no significant differences between any groups.

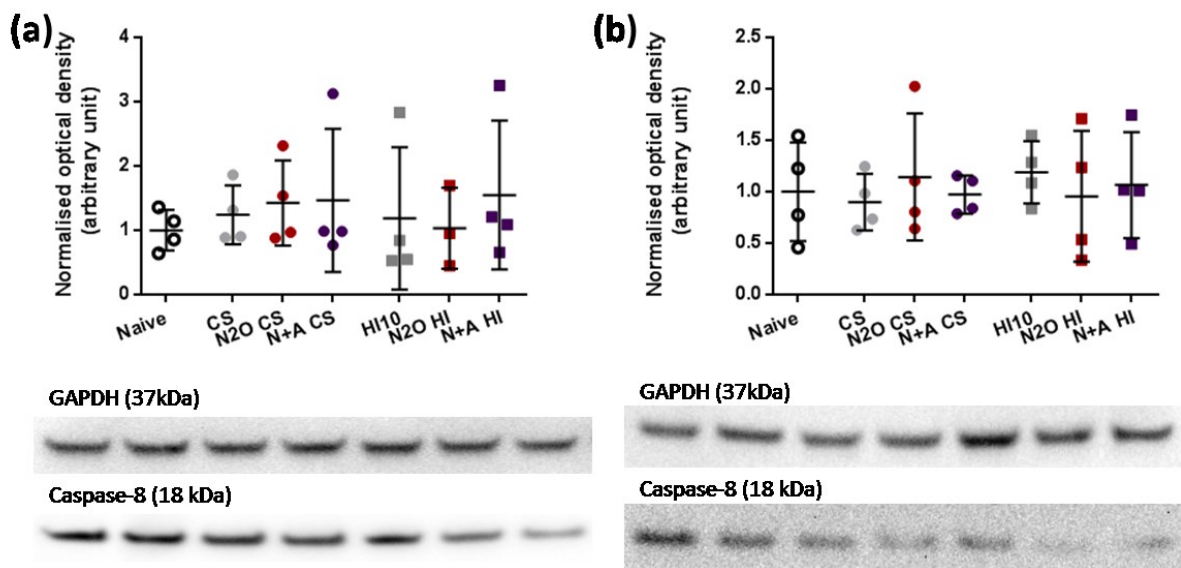


Figure 8.18 **Expression of cleaved caspase-8 on postnatal day 3.** The relative expression of the 18 kDa fragment of the pro-apoptotic protein cleaved caspase-8 was analysed by Western blot 3 days after birth. Both the cortex (a) and hippocampus (b) were analysed, but there were no significant differences between any groups.

8.5.5.2. Anti-apoptotic proteins

Only one anti-apoptotic protein yielded results for postnatal day 3 samples. Phosphorylated Erk 1/2 showed a statistically significant difference after Kruskal wallis analysis in the

hippocampus (P3 Hipp pErk 1/2; Kruskal Wallis, $p = 0.0372$, $n=4$, Figure 8.19), and Dunn's multiple comparison identified a significant decrease in pErk 1/2 between naive control animals and CS (naive vs. CS, *) and a significant increase in expression between CS alone and CS pre-treated with N_2O + argon (CS vs. N+A CS, *), as shown in Figure 8.19. There was, however, no similar significant difference in the cortical expression of pErk 1/2 (P3 Cort pErk 1/2; Kruskal Wallis, $p = 0.8953$, $n=3-4$). Bcl-2 was also measured by Western blot analysis; however bands could not be obtained for analysis.

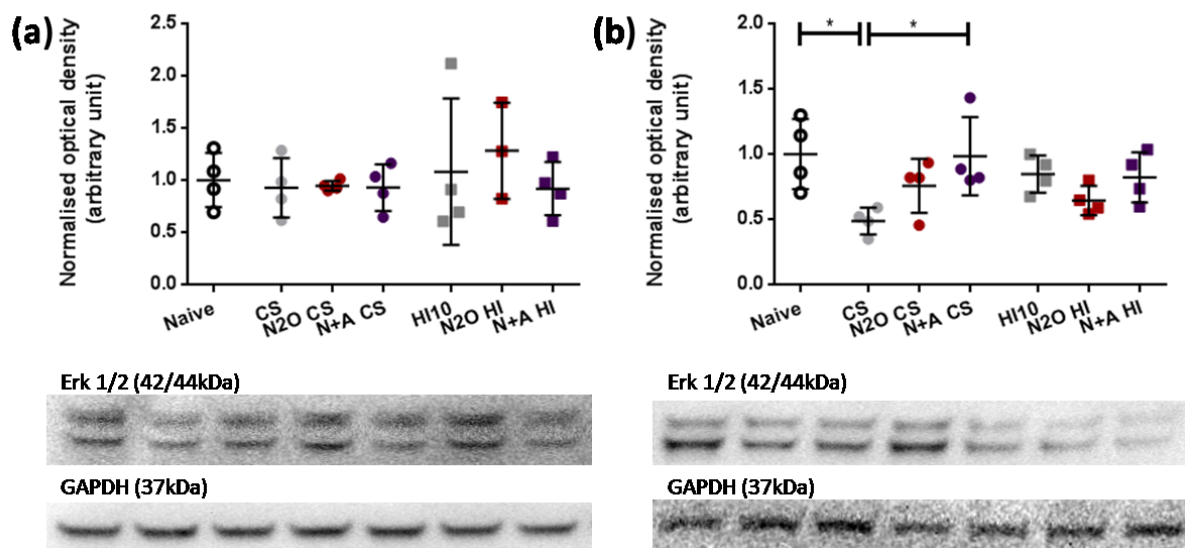


Figure 8.19 **Expression of Erk 1/2 on postnatal day 3.** The relative expression of the anti-apoptotic protein Erk 1/2 was analysed by Western blot 3 days after birth. Both the cortex (a) and hippocampus (b) were analysed. While the cortex did not show any significant changes, there was a significant decrease in expression in caesarean section pups relative to naïve controls. Further to this, pre-treatment with N_2O + argon could return expression to naïve levels.

8.5.6. Postnatal day 7 histological changes

Two different measures of histology were analysed from postnatal day 7 samples, TUNEL staining in both the hippocampus and cortex, and cresyl violet staining in the hippocampus. While there was no significant difference in the number of TUNEL positive cells in the hippocampus between treatment groups (P7 Hipp TUNEL; One way ANOVA, $p = 0.6075$,

n=5-7, Figure 8.21), there was a significant difference between HI alone and HI with either N₂O alone or N₂O + argon pre-treatment in the cortex (P7 Cortex TUNEL; One way ANOVA, $p = 0.0169$, $n=4-7$, Dunn's multiple comparison; CS vs HI, HI vs N₂O HI and HI vs N+A HI = *, Figure 8.20). In the hippocampus, the numbers of shrunken pyknotic nuclei were counted in the CA1 and CA3 regions. The difference between groups did not reach significance (P7 CA1 damaged nuclei; Kruskal Wallis, $p = 0.2964$, $n=3-4$; P7 CA3 damaged nuclei; Kruskal Wallis, $p = 0.7311$, $n=3-4$, Figure 8.22).

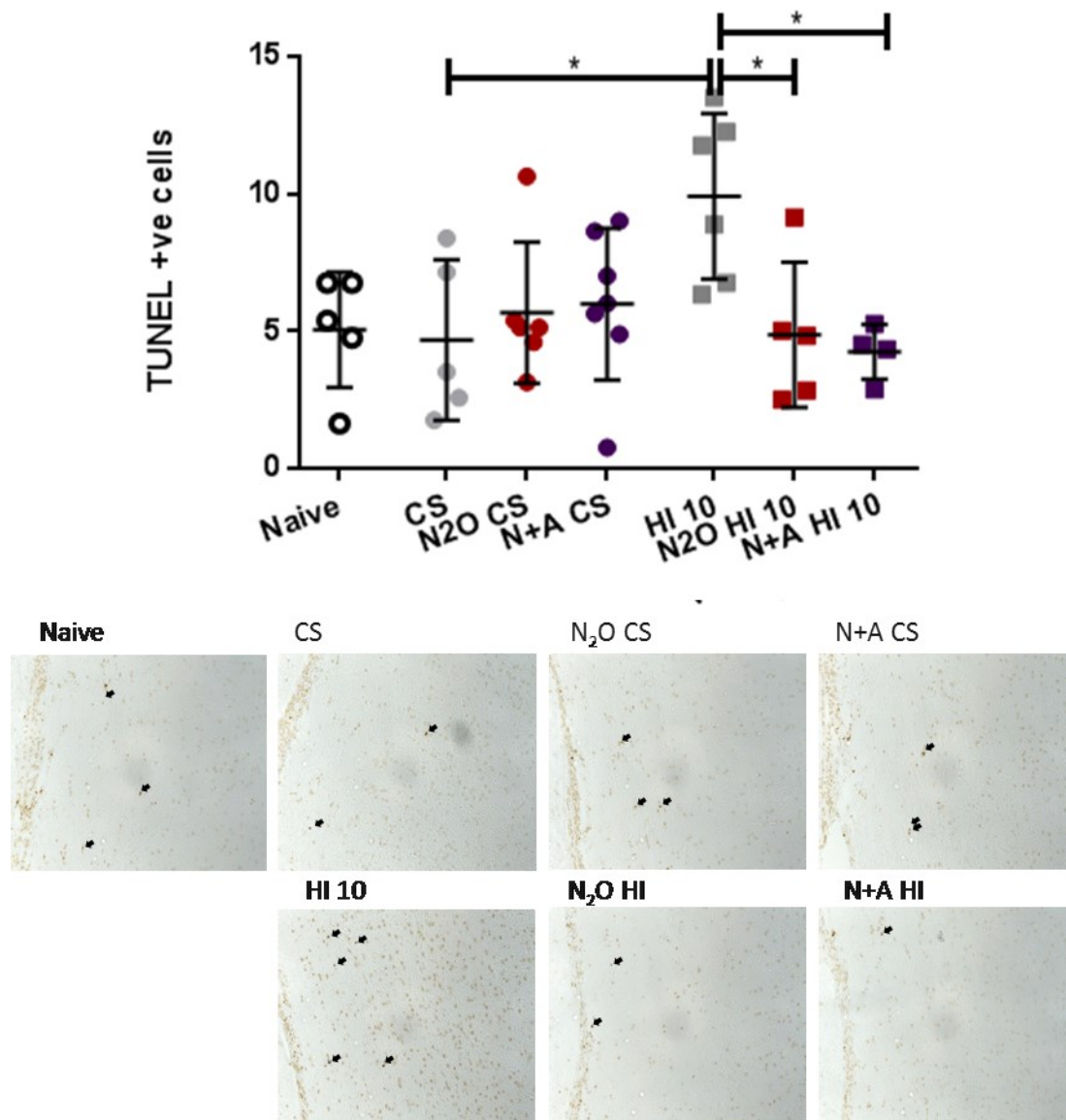


Figure 8.20 **TUNEL positive cells in the cortex.** The number of TUNEL positive cells in the cortex of postnatal day 7 rats was counted to assess the effects of prenatal gas exposure on hypoxic outcomes. An insult of 10 minutes hypoxia resulted in a significantly increased number of TUNEL positive cells in the cortex, while pre-treatment with either N₂O alone or N₂O with argon was able to reduce this effect. Representative images for each group show TUNEL positive cells, marked with a black arrow.

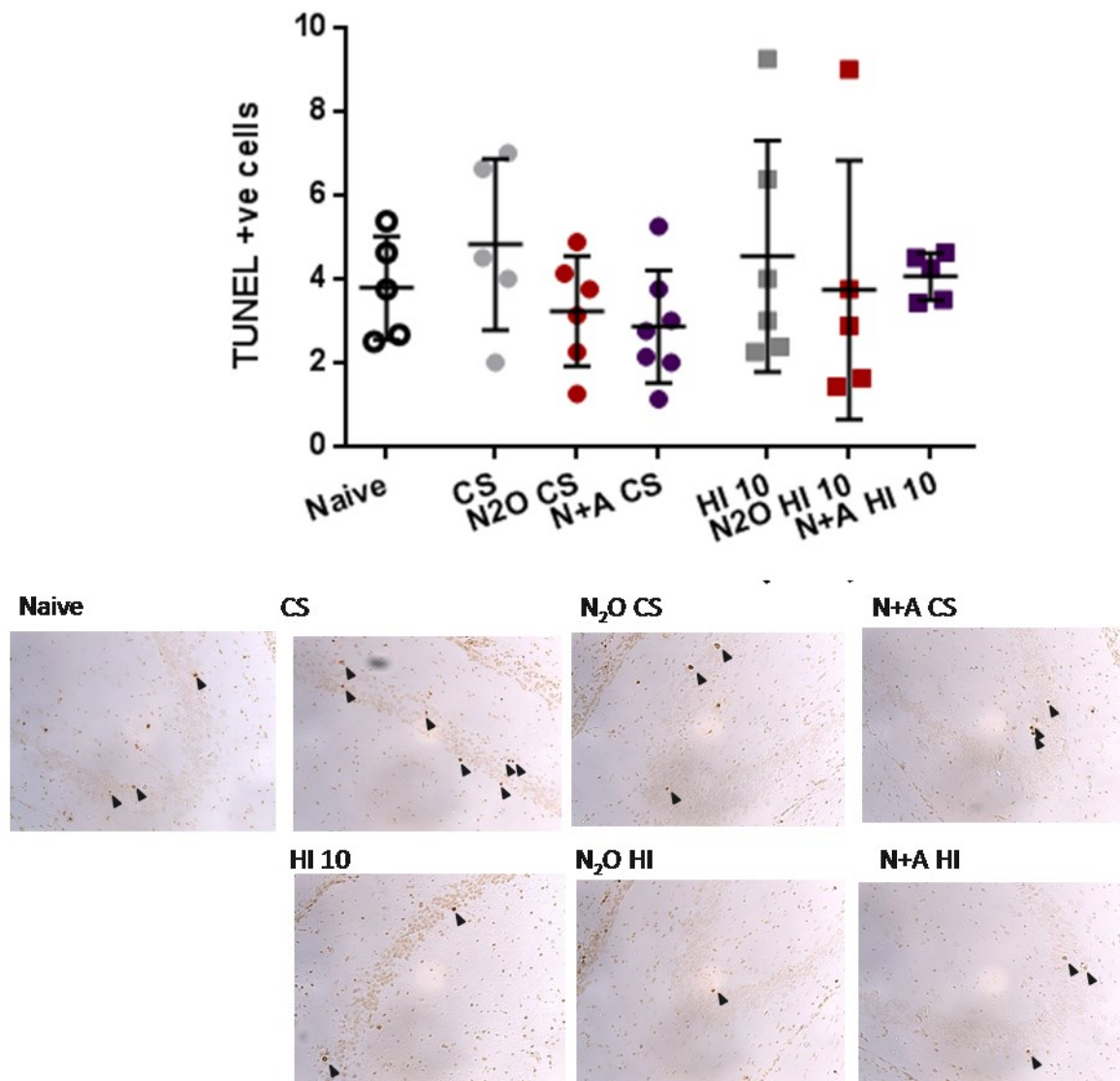


Figure 8.21. **TUNEL positive cells in the hippocampus.** The number of TUNEL positive cells in the pyramidal cell layer of the hippocampus was assessed following perinatal hypoxia or caesarean section with different pre-treatments. There was no significant difference between any of the groups. Representative images for each group show TUNEL positive cells, marked with a black arrow.

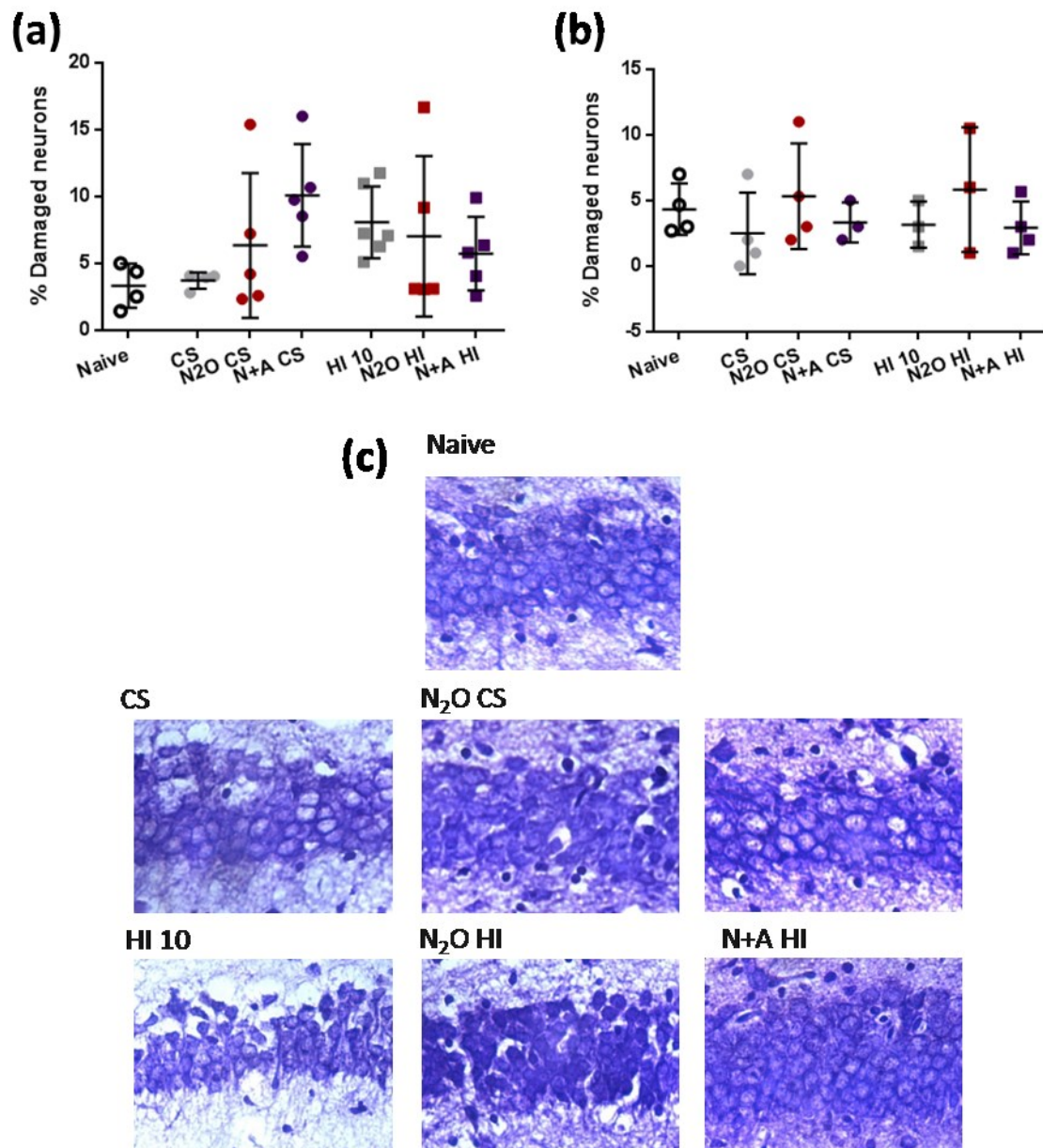


Figure 8.22 **Cresyl violet staining of the hippocampus.** Cresyl violet staining was performed and the number of pyknotic nuclei visible in the pyramidal cell layer of the CA1(a) and CA3 (b) regions were counted. Representative images of the CA1 region are shown (c). There were no significant differences between any of the treatment groups.

8.5.1. Ranking data sets

As a measure to help assess the potential toxicity of any of the groups over such a wide array of outcome measures, the data have been ranked from least to most damage according to the mean value of each group. In this way, each group was assigned a number

between 1 (least damage) to 7 (worst damage). This can help to draw trends between groups to help discuss the many outcomes.

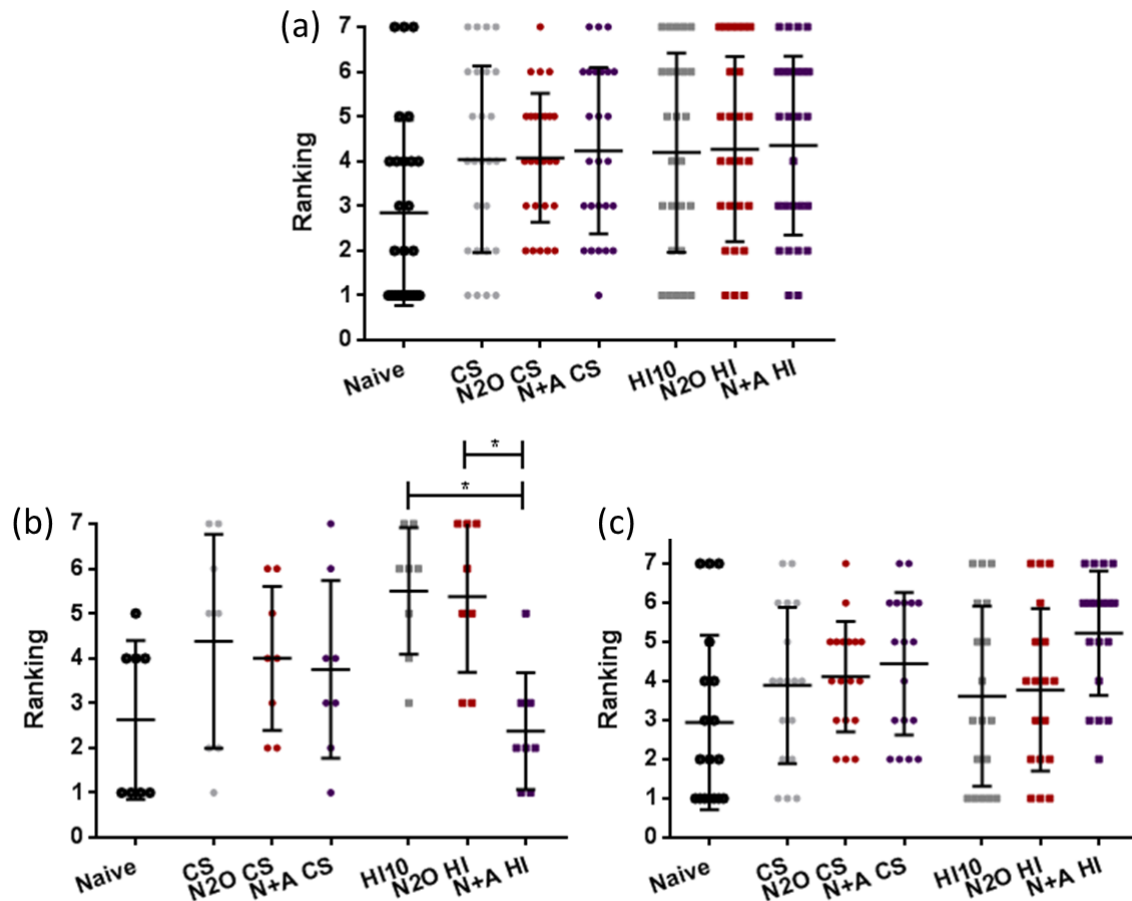


Figure 8.23 Ranking of all data sets. All data sets were ranked according to their mean value. (a) The mean ranking for all outcome measures shows naïve having the lowest value, while N₂O + argon has the worst ranking overall. (b) When analysing only physiological and histological outcomes (weight, mortality and histology), there is a marked difference in trend, with N₂O + argon showing the second lowest damage score, and having a statistically lower damage ranking than 10 minutes hypoxia, which was the worst damaged. (c) When analysing Western blot data, N₂O + argon had the worst damage ranking, with naïve having the best.

As can be seen from Figure 8.23a, none of the insults of treatments show any marked increase in damage compared to each other, however all rank higher than naïve alone (Ranking all samples; Kruskal-Wallis, $p = 0.0564$, $n=25$, Dunn's multiple comparison = no significant differences, Figure 8.23a). However, when the data is broken down into two groups, physiological and histological data versus Western blot data, there is a clear difference between the two. When analysing only physiological and histological changes

(body weight, mortality and tissue histology data), naïve samples had the lowest damage score, as expected, and 10 minutes hypoxia was the worst. Interestingly, examining only these measures there is a statistically significant difference between HI10 and N₂O + Argon Hypoxia samples, indicating that the administration of N₂O + argon before hypoxic insult could be protective (Ranking physiology and histology samples; Kruskal-Wallis, $p = 0.0028^{**}$, $n=7$, Dunn's multiple comparison = HI10 vs N₂O + Argon HI10 = *, N₂O HI10 vs N₂O + argon HI10 = *, Figure 8.23b). However, when the Western blot data of proteins was analysed for both postnatal days 1 and 3, the opposite trend was seen, with N₂O + argon before hypoxia increasing the damage score, indicating an increase in pro-apoptotic proteins and a decrease in anti-apoptotic proteins (Ranking WB samples; Kruskal-Wallis, $p = 0.0353^*$, $n=18$, Dunn's multiple comparison = no significant differences, Figure 8.23c).

8.6. Discussion

The aim of this chapter was to expand the data from previous chapters regarding the potential neurotoxicity of N₂O and the ability of argon to counteract this. To give a more clinically relevant scenario, a model of perinatal hypoxia was used in rat pups still *in utero*. From the data presented in this chapter, it is clear that the model used within did not give sufficient hypoxic injury to reliably study the effects of our gases of interest; however some discussion points do arise from the data.

As a whole, the data shows little coherent story either in favour of the theory of argon neuroprotection or against it. The largest difficulty in this regard is the lack of significant damage wrought by the ten minutes hypoxic insult given to the pups. This work had been based off previous work done in the laboratory by a different researcher. It was found in

their work that 10 minutes hypoxia gave increased cleaved caspase-3 expression and a decreased number of healthy cells in the hippocampus (Yang et al., 2011, Yang et al., 2012). For this reason, the work carried out in this thesis initially began using 10 minutes hypoxia alone. However, upon discovering little insult, a hypoxic time course analysis was carried out to determine the optimal hypoxic time to use for the remainder of the work. In this study, pups without any pre-treatment were subjected to 10, 15, 17 or 19 minutes of hypoxia, and samples were analysed for histological changes, namely TUNEL staining in the cortex and hippocampus, and damaged nuclei in the hippocampus at postnatal day 7 (Figure 8.6 and Figure 8.7). For the longer time points, 17 and 19 minutes, pup mortality at 24 hours was very low, at 58% and 13.8% respectively, as seen in Figure 8.5. Due to the large sample numbers needed for the work it was determined that reaching appropriate sample numbers for each treatment group, time point and analysis measure would be too difficult with such low pup numbers, and would have exceeded the mortality threshold set for our project license, so these time points were not continued for histological analysis. When 10 and 15 minutes hypoxia were compared using histological methods, neither time point showed a significant difference from their caesarean section counterparts (see Figure 8.6 and Figure 8.7). However, there was a slightly higher degree of insult seen for 10 minutes hypoxia versus 15 minutes, and since the majority of the samples had already been collected for this time point, and the data suggested it had the higher likelihood of reaching significance with added samples, the work was continued with 10 minutes hypoxia. Unfortunately, only in one outcome measure did 10 minutes hypoxia significantly differ from caesarean section alone, with a significant increase in the number of TUNEL positive cells seen in the cortex (Figure 8.20). Interestingly, this was not mirrored in the hippocampus (Figure 8.21), where the two were not different from each other.

Histological changes at postnatal day 7 were analysed, namely TUNEL staining for DNA damage and cresyl violet staining, a commonly used stain for neuronal pyknosis. Pyknosis is the condensation of nuclear material following apoptosis or necrosis, so counting pyknotic cells gives an indication of cells at the late stages of cell death. In the cortex, it was found that hypoxia alone induced a non-significant increase in TUNEL positive cells, while pre-treatment with N₂O alone or N₂O + argon induced a significant decrease in positive cell staining, which implies this pre-treatment somehow protects the neurons from DNA damage (Figure 8.20). The cortical area represented in the sections analysed in this study were from the somatosensory cortex, encompassing all layers of the cortex from the cortical surface to the membrane of the lateral ventricle. An area 600µm mediolateral and 70µm anterior-posterior was analysed in each brain, with 8µm slices analysed. This of course means that these results cannot be extrapolated to the cortex as a whole, as it is a brain area which encompasses many functions and might be expected to have different vulnerabilities dependent on various inputs. What is difficult to reconcile is the differences seen between the vulnerability of different brain areas to insult. For TUNEL staining, while the cortex showed a significant change in cell death (Figure 8.20), the hippocampus showed no such difference (Figure 8.21), and similarly, for protein changes the hippocampus and cortex were differently susceptible to changes (Figure 8.19). This may be in part due to the developmental stage of these animals and the concurrent neurodevelopmental differences in the brain regions (McQuillen and Ferriero, 2004). In work previously published on different developmental vulnerability to hypoxia, it was found that the hippocampus is generally more resistant to damage in younger pups (postnatal day 2) compared with week old pups (Towfighi et al., 1997). This may explain why the cortex had higher levels of cell death but did not have significantly increased anti-apoptotic protein expression.

One surprising outcome from the data was a lack of significant protein changes in either the cortex or the hippocampus at either 24 hours or 72 hours after insult, with the exception of Erk 1/2 (Figure 8.19). From reading the literature, and the preliminary data from primary cells (Chapter 5), the hypothesis was that protein changes involved with protecting or damaging mitochondria, leading to apoptosis, would be found as the cause of hypoxic brain injury, and be a pathway by which argon could have its neuroprotective effects. However there are a number of reasons for this lack of findings. The pathway investigated here is primarily the intrinsic apoptotic pathway which, as mentioned above, relates to mechanisms by which the mitochondria are damaged and release internal proteins which act as part of the apoptotic cascade, eventually leading to caspase-3 cleavage and cell death. However, there is also an extrinsic pathway, centred more closely on proteins not related to the mitochondria, but instead is activated by so-called “death receptors”, located on the cell surface. When these receptors are activated, they initiate a downstream cascade involving caspase-8 cleavage, resulting in caspase-3 cleavage and ultimately apoptosis. However, in this study there was no difference in caspase-8 cleavage seen in Western blot analysis, indicating that extrinsic pathway may also not have been activated (Figure 8.13 and Figure 8.18). Besides these two pathways, there are also mechanisms which are initiated by inflammation (Thornton and Hagberg, 2015) or changes in intracellular calcium concentrations, such as calpain mediated apoptosis (Momeni, 2011). Inhibitors of calpain have been shown to protect against hypoxia induced neurotoxicity (Rami and Kriegstein, 1993), so this pathway may underlie the neurotoxicity seen in this data. It is possible that these other pathways are involved in neuronal death following hypoxia, however it is unlikely that if one of these was activated, the intrinsic and extrinsic pathways would not also have shown some changes. It is more likely, therefore that the changes in TUNEL

positive cells seen at postnatal day seven, indicative of DNA damage and cell death, are a late stage result of the insult and it is possible that the molecular pathways we examined were done at too early a time point to see changes. On the other hand the early changes seen in terms of postnatal weight may possibly have been a reflection of physical impairment of the pups following insult, which may have hindered their ability to reach the dam and feed adequately, while perhaps not relating specifically to neuronal damage. Even if this latter is true, it is interesting that while most groups get closer to naive pups in weight over the 7 days, the N₂O alone pre-treated hypoxia group continue to lose weight, perhaps implying a longer term impairment to these animals. Interestingly, very few papers published using this model of perinatal hypoxia show protein changes which may underlie the damage seen. Three papers have shown increases in cleaved caspase-3 after hypoxia (Mitchell and Snyder-Keller, 2003, Yang et al., 2012, Yang et al., 2011). As well as this, one study using 20 minutes hypoxia found increased levels of both Bad and Bcl-2, and also an increase in Erk 2 (Morales et al., 2008), which would imply the hypoxic system might instigate neuroprotective as well as apoptotic pathways. However these results were found 7 days after hypoxic insult, a time-point we did not include for protein analysis. It is interesting then that this is some of the first data to look what underlies the apoptosis so often reported (Morales et al., 2008, Neira-Pena et al., 2015, Van de Berg et al., 2002, Yang et al., 2011). It is possible therefore that the intrinsic mitochondrial pathway of apoptosis is not a major cause of cell death in this particular model of hypoxia. However, due to the lack of concrete markers of cell death at later time points, it is difficult to extrapolate information from this data.

This study deviates somewhat from published studies using the same model, as the surgery for these dams was carried out on gestational day 21, the day before the expected delivery

date. In this study, dams were consistently found to give birth overnight/early morning on final day of gestation. To ensure sufficient samples were collected without the need to cull dams giving birth early, surgery was moved to the preceding evening after 7pm to ensure enough samples could be collected for analysis. By keeping the time as late as possible, the time discrepancy between caesarean section and possible natural birth was kept to a minimum, to ensure pups were developmentally mature enough. Due to the short gestational duration for rats, even one complete day represents a large proportion of the development, so limiting prematurity was of utmost importance. Even moving the surgery to the day before there were instances of dams birthing before this time, so this gave confidence that this time point did not differ significantly from the possible natural birth times of the dams, therefore there was no expectation that this would affect results. Dams that gave birth early could not be used for this study, but pups were instead used for *in vivo* toxicity studies as described in Chapter 7.

To help facilitate the discussion of the data, which has many factors and can be difficult to draw conclusions on the bigger picture, a rank analysis of all data was performed. In this analysis, for each outcome, the seven treatment groups were ranked from least to most damaged, with the least damaged group scoring 1, and the most damaged group scoring 7. These scores were then plotted, showing the overall cumulative damage of each treatment across all outcome measure. As can be seen from Figure 8.23a, when all outcome measures were ranked, no specific insult or pre-treatment was actually the most damaging treatment, with naïve being the lowest and all other groups showing very similar rates of damage. This data is at odds with the initial hypothesis, and the *in vitro* data previously discussed in chapter 5, where we expected some changes with the treatments. However, when the data was split into either Western blot data versus physiological and histological changes, there is

a split in the effect of the treatments (see Figure 8.23a verses b). Of the 26 outcome measures included in the ranking analysis, 18 were Western blot data, and 8 were physiological and histological data, encompassing 24 hour survival, postnatal weight at days 1, 3 and 7, and TUNEL and cresyl violet staining. The Western blot data shows a very interesting trend of N₂O plus argon pre-treatment before either CS or hypoxia inducing the highest levels of damage, a complete contradiction to the expected benefits of argon addition. However, when analysing the physiological and histological changes, there is a very clear difference between the groups, with 10 minutes hypoxia having the highest damage score, and pre-treatment with N₂O plus argon causing a significant decrease in the damage score, bringing it level with the naïve data. While these two data trends are quite different from each other, one data set may be more reliable than the other for comparison. While the Western blot data shows a trend to N₂O plus argon inducing damage, by looking more closely at the data (see Figure 8.11-8.19), it can be seen that the protein changes are on a rather small scale, with the majority of postnatal day 1 and 3 protein changes being either less than double or half the quantity of naïve samples. Although attempts were made to quantify cleaved caspase-3 expression in these protein samples, bands could never be identified and so no measurement of the final outcomes of these protein changes could be discovered. Therefore it is difficult to determine whether these relatively small protein changes, which never reached significance in any brain area at any time point, could be influencing the changes in the brain. On the other hand, from Figure 8.23b, we can see a significant decrease in the damage done after the administration of N₂O plus argon before 10 minutes hypoxia in physiological and histological data. These include changes such as actual cell death in the brain, mortality rates and body weight. These are much more concrete outcomes to measure as they are the endpoints of many different physiological

changes, as opposed to minor parts such as for the protein changes. As well as statistically significant changes in cell death within the brain, at postnatal days 1 and 3 both HI10 and N₂O pre-treated HI10 pups weighed between 10-15% less than their naïve counterparts, while N₂O + argon pre-treated HI10 were just 1% lower weight. By postnatal day 7, N₂O pre-treated HI10 reached 17% lower weight than naïve, although HI10 alone had begun catching up to naïve, weighing just 6.7% less. These body weight changes, shown in Figure 8.10, even if due to physical inability to feed from the dam, would most likely have implications due to nutrient uptake. However, as none of the data is compelling in its own right, the analysis of damage ranking cannot be used as a final outcome measure; instead it can only hint at possible changes which may be worth investigating in more depth.

In summary, this model of perinatal hypoxia did show some very limited evidence of neuronal damage, as did caesarean section alone. In some outcome measures, N₂O + argon administered before the initial insult was able to significantly decrease the severity of the outcome compared to either caesarean section or hypoxia. To gather more data from this model, a more consistently damaging hypoxia model would need to be established. This model was tried using 15 minutes of hypoxia, but paradoxically this time point showed less damage than 10 minutes hypoxia (see Section 8.5.1) so, in line with previous publications from this group using the same time point, 10 minutes was used for this thesis. While the preliminary data showed a trend to increased cell damage in the cortex and hippocampus, when the work was expanded there was no great increase in the levels of damage seen, resulting in a relatively weak damage model. The strength of this model in general use is it more accurately reflects a milder hypoxic insult at birth, unlike some severe perinatal hypoxia models, but the limitation of this is a lower threshold of injury against which to compare treatment options. However, it could be argued that administration of N₂O before

a mild hypoxic insult does not exacerbate the injury seen, which was another outcome measure this thesis wanted to address. In this way, when combined with the data from chapters 6 and 7, there appears even less evidence for the neurotoxicity of N₂O. It is also useful to have some early evidence that the addition of argon to N₂O as a pre-treatment was able to give improved outcomes. This is particularly interesting as this is the lowest concentration of argon so far shown to induce neuroprotection after hypoxic insult *in vivo*, expanding our knowledge about the ranges at which argon can have a biological effect. Future work to use these gases in a more robust injury model could pave the way for a broader use for argon.

Chapter 9.

Discussion

This study used four model systems to assess the neuroprotective benefit of argon gas against two different insults, either N₂O in the naïve models without hypoxia, or against hypoxia itself. In the hypoxia models, the effects of N₂O were assessed as, after hypoxia, N₂O could have had either neuroprotective or additive neurotoxic effects due to its action as an NMDA receptor antagonist. It was found in primary neuronal cultures that N₂O had the ability to induce cell death via apoptosis, and argon was able to counteract this toxicity. However, in tumour cell lines, these effects could not be seen. As well as this, in a hypoxic model in one cell line, no effect of N₂O, either with or without argon, was seen. In a postnatal rat pup model of gas exposure without hypoxic insult, again N₂O showed no evidence of neurotoxicity, and argon, when administered alongside N₂O, induced no protein changes in the brains of these pups. Finally, a perinatal hypoxia model was used to assess the effects of these two gases in a more in depth model. Here it was found that adding argon to N₂O gas exposure before birth had a positive effect on pup mortality, postnatal weight and cell death in the brain, however many of these changes were merely trends and did not reach significance. N₂O alone, while having no significantly negative effect on pups, generally did not appear to have any beneficial effect on the pups either.

There are a number of interesting discussion points arising from this work which warrant examination and which will be expanded upon in this chapter.

9.1. Main findings of the thesis

The main findings of this thesis point towards N₂O having a net negative effect on both cells grown in culture and in an in vivo model of perinatal hypoxia. As well as this, this thesis provides the first data of neuroprotective benefits of argon at low concentrations against both anaesthetic and hypoxic insult. N₂O was shown to increase intracellular cleaved

caspase-3 (Figure 5.6), and induce significant propidium iodide staining (Figure 5.5), a marker of apoptosis, in a primary neuronal culture. In an in vivo hypoxic model, N₂O was also weakly associated with decreased pup survival and weight in the week following hypoxic insult (Figure 8.9, Figure 8.10), although no protein changes in the brain could be found to explain these changes. It was also associated with a non-significant increase in damaged neurons in the CA1 region of the hippocampus 7 days after birth (Figure 8.22). Paradoxically, N₂O alone was significantly associated with a decrease in TUNEL positive cells in the cortex after hypoxia, a measure of apoptosis (Figure 8.20). This may reflect the non-heterogeneous effects of different insults in different brain areas. Interestingly, when argon was added to N₂O in all the models used, there was no evidence of any neurotoxicity using any outcome measure. This points to a high safety profile for argon, even in combination with another gas which has shown neurotoxic effects.

An interesting finding in this work is the evidence suggesting caesarean section can have a negative effect on pups, even without a pre-treatment. Animals who underwent caesarean section alone showed evidence of decreased weight on the first postnatal day, as well as a significant decrease in the anti-apoptotic protein Erk 1/2 when measured 3 days after birth (Figure 8.19). As will be described later in this chapter, much of the published data using this model of perinatal hypoxia do not report comparisons between naive controls and caesarean section controls, and of those that do, a number do show significant differences between the two. As there is conflicting data in the literature regarding caesarean sections ability to induce neurotoxicity, this underlies the need to present both control data sets, and only compare hypoxic insult to caesarean section, not to naive control data.

Some positive indications for N₂O from this thesis comes from the *in vivo* model of anaesthetic neurotoxicity in naive animals (Chapter 7). Here, even with four hours exposure at 50% N₂O, animals showed no evidence of neurotoxicity. This is a subject area with much controversy currently, where researchers show toxicity after exposure to some of the most commonly used anaesthetic agents, and clinicians discuss the relevance of possible toxicity where anaesthetics are often inevitable to treat much more serious medical conditions. N₂O is no stranger to such controversy in this field, and was one of the first agents to be questioned in this regard, however the evidence has been mixed, and the data presented in this thesis does suggest that short term exposure does not have any short term negative consequence to a neonatal rat pup. However, longer time points would be necessary to check apoptotic outcomes as, from what was learned in Chapter 8, there may be evidence of apoptosis despite no protein changes in the mitochondrial pathway of apoptosis.

Overall, this thesis suggests that the more vulnerable a model to neurotoxic damage, the great the impact N₂O and argon will have. In both the primary neuronal culture and the *in vivo* model of hypoxia, N₂O had evidence of neurotoxic effects which could be reversed by the addition of argon, while in the more robust immortalised cell lines and naive pups *in vivo*, N₂O had no obvious toxic effects, and argon showed no changes.

9.2. Comparison with published data

In some aspects, this thesis confirms and expands upon much of the published data, in particular regarding the neurotoxicity of N₂O and definitely in regards to the neuroprotective effects of argon. However, in other ways, there are contradictions in what is reported here versus the literature, for example regarding the expected pathways of neurotoxicity. As mentioned, N₂O is generally found to be neurotoxic in models of the

nervous system, particularly when given in combination with other anaesthetic agents such as isoflurane (Lu et al., 2006, Shu et al., 2010, Yon et al., 2005). This thesis data expands upon this knowledge by using N₂O alone and applying it to both an *in vitro* primary culture model and an *in vivo* hypoxic model. This thesis provides the first data to report neurotoxicity of N₂O in a primary neuronal cell culture model, and outlines the lowest concentration to show an effect in combination with hypoxia, where the other reports used 70% N₂O (Taninishi et al., 2008, Baughman et al., 1988, Hoffman et al., 1993). Despite not finding neurotoxicity in an *in vivo* model of neonatal anaesthesia, this does not necessarily contradict published data. Another study which found neurotoxicity when N₂O was combined with isoflurane showed no toxicity of N₂O alone at short (2-8 hours) or long (24 hour) time points (Yon et al., 2005). It is therefore likely that *in vivo*, N₂O is neurotoxic only in certain situations, such as when combined with another anaesthetic or hypoxic insult.

Argon has generally been reported to show neuroprotective benefits in models of hypoxia (Fahlenkamp et al., 2014, Ryang et al., 2011, Zhuang et al., 2012) and traumatic brain injury (Harris et al., 2013, Loetscher et al., 2009), both *in vitro* and *in vivo* in both rodents and porcine models (Alderliesten et al., 2014). One of the most recent reports of argon neuroprotection addressed a hypoxia model in piglets, where neuroprotection was found with 50% argon, including a reduction in TUNEL positive cells (Broad et al., 2016). However, unlike most previous publications regarding argon protection, this thesis has shown biological effects *in vivo* at 20-29% concentration, whereas the majority of other data is between 50-70%. Two studies report some protection after 25% argon exposure; either following traumatic brain injury or oxygen glucose deprivation in a hippocampal slice model (Loetscher et al., 2009), or ischaemia reperfusion injury in rat retinas, although only at the shortest time point (Ulbrich et al., 2014). However, the latter paper also showed a

significant decrease in Bcl-2 expression following argon exposure, which is in contrast with the data in this thesis and other published data on the mechanisms of action of argon (Rizvi et al., 2010, Zhuang et al., 2012). As well as the agreement of this data with data regarding protective action *via* Bcl-2 upregulation (Zhuang et al., 2012), this work also shows argon increasing intracellular concentrations of phosphorylated Erk 1/2, a pathway becoming increasingly implicated in argons actions (Fahlenkamp et al., 2012, Ulbrich et al., 2015b, Ulbrich et al., 2015a). New research suggests Toll-Like receptors are the likely site of action for argon, where activation of these receptors induces Erk 1/2 phosphorylation (Ulbrich et al., 2015a).

An interesting finding in this work relates to the toxicity seen following caesarean section (CS) in perinatal pups without preconditioning. Currently, there are few *in vivo* studies investigating the effects of caesarean section on neurodevelopment, but CS has been linked in humans to disorders such as type-1 diabetes and asthma (Hyde and Modi, 2012), and there are possible correlations with ADHD and autism (Curran et al., 2015). An interesting theory behind the possible cause between caesarean section and neurodevelopmental delays posits that natural brain maturation which continues until birth and beyond in humans can be interrupted by elective caesarean section, removing the infant from the uterine environment at an earlier developmental stage than perhaps if left to progress to natural labour (Kapellou, 2011). As the rates of elective caesarean sections are increasing worldwide (Boyle and Reddy, 2012, Mittal et al., 2014, Feng et al., 2012b, Betran et al., 2016), this could suggest a greater burden on healthcare systems by performing caesarean sections beyond those medically necessary. *In vivo*, caesarean section has been shown to alter dopaminergic receptor expression (El-Khodir and Boksa, 2001), and it has been suggested that many of the negative effects of caesarean section of subsequent

neurodevelopment may be as a result of increased stress during the perinatal period, even suggesting that the well documented alterations to the gut microbiome following CS may relate to neurodevelopmental problems (Gur et al., 2015). However neither of these mechanisms appear to be influential here, as animals were born as close to the time of natural birth as possible, and naïve dams did not undergo labour or the stress of anaesthesia but pups still showed differences from naïve or preconditioned animals. While the differences seen here relate only to lower weight after birth and evidence of some disruption of normal molecular pathways, namely Erk 1/2 phosphorylation, it is consistent with evidence from other studies using this model of perinatal hypoxia. Of 54 studies using this model of perinatal hypoxia found in the literature, only 17 compare naïve pups to caesarean section delivered pups. Of these 17 studies, 3 give evidence of significant differences between naïve and CS delivered pups (Brake et al., 1997a, Brake et al., 1997b, El-Khodori and Boksa, 1998). Perhaps, then, it is worthwhile to consider the necessity of greater clinical investigation into the effects of caesarean section on neurodevelopment, and to examine the clinical use of caesarean section where not necessarily required medically.

9.3. The actual toxicity of anaesthetics

This study brings into question a discussion which has been ongoing for many years now; namely, the actual toxicity of anaesthetic agents. In the vast body of research in the field, there are many reports on either side of the debate. However, most of the commonly administered anaesthetic agents have been reported to show some levels of neurotoxicity. While the debate currently centres upon whether or not these anaesthetics are indeed neurotoxic, an important consideration which is often missed is the clinical relevance of the parameters studied. As has been mentioned by many clinicians, the cause of interest in this

topic was not a body of evidence from the clinical driving *in vivo* research, but instead *in vivo* research causing concern about a field which has never seen a problem previously (Davidson et al., 2008). In many of the current studies, in particular for N₂O, very high concentrations or long durations are used, which might not necessarily reflect the clinical setting. Tied with this is the fact that many studies focus too closely on just the anaesthetic, without taking into consideration other important factors such as coinciding tissue damage due to surgery, repeated doses, diseases which predispose the patient to need anaesthetic or surgical intervention in the first place, or any other factors which are commonly associated with the settings under which most people are exposed to surgery (Sanders et al., 2013, Jevtovic-Todorovic et al., 2013, Anand and Soriano, 2004, Hudson and Hemmings, 2011).

One interesting area where the toxicity of anaesthetics is being closely studied is in the treatment of cancer. There has recently been a greater interest in the effects of anaesthetic agents on cancer cells, where some can increase metastasis and proliferation, while others can decrease these (Mammoto et al., 2002, Tavare et al., 2012, Zhao et al., 2014). In this case, the choice of anaesthetic used, for example during tumour resection, could play an important role in patient outcomes (Xuan et al., 2015). For similar reasons, if some anaesthetics are shown to increase or decrease neurotoxicity, the choice of agent used in patients at risk of neuronal damage, such as after traumatic brain injury or stroke could have significant effects (Hancock and Nathanson, 2004, Anastasian, 2014). It may then be more important to look at anaesthetic effects in models of interest, such as studied in Chapter 8. By examining the neurotoxicity of N₂O administered before perinatal hypoxia, a setting which could be regularly occurring clinically in the UK due to the high use of N₂O as a labour analgesic, a real clinical evaluation of the gas can be more readily performed.

9.4. Perinatal hypoxia model

The model of hypoxia used in Chapter 8 of this thesis is a widely used model of global hypoxia, designed to mimic oxygen deprivation during the birth process (Weitzdoerfer et al., 2004). There are a number of different models used around the perinatal period to induce hypoxic ischaemic brain injury, each with strengths and limitations, and each model gives insight into various aspects of perinatal hypoxia in greater or lesser detail (Yager and Ashwal, 2009). The model used in this thesis is primarily referred to as perinatal asphyxia, where the entire uterine oxygen supply to pups is cut off during the birth process, *i.e.* before pups begin breathing air independently. This closely mimics hypoxic incidents before and during the birth process in humans, such as a lack of maternal blood supply which limits oxygenation, rather than infants already breathing who have some pulmonary obstruction. The limitation of this model lies in the developmental stage of pups during the perinatal period compared with human neurodevelopment. While there is much controversy over correlations between rodent and human neurodevelopmental equivalence, there can be little doubt that a term rat pup and human neonate are quite different in most neurodevelopmental respects. However, despite these neurodevelopmental differences, there are differences between pups still *in utero*, truly newborn animals and those at a similar developmental stage to human term infants that have been feeding, breathing and interacting with the extra-uterine environment for a number of days, as is the case for some of the later neonatal models. For example, it has been reported that the act of beginning breathing in newborn pups alters noradrenaline levels in the brain (Lagercrantz et al., 1992). While these are primarily involved in brain areas involved with breathing rhythm, these changes could have wider effects within the brain. As well as the respiratory system, there

are dramatic changes in many other body systems between resting state *in utero* and the extra-uterine environment, such as temperature regulation, metabolism and digestion (Ward Platt and Deshpande, 2005), meaning a vastly different systemic environment between these two states. Therefore, while models which assess postnatal days 3-10 may capture the neurodevelopmental stage of the term infant, they cannot take into consideration other aspects of the perinatal brain which may be important when considering hypoxia *in utero*.

The most commonly used model of neonatal hypoxia is the Rice-Vannucci model of arterial ligation in combination with a hypoxic environment (Rice et al., 1981). This model is primarily carried out on postnatal day 7 (P7) pups, but some studies report ages between P3 and P10 (Matchett et al., 2009, Alexander et al., 2014). While, as discussed above, these models aim to mimic hypoxia during the neurodevelopmental equivalent to a term infant, they differ substantially from true perinatal hypoxia. Besides the physiological differences described above, there is also the severity of this model in comparison with perinatal hypoxia. The Rice-Vannucci model involves anaesthetising pups at a young age, performing an invasive arterial ligation procedure and exposing the pups to an environment of low oxygen, not full hypoxia. This results in a severe unilateral lesion of the brain, more similar to a stroke model in terms of the severity and focal damage wrought. In comparison with perinatal hypoxia in humans, which is generally global and a more diffuse damage, it can only help to assess the general neuroprotective benefits of different agents, and to give information on optimal treatment times. It cannot, however, encapsulate the physiological state of pups during the birth process, as discussed above. As well as this, using anaesthetic during the surgery to induce hypoxia could have confounding effects when studying neurotoxicity and neuroprotection.

9.5. Limitations of this work

This thesis has shown evidence that N₂O can have neurotoxic effects, while the addition of argon can reverse these effects and induce protection. However, there are some caveats to these findings which must be considered when interpreting the data.

A serious limitation in the data stems from the inability of the chosen hypoxic models to induce a high level of neurotoxicity. This in turn leaves little room to interpret the neuroprotective abilities of argon, or indeed N₂O, against the insult. As mentioned above, the model of perinatal hypoxia used in this study is designed to be less damaging than the more commonly used Rice-Vannucci model, to more closely mimic the global damage seen in infants during perinatal hypoxia. However, with the model in this thesis, it was unable to induce a high level of brain damage, even when using a longer time point of hypoxia. As seen in Section 8.5.1, time points of 10, 15, 17 and 19 minutes hypoxia were assessed for pup mortality, commonly used as an initial indicator of pup health with this model. However, longer hypoxic time points of 17 and 19 minutes reduced pup survival to a degree which would have made sample collection for all outcome measures impossible under the circumstances. Further investigations of 10 versus 15 minutes were carried out, but as described in Section 8.5.1, 10 minutes actually showed the worst outcomes compared to both controls and 15 minutes hypoxia. As 10 minutes was also previously used in publications from our group, it was decided that the remaining work from this thesis should also use 10 minutes hypoxia. Despite not showing a very severe neurotoxic insult, it still allows us to draw conclusions regarding the additive neurotoxic effects of N₂O combined with hypoxia, to determine whether the addition of N₂O worsens neuronal outcomes. From

this study, it doesn't appear as if N₂O has a dramatic effect in addition to hypoxia, but there is some evidence that it worsens rather than improves outcomes.

One design problem with this study revolves around the tumour cell line models in Chapter 6. While these models were initially set up to allow high throughput of cells to validate the primary cell culture model and expand the data by including a hypoxic insult, this could not be achieved to the intended levels. The hypoxic insult chosen was based off previous work in this group from primary neurons, but as tumourigenic cells have altered physiological responses, particularly to hypoxia, a greater insult could have been used. As tumours often become hypoxic, these cells have altered physiological pathways to protect against and thrive under hypoxic conditions. By giving a 75 minute oxygen glucose deprivation insult, it is likely that the model is not reflective of hypoxic insult in the brain. However, previous data with SH-SY5Y cells has shown damage using between 60-90 minutes OGD (Pei and Cheung, 2003, Lee et al., 2010), so there is still room for interpretation of the data in light of this. With the data available from this thesis, again it is only possible to draw conclusions on the additive effect of N₂O and hypoxia in these cells, and it does not appear that N₂O worsens the conditions of the cells. Ideally, longer time points would have been carried out, however due to limitations in the stocks of SH-SY5Y cells which were eradicated by widespread infection in the shared laboratory space; it was not possible to locate a new stock of cells in time to repeat the work using longer time points.

From the data presented, another limitation was the lack of power available in the behavioural data. Due to issues outside of the project control, it was not possible to continue behavioural testing to reach high enough animal numbers for interpretation. The results have been presented as supplementary data (Section 11.1), however with most

groups at very low numbers and one group missing from analysis, it cannot be interpreted in any meaningful way, hence exclusion from the main thesis text. Behavioural data is an important aspect of this genre of research, as it is important not only to investigate molecular changes in the brain after insult, but to attempt to correlate this with changes in phenotype, particularly behaviour. Morris watermaze was chosen as the behavioural test for this work as it was hypothesised that hypoxia and N₂O would affect learning and memory pathways. However, as this could not be confirmed in this model, no further implications of the changes seen after perinatal hypoxia can be made beyond that discussed previously.

A final limitation of the work regards the use of a pre-treatment agent against perinatal hypoxia. It must be noted that very few prophylactic treatment options have been investigated for this type of insult, as it is often difficult to predict the occurrence of this type of injury clinically. The work in Chapter 8 uses a four hour pre-treatment with N₂O with or without argon. While N₂O is a very commonly used labour analgesic, with a rate of use in over 70% of labouring mothers in the UK, it would not be administered in the manner described in this thesis, with the mother inhaling the gas periodically throughout labour, as opposed to continuously administered as in this work. However, labour can continue for extended periods of time and it would not be unusual for mothers to use N₂O on and off for periods well over four hours. The addition of argon to this gas mixture is also not outside the realms of clinical possibilities, as it has been proven safe to use in humans previously, and in the work described here, the analgesic dose of N₂O (50%) and safe concentration of O₂ (30%) still leaves room for 20% argon, which could be enough for pre-treatment effects in the case of hypoxic ischaemic insult. By protecting the brain before insult, this could give clinicians more time after birth to start infants on another neuroprotective treatment with

the knowledge that primary injury may have been lessened. However, introducing a new gas to this commonly used labour analgesia for the protection of the small subset of births that may go on to have hypoxic insult could prove difficult.

9.6. Future experiments needed

The biggest complement to this work would be to repeat the data using a stronger insult in both *in vitro* and *in vivo* hypoxia models. This would allow expansion of data sets, and confirmation of trends seen in this work. As well as this, investigations into different pathways by which N₂O and argon could be having the neurotoxic and protective roles respectively would be worthwhile. Utilising different techniques such as PCR to analyse alterations in RNA expression could find earlier changes that were not found by protein analysis in this thesis. As well as this, reactive oxygen species, inflammatory pathways and cytokines could all be investigated to consider the overall picture of brain health in the model, and attempt to understand different brain damage pathways. The main limitation to completing these assays in the work here was the numbers of animals to be used. As it stood, with the many treatment groups and time points, it would have been difficult to collect whole new data sets for these investigations, as PCR and ROS investigation need different tissue processing than carried out in this work, hence possibly doubling the number of samples needed.

Another useful experiment could be to use argon as a treatment after perinatal hypoxia, as has often been described for xenon. This could give greater insight into the flexibility of argon treatment at different stages surrounding insult, and help to determine optimal treatment times. Similarly, by using a different model of neonatal hypoxia, such as the Rice-Vannucci model, the prophylactic effects of argon could be investigated at a

neurodevelopmental stage more similar to a term human infant, therefore complementing and developing this data by taking into consideration developmental stage as well mimicking true perinatal hypoxia during the birth process. As shown in Table 2.5, no studies thus far have examined the preconditioning effects of argon in a neuroprotective model, so this thesis presents the first evidence of protective capabilities. Expanding this work with models which cause more profound neuronal damage could help expand this work and really test the limits of argon's neuroprotective abilities.

9.7. Conclusions

This thesis attempted to obtain evidence that N₂O could have neurotoxic effects in vulnerable models both *in vitro* and *in vivo*. As well as this, it sought to gather evidence that argon has neuroprotective abilities in combination with N₂O. Unfortunately, due to limitations described above, the *in vivo* work was unable to definitively address these issues. However, from the *in vitro* work, there was some evidence that N₂O could indeed have neurotoxicity, and it was also seen that the neuroprotective effects of argon are possibly via protection of the mitochondria and upregulation of Bcl-2 expression and Erk 1/2 phosphorylation. On the other hand, without prior insult, N₂O was not shown to induce neurotoxicity *in vivo*, suggesting that exposure for a short period in healthy patients is unlikely to cause any serious damage. At this moment in time, no definitive conclusions can be drawn regarding N₂O actions with hypoxia besides to say it does not appear to significantly exacerbate any injury.

Clinically, this work and the similar data should be used as a call to design more complete trials to truly understand the effects of the anaesthetic agents used so often on vulnerable patients. While *in vitro* and *in vivo* work is important to understand mechanisms of action,

and highlight the possible implications for these drugs, nothing can be done until clinical investigations determine the true extent of any neurotoxicity. While doubts can be cast upon the relevance of animal models on human experiences, with the mounting evidence showing much greater impact of what were once thought to be benign agents, well planned studies are needed to determine what anaesthetics and what situations could be causing long term damage to children.

Chapter 10.

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Chapter 11.

Supplementary data

11.1. Behavioural data

In this study, a widely used model of perinatal hypoxia was utilised, where the entire uterus was excised, one half clamped and placed in a water bath at 37°C to induce hypoxia, and the other half birthed immediately as caesarean section control pups. For the preconditioning model, dams were exposed to four hours of gas immediately before caesarean section. This model hopes to mimic the effects of N₂O during labour, combined with hypoxia at the time of birth, to give a clinically relevant model. As well as this, by using both uterine horns for different insults, caesarean section pups can act as a control for hypoxia treated animals, undergoing the same maternal effects. Morris water maze testing was used to assess the spatial learning and memory of the animals (Morris, 1984). Pups used for behavioural testing were weaned from the surrogate dam at postnatal day 21 and separated by sex. On postnatal day 45, the behavioural training protocol was started.

11.1.1. Methods

Perinatal hypoxia was induced in gravid dams on the final day of gestation. Dams were exposed to the gas of interest, outlined in Figure 11.1, for four hours, before culling of the dam.

	Naïve	N ₂ O treatment	N ₂ O + Argon treatment
O ₂	21%	21%	21%
N ₂	78%	24%	-
N ₂ O	-	50%	50%
Argon	0.9%	-	24%

Figure 11.1. The percent composition of the gases administered to gravid dams before birth of the pups.

Immediately after culling, the uterus was exposed and the two uterine horns clamped. One was removed and placed in a 37°C water bath for 10 minutes to induce hypoxia, while the other was excised and pups born by caesarean section. After the hypoxic insult, hypoxic pups were also removed from the uterus by caesarean section. Pups were left with a surrogate dam for 21 days, at which time they were weaned and males and females separated from each other. On postnatal day 45, behavioural testing commenced. The timeline of the project is outlined in Figure 11.2.

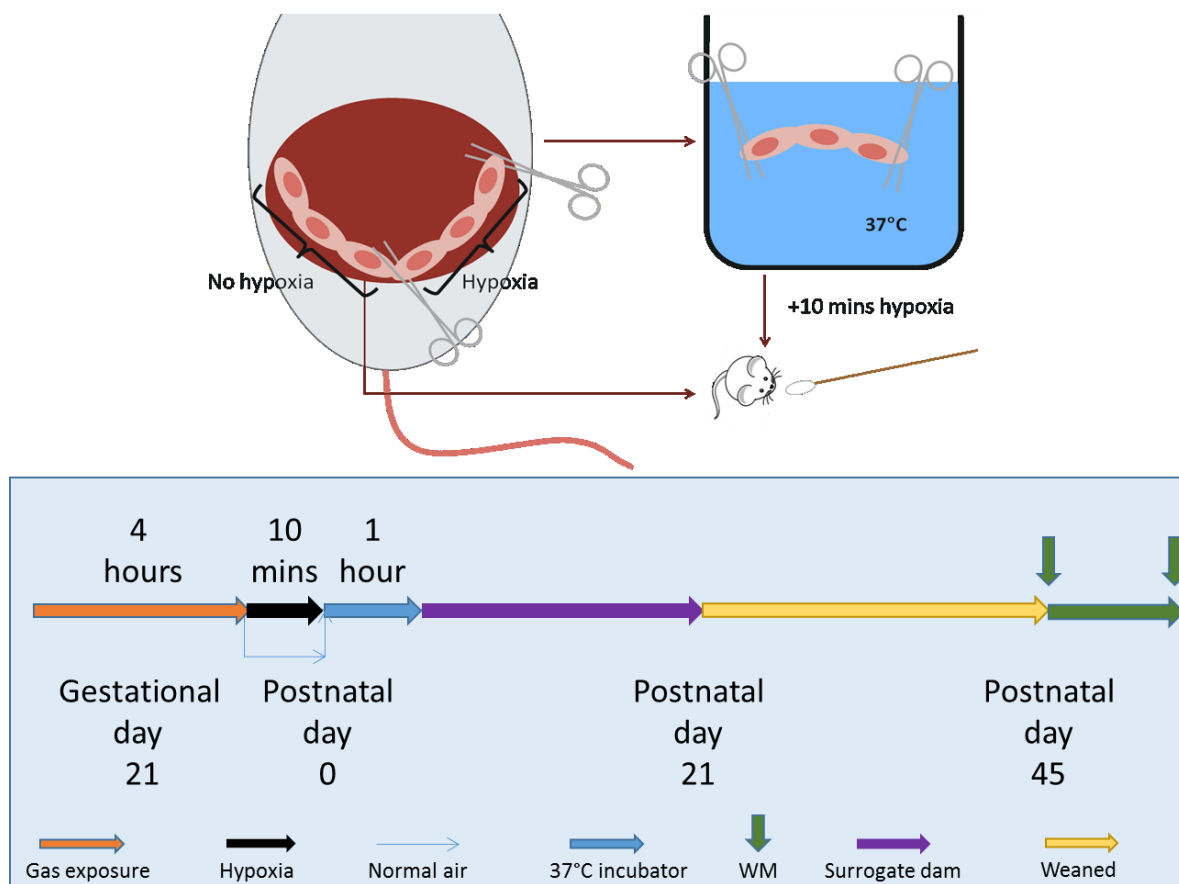


Figure 11.2. The timeline of pups from *in utero* gas exposure to completion of water maze (WM) trials.

Each day before commencing the behavioural protocol, the animals were brought to the room in their cages for 30 minutes to habituate to the new surroundings. The water maze

procedure used was a 6 day protocol, with five training days followed by one test day. On days 1, 3 and 5, pups were weighed to ensure there was no weight loss caused by stress during the protocol. A permanent marker was used to mark the base of the tail of different animals to identify them easily during testing.

11.1.1.1. Morris water maze

The Morris water maze is a learning and memory task, wherein the animals need to find and remember the location of a hidden platform in a large round water tank (D'Hooge and Deyn, 2001, Morris, 1984). Visual cues are placed around the arena to provide location information. This training phase is continued for five days, and on the sixth day the platform is removed and the memory of the animal is assessed by their duration spent swimming in the location where the platform was. The tank used for training was a round, 1.1m diameter tank made of white plastic. A black plastic base and wall insert were used to provide a uniform dark background for the white animals, to make detection using camera software easier. The tank was filled to a level 2cm above the level of the platform, to ensure the platform was not visible but, once found, the animal could comfortably mount. The water was maintained at a temperature of $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ to prevent hypothermia in the animals. Four marks on the outside of the tank marked the North, South, East and West points of the tank (not visible from inside the tank) and for each experiment (i.e. 5 days learning) the platform was always placed in the centre of one of the four quadrants (See Figure 11.3).

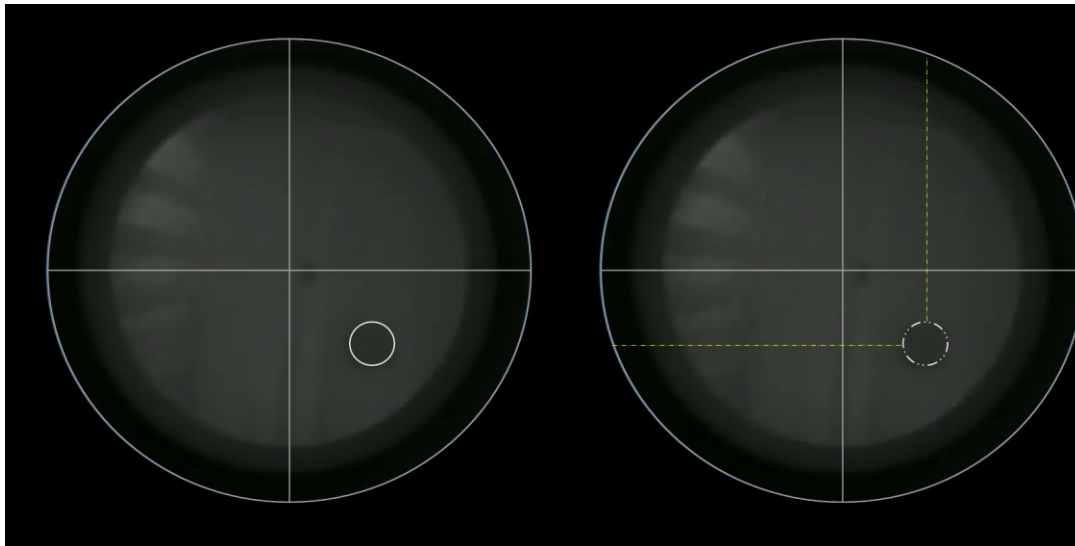


Figure 11.3 **The layout of the Morris water maze.** The water maze tank is a 1.1m^2 tank filled with water to a height just above the height of a platform. (a) The cardinal directions were marked on the outside of the tank to guide the experimenter when placing the animals in the tank. Each animal was trained 4 times per day, with an entry to the tank from each of the cardinal points. On each training day the order of entry point was randomised to prevent learning by association. (b) On the test day, when the platform had been removed, animals were placed in the tank from one of two points, A or B, again to ensure learning had not just come from association with the entry point.

Animals were introduced to the tank from one of the cardinal points, with the animal held around the front shoulders and placed tail first to prevent shock, and facing the wall to prevent seeing any visual cues which could be associated with the experimenter placing them in the tank. The animal was left to swim freely for 60 seconds, or until they reached the platform, with the experimenter staying out of sight. If within 60 seconds the animal did not find the platform, the experimenter would lead them to the platform. Once on the platform, the animal was left for 5 seconds to look around. The animal was then removed, dried and placed in a 37°C heat box to dry off and to keep separate from animals not yet tested. Each animal was given 4 trials a day, with each trial having a different entry point, randomised to prevent learning of a set swim pattern. There was a 15 minute break given between each trial, so each animal spent approximately one hour per day in the training protocol.

On the final day, the test day, the platform was removed and the animal placed from one of two start points equidistant to the previous location to the platform, with each animal alternated between the two start points, as shown in Figure 11.3. The animal was allowed to swim freely for 60 seconds, before being removed to dry off. Each animal was tested only once.

All training and testing was recorded by a Sony ExWaveHAD video camera placed directly above the water maze, and each trial was recorded and stored for later analysis. Using EthoVision (Tracksys, Nottingham, UK), the visual field of the tank, quadrants and platform were outlined on the video recordings. Utilising the colour difference between the animals and water, the software tracked the midpoint of each animal through each trial, calculating duration spent in each area, latency to platform, and swim speed.

11.1.1.2. Rotarod

The rotarod test is a measure of gait and motor function in animals (Hamm et al., 1994). As this hypoxia model is one of global hypoxia mimicking disabilities like cerebral palsy, it was important to consider any loss in motor ability as well as cognitive function. In the rotarod test, a rotating drum is suspended above a cage-like base made of metal bars. The drum has a grooved, non-slip plastic covering to ensure the animals have some grip. The base is connected to a timer which is started by the drum rotating, controlled by the experimenter, and stopped when the animal falls onto the wire floor. This gives an accurate latency from starting the trial to the animal falling off. The drum was set to rotate at a speed of 30 revolutions per minute. Each animal was tested three times and the average latency to fall was calculated.

11.1.2. Training

Training was assessed by measuring latency to platform each day and obtaining an area under the curve for each animal. As can be seen from Figure 11.4a, N₂O treated animals who underwent 10 minutes hypoxia actually had the smallest area under the curve, which suggests that they learned the fastest of all groups. Hypoxia alone appears better than naïve, while caesarean section (CS) and either CS or hypoxia with argon pretreatment performed worse than naïve. However, when these results are broken down to compare animals tested within the same batch (Figure 11.5b-d), this difference in N₂O + hypoxia group fails to continue. This breakdown into groups was performed as the first batch of watermaze training was performed with a slightly different protocol, and to ensure this did not bias the results the data was separated out. Either CS or hypoxia combined with N₂O + argon actually appear to perform better than their naïve counterparts. However the trend for CS hypoxia alone appear to take longer to learn platform location holds true, indicating that the only group which performs worse in learning the task than naïve are caesarean section.

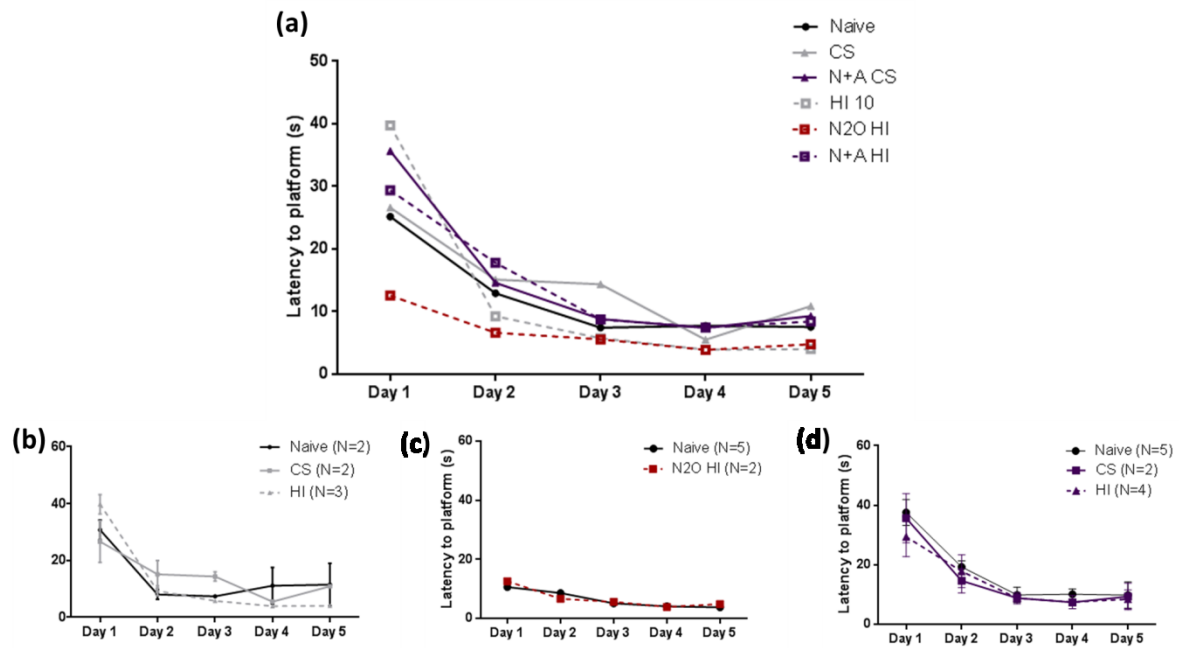


Figure 11.4 **Latency to find the platform over 5 training days.** Over the course of 5 training days, the latency of time to find the submerged platform decreased as animals learned the location of the platform. Generally the animals learned this quite quickly and there was no significant difference between groups. (a) represents all data compiled while the lower graphs show animals with no pre-treatment (b), N₂O pre-treatment (c) or N₂O plus argon pre-treatment (d) with their naive controls to show when matched with their naive there was no difference.

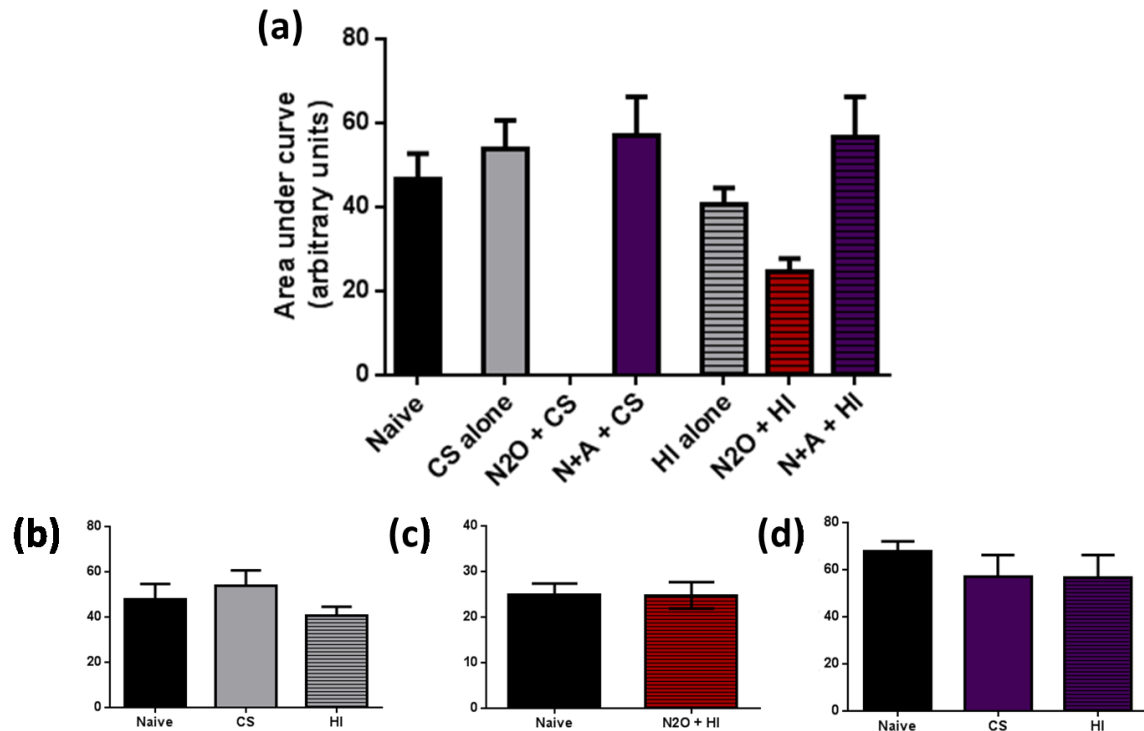


Figure 11.5 **Area under the curve for training for each of the treatment groups.** The data from latency to platform during training was converted to a figure of area under the curve to give a numerical value to learning. Again, although the compiled data appears to show some differences (a), this cannot be seen once each treatment is compared to its own control. This is shown for no pre-treatment (b), N₂O pre-treatment (c) or N₂O plus argon pre-treatment (d).

11.1.3. Probe trial

When required to locate the platform after its removal, animals who received 10 minutes hypoxia at birth actually spent more time in the target quadrant than any of the other treated animals, which would indicate an improved memory Figure 11.6a. However, similar to what can be seen when analysing training data, when each group is compared directly to their counterpart naïve controls, only caesarean section animals show evidence of performing worse, as seen in Figure 11.6b-d. Both N₂O + argon hypoxia and hypoxia alone treated animals performed slightly better than their naïve counterparts.

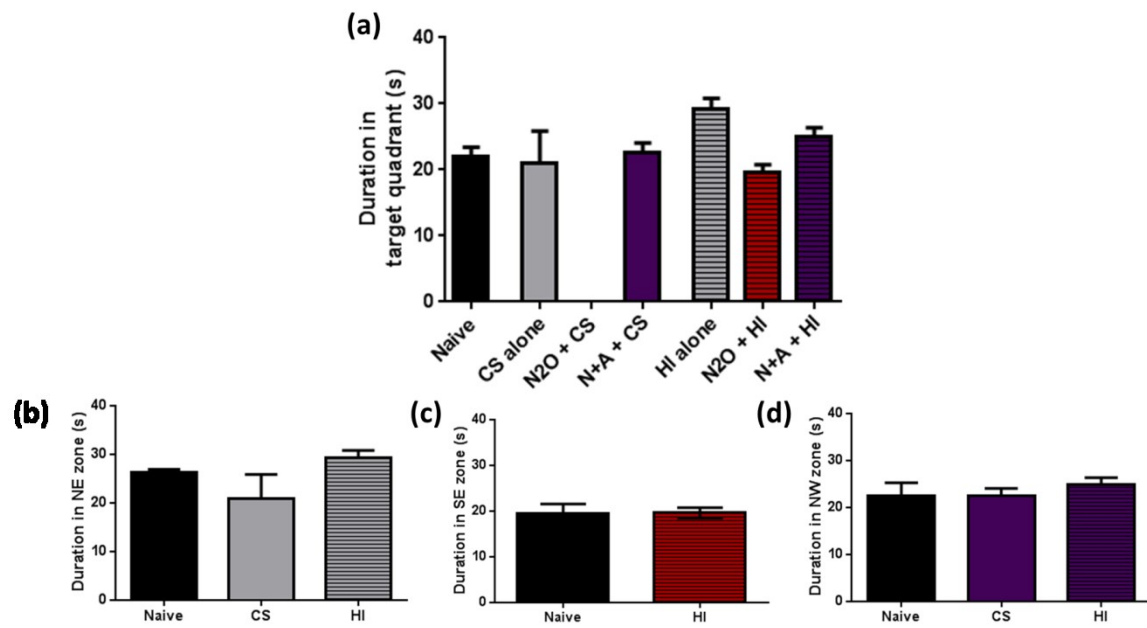


Figure 11.6 **Duration spent in the target quadrant.** After 5 days training, animals had one trial to assess their memory. The duration spent swimming in the quadrant where the platform had been during training was assessed as an index of memory. There was no significant difference between any of the groups when compared to their own naive, regardless of whether they had no pre-treatment (b), N₂O pre-treatment (c) or N₂O plus argon pre-treatment (d).

11.1.4. Rotarod

The rotarod test shows a variable level of physical ability between the different treatment groups, with both 10 minute hypoxia and N₂O + argon caesarean section treated animals showing a very short latency to fall when compared with the other groups. However, when broken down to analyse animals trained and tested together, these results are less clear for hypoxia alone, while caesarean section alone actually appears to perform better than naïve, although it is worth noting that the naïve animals in this group perform particularly poorly when compared with others.

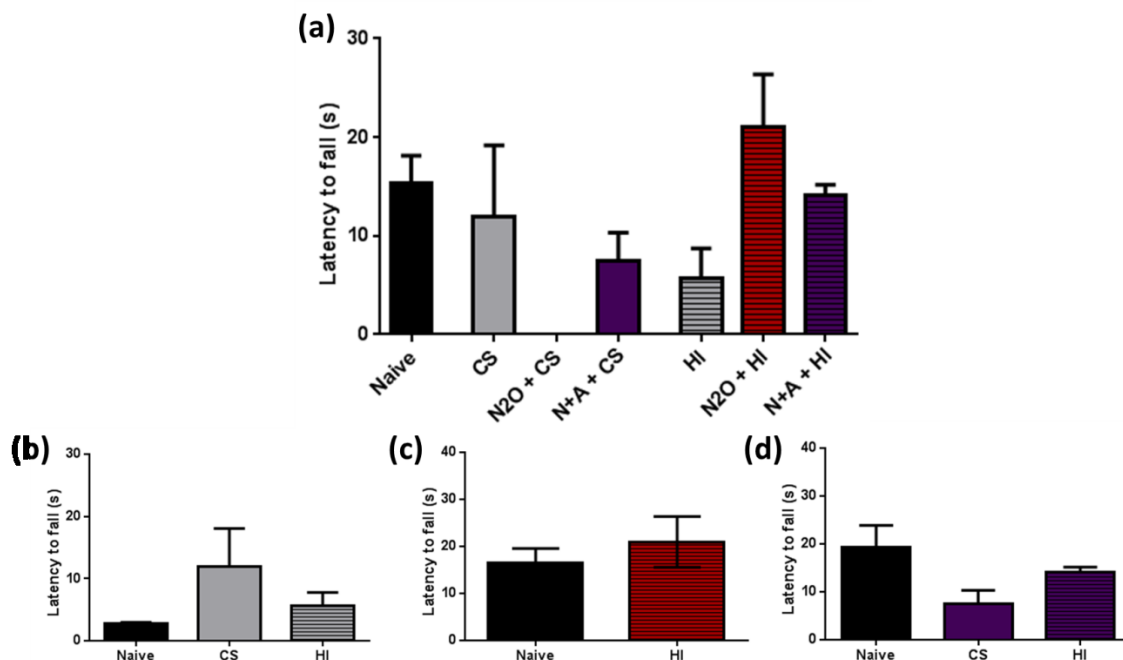


Figure 11.7 **Time spent on the rotarod before falling.** Rotarod performance was assessed to determine whether there was any particular physical deficit in the animals which may have affected their ability to swim. A decreased latency to fall is associated with reduced motor function. There was no significant difference between any of the groups, namely, no pre-treatment (b), N₂O pre-treatment (c) or N₂O plus argon pre-treatment (d) with their naïve controls.