

# Imperial College London

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## **Neurodegeneration induced by general anaesthetics in the developing brain: Mechanism and prevention**

A thesis submitted for the degree of  
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## Abstract

In the early development of brain, there is an important period called the synaptogenesis period (also known as the brain growth-spurt period) which occurs in different mammalian species at different times relative to birth. In rodents, it begins a day or two before birth and ends 2 weeks after birth, whereas in humans it starts at the beginning of the third trimester and ends several years after birth. This is a critical period during neural development as neurons are establishing communications with their environment and other neurons to ensure that they develop and function appropriately; disruption at this stage by toxic agents including anaesthetic agents may compromise the normal neuronal development of the central nervous system.

During the course of my PhD study, I found that inhalational agents isoflurane (ISO) alone or in combination with nitrous oxide ( $N_2O$ ) caused a wide spread neurodegeneration in the brain including the cingulate cortex, hippocampus, substantia nigra and basal forebrain in postnatal day 7 Sprague-Dawley rat pups. In those brain regions, the cells involved included glutamatergic, GABAergic and dopaminergic neurons. However, the cholinergic neurons in the basal forebrain were spared from anaesthesia-induced neuroapoptosis. My studies also showed that the neurodegeneration was not only confined to the higher centres, but was also observed in the spinal cord. These data indicated that the anaesthetics studied acted at molecular level and induced neuronal apoptosis by activating the intrinsic apoptotic pathway.

It has been well documented that preconditioning with hypoxia or other interventions including pharmacological agents can protect against subsequent brain injury induced by hypoxia/ischemia. However, my data showed that hypoxic preconditioning exacerbated N<sub>2</sub>O+ISO-induced neonatal neurodegeneration, while xenon preconditioning protected against anaesthetics-induced neuroapoptosis and subsequent neurocognitive impairment.

Most studies in this field including my own study described above are more relevant to the clinical situation where neonates are managed in the critical care unit, i.e. in the absence of surgery. For this reason, I implemented another experimental setting to mimic the clinical situation in the operating room where neonates receiving anaesthetic drugs are also subjected to nociceptive surgical stimuli. Therefore, in another experiment, rat pups in addition to receiving anaesthetic drugs were also subjected to standardised nociceptive stimuli. The results suggested that nociceptive stimuli enhanced neuronal apoptosis induced by anaesthetic drugs in the brain and the spinal cord and consequently impaired neurological function.

Were the data reported in my thesis to be extrapolated to the clinical setting, it would have very important implications for the clinical management of paediatric population receiving general anaesthesia.

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## ABBREVIATIONS AND ACRONYMS

|         |                                                    |
|---------|----------------------------------------------------|
| AAS     | antimycotic solution                               |
| Ach     | acetylcholine                                      |
| ADP     | adenosine diphosphate                              |
| AMPA    | á-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate |
| APAF -1 | apoptotic protease activating factor 1             |
| ATP     | adenosine-5'-triphosphate                          |
| AUC     | arbitrary unit                                     |
| BAX     | promotes apoptosis by competing with Bcl-2 proper  |
| BBB     | blood-brain barrier                                |
| BCL-2   | apoptosis regulator                                |
| BDNF    | brain-derived neurotrophic factor protein          |
| BID     | BH3 interacting domain death agonist               |
| cAMP    | cyclic adenosine monophosphate                     |
| CCI     | chronic constriction injury                        |
| CFLIR   | c-Fos-like immunoreactivity                        |
| ChAT    | choline acetyltransferase                          |
| CNS     | central nervous system                             |
| CREB    | cAMP response element-binding protein              |
| DAB     | 3, 3'-diamino-benzidine                            |
| ECL     | enhanced chemiluminescent                          |
| ERK     | extracellular-signal-regulated kinases             |
| FAS     | fetal alcohol syndrome                             |

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|                  |                                     |
|------------------|-------------------------------------|
| GABA             | gamma-aminobutyric acid             |
| GAD67            | glutamic acid decarboxylase-67      |
| HBSS             | hanks-balanced salt solution        |
| HIF-1 $\alpha$   | hypoxia-inducible factor-1 $\alpha$ |
| HSG              | high sucrose-glucose                |
| IL-beta          | interleukin beta                    |
| IR               | immunoreactive                      |
| KA               | kainic acid                         |
| LTP              | long-term potentiation              |
| MAC              | minimum alveolar concentration      |
| MAP              | mitogen-activated protein           |
| N <sub>2</sub> O | nitrous oxide.                      |
| NDS              | normal donkey serum                 |
| NGS              | normal goat serum                   |
| NMDA             | N-Methyl-D-aspartate                |
| OGD              | oxygen-glucose deprivation          |
| P53              | tumor protein 53                    |
| PAG              | periaqueductal gray                 |
| PBS              | phosphate buffered saline           |
| PCD              | physiological cell death            |
| PND              | post-natal day                      |
| PT               | permeability transition             |
| PTZ              | pentylentetrazol                    |
| SNI              | spared nerve injury                 |

|                  |                                   |
|------------------|-----------------------------------|
| TBS-T            | tris buffered saline              |
| TIA <sub>s</sub> | transient ischaemic attacks       |
| TNF- $\alpha$    | tumor necrosis factor             |
| tPA              | tissue plasminogen activator      |
| vGLUT1           | vesicular glutamate transporter 1 |

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# **CHAPTER 1**

## **INTRODUCTION**

Practitioners have long held the illusory hope that the constellation of profound behavioural effects comprising general anaesthesia is entirely reversible and that the central nervous system (CNS) is returned to its pristine state once the anaesthetic agent is eliminated from the active site. However, there is increasing evidence to suggest that general anaesthetics exert long-term effects on the CNS following administration depending on the class of agent and the age of the exposed subject.

Alcohol abuse during pregnancy can cause neurobehavioral disturbances in children ranging from hyperactivity and learning disabilities to depression and psychosis and this clinical condition is called Foetal Alcohol Syndrome (FAS). This syndrome has been well documented by several elegant laboratory works in rodents (Jevtovic-Todorovic et al., 2000; Olney et al., 2004). It is thought that the brain is particularly sensitive to toxins including ethanol during the period of synaptogenesis, also known as the Brain Growth Spurt period. In rodents, synaptogenesis occurs predominantly as a postnatal event, extending from approximately 2 days before birth to 2 weeks after birth, whereas in humans, it begins during the third trimester of pregnancy and lasts until 2 to 3 years after birth (Dobbing and Sands, 1979; Dobbing and Sands, 1993). Thus, in theory, humans may be most susceptible to the toxicity of general anaesthesia from the pre-partum period through infancy. In rodents, the developing brain is particularly vulnerable to anaesthesia-induced neurotoxicity at 7-days-of-age and vulnerability rapidly subsides with increasing age (Loepke and Soriano SG, 2008).

The targets for the behavioural properties of general anaesthetics reside within the central nervous system. Until relatively recently, it had been thought that general

anaesthetics exert a non-specific reversible effect on excitable membranes within the CNS which are returned to their pristine state once the anaesthetic is eliminated from this compartment. However, anaesthetics are now known to modulate specific ligand-gated ion channels, principally GABA<sub>A</sub> receptor and the NMDA subtype of the glutamate receptor, thereby disrupting synaptic function (Franks, 2006). The possibility that these synaptic actions could result in long-term consequences was diligently pursued by the investigators that had originally identified the deleterious effects of alcohol on the developing brain (Ikonomidou et al., 2000). Alcohol is similar to anaesthetics, both in its' behavioural and molecular effects; it has been shown that post-natal exposure of rodents to standard anaesthetics produced similar morphologic and functional impairment to that seen with alcohol exposure producing the foetal alcohol syndrome (Jevtovic-Todorovic et al., 2003a). Indeed, anaesthetics include N-methyl-D-aspartate glutamate receptor antagonists (e.g., ketamine, nitrous oxide) and agents with  $\gamma$ -aminobutyric acid A mimetic effects (e.g., pentobarbital, diazepam, isoflurane, halothane, propofol) alone or in combination can cause a wide spread neurodegeneration as seen from *in vitro* to *in vivo* settings and from rodents to monkeys (Jevtovic-Todorovic et al., 2003a Istaphanous GK and Loepke AW 2009). However, this is still a novel area of research. There are many questions that need to be addressed. Hence, The aims of my research work in this PhD thesis are as follows:

- 1) To establish a novel strategy which can be used to study the neurodegenerative effects of anaesthetic drugs in the developing brain *in vitro* and *in vivo* settings;
- 2) To answer the question of whether this neurodegeneration occurs only in the CNS in the brain or also occurs in the spinal cord;
- 3) To determine which category of neurons are more vulnerable to this novel anaesthesia strategy;
- 4) To explore any potential

strategies which can prevent or attenuate such neurodegeneration; 5) To see whether this neurodegeneration still occurs in the presence of surgical nociceptive stimuli as previous studies were done in its absence.

## **CHAPTER 2**

## **BACKGROUND**

## 2.1 Foetal Alcohol Syndrome

Foetal alcohol syndrome (FAS) refers to a constellation of physical abnormalities, most obvious in the features of the face and in the reduced size of the newborn, and problems of behaviour and cognition, in children born to mothers who drank heavily during pregnancy (Clarren and Smith, 1978). Rates of FAS in most studies are similar—in the order of 0.5 to 3 cases per 1,000 births.

The defects of FAS may or may not be apparent or easily diagnosed at birth. Although the manifestations of the damage might change with age, FAS never completely disappears and, as with many developmental disabilities, there is no cure, but there may be some amelioration in some individuals (Forrest and du Florey, 1991). FAS do not refer to signs of acute alcohol exposure or withdrawal at birth. Newborns can have blood alcohol levels high enough to affect acutely their central nervous system function and not have FAS (Abel et al., 1983).

Alcohol was also used as an anaesthetic drug in the last century in dental paediatric anaesthesia. It acts on NMDA and GABA receptors. This may be the reason encouraging scientists to investigate the potential neurotoxicity of anaesthetics in the developing brain in nowadays.

Currently used anaesthetics act by modulating the activity of the gamma-aminobutyric acid and glutamate neurotransmitter systems. It has been hypothesised that during synaptogenesis, neurones with N-Methyl-D-aspartate (NMDA) receptors are

sensitive to both over- and under-stimulation. Abnormal inhibition of neuronal activity, either via blockade of the NMDA glutamate receptor or by excessive stimulation of the GABA<sub>A</sub> receptor, triggers neuronal apoptosis. Only a transient disturbance lasting a few hours can trigger apoptotic neurodegeneration (Olney et al., 2002d).

## **2.2 Molecular mechanism of anaesthetics**

The list of CNS transmitters is rapidly expanding. In addition to the classic transmitters (e.g., acetylcholine), endogenous amino acids and peptides may act as neurotransmitters or modulate the action of other neurotransmitters. Furthermore, the postsynaptic action of neurotransmitters may result in the formation of second messengers that mediate changes in neuronal transmission. Limited information is available concerning the relationship between the levels of neuroregulators in the CNS and the inhaled anaesthetics requirement.

### **2.2.1 Acetylcholine**

Inhaled anaesthetics do not alter acetylcholine concentrations in macroscopic structures (e.g., cortex) of rat brain (Ngai et al., 1978) but may increase (Taguchi et al., 1991) or decrease (Keifer et al., 1996) acetylcholine content in specific brain nuclei. Inhaled agents decrease acetylcholine turnover rate, with the magnitude of the decrease varying with the brain region examined (Ngai et al., 1978). Synthesis of acetylcholine in brain is impaired by inhaled agents and is associated with an anaesthetics-induced inhibition of choline uptake (Griffiths et al., 1994). A

hypothesis that depressed cholinergic neurotransmission plays a role in the mediation of the anaesthesia state is supported by the finding that intracerebroventricular injection of hemicholinium-3 (which reduces synaptic levels of acetylcholine) decreases isoflurane MAC\* in rats (Zuker, 1991).

### 2.2.2 GABA

Although rats administered 1 to 4 percent atm halothane for 30 minutes exhibit no change in whole brain GABA content (Arai et al., 1990), anaesthesia may alter GABA levels in selected regions of the brain. Treatment of rat cerebral cortex slices with 3 percent atm halothane inhibits the metabolism and increases the uptake or release of GABA (Cheng et al., 1981). If the accumulation of GABA in inhibitory neurons is associated with an increase in inhibitory activity, the resulting decrease in synaptic transmission might contribute to the anaesthetic state (Cheng et al., 1981). Findings consistent with this hypothesis include the production of the state of general anaesthesia by a GABA analogue (Cheng et al., 1985), and the ability of GABA antagonists to lessen the antinociceptive action of halothane in rodents (Mason et al., 1996).

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\*MAC- **Minimum alveolar concentration** is defined as the concentration of the vapour in the lungs that is needed to prevent movement (motor response) in 50% of subjects in response to surgical (pain) stimulus. If MAC increased by 10 % of its value allows anaesthetising 100% of the subjects. (Miller et al., 1973)

### 2.2.3 Excitatory Amino Acids

A role for excitatory amino acids in anaesthesia is implied by the depression in glutamate-induced neurotransmission by inhaled anaesthetics and the decreased requirements of volatile anaesthetics after administering inhibitors of excitatory amino acid transmission (Hudspith et al., 1997). Anaesthesia does not result from a depletion of excitatory amino acids because rats anaesthetised with halothane have an increased whole brain content of aspartate and glutamate (Arai et al., 1990). It has been suggested that the ability of enflurane to enhance glutamate release from synaptosomes may provide an explanation for clinically observed enflurane-induced convulsion (Hirose et al., 1992).

NMDA (N-methyl-D-aspartic acid) is an amino acid derivative acting as a specific agonist at the NMDA receptor, and therefore mimics the action of the neurotransmitter glutamate on that receptor. In contrast to glutamate, NMDA binds to and regulates the NMDA receptor only, with no effect on other glutamate receptors.

### 2.2.4 Calcium

Calcium has been considered as a neuroregulator because there is evidence that inhaled anaesthetics alter intracellular concentrations of calcium and because changes in intracellular calcium may influence neuronal excitability (Kress and Tas, 1993) (e.g., *via* a calcium-dependent release of neurotransmitter). Inhaled anaesthetics usually (Kress and Tas, 1993) but not always (Miao et al., 1995) increase

resting cytoplasmic calcium concentration, and it has been proposed that the depressant action of halothane in rat hippocampal slices involves enhancement of GABA-mediated inhibition through release of intraneuronal calcium (Mody et al., 1991). In contrast, increases in intraneuronal calcium evoked by various stimuli are often prevented by inhaled agents. The mechanisms for the attenuation of the rise in calcium level by inhaled anaesthetics may depend on the anaesthetic and tissue examined and includes impairment of calcium influx into cells (Kress and Tas, 1993; Miao et al., 1995) and modulation of intracellular calcium release *via* action on inositol triphosphate-dependent pathways (Hossain et al., 1994).

### 2.2.5 Presynaptic Action

A presynaptic site of inhaled anaesthetic action may be implied from electrophysiologic recordings that compare postsynaptic potentials obtained *via* presynaptic stimulation versus those generated by direct application of neurotransmitter. For example, in mouse hippocampal slices, glutamatergic excitatory postsynaptic currents are reduced by 50 percent by -1 MAC halothane, whereas halothane (even at concentrations of -5 MAC) has no effect on the currents induced by bath application of glutamatergic agonists. (Perouansky et al., 1995)

A presynaptic site of action is also implied from studies that demonstrate an influence of anaesthetics on neurotransmitter release (Griffiths and Norman, 1993). Clinical concentrations of inhaled agents reduce depolarization-evoked release of norepinephrine but not acetylcholine from slices of rat cerebral cortex (Brzil et al.,

1989). In the rat striatum, isoflurane and halothane increase spontaneous dopamine release (Mantz et al., 1994) but decrease nicotine-evoked release of dopamine and have no effect on potassium-induced GABA release (Salord et al., 1997). Inhaled agents do not alter basal glutamate release in guinea pig (Miao et al., 1995) or rat (Schlame and Hemmings, 1995) cerebrocortical synaptosomes but are reported to either depress (in the guinea pig) (Miao et al., 1995) or have no effect on (in the rat) (Schlame and Hemmings, 1995) potassium evoked glutamate release from synaptosomes. Thus, the ability of anaesthetics to alter neurotransmitter release depends on the biologic preparation examined, the method employed to evoke neurotransmitter release, the neurotransmitter, and the anaesthetic drug and its concentration.

In addition to an effect on the presynaptic release of neurotransmitter, it is conceivable that inhaled anaesthetics may alter the duration of neurotransmitter action by influencing the reuptake of neurotransmitter into nerve terminals. Halothane and isoflurane inhibit the uptake of choline (Griffiths et al., 1994), 5-hydroxytryptamine, and dopamine (El-Maghrabi and Eckenhoff, 1993) (but not GABA) (Mantz et al., 1997; Miyazaki et al., 1997) by rat brain synaptosomes in a concentration dependent manner. An anaesthetics-induced increase in the uptake of glutamate (Miyazaki et al 1997; Larsen et al., 1997) has been speculated to decrease excitatory transmission and contribute to action of volatile agents.

### **2.2.6 Postsynaptic Action**

There is evidence showing the postsynaptic effects of inhaled anaesthetics on both excitatory and inhibitory neurons. Postsynaptic sites of action of anaesthetics may be studied by application of putative neurotransmitters thought to act directly on postsynaptic membrane receptors (Pocock and Richards, 1993). Depending upon the particular neuronal preparation and neurotransmitter studied, anaesthetics may depress, have little influence, or enhance the postsynaptic response. In the lamprey model, halothane decreases synaptic response to bath applied glutamate to the same extent as the decrease in the excitatory postsynaptic potential, implying a postsynaptic site of action (Yamamura et al., 1997). Halothane and isoflurane reduce the depolarizing responses to iontophoretic applications of acetylcholine or glutamate to dendrites in guinea pig neocortical slices, with the acetylcholine response being depressed more than the glutamate response (Puil and El-Beheiry E, 1990). In contrast, halothane and isoflurane have little or no effect on the response induced by iontophoretic application of GABA to perikaryons in these neocortical slices (Puil and El-Beheiry E, 1990). In hippocampal neurons (Zimmerman et al., 1994) and in neurons dissociated from the nucleus tractus solitarius of rats (Wakamori et al., 1991), volatile anaesthetics enhance the currents produced by application of GABA.

A postsynaptic action is also evident from the ability of clinical concentrations of inhaled agents to modulate current flow through a variety of ion channels found on postsynaptic membranes (Harris et al., 1995). The enhancement neuromuscular blockade by inhaled anesthetics observed in the clinical setting probably results from

a reduced effectiveness of acetylcholine at postsynaptic motor endplate (Wachtel, 1995).

In summary, inhaled anesthetics act on synapses, including afferent axons at the nerve terminal. Inhaled agents alter axonal and synaptic transmission in isolated mono- and polysynaptic neuronal systems and may have both pre- and postsynaptic effects. Clinical concentrations of inhaled agents can depress, leave unchanged or enhance presynaptic neurotransmitter release and the postsynaptic response. The effect depends on the biologic preparation, the frequency of neuronal transmission, the particular neurotransmitter and the anaesthetics examined.

## **2.3. Inhaled anaesthetics action in the CNS**

### **2.3.1 Brain**

Inhaled anaesthetics can act by altering neuronal activity in selected regions of the CNS. Because the brain stem reticular formation plays a role in altering the state of consciousness and alertness and in regulating motor activity, it is often suggested that this structure is an important site of anaesthetics action. The effect of anaesthesia on neuronal activity in the reticular formation is variable and can be increased, unchanged, or decreased, depending on the agent and the neuronal unit examined (Ogawa et al., 1992).

General anaesthetics interrupt transmission in the CNS at sites other than the reticular formation. Clinical concentrations of inhaled agents may alter spontaneous

and evoked activity of mammalian cerebral cortex (Berg-Johnsen and Langmoen, 1990) and hippocampus (MacIver and Roth, 1988). Although inhaled anaesthetics usually depress the excitability of brain neuron, situation can be found in which anaesthetics enhance excitability (MacIver and Roth, 1988). In addition, inhibitory transmission may be influenced by volatile agents. Volatile anaesthetics may either enhance the amplitude and duration of inhibitory postsynaptic currents or selectively depress inhibitory postsynaptic potentials (Fujiwara et al., 1988). Neuronal pathways between various brain regions and consisting of both excitatory and inhibitory components may also be influenced by inhaled agents. The transfer of sensory information from the thalamus to certain cortical regions is thought to be particularly sensitive to anaesthetics (Angel, 1993).

### **2.3.2 Spinal cord**

Both excitatory and inhibitory neurotransmission in mammalian spinal cord may be altered by inhaled anaesthetics (Collins et al., 1995). Anaesthetics effects depend on the concentration of the agent and on the particular spinal cord pathway examined. Inhaled anaesthetics alter responses of spinal dorsal horn to both noxious and nonnoxious stimuli (Collins et al., 1995). Volatile anaesthetics also depress the F-wave amplitude (a measure of excitability of spinal motor neurons) in rats (Rampil and Kings, 1996) and humans (Zhou et al., 1997). Thus, both a reduction in the sensory processing and an inhibition of motor neuron excitation are likely to be involved in anaesthetic-induced lack of movement in response to a noxious stimulus. In addition to a direct action on the spinal cord, inhaled agents may indirectly

influence activity of spinal neurons by altering the tonic input received from descending modulatory systems from the brain (Collins et al., 1995; Borges and Antognini, 1994).

### **2.3.3 Glutamatergic and GABAergic transmission systems in CNS**

Once fully functional, a network of glutamatergic neurones forms virtually the entire basis for excitatory transmission in the CNS - functioning N-methyl-D-aspartate (NMDA) receptors are essential for survival (Forrest et al., 1994; Li et al., 1994). The initial categorisation of glutamate receptors is into ionotropic ion channels (NMDA, AMPA and KA) and metabotropic 2nd messenger systems (Dingledine and McBain, 1999), whilst the main ligands involved are glutamate, aspartate and glycine. As with most body systems vulnerable to over-activation, the glutamatergic system is extensively regulated: by local glial cells (Oliet et al., 2001) and molecules such as glutamate,  $Mg^{2+}$ ,  $Zn^{2+}$ ,  $H^+$  and the essential co-agonist glycine (Danysz and Parsons, 1998). The NMDA receptor has been associated with cognitive impairment (Malhotra et al., 1996), pain (Nakata et al., 2000), drug neurotoxicity (Huber et al., 2001), schizophrenia (Farber et al., 1995), synaptic plasticity (Kato et al., 1999; Philpot et al., 2001), protein synthesis (Scheetz et al., 2000) and dependency states (Tsai and Coyle, 1998; Tokuyama et al., 2001).

Aminobutyric acid (GABA) is the principle inhibitory neurotransmitter in the CNS (Olsen and DeLorey, 1999). GABAergic neurones are widespread throughout the nervous system, and hence any systemic disruption (e.g. anaesthesia or

anticonvulsive therapy) could have enormously complex ramifications (Davis and Wu, 2001). One irregularity in the supposition that GABA is inhibitory does exist in specialised electrophysiological conditions in the embryo, where GABA in fact becomes excitatory (Olsen and DeLorey, 1999).

## **2.4 Neurodevelopment**

### **2.4.1 Normal neurodevelopment**

Normal neurodevelopment is a carefully regulated sequence of events including proliferation, differentiation, migration and synaptogenesis (Butler, 1999). Glutamate is thought to have a role in all of these processes (Ikonomidou and Lechoslaw, 2002), for example high concentrations of glutamate at migration target zones suggest a role as a neuronal chemoattractant (Behar et al., 1999) along with the NMDA receptor used to detect it (Komuro and Rakie, 1993). The intriguing finding of specific NMDA receptor subtypes (e.g. NR2B and NR2D) in different anatomical regions may shed light on the precise nature of migration control (Behar et al., 1999). From work by the same group, it is also apparent that different species employ different mediators in migration control – currently either GABA (rats) or glutamate (mice) (Behar et al., 2001).

### 2.4.2 Synaptogenesis

Synaptogenesis (the brain growth spurt) is a period of a rapid establishment of synapses, characterised by a high level of physiological cell death (up to 1% (Olney et al., 2002b)). This includes the formation of extensive corticothalamic and thalamocortical projections (Molnar and Blakemore, 1995). Despite the immense complexity of inter-species embryology, it has been shown that comparisons can be made because milestones in neurodevelopment tend to occur in the same sequence (Clancy et al., 2001). This permits an extrapolation of the period of peak synaptogenic activity from the 7 day old rat pup (Olney, 2002a) to a 0-8 month old human being (Ikonomidou et al., 1999; Jevtovic-Todorovic et al., 2003a). However, based on analysis of NMDA receptor subtypes, it is more probable that humans experience an extended period of synaptogenesis which from the beginning of the 3rd trimester of pregnancy to several years old (Dobbing and Sands, 1979; Jevtovic-Todorovic et al., 2003a).

Synapses are morphologically distinct sub cellular junctional structures, composed of a presynaptic terminal, a postsynaptic target and the synaptic cleft aligning pre- and post-synaptic specializations (Cowan et al, 2001; Pappas et al, 1972). The presynaptic terminal is characterized by a cluster of synaptic vesicles surrounding the electron-dense membrane specializations. The postsynaptic site contains densely packed ion channels and signal transduction molecules. Both the pre- and post-synaptic specializations display variable appearances depending on the organisms and neuronal types.

Anesthetics may disrupt normal synaptic transmission by interfering with the release of neurotransmitter from the presynaptic nerve terminals into the synaptic cleft by altering the reuptake of neurotransmitter to receptor sites on the postsynaptic membrane, or by influencing the ionic conductance change that follows activation of the post-synaptic receptor by neurotransmitter.

## 2.5 Apoptosis

Apoptosis is a highly regulated form of cell death that was first characterised by Kerr (Kerr et al, 1972). It is also named as the programmed cell death and involves a series of biochemical events that lead to a variety of morphological changes, including blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (Bursch et al, 2000). Processes of disposal of cellular debris whose results do not damage the organism differentiate apoptosis from necrosis.

Redundant or unsuccessful neurones are removed from the developing CNS by this process. In contrast to necrosis, which is a form of traumatic cell death that results from acute cellular injury, apoptosis, in general, confers advantages during an organism's life cycle. Between 50 billion and 70 billion cells die each day due to apoptosis in the average human adult. For an average child between the ages of 8 and 14, approximately 20 billion to 30 billion cells die a day. In a year, this amounts to the proliferation and subsequent destruction of a mass of cells equal to an

individual's body weight (Werlen et al., 2003). In addition to its importance as a biological phenomenon, defective apoptotic processes have been implicated in an extensive variety of diseases. Excessive apoptosis causes hypotrophy, such as in ischemic damage, whereas an insufficient amount results in uncontrolled cell proliferation, such as cancer (Wang et al., 1995).

### **2.5.1. Apoptosis in the developing nervous system**

Control of physiological cell death (PCD) in the immature CNS is currently thought to be governed by the neurotrophic hypothesis – whereby neurones which fail to reach their survival promoting synaptic targets (Sherrard and Bower, 1998) initiate a specialised form of cell suicide secondary to withdrawal of environmental trophic support (Young et al., 1999) (*via* both neurotrophins and electrical stimulation) (Brenneman et al., 1990). Due to the complex divergent and convergent nature of the “survival pathway” many ligands and mechanisms are involved in maintaining neuronal survival. The cytosol and mitochondria of neurones field a balanced assortment of molecules which are either anti-apoptotic (e.g. Bcl-2 and cAMP response binding protein) or pro-apoptotic (e.g. Bad, Bax and the caspase family) which determine cell fate. Bcl-2 and its associated peptides are thought to be particularly important in the developing CNS (Yuan and Yanker, 2000), as evidenced by the high levels of expression in the neonate and the fact that experimental over-expression of Bcl-2 can both override lack of trophic support (Garcia et al., 1992), and even prevent PCD altogether (Martinou et al., 1994). A variant of Bcl-2 (Bcl-XL)

may have a specialised role in maintaining developing neurones before they have found their synaptic targets (Motoyama et al., 1995).

## **2.5.2 Apoptosis pathway**

Two major apoptotic pathways within this have been studied extensively: the intrinsic and extrinsic pathway. The intrinsic apoptotic pathway involves the down-regulation of anti-apoptotic proteins from the Bcl-2 family (e.g Bcl- 2) and various changes at the level of the mitochondria which include: an increase in mitochondrial permeability due to the translocation of Bax from the cytosol to the outer membrane of the mitochondria, release of cytochrome c from the mitochondria and formation of the apoptosome complex (cytochrome c, apoptotic protease activating factor 1 (APAF -1) and procaspase-9) which activates caspase-9. Subsequently, the executioner caspases-3,-6 and -7 are activated, culminating in cell death (Hengartner, 2000).

### **2.5.2.1 Markers of the Common Apoptotic Pathway**

Caspase-3 is considered to be the major executioner in neuronal apoptosis (Yakovlev and Faden, 2004; Yakovlev et al., 2001) and caspase-3 gene activity and protein is down regulated during brain development, which parallels the repressed apoptotic potential in the mature brain (Yakovlev et al., 2001). Hence, in addition to anaesthetics enhancing apoptotic neurodegeneration by disrupting physiological synaptic activity, which in turn may reduce neurotrophin-initiated survival signals,

the neurons in neonatal (in comparison to adult) rodents may be more vulnerable to death due to relative up-regulation of vital components of the apoptotic machinery (e.g.caspase-3 activity).

### **2.5.2.2 Markers of the intrinsic apoptotic pathway**

The release of cytochrome c from the mitochondria into the cytosol is one of the principal components of intrinsic pathway activation. Once released, cytochrome c binds to Apaf-1 and activates the apoptosome. However, cytochrome c translocation does not necessarily determine cell fate, since activation of the apoptosome is also regulated by other factors (Yakovlev and Faden, 2004; Cozzolino et al., 2004).

### **2.5.2.3 Markers of the extrinsic apoptotic pathway**

A representative marker of extrinsic pathway involvement is the activation of caspase-8.

The probability of extrinsic pathway involvement is in line with recent findings suggesting that the anaesthetic cocktail of isoflurane, N<sub>2</sub>O and midazolam induces neuronal death in the developing rat brain by both apoptotic pathways (Yon et al., 2005). In that setting, the intrinsic pathway was involved within 2 hours of anaesthetic exposure, whereas 4 hours was needed to activate the extrinsic pathway.

## 2.6 Types of neurodegeneration

### 2.6.1 Excitotoxic neurodegeneration

The term 'excitotoxicity' was coined to describe the neuronal toxicity of high glutamate concentrations at the NMDA receptor (Olney, 1969). Following an initial insult (be it trauma) (Pohl et al., 1999), hypoglycaemia (Frankiewicz et al., 2000), and ischaemia (Martin et al., 1998), it is thought that neurones switch to less efficient anaerobic respiration. The ensuing lack of ATP deranges ion pump function – specifically resulting in reversal of the pumps designed to clear glutamate from the synapse (hence producing high synaptic glutamate concentrations) (Jevtovic-Todorovic and Olney, 2003b). This is the mechanism by which neurones can be damaged long after the insult has subsided. Specific isoforms of protein kinase C have been shown to modulate this process (Wagney et al., 2001) - this is almost certainly linked to the NMDA receptor's ability to regulate intracellular  $\text{Ca}^{2+}$  accumulation (Kubo et al., 2001). At the genetic level, high levels of glutamate stimulation upregulate genes connected to convulsion and ischaemia (e.g. zif/268 and c-jun) (Beas-Zárate et al., 2001).

### 2.6.2 Neurotoxic neurodegeneration

Assessment of the potential neuroprotective effect of NMDA receptor blockade in adult rats showed that excitotoxic damage could be reduced by agents such as MK801 (a powerful experimental NMDA receptor antagonist). However, the very same experiments (Pohl et al., 1999) highlighted a major predicament with use of

MK801. It was found that this drug caused cells of the posterior cingulate and retrosplenial cortices to acutely degenerate. Subsequently, both N<sub>2</sub>O (Jevtovic-Todorovic et al., 1998) and ketamine (Nagata et al., 2001) have been shown to have this undesirable property. It was hypothesised that in this specific area of the brain glutamate is in fact inhibitory – both by regulation of inhibitory tone of excitatory inputs and local autoregulation by GABAergic neurones (Jevtovic-Todorovic and Olney, 2003). In terms of CNS function, this neurotoxicity disrupts the extended hippocampal circuit of memory (of which the PC/RSC is a component) (Aggleton and Brown, 1999), produces the psychomimetic side effects seen with clinical use of NMDA receptor antagonists (Farber et al., 1995), and may be a predisposing factor to adult schizophrenia (Malhotra et al., 1996; Newcomer and Krystal, 2001).

### **2.6.3 Neurodegeneration in neonates**

In 1999, data were published stating that use of NMDA receptor antagonists in neonatal rats produced specific patterns of neurodegeneration (distinct from glial cells) (Ikonomidou et al., 1999). On electron microscopy, this neurodegeneration was identical to apoptotic cell death, and most evident in the laterodorsal thalamic nucleus, one of the areas of the brain implicated in learning and memory (Goen et al., 2002). This phenomenon has since been demonstrated in other brain regions with other drugs (Monti and Contestabile, 2000).

Jevtovic-Todorovic and co-workers showed in their later work showed that neonatal rats are vulnerable to harmful side effects of anaesthesia during the synaptogenic period. They demonstrated up to a 68 fold increase in the number of degenerating neurones above baseline in areas such as the laterodorsal and anteroventral thalamic nuclei (and to some extent layer II of the parietal cortex) after exposure to anaesthetic agents (Jevtovic-Todorovic et al., 2003a) – which resulted in a functional neurological deficit in behaviour tests later in life. Specifically, the GABAmimetic gas isoflurane (Gyulai et al., 2001), produced dose-dependent neurodegeneration in its own right, with synergistic neurodegeneration with the successive addition of midazolam (a double GABAmimetic cocktail) and then N<sub>2</sub>O (a triple cocktail) (Jevtovic-Todorovic et al., 2003a). This process has been shown to occur with exposure to GABAmimetics in areas other than anaesthesia, such as anticonvulsant therapy and maternal drug abuse in rats (Bittigau et al., 2002; Farber and Olney, 2003).

A clinical manifestation of this type of neurodegeneration is detected in 1 to 2 infants per 1000 livebirths as Fetal Alcohol Syndrome (FAS) (Moore and Persaud, 1998) - characterised by abnormal facial features, microencephaly and mental retardation (Olney et al., 2002c). It is thought that binge drinking by pregnant mothers produces very high levels of ethanol (a dual GABAmimetic and NMDA receptor antagonist (Farber and Olney, 2003)) in the fetal brain, which in turn triggers the type of neurodegeneration discussed above (Ikonomidou et al., 2000). It is worth noting that this mechanism of action closely resembles that of current anaesthetic procedures.

Both the NMDA receptor and the GABA receptor have been linked to the mechanism of this apoptotic cell death. Activation of the NMDA receptor can enhance neuronal survival both post-translationally (*via* the PI (3) K-Akt pathway (Brunet et al., 2001)) and genomically (*via* intermediate early genes (Wisden et al., 1990)). Indeed, the NMDA receptor appears to control several key molecules that are either anti-apoptotic in their own right or produce an anti-apoptotic effect by regulating other systems, such as NF- $\kappa$ B (Lipsky et al., 2001; Bhakar et al., 2002) (which is pro-inflammatory to glial cells (Hardingham and Bading, 2003)) and CREB (Hardingham et al., 2002) (which controls a self-supporting pro-survival cascade (Hardingham and Bading, 2003)). Also, the electrical stimulation cells needed to survive is thought to be  $\text{Na}^+$  and  $\text{Ca}^{2+}$  mediated *via* the NMDA receptor. On the other hand, high concentrations of GABA (e.g. with use of the anticonvulsive valproate) also directly suppresses neurotrophins such as BDNF, and attenuates other cell survival cascades such as ERK, Akt and c-Raf (Bittigau et al., 2002). It would be reasonable to suppose that sustained blockade of the NMDA receptor, combined with facilitation of GABAergic transmission, and could shift the cell balance towards pro-apoptosis.

#### **2.6.4 Characterising neurodegeneration**

The Cupric-Silver technique (DeOlmos Silver Staining) has repeatedly been shown to be excellent for highlighting the density and distribution of neurodegeneration (Beltramino et al., 1993; Jevtovic-Todorovic et al., 2003b). The process highlights argyrophilia (a generalised CNS response to injury (O'Callaghan and Jensen, 1992)) to reveal cumulative neurodegeneration, so issues surrounding the small timeframe of

marker expression, as in other techniques identifying gene products and enzyme activation, do not apply (DeOlmos and Ingram, 1971). The downfall of silver staining is the difficulty in accurately quantifying neuronal injury from fragments.

At the end of the apoptotic signalling cascade, caspase-9 activates caspase-3 (a cysteine protease), and thus caspase-3 is a marker of those cells that are downstream of the apoptotic commitment point. While broadly paralleling silver staining, caspase-3 immunohistochemistry is superior for both quantification purposes and characterisation of physiological cell death (Olney et al., 2002b).

C-fos is one of the immediate early genes that have a role in linking cytoplasmic events to nuclear gene transcription (Walton et al., 1998). As a regulator of gene expression, c-Fos indicates a state of neuronal activation, a result of several possible different external stimuli, including apoptotic cell death (Dragunow and Preston, 1995) and pain (reviewed in Duckhyun and Barr, 1995). C-fos has previously been shown to be a sensitive marker of the neurotoxicity of NMDA receptor antagonists in adult rats (Ma et al., 2002) and is valid for assessment of NMDA receptor activation (Hasegawa et al., 1998).

All intrinsic and extrinsic Apoptotic Pathway involves a cascade of events that culminate in apoptotic cell death. Although each pathway is characterised by the input of a multitude of proteins, caspase-3, both cytochrome C and caspase-9, and caspase-8 are characteristic of the activation of the common, intrinsic and extrinsic pathway respectively.

## 2.7 Neurons and brain structures involved in learning and memory

Neurons are the principle cells within the central nervous system (CNS) and based on morphology, location, connectivity and chemistry, there are hundreds, if not thousands of individual neuronal cell types (Masland, 2004). Synaptic transmission between different neurons provides the nervous system with its remarkable functional capacity and neurotransmitters are the diverse chemical substances that participate in such process. Thus far, more than a hundred neurotransmitters have been implicated in synaptic transmission and some of the neurons can be classified as glutamatergic, GABAergic, dopaminergic and cholinergic neurons based on the neurotransmitters they produce (Steven and Hyman, 2005).

Glutamate is the major excitatory neurotransmitter in the CNS of mammals (Shepherd et al., 2007). Although best known for its role at synapses in the mature nervous system, glutamate is also of vital importance during development where it regulates neurogenesis, neurite outgrowth, synaptogenesis and long-term potentiation (LTP) (Suzuki et al., 2006; Mattson, 1996; Kullmann and Lamsa, 2007). LTP is the ability to execute cumulative increases in synapse strength that lead to long-term memory (Lynch, 2004). However, these glutamate-mediated processes could potentially be inhibited by general anaesthetics (Lingamaneni et al., 2001) possibly *via* a process that involves site-specific phosphorylation (Snyder et al., 2007).

In contrast to glutamate, Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the CNS of mammals and GABAergic neurons also play a part in learning and memory formation and consolidation (Lüscher and Keller, 2004; Das, 2003) and activation of gamma-aminobutyric acid type A (GABA<sub>A</sub>) receptors has been shown to downregulate memory consolidation processes (Brioni, 1993). Both glutamatergic and GABAergic neurones are widely distributed within the CNS (Foster and Kemp, 2005). The hippocampus and neocortex (including cingulate cortex) are crucial brain structures for learning and memory (Squire et al., 2004; Wiltgen et al., 2004; Frankland and Bontempi, 2005; Blum et al., 2006), which contain mainly glutamatergic and GABAergic neurons (Sem'yanov, 2005; DeFelipe et al., 2002). So far behavioural studies in animals have suggested permanent deficits in hippocampus-dependent learning post anaesthetic exposure (Sall et al., 2009; Satomoto et al., 2009). Therefore, depletion of glutamatergic and GABAergic neurons in the hippocampus and neocortex may well contribute to learning and memory deficits observed after anaesthetic exposure.

Acetylcholine (ACh) has been implicated in cognitive processing, arousal, and attention in the brain (Picciotto et al., 2002). Studies thus far have also demonstrated the importance of cholinergic system in learning and memory (Hasselmo, 2006). Blockade of muscarinic cholinergic receptors impairs the encoding of new memories in human subjects (Atri et al., 2004; Hasselmo and McGaughy, 2004). Conversely, drugs that activate nicotinic receptors enhance the encoding of new information (Buccafusco 2005). In humans memory deficits associated with Alzheimer's disease and normal aging can be attributed to degeneration of

cholinergic basal forebrain neurons, especially in the nucleus basalis of Meynert (Muir, 1997; Wu et al., 2005). Moreover, aged rats that demonstrate impairments in learning and memory tasks are also characterised as having smaller numbers of neurons and greater atrophy of surviving cholinergic neurons in the basal forebrain (Koh and Loy 1988; Koh et al. 1989). Within the basal forebrain cholinergic system, cholinergic neurons primarily project to the hippocampus and neocortex (Reiner and Fibiger 2001) and inhalational anaesthetic agent, isoflurane, has been shown to suppress the release of Ach in the rat cerebral cortex (Shichino et al. 1998) and dorsal hippocampus (Whittington and Virag 2006) *in vivo*. However, whether such suppression is due to anaesthesia-induced cholinergic neuron death in the basal forebrain remains elusive.

In the field of catecholamine transmitters, dopamine pathways have been recognised as playing a critical role in cognition and emotion, and recently there has been a large increase in the experimental evidence for a role of dopamine in learning and memory (Packard and Knowlton 2002; Jay, 2003; González and Feria, 2008; Olvera-Cortés et al., 2008; Nieoullon and Coquerel, 2003). Neurochemically the hippocampus, neocortex and striatum receive midbrain dopaminergic projections from mainly the substantia nigra and ventral tegmental area and release of dopamine in these regions can induce an enhancement of long-term potentiation (Jay, 2003). Animal studies have shown that intra-hippocampal or intra-striatal injections of various dopamine receptor agonists could enhance learning and memory consolidation (Packard and White, 1991; Wilkerson and Levin, 1999; Packard et al., 1996), whereas the depletion of dopamine leads to the opposite

(Wilkerson and Levin, 1999; Roeltgen and Schneider, 1994; Adriani et al., 1998). In Parkinson's disease, cell death in the substantia nigra leads to a loss of dopaminergic output (Schapira, 2009) and behavioural studies in patients with Parkinson's disease have demonstrated that the circuitry required for learning the stimulus-response associations is disrupted (Knowlton et al., 1996). Several *in vivo* studies thus far have indicated that anaesthetics could potentially have an impact on dopaminergic neurons as the level of dopamine in the striatum could be increased by anaesthetic exposure (Adachi et al., 2000; Adachi et al., 2005). Also, general anaesthetics with the neurotoxin, 6-hydroxydopamine could significantly increase the extent of nigrostriatal dopaminergic neuronal loss (Datla et al., 2006). Therefore, anaesthetic exposure during the period of rapid synaptogenesis may lead to increased dopaminergic neuronal cell death in the substantia nigra and ventral tegmental area, which could contribute to the learning and memory deficits observed later in life.

## 2.8 Preconditioning

The phenomenon of preconditioning was first described by Murry and has since evoked great interest because of its potential as a pre-emptive treatment against cell, tissue and organ damage (Murry et al, 1986; Grimm et al, 2005). There is no established definition for preconditioning but it can be thought of as the brief presentation of a sub-lethal stressor providing protection, presumably by rendering vulnerable tissue more "tolerant" to a greater injury (Yellon et al, 1998). This occurs due to transient augmentation of endogenous adaptive responses that induce protection (Gidday, 2006).

Adaptation to a preconditioned state is biphasic, namely acute and delayed. Acute preconditioning occurs within minutes but is relatively short lived, providing protection as a result of adaptation of membrane receptors, ion channel permeabilities, protein phosphorylation and other post-translational modifications (Gidday, 2006). Delayed or “classical” preconditioning requires many hours or days to fully manifest. These late effects are the result of gene up-or down-regulation (Steiger and Hanggi, 2007) and are dependent on denovo protein synthesis (Meldrum, 1997). Therefore delayed preconditioning can be thought to genetically “reprogramme” the cell’s response to a particular insult (Gidday, 2006). Commonly known preconditioning agents include hypoxia, ischaemia and pharmacological agents.

### **2.8.1. Cross tolerance**

While the original studies involved hypoxia or ischaemia, the early elucidation of the molecular mechanisms involved in the process of HI preconditioning raised the possibility of manipulating those targets pharmacologically. Therefore, a plethora of agents have been investigated; the most clinically promising of these are anaesthetic agents because of their possible application in elective, high-risk surgical patients. The putative therapeutic utility of anaesthetic preconditioning, especially for injury to the CNS, exceeds that induced by a strategy involving hypoxia and/or ischaemia because the latter strategy has a narrower safety margin. These studies introduced the notion of “cross tolerance”, where preconditioning against HI injury may be induced not only by hypoxia or ischaemia but also by a diverse variety of metabolic

or physical stressors, or pharmacological agents. However, the cross-induction of pharmacological-induced tolerance may differ in efficacy compared with hypoxic or ischaemic preconditioning and the protection is often governed by multiple mechanisms.

### **2.8.2 Anaesthetic preconditioning**

A notable form of pharmacological preconditioning is anaesthetic preconditioning where certain inhalational anaesthetics, such as halothane, isoflurane and sevoflurane, have been demonstrated as effective inducers of acute and delayed preconditioning (Piriou et al., 2002). Anaesthetic preconditioning has also been successfully trialled in humans and is a promising therapy for surgical patients at high risk of developing ischaemic complications. Particularly relevant to this study, neuroprotection by preconditioning with xenon has recently been demonstrated in our laboratory (Ma et al., 2006), although the underlying molecular mechanisms remain to be elucidated.

Pre-exposure to xenon for 2 hours resulted in a decrease in the release of lactate dehydrogenase (a marker for neuronal injury) following oxygen-glucose deprivation of cultured neuronal-glial cells (Ma et al., 2003a). This delayed preconditioning effect of xenon indicates a role for the anaesthetic in protecting against neonatal asphyxia. What remains to be elucidated is whether these neuroprotective properties are observed when neuronal death is induced by other insults, such as anaesthetics, during the period of synaptogenesis.

### 2.8.3 Hypoxic/Ischaemia preconditioning

Hypoxic preconditioning refers to a period of hypoxia followed by a period of time, often between 1 to 2 days, during which there is protection from an otherwise lethal insult (Sharp et al., 2004). Like ischemic preconditioning, it requires the synthesis of new RNA and protein, similar to the mechanism of xenon preconditioning (a reference needed). Hypoxia – induced tolerance in the brain has been reported to be blocked by the presence of inhibitor of RNA and protein synthesis (Gage and Station, 1996). This suggests that an increase in transcription and translation are necessary for protection. Recent studies have demonstrated that hypoxia can damage mitochondrial DNA in brain cells (Wang et al., 2000) which stimulate protective mechanisms. These could play an important role in the cellular protection afforded by hypoxic preconditioning. Indeed, a study by (Gidday et al , 2005) first showed that exposure of neonatal rat pups to hypoxia alone (8% oxygen for 3 hours) provided a protection against injury induced by combined hypoxic/ischemia, as well as several other type of injury such as oedema and seizures (Gage and Station, 1996; Miller, 2001).

## 2.9 Neuroprotection by xenon

Xenon, deriving its name from the Greek for “stranger”, is an inert and extremely rare noble gas, constituting no more than 0.0875 parts per million of the atmosphere. Xenon was first demonstrated to possess anaesthetic properties in humans in 1951 by Colleen and Gross. It is a small apolar atom with a low blood/gas

partition coefficient; it is able to easily pass through the blood brain barrier and produce a rapid onset and offset of action (Goto et al., 1998), an advantageous property for an inhalational anaesthetic drug. Xenon does not affect heart rate, mean arterial blood pressure (Dingley et al., 2001) or cardiac contractility (Luttropp et al., 1993) nor does it impair the neurohormonal stress response to surgery (Burov et al., 1995). Animal studies conducted by (Lane et al., 1980) demonstrated xenon to be devoid of any fetotoxicity. Thus, its safety and efficacy profile in clinical anaesthesia appears unrivalled; its scarcity in the atmosphere, and hence the substantial expense involved in its purification, is the only factor limiting its widespread use (Sanders et al., 2003).

Franks and co-workers, working in this laboratory, were the first to describe in a seminal paper that xenon is a potent antagonist of the NMDA receptor (Franks et al., 1998). In a series of *in vitro* studies xenon reduced injury in a mouse neuronal –glial cell co-culture induced by either NMDA, glutamate, oxygen deprivation or oxygen-glucose deprivation(OGD) (Wilhelm et al., 2002); this finding has been corroborated with the results of others studies in a different neural crest cell line (Petzalt et al., 2004).

The neuroprotective effects of xenon have also been demonstrated *in vivo* models of acute neuronal injury involving administration of excitotoxins to rats (Ma et al., 2002), anaesthetics-induced apoptotic neurodegeneration in neonate, cardiopulmonary bypass in rats (Ma et al., 2003b), middle cerebral artery occlusion

in mice (Homi et al., 2003), cardiac arrest in pigs (Schmidt et al., 2005), and hypoxia-ischaemia in neonatal rats (Ma et al., 2006).

Xenon is a more efficacious neuroprotective agent than gavestinel or dizocilpine (MK 801) (Ma et al., 2005b), two other NMDA antagonists that have been clinically tested. Importantly, xenon lacks the toxicity that afflicts other NMDA receptor antagonists (Ma et al, 2002). These studies establish that xenon has a potent neuroprotective effect once injury has occurred. Furthermore, xenon is able to prevent neurotoxic damage caused by other NMDA antagonists (Nagata et al, 2001).

## **2.10 Target protein in the pathway of protect apoptosis**

### **2.10.1 Bcl-2 family**

Bcl-2 closely related anti-apoptotic homologues such as Bcl-x exert control of mitochondrial permeability by stimulating ADP/ATP exchange, stabilizing the mitochondrial inner transmembrane potential, and preventing the opening of a permeability transition pore that enables the release of cytochrome c. There are similarly structured proapoptotic Bcl-2 family members such as Bax or Bak that can promote mitochondrial permeability *via* their direct effects on the mitochondria or indirectly through inhibition of Bcl-2 (Dejean et al, 2006). All of these Bcl-2 family members are present in outer mitochondrial membranes as dimmers with a structure analogous to that of diphtheria toxin where they directly control membrane permeability in ion channel fashion (Martinez-Caballero et al., 2005). Likewise, in

mammals similar cytoplasmic proapoptotic Bcl-2 family members such as Bad or Bik have been identified with only limited homology to Bcl-2. Following apoptotic stimulation they translocate to the mitochondria and block the protective regulatory activity of Bcl-2 by binding to the conserved Bcl-2 surface. A third type of Bcl-2 family protein is exemplified by the cytoplasmic protein BID which has limited sequence homology to Bcl-2. When BID is cleaved by activated caspase-8, it assumes a structure like Bcl-2, translocates to the mitochondria, and independently inserts and induces channel formation in the membrane (Desagher et al., 2000; Cory et al., 2003; Green et al., 2004).

### **2.10.2 Cytochrome c**

There is considerable evidence that disruption of mitochondrial functions (e.g. loss of transmembrane potential, permeability transition (PT) and release of cytochrome c leading to impaired electron transport) are important events in many apoptotic cell deaths. The release of cytochrome c induced by a variety of death stimuli results in the activation of a complex of apoptosis-activating factor 1 (Apaf-1) and caspase-9, leading to the cytochrome c dependent processing of pro-caspase-3 and apoptosis in a number of scenarios. There is evidence that caspase-3 activation can also occur independently of mitochondrial cytochrome c release. For example, two cell type-dependent death pathways emanating from the Fas receptor have been discovered. In type II cells, apoptotic cell death was inhibited by Bcl-2 and depended on the loss of mitochondrial functions; whereas in type I cells, apoptosis (involving caspase-3

activation) was not blocked by Bcl-2 even though Bcl-2 prevented disruption of mitochondrial functions including cytochrome c release.

In multiple myeloma cells, there exist cytochrome c-dependent and -independent mechanisms of apoptosis, both of which involve caspase-3 activation. Once caspase-3 is activated, downstream death substrates are cleaved irrespective of the involvement of cytochrome c. But caspase-3 might also amplify the upstream death cascade, including cytochrome c release from mitochondria, by cleaving Bcl-2, converting it from an anti-apoptotic to a pro-apoptotic protein. Together these results indicate that mitochondria certainly play a role in apoptosis, but also mean that in some situations caspase-3 is activated when the mitochondrial step is completely bypassed. Furthermore, the release of cytochrome c can theoretically occur downstream of caspase-3 in some instances, and apoptotic cell death does not always involve the cytochrome c-dependent activation of caspase-3. Curiously, the introduction of some pro-apoptotic proteins into cells causes death independently of caspases. Enforced expression of pro-apoptotic members of the Bcl-2 family (e.g. Bax) kills mammalian cells, accompanied by membrane alterations and DNA condensation, but without DNA fragmentation and caspase activation. These studies on the expression of Bax like proteins are consistent with the conclusion that DNA fragmentation requires functional caspase-3 and, importantly, imply that not all morphological changes in dying cells require caspases. The fact that Bax can cause cytochrome c release and promote mitochondrial PT or puncture the mitochondrion in a caspase-independent fashion further emphasizes the importance of the

disruption of mitochondrial functions in apoptosis ( Jiang et al., 2004; Liu et al., 1996; Zou et al., 1997; Li et al., 1997; Yang et al., 1997).

### **2.10.3 P53**

P53 is a transcription factor that regulates the cell cycle and hence functions as a tumor suppressor. It is important in multicellular organisms as it helps to suppress cancer. P53 has been described as "the guardian of the genome", "the guardian angel gene", or the "master watchman", referring to its role in conserving stability by preventing genome mutation. P53 has many anti-cancer mechanisms: It can activate DNA repair proteins when DNA has sustained damage. It can also hold the cell cycle at the G1/S regulation point on DNA damage recognition (if it holds the cell here for long enough, the DNA repair proteins will have time to fix the damage and the cell will be allowed to continue the cell cycle). It can initiate apoptosis, the programmed cell death, if the DNA damage proves to be irreparable.

P53 is also a pivotal molecule regulating the death of neurons both after acute injury and during the development. The p53 tumour suppressor gene is involved in the regulation of apoptosis in a number of different paradigms. As a tumour suppressor, the best understood function for p53 is to mediate cell cycle arrest or apoptosis after DNA damage, thereby preventing the propagation of damaged cells (Ko et al., 1996). Aberrant cell cycle regulation during development, such as the widespread CNS defect in Rib null transgenic mice, evokes a p53-mediated apoptosis (Clarke et al., 1993; Jacks et al., 1994; Lee et al., 1993).

#### **2.10.4 MAP Kinase and phosphate MAP Kinase**

Mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens) and regulate various cellular activities, such as gene expression, mitosis, differentiation, and cell survival/apoptosis. The MAPK/ERK pathway is a signal transduction pathway that couples intracellular responses to the binding of growth factors to cell surface receptors. This pathway is very complex and includes many protein components (Orton et al., 2005). Protein phosphorylation presents a mechanism by which the activity of numerous proteins in the form of enzymes, receptors, transporters, docking and scaffolding proteins is switched on or off across a wide array of biological processes from cell growth and differentiation through apoptosis to disease. Human genome may encode as many as 2000 protein kinase to phosphorylate amino acid residues serine, threonine and tyrosine within these proteins in various tissues, cell lines and organelles.

### **2.11 Central pain pathway**

The pathways that carry information about noxious stimuli to the brain, as might be expected for such an important and multifaceted system, are also complex. It helps in understanding this complexity to distinguish two components of pain: the sensory discriminative component, which signals the location, intensity, and quality of the

noxious stimulation and the affective motivational component of pain—which signals the unpleasant quality of the experience, and enables the autonomic activation that follows a noxious stimulus (the classic fight-or-flight reaction).

The discriminative component is thought to depend on pathways that target the traditional somato sensory areas of cortex, while the affective motivational component is thought to depend on additional cortical and brainstem pathways.

Pathways responsible for the discriminative component of pain originate with other sensory neurons, in dorsal root ganglia and, like other sensory nerve cells the central axons of nociceptive nerve cells enter the spinal cord *via* the dorsal roots. When these centrally projecting axons reach the dorsal horn of the spinal cord, they branch into ascending and descending collaterals, forming the dorsolateral tract of Lissauer. Axons in Lissauer's tract typically run up and down for one or two spinal cord segments before they penetrate the gray matter of the dorsal horn. Once within the dorsal horn, the axons give off branches that contact neurons located in several of Rexed's laminae (these laminae are the descriptive divisions of the spinal gray matter in cross section, again named after the neuroanatomist who described these details in the 1950s).

The axons of these second-order neurons in the dorsal horn of the spinal cord cross the midline and ascend all the way to the brainstem and thalamus in the anterolateral (also called ventrolateral) quadrant of the contralateral half of the spinal cord. These fibers form the spinothalamic tract, the major ascending pathway

for information about pain and temperature. This overall pathway is also referred to as the anterolateral system, much as the mechanosensory pathway is referred to as the dorsal column–medial lemniscus system.

The location of the spinothalamic tract is particularly important clinically because of the characteristic sensory deficits that follow certain spinal cord injuries. Since the mechanosensory pathway ascends ipsilaterally in the cord, a unilateral spinal lesion will produce sensory loss of touch, pressure, vibration, and proprioception below the lesion on the same side. The path-ways for pain and temperature, however, cross the midline to ascend on the opposite side of the cord. Therefore, diminished sensation of pain below the lesion will be observed on the side opposite the mechanosensory loss (and the lesion). This pattern is referred to as a dissociated sensory loss and together with local dermatomal signs.

## **2.11.1 c-Fos**

### **2.11.1.1 c-Fos expression in nociceptive pathways in the brain**

While the majority of studies on c-Fos and nociception have focused on expression in the spinal cord, several studies have used c-Fos expression to investigate nociceptive processing in the brain. Elizabeth Bullitt (Bullitt, 1989; Bullitt, 1990) examined c-Fos expression in the brainstem, hypothalamus, and thalamus in rats subjected to noxious mechanical stimulation (paw pinch) or noxious thermal stimulation (immersing a paw in hot water). She reported that these forms of

noxious stimulation induced c-Fos expression in the dorsal reticular nucleus, central gray, dorsal raphe, parabrachial nucleus, inferior colliculus, the lateral hypothalamus, and the midline and intralaminar thalamic nuclei. Other studies have confirmed that c-Fos is expressed in neurons in the dorsal and lateral reticular nuclei (Carstens et al., 1995; Lanteri–Minet et al., 1994), the cuneiform nucleus (Lanteri–Minet et al., 1994), the parabrachial nucleus (Bellavance et al., 1996; Hermanson et al., 1996; Lanteri–Minet et al., 1994; Pertovaara et al., 1993), the PAG (Keay et al., 1993; Lanteri–Minet et al., 1994), the hypothalamus (Pan et al., 1994), and the medial thalamus (Pertovaara et al., 1993). This distribution of c-Fos expression is much as one would expect based on tracing and electrophysiological recording studies. However, rather than simply confirming what had already been established by electrophysiological recording techniques, these studies have shown that c-Fos expression can be used to identify nociceptive processing in cell groups of restricted extent (e.g., in the various nuclei in the intralaminar and midline thalamus), which would be almost impossible using electrophysiological recording. Thus, c-Fos can be used to make fine-grained delineations about the precise areas that are activated by noxious stimulation, as well as revealing the overall distribution of neuronal populations that respond to that stimulation. An excellent illustration of this can be seen in the work of Keay and Bandler (Keay et al., 1993), who used c-Fos expression to investigate which regions of the PAG are activated by different types of noxious stimuli. They found that cutaneous noxious stimulation (produced by radiant heat) induced c-fos expression predominantly in the lateral and dorsolateral segments of the PAG, whereas the c-Fos induced by deep noxious stimulation (injection of dilute formalin

into the muscles of the neck) showed a strong predilection for the ventrolateral quadrant of the PAG.

Several other brain regions, not traditionally viewed as contributing to nociception, have been shown to contain neurons which express c-Fos in response to noxious stimulation. For example, numerous studies have reported that noxious stimuli elicit c-Fos expression in the locus coeruleus (Jones et al., 1995; Smith et al., 1994). While this structure was once believed to receive direct nociceptive input from the spinal cord, a recent investigation has established that neurons in the spinal cord do not project to this structure (Aston-Jones et al, 1994). Noxious stimulation has also been shown to elicit c-Fos expression in neurons in the superior colliculus, but this expression was seen bilaterally even though the noxious stimulus was applied unilaterally (Telford et al., 1996). Moreover, the distribution of Fos-labeled neurons in the superior colliculus did not differ when the noxious stimulus was applied to the hind paw or to the face, indicating that this structure does not encode information about the location of noxious stimulation.

Finally, peripheral noxious stimulation has been shown to induce c-Fos expression in cells of the anterior pituitary that was also immunoreactive for adrenocorticotrophic hormone and beta-endorphin (Pan et al., 1994; Pan et al., 1996). While c-Fos expression was induced by noxious stimulation in each of these structures, it is unlikely that they play a role in nociception. Rather, the observed c-Fos expression most likely reflects the role these structures play in the stress response elicited by pain.

### 2.11.1.2 Activation of c-Fos during development.

Gubits et al. (1988) studied the levels of c-Fos expression in the brain at different stages of development. They showed that higher levels of c-Fos mRNA occurred in the rat brain between birth and postnatal day 16 (P16). The highest levels of c-Fos mRNA were seen in meninges at embryonic day 18 (E18) and cerebral cortex and cerebellum at P30. At this stage (P30), only low levels of c-Fos mRNA were detected in the hippocampus and in striatum. The levels of c-Fos mRNA in the whole brain attained at P30 were maintained up to 1.5 years of age in male activation of c-Fos in the rat brain. A transient decrease in these levels was seen at P50, raising the question of whether this decrease is related to the onset of puberty in males. It was suggested that the expression of c-Fos could be related to synaptogenesis and differentiation rather than to mitosis. An *in situ* hybridization study by Caubert (1989) investigated the expression of c-fos mRNA in the developing brain. Around embryonic day 12 (E12), high levels of c-Fos were seen throughout the CNS. At later stages, the expression of c-Fos was confined to limited areas of the cerebellum, spinal cord, forebrain and retina (Pan et al., 1996). A fos-LacZ transgenic mouse had been recently used to study the developmental expression of the transgene (Smeyne et al., 1993). Expression of the transgene that was observed at P0 in olfactory bulb, hippocampus retrosplenial cortex and thalamic nuclei, decreased to adult levels by 3 weeks. This again suggests that c-Fos may play a role in the CNS during development (Smeyne et al., 1992). Northern blot analysis has shown low levels of c-fos mRNA which increases within P13-P30 (Gubits et al., 1988; McCormack et al., 1992). Developmental differences in expression of c-Fos in the brain have also

been observed after seizure induction (Schreiber et al., 1992; Jensen et al., 1993). The PTZ administration in rats resulted in increase in c-Fos immunoreactivity in superficial layers of retrosplenial, cingulate and neocortex in adults but only deep layers in P10 rats were affected (Jensen et al., 1993). Similarly, KA administration resulted in increase in c-Fos expression in limbic structures in adult animals but not in pups prior to P13 (Schreiber et al., 1992). The effects of PTZ, fluorothyl and hypoxia were studied in immature rats (postnatal day 10) and compared with that in adult rats. The CFLIR after PTZ or fluorothyl-induced seizure was elevated in amygdala, piriform cortex and hypothalamus at both ages; superficial layers of retrosplenial, cingulate and neocortex were stained in adults but staining was confined to deep layers of neocortex in P10 rats. Intense staining at 2 hr after seizure onset was reduced at 4 hr in adults but IR was not seen until 4-6 hr after seizure in P10 rats. The CFLIR within the dentate gyrus and hippocampus occurred only after prolonged seizures. Hypoxia, which produced seizures in P10 rats, induced CFLIR in the layer VI of neocortex but rarely involved limbic structures (Jensen et al., 1993).

Differential regulation of c-Fos expression during development after brain injury has been reported (Herrera et al., 1993). Rats that were inflicted with a mechanical cortical injury at post-natal day (PD) 10 or 15, and sacrificed 1.5 hr after, did not show an increase in c-fos immunoreactivity far from the wound, but some of these animals did show c-Fos immunoreactive (IR) nuclei close to the lesion (PD 15). At PD 22, cortical injury produced an increase in c-Fos IR nuclei in the piriform cortex ipsilateral but not contralateral to the lesion. This pattern was maintained up to PD 360 (Herrera et al., 1993). Similarly, the presence of c-Fos IR cells was observed in

the ipsilateral cingulate cortex from PD 22 onward. Ruppert and Wille (1987) showed that disruption of the cerebellum at P3 and up to P7 was followed by a massive increase in c-Fos mRNA (Ruppert and Wille, 1987). This effect was not seen in the adult animal with similar injury. This finding shows a clear differential regulation of c-Fos during development and may help to explain, at least partially, the different clinical outcomes seen in children and in adults after head trauma.

## **CHAPTER 3**

# **GENERAL METHODS AND EXPERIMENT MODELS**

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.

## **3.1. Animals**

### **3.1.1. Neonatal rats**

Postnatal day 7 (PND7) Sprague-Dawley (SD) rat pups (Harlan UK) were used for *in vivo*, *in vitro* and behavioural 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. The data of the birth was defined as day 0. On PND 7 which is the period of synatogenesis (Clancy et al, 2001), pups were used for experiments.

## **3.2 Monitoring**

All rats were kept normothermic throughout gas exposure using a water bath. After initial equilibration, the composition of the chamber gas was analysed by a gas monitor (Datex, Capnomac Ultima). Given the inert chemical characteristics of xenon, a specific 439 XE analyser (Air Products, Surrey UK) was used to monitor and verify the delivery of the anaesthetic concentration of xenon based on radiofrequency.

## **3.3 Gas Exposure**

### **3.3.1. Neonatal gas exposure *in vivo***

#### **3.3.1.1 Anaesthetics exposure for neuroapoptosis in the high centre brain study**

Seven day old Sprague-Dawley rats were exposed to oxygen along with one of several gas combinations (nitrogen, 70% nitrous oxide, 0.75% isoflurane, 70% N<sub>2</sub>O + 0.75% isoflurane,) for 6 hours (n = 3-5/group). The rats were sacrificed after anaesthesia, and their brains processed to assess the severity of apoptosis (using caspase-3 immunohistochemistry, western blotting).

#### **3.3.1.2 Anaesthetics exposure for neuroapoptosis in the spinal cord study**

Seven day old Sprague-Dawley rats were exposed to oxygen along with 70% N<sub>2</sub>O + 0.75% isoflurane for 6 hours (n = 3-5/group). The rats were sacrificed after anaesthesia, and their spinal cord processed to assess the severity of apoptosis (using caspase-3 immunohistochemistry).

### **3.3.1.3 Preconditioning**

#### **3.3.1.3.1. Preconditioning with hypoxic ischemia**

Seven day old Sprague Dawley rat pups were pre-treated with, 8% oxygen (hypoxia) or ischemia by ligating the right carotid artery. They were then exposed to the anaesthetic combination of 70% N<sub>2</sub>O +0.75% isoflurane for 6h. The brains were removed immediately after anaesthetic exposure and sectioned for Cresyl Violet (fissl) Staining.

#### **3.3.1.3.2. Preconditioning with anaesthetics**

Seven day old Sprague Dawley rat pups were pre-treated with one of the following: 70% xenon, 70% N<sub>2</sub>O, 8% oxygen (hypoxia). They were then exposed to the anaesthetic combination of 70% N<sub>2</sub>O + 0.75 % isoflurane for 6h. The brains were removed immediately after anaesthetic exposure and sectioned for immunohistochemistry of caspase 3, a marker of cell apoptosis. In other cohorts solely pre-treated, the brains were harvested for western blotting. 70% xenon balanced with O<sub>2</sub> was delivered through a customised-built anaesthetic machine modified for xenon delivery (Ohmeda, Surry, UK). In order to save xenon a closed-circuit was used (Figure 3.1).

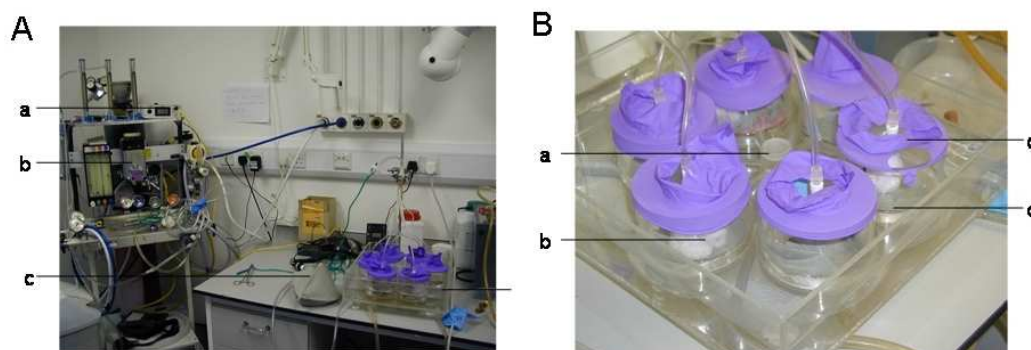


**Figure 3.1 Experiment set up.**

A. Gas delivery: (a) xenon monitor; (b) gas delivery equipment; (c) xenon closed circuit; (d) custom built anaesthetic chamber. B. Custom built anaesthetic chamber with 6 individual well, (a) main inflow of gas branching into 6 smaller tubes leading to wells; (b) silica gel and soda lime; (c) individual well with tube connected to lid to allow outflow of gas; (d) water bath kept normothermic (37°C).

Silica gel was added to the chamber to eliminate moisture and soda lime to absorb carbon dioxide. 70% N<sub>2</sub>O balanced with O<sub>2</sub>, and 8% O<sub>2</sub> balanced with nitrogen was delivered by a calibrated anaesthetic machine. Exposure times were chosen based on previous studies (Sharp et al, 2004; Ma et al, 2007). Gases for all other groups were delivered in a high-flow open-circuit, using a calibrated flow meter.

### 3.3.1.4. Nociceptive Stimulation during anaesthetics gas exposure

Separate cohorts (n= 4 per group) of 7-day- old, littermate Sprague-Dawley rat pups were randomly assigned to six groups: 1. Naïve (room air); 2. Anaesthetics alone (70% nitrous oxide and 0.75% isoflurane for 6 hrs); 3. Formalin injection alone (subcutaneous injection of 10 µl 5% formalin into the left hind paw); 4. Anaesthetics + formalin injection; 5. Surgical incision alone (1 cm midline cut to the left hind paw); 6. Anaesthetics + surgical incision. The remaining gas constituents were 25% oxygen in groups 2-6 and balanced with nitrogen where necessary. To comply with the

Animals Act which prevents animals from being subjected to pain without administration of a general anaesthetic drug, the surgical incision and formalin injection were conducted 10 minutes after the rats were anaesthetised with 70% nitrous oxide and 0.75% isoflurane in groups 3 and 5; they were then exposed to room air rather than anaesthetic reagents for 6 hours.

## **3.4 Tissue Fixation**

### **3.4.1 Brain perfusion**

Rats destined for immunohistochemistry study were terminated by administering an overdose of pentobarbitone (100mg/kg) intraperitoneally. One vial of Heparin (Elkins-Sinn, 10,000 units /ml) was mixed with 1-liter saline. Fill 60 cc syringes with saline and 4% paraformaldehyde saline can be Hank's Balanced Salt Solution, sterile saline, or PBS. The rats were mounted onto the rack in the sink using butterfly clamps. A cross-sectional incision was made beginning just below the sternum and cutting through the abdominal muscles to the beginning of the rib cage. The sternum was held with a large haemostat and the thoracic cage was pulled over the head while cutting along each side of the thoracic cage with scissors. The diaphragm was cut with scissors. A small haemostat clip was applied to the tip of the heart and laid across the abdominal region to hold the heart in a firm position. The perfusion cannula was inserted into the left ventricle. A small incision was made in the right ventricle with scissors. 60ml of saline with Heparine was infused followed by 60ml of 4% paraformaldehyde. The hemostats and the perfusion cannula were removed

after infusion of the solutions. Thereafter, in the sink, the rats were decapitated using the guillotine. An incision was made with a scalpel from anterior to the eyes to just posterior of the ears in order to completely expose the cranium. The patriotism was scraped to the side and the muscles and tendons detached around the cranium. The skull was removed with rongeurs by working from posterior cerebellum to anterior olfactory bulb. After the entire cortex was exposed, the dura was carefully cut with scissors and forceps. Using a flat-ended spatula, the olfactory bulbs were severed and afterwards the spatula was placed under the anterior cortex and the brain was gently lifted. The optic nerves were severed and the brain was removed and placed in 4% Para formaldehyde for 24 hours. It was then immersed in 30% sucrose with PB and 1% sodium azide, and refrigerated until the brain sank. It was then embedded and frozen into O.C.T solution and sliced coronally into section 30um thick using a cryostat (Bright Instrument Company Ltd., Huntingdon, UK).

### **3.4.2 Paraffin embedding and sectioning**

On the day of embedding, the paraffin was melted in the vacuum oven for later use. The 70% E/S was decanted from the storage vial, care was taken to avoid disturbing the brain. 95% ethanol was added to dehydrate the embryos and rock on a nutator. The 95% ethanol was decanted and 100% ethanol was added – rock on nutator. Immerse the brains in infiltrating paraffin for one hour. The brains were immersed in the embedding paraffin for another hour. To begin with, the tissue-embedding hot plate was turned on for pre-warming. Metal mould(s) with paraffin were carefully cleaned with ethanol using Kim wipes. After drying, the mould were filled with

embedding paraffin liquid and placed into the vacuum oven for pre-warming and for dissipating air bubbles. This usually took about 10 min. The brains were transferred (from the embedding paraffin) to the pre-warmed wax mold. Brains were oriented to stand on their head in the mold with their longitudinal axis in an upright position and their body axis perpendicular to the bottom surface. The mold was placed on the hot plate and gradually moved to cooler regions of the plate. After a few minutes the mold was removed from the hot plate and placed on the cold plate. Once all of the brains in the mold were standing on their heads in similar orientation and at some distance from each other, a bit more paraffin was gently added. More paraffin was added to fill the mold. Once solidified, the wax mold was placed at -20°C to harden overnight. 100% EtOH was used to clean a container of the water bath which was used for spreading the sections. The container was filled, to 80% of the container height, with sterile ddH<sub>2</sub>O and the water warmed to 39 - 42°C. The suitable temperature differs among tissue blocks, but 40 - 42°C is a common starting point.

The lock handle was released on the right side. We set the section thickness to 5mm on the control panel. The wax block was mounted onto the chuck holder and the angle control knobs was loosened to allow the relative position of the chuck holder to be adjusted with respect to the cutting blade.

### **3.4.3 Brain harvesting**

Rat pups were sacrificed after finishing gas exposure and treatment, the brains were immediately removed. They were separated into cortex, hippocampus and hypothalamus. The tissues were frozen using liquid nitrogen at -80°C.

### **3.4.4 Hippocampal slice fixation**

The study protocol was approved by Home office (UK) and conforms to the United Kingdom Animals (Scientific Procedures) Act, 1986.

Organotypic hippocampal slices were prepared according to the interface culture method as modified by (Sullivan et al, 2001). In brief, neonatal rat pups (PND9/10) were anaesthetised using 0.5% isoflurane until quiescent and sacrificed by decapitation. The brain was quickly dissected and placed in ice cooled (4°C) dissection solution to reduce extraction-induced ischemia (200ml high sucrose-glucose (HSG) solution, 64ml Hanks-balanced salt solution (HBSS) (Gibco) and 0.6ml Antibiotic-Antimycotic solution (AAS) (Sigma)). All stages of slice preparation were carried out under sterile and ice-cooled conditions.

Excess tissues (cerebellum, olfactory bulbs and meninges) were removed and the brain cut into 400 µm sagittal slices using a McIlwain Tissue Chopper (Mickle Laboratory, Cambridge, UK). Under a microscope (Zeiss W-PI 10\*) and cooling light

(KL 1500, Schott), the slices were separated using fine forceps, avoiding contact with the hippocampus.

Slices with hippocampus were selected and placed onto 30mm diameter semiporous cell culture inserts (Falcon, Becton Dickinson Labware, Millipore, and Bedford, MA, USA). Five slices were positioned on each insert and placed in a 6-well Tissue Culture Tray (Multiwell TM Falcon, Becton Dickinson labware, Bedford, MA, USA) Growth media (1.5ml) (made up with: 282ml Medium Stock (Gibco/Life technologies), 32 ml Horse Serum, 32ml Foetal Bovine Serum and supplemented with 3.2ml Glutamine and 3.2 AAS (Gibco/Sigma)) was then transferred to each well.

The slices were incubated for 24 hours in humidified air at 37°C, enriched with 5% Carbon Dioxide to allow for recovery from the trauma induced by the extraction. The culture medium was replaced the following day with fresh, equilibrated medium.

The gases were delivered by a standard anaesthetic machine through the chambers at 2-3L/min. The gases were analysed using an S/5 spirometry module (Datex-Ohmeda, Bradford, UK) and xenon was monitored using a 439XE monitor (Air Products, UK) due to xenon's inert chemical characteristics. After 3 minutes of gas flow the chambers were sealed and placed in a 37°C (Galaxy R Carbon Dioxide Chamber, Wolf Laboratories).

## 3.5 Immunohistochemistry

### 3.5.1 Solutions

#### 0.2 M Phosphate buffer (PB) (2 L)

46 g  $\text{Na}_2\text{HPO}_4$

9.1 g  $\text{Na}_2\text{H}_2\text{PO}_4$

2 L  $\text{dH}_2\text{O}$

#### 0.1 M Phosphate-buffered saline (PBS) (2 L)

1 L 0.2 M PB

1 L  $\text{dH}_2\text{O}$

18 g NaCl

#### 0.1 M Phosphate-buffered saline with 0.3 % triton (PBS-T) (2 L)

2 L PBS

6 mL Triton X-100 (Promega)

#### 0.1M Phosphate-buffered saline with 0.3 % $\text{H}_2\text{O}_2$ / 70 % methanol (30 mL)

9 mL 0.1 M PBS

21 mL Methanol

300  $\mu\text{L}$  30%  $\text{H}_2\text{O}_2$

#### Heparinised phosphate-buffered saline (PBS-Hep) (2 L)

2 L PBS

2 mL (1000 U / mL) heparin sodium solution (Sigma)

#### 10 % paraformaldehyde (2 L)

Prepare under fume hood and store at 4 °C

1.6 mL  $\text{dH}_2\text{O}$  (heat to ~70 °C)

200 g paraformaldehyde (dissolve in water)

1 mL NaOH (to aid dissolution)

2 L dH<sub>2</sub>O

Cool solution to room temperature, titrate to pH to 7.4 with HCl and/or NaOH, filter and store at 4 °C.

#### **4 % Paraformaldehyde (2 L)**

Prepare under hood, make immediately before use and store at 4°C

1 L 0.2 M PB

0.8 L 10 % paraformaldehyde

Top up to 2 L with dH<sub>2</sub>O

#### **30 % Sucrose solution (2 L)**

600g sucrose

2 L 0.1 M PBS

#### **1 M Sodium hydroxide (NaOH) (2 L)**

50 g sodium hydroxide pellets

Dissolve to 2 L with dH<sub>2</sub>O

### **3.5.2 Immunostaining**

The mounted sections sections were washed in PBS (washing was performed 3 times for 5 minutes). Endogenous peroxidase activity may be found in many tissues, for example, due to the presence of red blood cells and, therefore, sections were quenched of peroxidise activity by pre-treatment in 0.3 % hydrogen peroxide / 70 % methanol in 0.1 M PBS for 30 minutes, and subsequently washed in PBS. Sections

were then incubated with a 3 % blocking solution for 30 minutes to suppress non-specific binding, followed by washing. Sections were incubated with the primary antibody diluted in 1 % goat serum in PBS-T) overnight for 16 hours at 4 °C, followed by washing. To test for the specificity of the antibodies used, control slides were incubated with 1 % goat serum in PBS-T only, without a primary antibody present. Sections were then incubated with a biotinylated secondary antibody.

### **3.5.2.1 Cleaved caspase-3 staining**

Activation of caspase-3 associated with apoptosis was established in neonatal model of acute neuronal injury. Thus activated caspase-3 is a relevant marker of apoptosis and it was used throughout my project. Slices were washed in PBS then quenched in a solution of 70% methanol and 0.3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for 30 minutes. Slices were washed again in PBS, with 3 changes, and incubated in 3% blocking solution (PBST(PBS containing 0.3% Triton-X (Promega corporation, Madison,WI)), and 1% normal goat serum (NGS) (Vector Laboratories Inc.,Burlingame,CA) for 1 hours at room temperature. After overnight incubation at 4°C with rabbit anti-cleaved caspase-3 antibody (1:1500) (New England Biolabs, Hertfordshire,UK) in blocking serum the sections were washed 3 times in PBST and incubated with biotinylated secondary antibody (1:200 goat anti-rabbit IgG antibody)(Chemicon International, Temecula, CA) for 1 hour at room temperature. Following a further 3 washes in PBST, sections were incubated in freshly prepared ABC (Avidin-Biotin-peroxidase Complex) solution from a Vectastain ABC kit ( Vector Laboratories Inc., Burlingame, CA , USA) for 1 hour. ABC solution was removed with PBS and fresh 3,

3'-diamino-benzidine (DAB) solution (distilled water, buffer, DAB stock,  $\text{H}_2\text{O}_2$  and nickel solution from a peroxidase substrate kit) solution from a peroxidase substrate kit) (Vector Laboratories Inc. Burlingame, CA) added. Sections were immersed for 6 minutes and immediately washed 3 times with PBS to end staining, followed by washes in double distilled water. Slices were then mounted onto Super Plus-coated glass slides, (VWR International, Lutterworth, UK) utilising the floating technique. Once mounted, thesections were dehydrated in 95% alcohol then 100% alcohol for 10 minute, followed by xylene. Vectamount was utilised to mount the cover glass prior to light microscope.

### **3.5.2.2 c-Fos staining**

Slices were washed in PBS then quenched in a solution of 70% methanol and 0.3% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) for 30 minutes. Slices were washed again in PBS, with three changes, and incubated in 3% blocking solution (PBST(PBS containing 0.3% Triton-X (Promega corporation, Madison,WI)), and 1% normal donkey serum (NDS) (Vector Laboratories Inc.,Burlingame,CA) for 1 hours at room temperature. After overnight incubation at 4°C with goat anti-c-fos antibody (1:1500) (New England Biolabs, Hertfordshire,UK) in blocking serum the sections were washed 3 times in PBST and incubated with biotinylated secondary antibody (1:200 donkey anti-goat IgG antibody)(Chemicon International, Temecula, CA) for 1 hour at room temperature. Following a further 3 washes in PBST, sections were incubated in freshly prepared ABC (Avidin-Biotin-peroxidase Complex) solution from a Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA , USA) for 1 hour. ABC solution was

removed with PBS and fresh 3, 3'-diamino-benzidine (DAB) solution (distilled water, buffer, DAB stock, H<sub>2</sub>O<sub>2</sub> and nickel solution from a peroxidase substrate kit) solution from a peroxidase substrate kit) (Vector Laboratories Inc. Burlingame, CA)) added. Sections were immersed for 6 minutes and immediately washed 3 times with PBS to end staining; this was followed by washes in distilled water. Slices were then mounted onto Super Plus-coated glass slides, (VWR International, Lutterworth, UK) utilising the floating technique. Once mounted, the sections were dehydrated in 95% alcohol then 100% alcohol for 10 minute, followed by xylene. Vectamount was utilised to mount the cover glass prior to light microscope.

### **3.5.2.3 Fluorescence double staining**

Double-labelling fluorescent immunohistochemistry was used in this study. Both caspase-3 and c-Fos were stained. This staining technique has been shown to be effective (Mason et al. 2000; Negoescu et al. 1994) and has been commonly used for visualization of two antigens in the same tissue sections in many studies (Wu et al. 2005; Taatjes et al. 2007; Dürr et al. 2007 ). The whole staining process in this study was conducted at room temperature.

#### **Step 1. Caspase-3 immunofluorescence staining**

Selected brain slices were washed in PBS for minimal 30 minutes, then incubated in 10% normal goat serum (NGS, Bioclear) diluted in PBST (0.3% Triton X-100 (Promega corporation, Madison,WI) in PBS) for 1 h. After that, sections were incubated overnight with rabbit anti-cleaved caspase-3 monoclonal antibody (1:2500, Cell Signalling) diluted in 1% NGS in PBST. The next day, sections were washed in PBS (x3,

10 mins each) and incubated 1 hour with a CyTM3 –conjugated goat-anti-rabbit secondary antibodies (1:200, IgG, Jackson ImmunoResearch) diluted in 1% NGS in PBST for two hrs. Sections were next washed in PBS (x3, 10mins each).

Step 2. c-Fos immunofluorescence staining anti-c-Fos antibody (1:1500) (New England Biolabs, Hertfordshire, UK). Selected brain slices were washed in PBS for minimal 30 minutes, then incubated in 10% normal donkey serum (NDS, Bioclear) diluted in PBST (0.3% Triton X-100 (Promega corporation, Madison,WI) in PBS) for 1 h. After that, Sections were incubated for 1 h in 1:200 biotinylated donkey anti-mouse secondary antibodies (IgG, Millipore) followed by 1 h in streptavidin Alexa Fluor 488.

### 3.5.3 Quantification

The number of DAB stained, (black) degenerated neurons expressing caspase-3 within the cortex or hippocampus was counted in 3 anonymous sections in each animal, -3 to -4 mm from bregma. Neuronal cells exhibiting intense black staining of the cell soma and dendritic or axonal processes were counted using a BX-60 light microscope (Olympus, Southall, UK) and example photomicrographs were taken with a Axiocam digital camera ( Zeiss, Gottingen, Germany). All parameters for imaging were standardised to maintain consistency.

### 3.5.4 Cresyl Violet (Nissl) Staining

Nissl is a term used by classical cytologists for the endoplasmic reticulum. Since all cells contain ER, a Nissl stain (cresyl violet) will stain all cell bodies in the brain. The staining procedure consists of sequentially dipping the mounted brain slices in about a dozen different solutions for specified amounts of time. An initial alcohol baths remove the lipids and fixative from the tissue, the sections are stained, followed by dehydrating the tissue in a series of alcohol baths.

The staining dishes were placed in fume hood and labelled in order 95% Ethanol, 70% Ethanol, 50% Ethanol, Distilled Water, Distilled Water, Cresyl Violet Stain, 50% Ethanol, 70% Acid Ethanol, 95% Ethanol, 95% Ethanol, 100% Ethanol. The slides were inserted into slide racks. The slide racks were passed through the following sequence of baths for the appropriate time indicated: 95% Ethanol 15min, 70% Ethanol 1min, 50% Ethanol 1min, Distilled Water 2min, Distilled Water 1min, Cresyl Violet Stain 5min, Distilled Water 1min, 50% Ethanol 1min, 70% Acid Ethanol 2min, 95% Ethanol 2min, 100% Ethanol 1min.

## 3.6 Western Blotting

### 3.6.1 Solutions

#### Tris-Hcl solution (500 mL)

12.1 g Trizma base

0.5 L dH<sub>2</sub>O

Titrate to pH 8.0 with HCl

**Tris-buffered saline (TBS) (1 L)**

8.8 g NaCl

50 mL Tris-HCl

Top up to 1L with dH<sub>2</sub>O

**Tris-buffered saline with 0.05 % Tween-20 (1 L)**

8.8 g NaCl

50 mL Tris-HCl

Top up to 1L with dH<sub>2</sub>O

0.5 mL Tween-20 (very viscous so add after dH<sub>2</sub>O)

### **3.6.2 Tissue homogenisation and cytosolic and membranous protein separation**

Rat pups were sacrificed, the brains were immediately removed and the cortices separated and frozen at -80°C. Samples were then lysed in cell lysis buffer (pH 7.5, 20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L Na<sub>2</sub>DTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 2 mmol/L DL-dithiothreitol, 1 mmol/L phenylmethanesulfonyl and 1 µg/ml leupeptin) so as to achieve a 0.2 g/ml concentration and homogenised (Polytron homogeniser by kinematica) on ice. The homogenised sample was centrifuged at 3000g for 10 minutes at 4°C to remove cellular debris. The supernatant was further centrifuged twice, initially at 11,000 g for 20 minutes at 4°C for western blotting of Bcl-2, and a second time at 20,000 g for 45 minutes at 4°C to separate cytosolic and

mitochondrial material for western blotting of cytosolic cytochrome c and cleaved caspase-3. Supernatant was stored at -80°C and protein concentration was determined using the Bradford Protein Assay (Bio-Ra, Herts, UK).

### **3.6.3 Protein quantification using the Bradford assay**

Bradford dye concentrate (Bio-Rad) was diluted with double distilled water (ddH<sub>2</sub>O) in a 1:4 ratio respectively, following manufacturer's instructions. To make standard protein samples, a known concentration of bovine serum albumin (Bio-rad) was serially diluted with ddH<sub>2</sub>O to make a range of concentrations from 1000, 500, 250, 125, 12.5 and 1.25 µg / mL. On ice, 10 µL of protein samples and 10 µL of protein standard samples were loaded in triplicate into a 96-microwell flat-bottomed clear plate. Triplicates of cell lysis buffer (10 µL) were also loaded to remove its possible contribution to total protein concentration, and ddH<sub>2</sub>O was used as blanks. Bradford dye (20 µL) was pipetted into each well and left to incubate for 5 minutes. The plate was then placed in a spectrophotometer to measure the optical density of each sample at 595 nm. The standard curve created from the protein standard samples allowed for determination of the protein sample concentration and their subsequent normalisation to each other.

### **3.6.4 Electrophoretic protein separation**

Based on the Bradford assay determination of protein concentration, protein samples were normalised to the same concentration and volume. Denaturing sample

buffer concentrate containing a tracking dye (Invitrogen) was added to the normalised protein samples in a 1: 3 ratio respectively and subsequently heated at 70°C for 10 minutes. 4 - 12 % gradient Bis-Tris gels (Invitrogen) were positioned in a gel holder and assembled into a Western blot tank that was then filled with running buffer (Invitrogen). Equal volumes of normalise protein samples were then loaded into the wells of the gel using a long fine-tipped pipette. Each well may hold a maximum of 25 µL of sample; 10 µL of mouse brain or kidney with sample buffer was loaded per well and this contained approximately 100 µg of total protein, where as 20 µL of cells with sample buffer were loaded per well and this contained approximately 60 µg of total protein. 5 µL of a biotinylated protein ladder (Cell Signalling Technology) was also loaded in an additional lane. The gel-loaded proteins were then fractionated by electrophoresis at 200 V for 35 minutes which was when the dye front had nearly reached the bottom of the gel.

### **3.6.5 Electrotransfer**

The proteins separated by electrophoresis were then transferred onto a nitrocellulose membrane that acts as a solid support. Pre-cut nitrocellulose membranes, filter papers and sponges were soaked in 10 % methanol transfer buffer (Invitrogen) for 5 minutes before the end of the electrophoretic run. According to the manufacturer's instructions, a gel and membrane sandwich was then created from negative to positive. Electrotransfer of proteins, from gel to nitrocellulose membrane as air pockets hinder the flow of electrical current, air bubbles were smoothed out between the gel and nitrocellulose membrane by gently pressing and

rolling together with a Pasteur pipette. The gel-membrane sandwich was then placed in a transfer box which was assembled into the Western blot tank. The transfer box was filled with transfer buffer and cool water was used to fill the rest of the tank to aid in heat dissipation. The transfer of proteins from gel onto membrane was run at 30 V for 60 minutes.

### **3.6.6 Immunodetection**

After electrotransfer, the nitrocellulose membrane was incubated with a blocking solution composed of 5 % non-fat dry milk in Tween-20-containing Tris buffered saline (TBS-T) for 3 hours on a gyrating shaker (Fisher Scientific) at room temperature. Subsequently, the blocked membrane was incubated overnight for 16 hours at 4°C on a gyrating shaker with the relevant primary antibodies; or mouse monoclonal antibody, 1 : 1000 (Novus Biologicals); or rabbit polyclonal antibody, 1 : 1000, (BD Biosciences). The primary antibodies were incubated at optimised dilutions in 5 % non-fat dry milk solution. After removing the antibody, 4 wash in TBS-T lasting 5 minutes each were performed at room temperature on a rapidly moving gyrating shaker. The membrane was then incubated with the appropriate goat-derived horseradish peroxidase-conjugated secondary antibody (Cell Signalling Technology) directed against the rabbit-derived primary antibodies at room temperature for 1.5 hours on a gyrating shaker. The secondary antibody was then removed and 5 washes in TBS-T lasting 5 minutes each were performed at room temperature on a rapidly moving gyrating shaker with an additional sixth wash for 5 minutes in TBS without tween-20, as this detergent interferes with the

chemiluminescent reaction. To maximise the visualising of immunoreactive bands, the enhanced chemiluminescent (ECL) reagent was mixed with the accompanying peroxidase reagent (Lumiglo kit (Cell SignallingTechnology)) only at the very last minute. The membrane was then incubated for 1 minute in the ECL solution and then placed protein-side up between two transparent acetate sheets, with trapped air bubbles gently rolled out using a Pasteur pipette. The acetate-covered membrane was then placed inside a film cassette and in a completely dark room exposed to radiographic film (ECL Hyperfilm (Amersham Biosciences)) for optimised lengths of time. After radiographic film capture of the immunoreactive bands, the membrane was removed from the acetate and washed 3 times in TBS-T lasting 5 minutes each at room temperature on a rapidly moving gyrating shaker. The membrane was then subjected to the same protocol as outlined above for detection of the house-keeping protein,  $\alpha$ -tubulin: it was re-blocked in blocking solution before incubation with the anti- $\alpha$ -tubulin antibody, washed, incubated with the secondary antibody, washed and visualised using the ECL system capture on radiographic film. The results were then quantified by densitometric analysis using Adobe Photoshop Elements 2.0 software, scaled relative to the control sample, and normalised with the housekeeping protein  $\alpha$ -tubulin.

### **3.7 Organotypic hippocampal slice culture**

Post-natal day 7(PND) SD rat pups (B&K Laboratories, Harlon) were provided and housed in a 24-hour diurnal enclosure with food and drink ad libitum. The date of birth for the animals was defined as 0-day-old and animals were sacrificed on PND 7

and 8 during synaptogenesis. The study protocol was approved by Home office (UK) and conforms to the United Kingdom Animals (Scientific Procedures) Act, 1986.

Organotypic hippocampal slices were prepared according to the interface culture method as modified by (Sullivan et al, 2001). In brief, neonatal rat pups (PND9/10) were anaesthetised using 0.5% isoflurane until quiescent and sacrificed by decapitation. The brain was quickly dissected and placed in ice cooled (4°C) dissection solution to reduce extraction-induced ischaemia (200ml high sucrose-glucose (HSG) solution, 64ml Hanks-balanced salt solution (HBSS) (Gibco) and 0.6ml Antibiotic-Antimycotic solution (AAS) (Sigma)). All stages of slice preparation were carried out under sterile and ice-cooled conditions.

Excess tissues (cerebellum, olfactory bulbs and meninges) were removed and the brain cut into 400  $\mu$ m sagittal slices using a McIlwain Tissue Chopper (Mickle Laboratory, Cambridge, UK). Under a microscope (Zeiss W-PI 10\*) and cooling light (KL 1500, Schott) the slices were separated using fine forceps, avoiding contact with the hippocampus.

Slices with hippocampus were selected and placed onto 30 mm diameter semi porous cell culture inserts (Falcon, Becton Dickinson Labware , Millipore, Bedford, MA,USA).Five slices were positioned on each insert and placed in a 6-well Tissue Culture Tray (Multiwell TM Falcon, Becton Dickinson labware, Bedford, MA,USA) Growth media (1.5 ml) (made up with: 282 ml Medium Stock (Gibco/Life

technologies), 32 ml Horse Serum, 32 ml Foetal Bovine Serum and supplemented with 3.2 ml Glutamine and 3.2 AAS (Gibco/Sigma)) was then transferred to each well.

The slices were incubated for 24 hours in humidified air at 37°C, enriched with 5% Carbon Dioxide to allow for recovery from the trauma induced by the extraction. The culture medium was replaced the following day with fresh, equilibrated medium.

### **3.8 Trace fear conditioning**

Seven day old SD rat pups were exposed to 8% oxygen balanced with nitrogen or 70% xenon and 30% oxygen for 2 hours. 24 hours after exposure, pups were treated with 70% nitrous oxide and 0.75% isoflurane for 6 hours. They were allowed to live up to 40 days. They were then tested for hippocampal-dependent memory and learning function in a contextual fear conditioning behavioural paradigm in which: On the first test day, the animals received both tone and shock six times during the 26-minute testing period (Conditioning Test); on the second day, they were exposed to the same environment but received neither tone nor shock for 8 minutes (Context Test); on the third day, they received tone three times during 18 minutes (Tone Test). The percentage of freezing time (Context results) and the area under curve (AUC) were derived from plots between the percentage freezing time and trial time in the Tone Test, and were used for statistical comparison (mean  $\pm$  SD, n = 4-6) (Figure 3.2).



**Figure 3.2 Behavioural tests –fear conditioning set up.**

### **3.9 Statistical analysis**

Both immunohistochemistry and western blotting results were expressed as mean  $\pm$  standard division of the mean. Statistical analysis comprised of one way analysis of variance (ANOVA) with post-hoc Student Newman-Keul's multiple comparison test. A p value of  $<0.05$  was considered statistically significant.

## **CHAPTER 4**

# **ANESTHESIA INDUCED APOPTOSIS IN NEONATES**

## 4.1 Introduction

Clinicians have long held the illusory hope that the constellation of profound behavioural effects comprising general anaesthesia is entirely reversible and that the central nervous system (CNS) returns to its pristine state once the anaesthetic agent is eliminated from the active site. However, as scientists have unravelled the targets in the CNS which may be responsible for the production of the ephemeral state of general anaesthesia, they have also found that perturbation of the normal functioning of these targets may produce long-lasting undesirable effects.

The work by Olney's team has helped elucidate the mechanisms underlying the deleterious effects of alcohol on the developing brain, which is responsible for the development of Foetal Alcohol Syndrome (FAS) (Ikonomidou et al., 2000). Olney and co-workers argued that as general anaesthetic drugs acted on the same receptors as alcohol, they might produce similar behavioural and molecular effects as the latter. Indeed, Olney and his colleagues showed that exposure of a few days' old rat pups to a standard anaesthetic cocktail produced similar morphologic and functional impairment as that seen with exposure to alcohol; these effects enhanced naturally occurring apoptosis (Jevtovic-Todorovic et al., 2003).

Several subsequent studies have confirmed the finding that this anaesthetic cocktail induces apoptotic neurodegeneration (Yon et al., 2005; Yon et al., 2006) and demonstrated that various other anaesthetic agents and cocktails also induce the same response when administered post-natally (Urraca et al., 2006; Takadera et al.,

2006; Young et al., 2005). As for this evidence, I postulate that common anaesthetics may also induce neurodegeneration in neonates. So, firstly, I use clinic anaesthetics in neonate animal to look for neurodegeneration evidence.

## 4.2 Method

### 4.2.1 Gas exposure

Seven day old Sprague-Dawley rats were exposed to oxygen along with one of several gas combinations (nitrogen, 70% nitrous oxide, 0.75% isoflurane, 70% N<sub>2</sub>O + 0.75% isoflurane,) for 6 hours (n = 3-5/group).

|                                           |
|-------------------------------------------|
| Naïve                                     |
| ISO(0.75%) 6 hours                        |
| N <sub>2</sub> O (70%) 6 hours            |
| N <sub>2</sub> O(70%) +ISO(0.75%) 6 hours |

Table 1. Seven day old Sprague-Dawley rats were exposed to different conditions presented above.

### 4.2.2 Immunohistochemistry

#### *In vivo*

The rats were sacrificed after anaesthesia, and their brains processed to assess the severity of apoptosis, caspase 3 as a marker. With the immunostaining of the brain slices was measured.

### ***In vitro***

Organotypic hippocampal slices were derived from 8/9 day post-natal C57B6 mice pups. The slices were treated as in the *in vivo* preparations. The slices were cryostat after 24 hours and immunohistochemistry of caspase-3, a member of the apoptotic common pathway, was performed. Photomicrographs were taken, CA1 and CA3 subregions were manually outlined and the number of caspase-3 positive cells counted.

#### **4.2.3 Western Blotting**

Gas delivery occurred for 6 hours after which the animals were immediately sacrificed and their cortices removed for western blotting of caspase-3, -8, -9, cytochrome c. Results were normalised to the control and the housekeeping protein  $\alpha$ -tubulin with densitometric analysis.

## **4.3 Results**

### **4.3.1. Cortical and hippocampus activated Caspase-3**

Neuronal cells exhibiting caspase-3 activation were readily distinguishable from the background as black cell body and axons. The staining established the level of background level of cortical activated capase-3 in rats exposed to air as  $19.3 \pm 6.4$  (mean  $\pm$  SD),  $n = 4$  (Fig. 4.1). Administration of 70% N<sub>2</sub>O in oxygen did not induce any significant increase in caspase-3 positive cells compared to naïve ( $22.5 \pm 5.9$ ,  $n =$

3;  $p > 0.05$  vs Naïve) whereas administration of 0.75% isoflurane in oxygen / air mixture produced a moderate increase in level of activated caspase-3 staining ( $76.5 \pm 11.4$ ,  $n = 5$ ;  $p < 0.01$  vs Naïve) (Figure 4.1). The combination of 70% N<sub>2</sub>O and isoflurane in oxygen significantly increased the number of caspase-3 positive cells compared with Naïve ( $232 \pm 20$   $p < 0.01$  vs Naïve) (Figure 4.1.A and Figure 4.1.B respectively).

The staining established the level of background level of activated capase-3 activation in hippocampus in rats exposed to air as  $5.2 \pm 1.8$  (mean  $\pm$  SD),  $n = 4$  (Fig. 4.1). 70% N<sub>2</sub>O in oxygen did not induced any significant increase in caspase-3 positive cells compared to naïve ( $3.7 \pm 1.4$ ,  $n = 3$ ;  $p > 0.05$  vs Naïve) whereas administration of 0.75% isoflurane in an oxygen / air mixture produced a moderate increase in the level of activated caspase-3 staining ( $22.1 \pm 9.6$ ,  $n = 5$ ;  $p < 0.01$  vs Naïve) (Figure 4.1). The combination of 70% N<sub>2</sub>O and 0.75% isoflurane in oxygen significantly increased the number of caspase-3 positive cells compare with Naïve ( $34.8 \pm 20.2$   $p < 0.01$  vs Naïve) (Figure 4.1.D) (Figure 4.1.E).

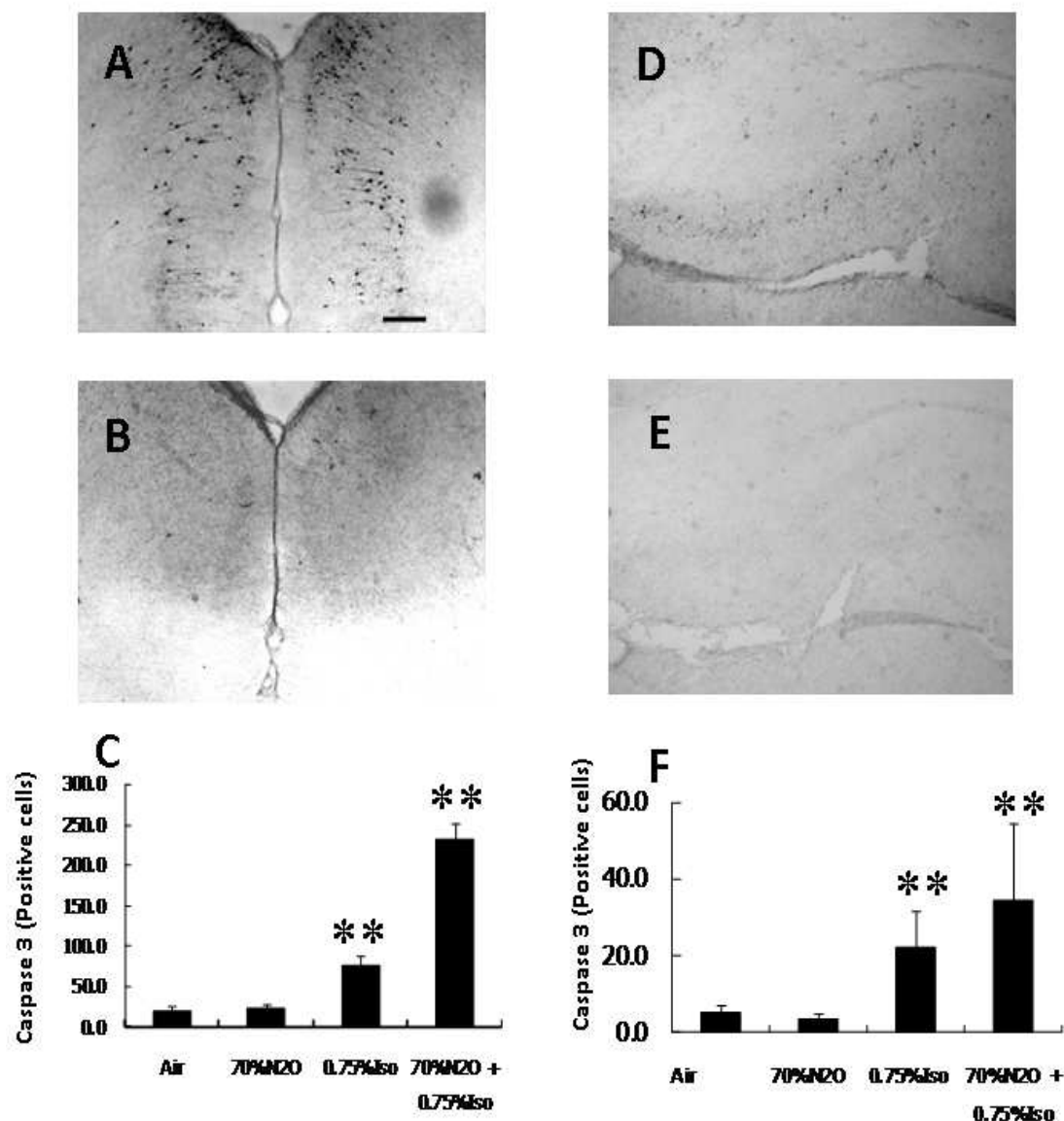


**Figure 4.1 Caspase 3 expressions induced by anesthetics *in vivo*.**

Apoptotic neurodegeneration induced by anesthetics (75% nitrous oxide (N<sub>2</sub>O), 0.75% isoflurane (Iso), 0.75% Iso plus 75% or 35% N<sub>2</sub>O) as measured with cleaved caspase-3 immunostaining in the cortex and the hippocampus of day 7 neonatal rats. (A) Photomicrograph of the cortex of a neonatal rat exposed to 0.75% Iso plus 75% N<sub>2</sub>O. (B) Photomicrograph of the cortex of a neonatal rat in Naïve group. (C) Data (mean  $\pm$  SD,  $n = 5$  or  $6$ ) of caspase 3–positive cells in the cortex from all treatment groups. (D) Photomicrograph of the hippocampus of a neonatal rat exposed to 0.75% Iso plus 75% N<sub>2</sub>O. (E) Photomicrograph of the hippocampus of a neonatal rat in Naïve group. (F) Data (mean  $\pm$  SD,  $n = 5$  or  $6$ ) of caspase 3–positive cells in the hippocampus from all treatment groups. \* $P < 0.01$ , \*\*  $P < 0.001$  versus air. Scale bar = 200  $\mu\text{m}$ .

### 4.3.2 Caspase 3 immunohistochemistry *in vitro*

Neuronal cells expressing cleaved caspase 3 were readily distinguishable in general, exhibiting an intense black staining of the cell soma and dendrites (Figure 4.2). The baseline background level of apoptosis in the negative control has a mean of  $28.0 \pm 8.0$  ( $n = 5$ ), which corresponded to cell death as a result of trauma due to dissection of the tissues and physiological apoptosis during synaptogenesis. Exposure to the individual agents nitrous oxide and isoflurane, resulted in statistically significant mean increase in cell death of  $39.0 \pm 8.0$  ( $n=5$ ) and  $45.0 \pm 9.0$  ( $n=5$ ), respectively compared to control ( $p < 0.01$ ). Combination of isoflurane with nitrous oxide resulted in a statistically significant increase in caspase 3 positive cells ( $60.0 \pm 11.0$ ,  $n=4$ ,  $p < 0.01$ ) (Figure 4.2).

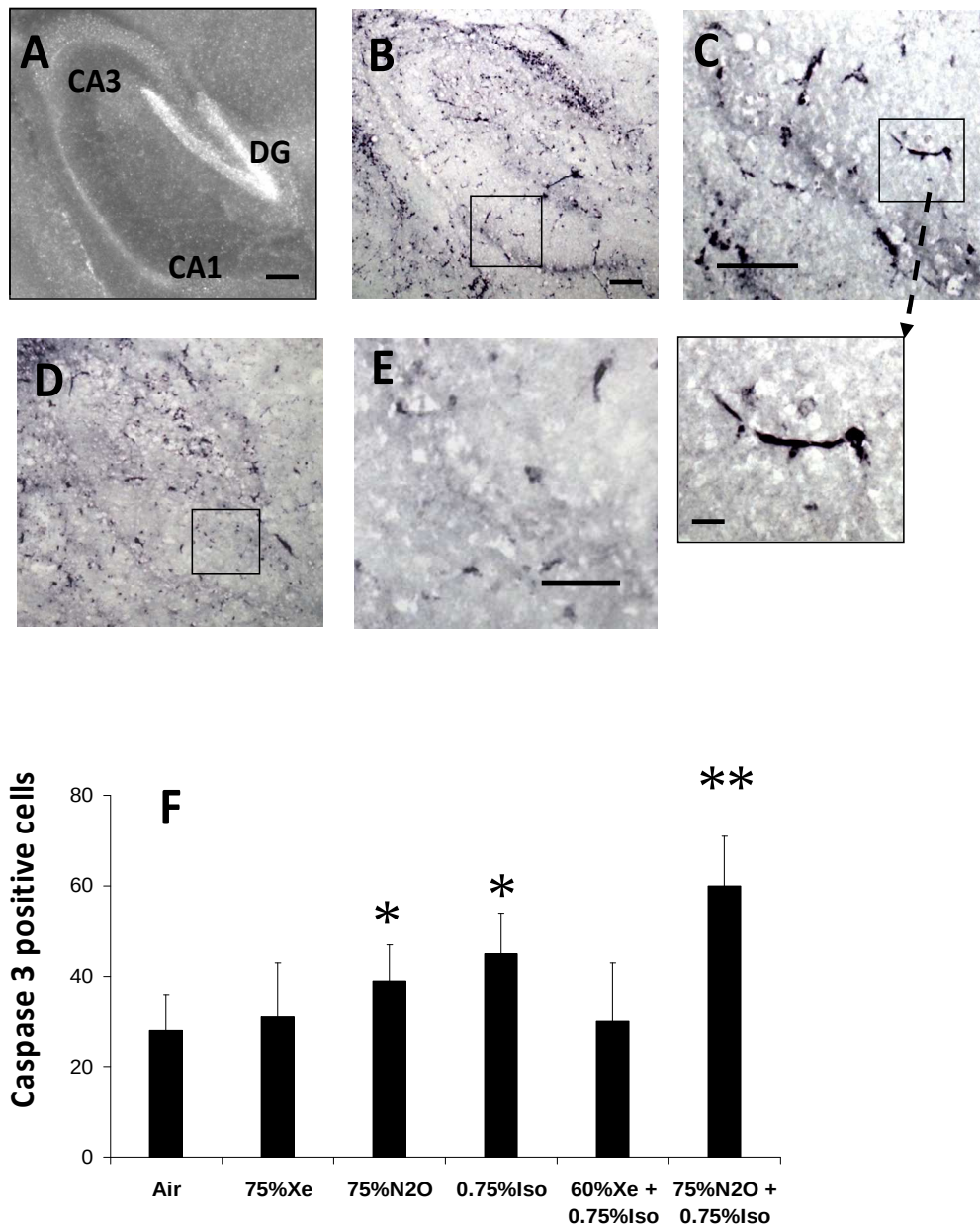


**Figure 4.2 Caspase 3 expression induced by anaesthetics *in vitro*.**

Exposure hippocampus slices *in vivo* to the individual agents isoflurane and nitrous oxide, resulted in a statistically significant ( $p < 0.01$ ) mean increase in cell death above control levels of  $40 \pm 13$  and  $39 \pm 15$ , respectively. Xenon and Iso combination ( $29 \pm 17$ ) have no effect compared with air ( $28 \pm 11$ ). Combination of isoflurane with nitrous oxide resulted in a statistically significant increase in caspase 3 positive cells ( $40.03 \pm 11.71$ ,  $n=6$ ,  $p < 0.01$ ).

#### 4.3.3.1 Immunoblotting of Caspase-3 expression

To investigate the involvement of the common apoptotic pathway, the expression of cleaved caspase-3 protein was measured. In agreement with previous studies using immunohistochemistry (Urraca et al., 2006), immunoblotting indicates that 70% N<sub>2</sub>O alone caused a significant increase in the expression of caspase-3 ( $1.00 \pm 0.03$   $p > 0.05$  vs air) (mean  $\pm$  SD) compared to the baseline. 0.75% isoflurane alone caused a slight increase in the expression of caspase-3 ( $1.31 \pm 0.08$ ,  $p > 0.05$  vs air), but when it was combined with 70% N<sub>2</sub>O it caused a significant increase in the latter ( $3.43 \pm 1.34$ ,  $p < 0.05$  vs air) (Figure 4.3 A).

#### 4.3.3.2 Immunoblotting of cytosolic cytochrome c expression

One of the key markers of intrinsic pathway activation is the release of cytochrome c from the mitochondria into the cytosol. 0.75% isoflurane alone caused no change in cytosolic cytochrome C expression compared to baseline ( $0.91 \pm 0.08$ ,  $p > 0.05$  vs air). However, 70% N<sub>2</sub>O produced a significant increase in cytosolic cytochrome c ( $3.36 \pm 1.33$ ,  $p < 0.05$  vs air). Combination of 0.75% isoflurane with 70% N<sub>2</sub>O induced a moderate increase in cytosolic cytochrome c expression ( $1.10 \pm 0.31$ ,  $p > 0.05$  vs air) (Figure 4.3 B).

#### **4.3.3.3 Immunoblotting of Caspase-9 expression**

Cleaved caspase-9 which is downstream of cytochrome c release was also investigated. 0.75% isoflurane in an oxygen / air mixture did not cause any significant change in caspase-9 expression when compared to control ( $0.94 \pm 0.10$ ,  $P > 0.05$ ). Both 70% N<sub>2</sub>O in oxygen and 70% N<sub>2</sub>O in oxygen combined with 0.75% isoflurane produced increases in caspase-9 expression ( $1.13 \pm 0.08$  and  $1.49 \pm 0.51$  respectively) but these were not statistically significant (Figure 4.3 C).

#### **4.3.3.4 Immunoblotting of Caspase-8 expression**

The expression of cleaved caspase-8 was used as a marker of activation of the extrinsic apoptotic pathway. Neither 0.75% isoflurane alone nor the combination of 70% N<sub>2</sub>O with 0.75% isoflurane produced any change in expression of cleaved caspase-8 expression compared to the baseline ( $0.96 \pm 0.18$  and  $0.92 \pm 0.24$  respectively). However, both 70% N<sub>2</sub>O alone and when combined with 0.75% isoflurane produced moderate increases in caspase-8 expression ( $1.05 \pm 0.21$  and  $1.27 \pm 0.29$  respectively) but these were not statistically significant (Figure 4.3 D).

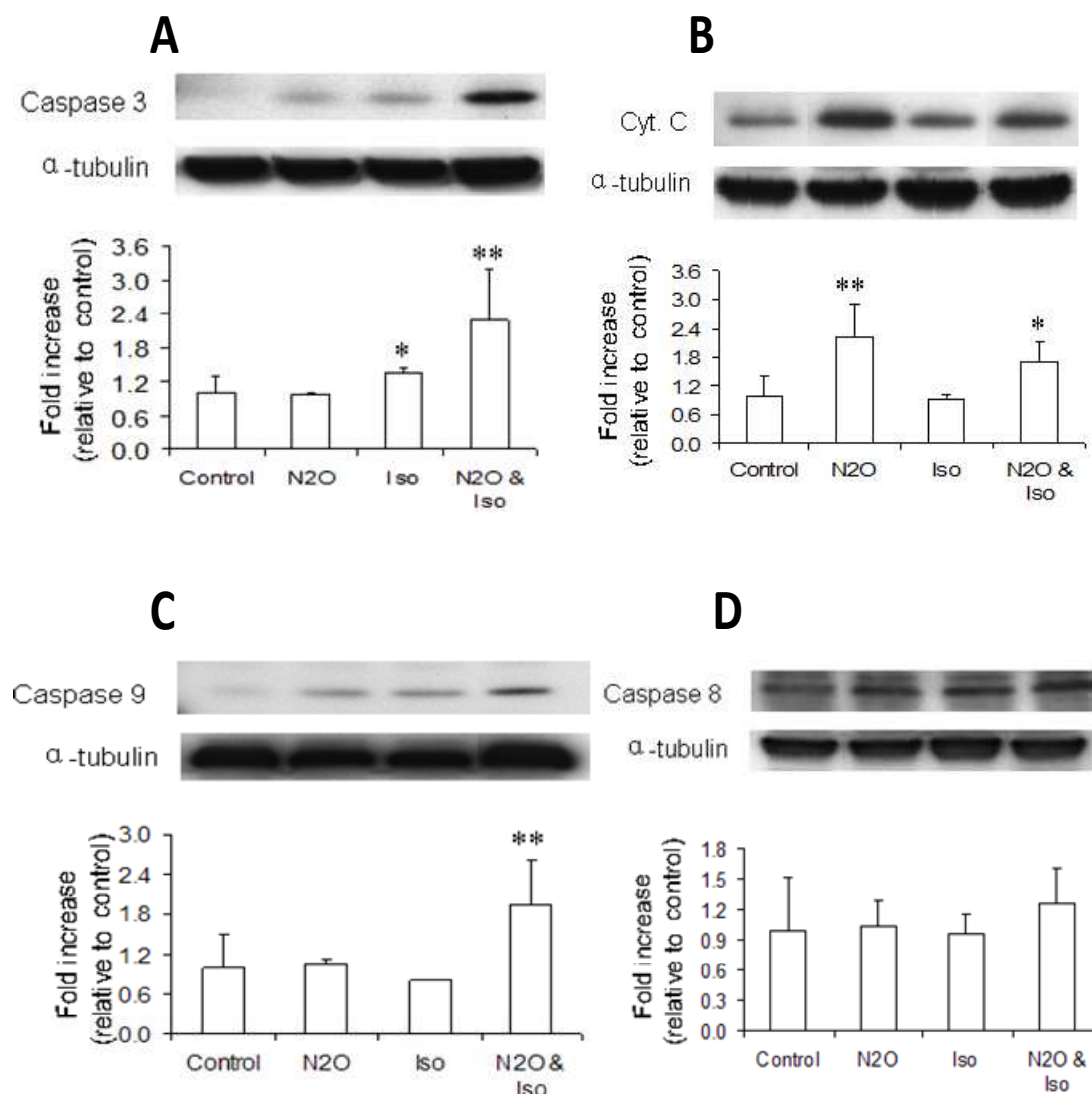


**Figure 4.3 Apoptotic neurodegeneration induced by anesthetics *in vivo*.**

Effects of anesthetic exposure (75% nitrous oxide (N2O), 0.75% isoflurane (Iso), 0.75% Iso plus 75% N2O) for 6 h in day 7 rats on expression of signaling components of apoptotic pathways. Representative immunoblotting images and mean  $\pm$  SD ( $n = 4$ ) of expression of caspase 3 (A), cytosolic cytochrome c (B), caspase 9 (C), and caspase 8 (D) induced by each anaesthetic exposure as reflected by the band density normalized with alpha-tubulin. Control = air exposure; Cyt. C = cytochrome c. \*  $P < 0.05$ , \*\*  $P < 0.01$  versus control.

## 4.4 Discussion

Neuronal cell death can be induced in several ways and these can be identified by their typical morphological appearances (Cozzolino et al., 2004). Broadly, although currently it is still debated as to whether these represent two distinct processes or whether they are just two poles at either end of a continuum, they can be classified as either apoptotic or excitotoxic neuronal degeneration (Yakovlev et al., 2001; Yakovlev et al., 2004). Anaesthetic drugs induced neurodegeneration during the period of synaptogenesis is probably mediated *via* the apoptotic pathway and this may be attributed to their excessive depression of neuronal activity (Cozzolino et al 2004).

There is increasing evidence supporting the view that intracellular calcium ( $\text{Ca}^{2+}$ ) transients are very important for maintaining and protecting healthy synapses (Papadia et al., 2005); it is therefore not surprising that blockade of NMDA subtype of glutamate receptors (which is a main source of these  $\text{Ca}^{2+}$  ions) during the critical period of synaptogenesis can result in neuronal damage. Inhibiting  $\text{Ca}^{2+}$  entry through synaptic NMDA receptors reduces cAMP response element binding (CREB) activity and this has numerous repercussions including diminished neurotrophin expression (e.g. BDNF) (Hansen et al., 2004; Dziejko et al., 2004). It removes proteins which are essential for the survival of neurons and the growth of synapses and hence lead to neuronal death (Huang et al., 2001). Furthermore, NMDA antagonists cause a reduction of extracellular signal-related protein kinase 1/2 (ERK1/2) catalytic activity, and the transcriptional reduction of the anti-apoptotic

protein Bcl2, all of which contribute to a pro-apoptotic environment (Asimiadou et al., 2005; Bittigau et al., 2002).

However, NMDA antagonists are not the only culprits, as exposure to isoflurane alone (a GABA<sub>A</sub> mimetic) may also induce apoptotic neurodegeneration (Hardingham et al., 2005). Activation of GABA<sub>A</sub> receptors during certain periods of the development of the CNS has a depolarising instead of a hyperpolarizing effect on neural membranes (Wang, et al., 2008). However, in line with the importance of Ca<sup>2+</sup> transients and with recent suggestions that proper balance between excitation and inhibition may be critical for cell survival in the CNS several authors (Hardingham et al., 2003; Xu et al., 2000) have suggested that the primary inhibitory brain neurotransmitter GABA produced its neurotoxic by inhibiting neuronal depolarisation and consequently depressed Ca<sup>2+</sup> influx levels resulting in reduced synaptic activity and thus increased the probability of neuronal death (Xu et al., 2000).

The toxic effects produced as a result of drugs interacting with GABAA and/or NMDA subtypes of glutamate receptors have been demonstrated with numerous agents (Ikonomidou et al., 2000; Ikonomidou et al., 1999; Bittigau et al., 2002). There is another interesting finding in the result that N2O alone can also induce caspase expression in vivo. This is relevant to clinic applications, because 70%N2O is common used in human neonatal surgery or delivery period nowadays. I think it is deserve more to detect the pathway of the damage.

## **CHAPTER 5**

# **ANESTHESIA PROVOKES SPINAL CORD INJURY IN NEONATAL RATS**

## 5.1 Introduction

Anaesthesia induces neuroapoptosis throughout the brain in the neonatal rat; this discussed in chapter 4 where rats received either 70% N<sub>2</sub>O or 0.75% ISO in oxygen and air mixture or both anaesthetic drugs. The current hypothesis suggests that inhibition of synaptic activity during the critical period of synaptogenesis results in a reduction in the release of neurotrophic factors at a time when neurons are busy establishing new synaptic contacts and lack of such contact will trigger apoptotic neuronal degeneration in the cells concerned as they are thought to be not essential (Antognini et al., 2002; Rampil et al., 1993). The mechanism of apoptotic injury and the extent of its spatial distribution in central nervous system remain largely unknown.

In this chapter, I shall therefore focus on the spinal cord as a possible site of injury produced by isoflurane and nitrous oxide anaesthesia drugs administered together in a combination similar to the one that produced robust neuroapoptosis in neonatal brain (Jevtovic-Todorovic et al., 2003; Antognini et al., 1993; Rampil et al., 1993). I postulate that anaesthetic treatment would not induce apoptosis in the spinal cord of neonatal rat.

## 5.2 Method

Seven day old Sprague-Dawley rats were exposed to oxygen along with 70% N<sub>2</sub>O + 0.75% isoflurane for 6 hours (n = 3-5/group) (Table 2). Three animals per group were sacrificed at the end of gas exposure; they were perfused transcardially with heparinised saline followed by paraformaldehyde 4% in 0.1M buffer. The spinal cords after they were removed by pneumatic injection were stored overnight at 4°C in paraformaldehyde and then transferred to sucrose solution (30%) with phosphate buffer and sodium azide 1%. They were kept refrigerated until they were sectioned at the lumbar level (Cheng et al., 2000). The sections were then incubated at 4°C with rabbit anti-cleaved caspase-3 (1:2500; New England Biolab, Hitchin, UK). The numbers of caspase-3 immunoreactive neurons were counted in four lumbar spinal cord sections per animal by an investigator blinded to the experimental protocol.

|                                           |
|-------------------------------------------|
| Naïve                                     |
| N <sub>2</sub> O(70%) +ISO(0.75%) 6 hours |

**Table 2. Seven day old Sprague-Dawley rats were exposed to different conditions presented above.**

## 5.3 Results

Anaesthesia with nitrous oxide and isoflurane for 6 hours increased the number of caspase-3 positive cells relative to air exposure ( $p < 0.01$ ,  $n = 3$  per group) (Figure 5.1).

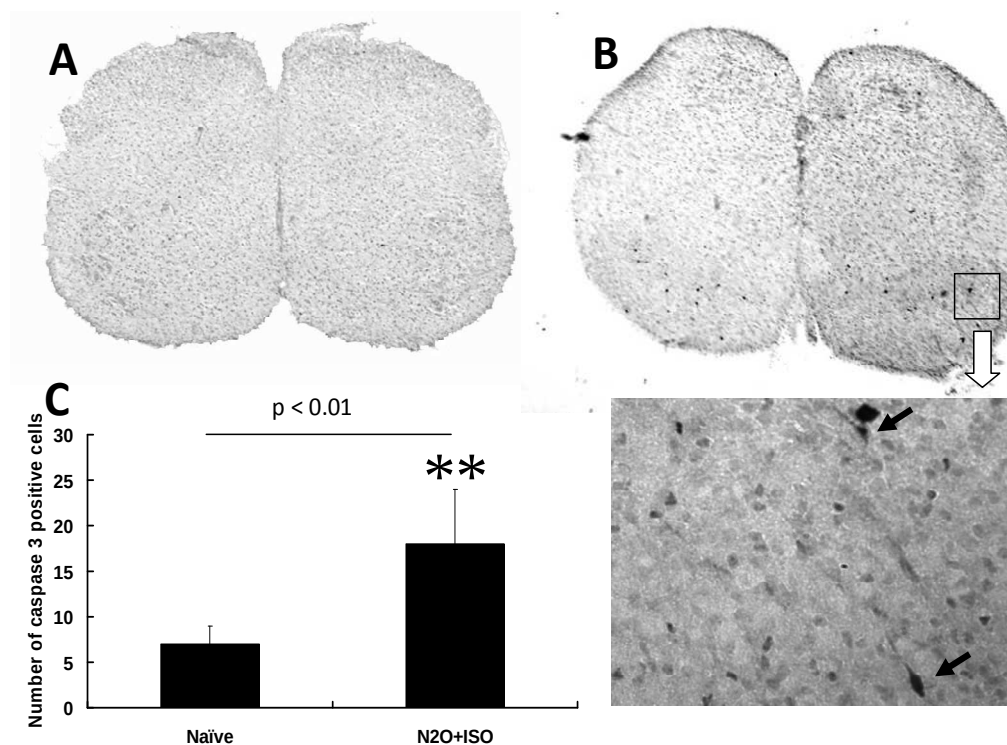


**Figure 5.1 Apoptotic neurodegeneration induced by anesthetics in spinal cord**

Apoptotic neurodegeneration induced by 75% Nitrous oxide + 0.75% isoflurane as measured with caspase-3 immunostaining in the lumbar enlargement of spinal cord of 7-day-old neonatal rats ( $n = 3$  per group). A, Air control; B, 75% Nitrous oxide + 0.75% isoflurane in oxygen; C, Mean (mean  $\pm$  SD;  $n = 3$ ). Black arrows = caspase 3 positive cells.

## 5.4 Discussion

The results demonstrate that exposure to an isoflurane-nitrous oxide based anaesthetic provokes apoptosis in the neonatal rat spinal cord as evidenced by the increase in cleaved-caspase 3 immunoreactivity. Furthermore, the current work, described here, demonstrates that the apoptotic injury is distributed over a wider area in the central nervous system than has previously been reported in other studies (Antognini et al., 1993; Antognini et al., 2002; Rampil et al., 1993; Rampil et al., 1994; De Jong and Wagman, 1968).

In previous studies, I used caspase-3 immunohistochemistry to identify the apoptotic injury as it is an early marker of cellular apoptosis and also it is considered to be a reliable marker for apoptosis in previously published studies where anesthetic drug induced injury in the brain were evaluated (Jevtovic-Todorovic et al., 2003). Therefore, in this study, we use caspase- 3 immunohistochemistry technique to identify neuronal apoptosis in the spinal cord.

The observed vulnerability of the ventral horn of the spinal cord to anaesthetic injury is in keeping with the ability of anaesthetic agents in suppressing neurotransmission in this region (Paxinos and Watson, 1998). Previous studies suggest that anaesthetic drugs must produce adequate depth of anaesthesia to produce their neurotoxic effects in the spinal cord (Antognini et al., 2002). This may indicate that the same mechanisms which produce the state of general anaesthesia by anaesthetic drugs

may also explain the apoptosis-induced cell death by these drugs (Antognini et al., 2002). Indeed, escalating doses of isoflurane provoke increasing apoptosis in the brain (Jevtovic-Todorovic et al., 2003).

However, it is also possible that both anaesthetic agents inhibit the apoptotic injury and increase the depth of anaesthesia through differing mechanisms. Nonetheless, the data presented here appear to support further the link between the ability for anaesthetic agents in inhibiting neuronal activity and provoking cellular death.

Anaesthetic-induced neurodegeneration in the spinal cord may have clinical significance despite this result uncover a functional endpoint for the injury. A fairly recent randomized controlled trial (the GAS study) was initiated to compare the effects of exposure of regional to general anaesthesia on neurodevelopment in rats during the neonatal period (Devor et al., 2001). The data suggest that the subjects in both arms of the trial may be vulnerable to spinal cord injury. The potential vulnerability of the neonatal spinal cord to neuroapoptosis induced by anaesthetic agents was, hitherto, unknown. Thus clinical trials may need to consider the pro-apoptotic potential of both general (Antognini et al., 1993; Antogini et al., 2002; Rampil et al., 1993; Rampil et al., 1994; De Jong et al., 1968) as well as local anaesthetics (Stabernack et al., 2005; Kingery et al., 2002) as the anaesthetic injury in the neonate is both dose- and duration-dependent, the potent inhibitory effects on neurotransmission of regional anaesthesia may also induce neuroapoptosis in the spinal cord. Indeed, because of the intensity and duration of action of regional

anaesthetics, the injury in the spinal cord after spinal anaesthesia may be more pronounced than after general anaesthesia. Functional neurodevelopmental assessments following regional anaesthetic treatment will also be required to address the potential impact of the findings on humans. As the methods employed in preclinical studies may be insensitive to the subtle changes induced in the developing nervous system in humans, future clinical trials should also consider investigation of sensory and motor responses during development of humans following exposure to general anaesthetic drugs in the neonatal period.

## **CHAPTER 6**

# **ANAESTHESIA-INDUCED NEURODEGENERATION IN DIFFERENT REGIONS OF THE DEVELOPING BRAIN**

## 6.1 Introduction

Neurons are the principle cells in the central nervous system; they can be classified, based on the neurotransmitters they release, for example glutamatergic, GABAergic, dopaminergic and cholinergic neurons (Steven and Hyman 2005). Neocortex and hippocampus (including cingulate cortex) are crucial brain structures involved in learning and memory (Squire et al., 2004; Wiltgen et al., 2004; Frankland and Bontempi, 2005; Blum et al., 2006) and they have mainly glutamatergic and GABAergic neurons (Sem'yanov, 2005; DeFelipe et al., 2002). These two types of neurones are widely distributed in the CNS (Foster and Kemp, 2006). Acetylcholine (ACh) is also critical for cognitive processing, arousal, and for attention in the brain (Karczmar 2010). Based on behavioural studies conducted in animals so far, general anaesthetics are believed to elicit a hippocampus-dependent learning deficit (Sall et al., 2009; Satomoto et al., 2009).

In humans, memory deficits associated with Alzheimer's disease and normal aging can be attributed to degeneration of cholinergic neurons in the basal forebrain region, particularly in the nucleus basalis of Meynert (Muir, 1997; Wu et al., 2005). Within the basal forebrain cholinergic system, cholinergic neurons primarily project to the hippocampus and neocortex (Phillis 2005). Isoflurane suppresses the release of ACh in these two brain regions in rats (Shichino et al., 1998; Whittington and Virag, 2006), the mechanism by which this is achieved remains unknown; such an effect may be due to anaesthesia-induced cholinergic neuron death in the basal forebrain.

Up to now, most research studies have been conducted on catecholamine neurotransmitters. More recently, an increasing number of studies have been published supporting the view that dopamine pathways play an important role in learning and memory and they also have a significant impact on cognition and emotion (Packard and Knowlton, 2002; Jay, 2003; González and Feria, 2008; Olvera-Cortés et al., 2008; Nieoullon and Coquerel, 2003). Neurochemically the hippocampus, neocortex and striatum receive midbrain dopaminergic projections mainly from the substantia nigra with some from the ventral tegmental area (Björklund and Dunnett 2007). The release of dopamine in these regions can enhance long-term potentiation (Jay, 2003). In Parkinson's disease, cell death in the substantia nigra leads to a reduction in dopaminergic output (Schapira, 2009) and behavioural studies in patients with Parkinson's disease have demonstrated that the circuitry required for learning the stimulus-response associations is disrupted (Knowlton et al., 1996).

Furthermore, several *in vivo* studies have indicated that anaesthetic drugs may have an impact on dopaminergic neurons. For example, it has been shown that the level of dopamine in the striatum rises following anaesthetic exposure (Adachi et al., 2000; Adachi et al., 2005) and general anaesthetics with the neurotoxin, 6-hydroxydopamine could significantly increase the extent of nigrostriatal dopaminergic neuronal loss (Datla et al., 2006). Therefore, we hypothesise that anaesthetic exposure during the period of rapid synaptogenesis may lead to an increase in cell death in the four types of neurons described above in specific brain regions, which could contribute to learning and memory deficits observed later on in life.

Animal studies have shown that anaesthetic drugs induced neuronal cell death is mediated *via* the apoptotic pathway (Ma et al., 2007; Sanders et al., 2008; Johnson et al., 2008; Wei et al., 2008; Lu et al., 2006). The study, described in this chapter, was designed to investigate whether gaseous or volatile anaesthetics (70% nitrous oxide and 0.75% isoflurane) would induce cell apoptosis in glutamatergic, GABAergic, dopaminergic and cholinergic neurones in specific brain regions of 7-day-old rats.

## 6.2 Method

### 6.2.1 *In vivo* experiment

Separate cohorts (n= 4-6 per group) of 7-day- old littermate Sprague-Dawley rat pups were randomly assigned to two groups: Naïve (room air); Anaesthetics (70% nitrous oxide and 0.75% isoflurane for 6 hrs) (Table 2).

|                                           |
|-------------------------------------------|
| Naïve                                     |
| N <sub>2</sub> O(70%) +ISO(0.75%) 6 hours |

**Table 3. Seven day old Sprague-Dawley rats were exposed to different conditions presented above.**

The remaining gas constituents were 25% oxygen in groups 2-6 and balanced with nitrogen where necessary. According to the Animals Act, surgical incision and formalin injection were conducted 10 minutes after rats were anaesthetised with 70% nitrous oxide and 0.75% isoflurane and rats in group 3 and group 5 were then exposed to room air rather than anaesthetic agents for 6 hours. Naive rats were placed in exposure chambers for 6 hr without exposure to anaesthetic agents or

noxious stimuli but otherwise similar conditions as those for the other studied groups were maintained.

For glutamatergic and GABAergic neurons, the cingulate cortex (Bregma 2.04-1.08mm) and CA1 subarea of the hippocampus (Bregma -5.40mm) were examined.

For cholinergic neurones, the entire basal forebrain was examined (Bregma 0.60mm).

For dopaminergic neurones, the substantial nigra was examined (Bregma -5.04mm).

### **6.2.2 Double-labelling fluorescent immunohistochemistry**

Double-labelling fluorescent immunohistochemistry was used in this study. Both caspase-3 and one specific neuronal-cell-type marker were stained. This staining technique has been shown to be effective (Mason et al., 2000; Negoescu et al., 1994) and is being used commonly in many studies for visualization of two antigens in the same tissue sections (Wu et al., 2005; Taatjes et al., 2007; Dürr et al., 2007).

The whole staining process in this study was conducted at the room temperature.

#### **6.2.2.1. Caspase-3 immunofluorescence staining**

Selected brain slices were washed in PBS for minimal 30 minutes, then incubated in 10% normal goat serum (NGS, Bioclear) diluted in PBST (0.3% Triton X-100 (Promega

corporation, Madison, WI) in PBS) for 1 h. After that, sections were incubated overnight with rabbit anti-cleaved caspase-3 monoclonal antibody (1:2500, Cell Signalling) diluted in 1% NGS in PBST. The next day, sections were washed in PBS (x3, 10mins each) and incubated for 1 hour with a CyTM3-conjugated goat-anti-rabbit secondary antibodies (1:200, IgG, Jackson ImmunoResearch) diluted in 1% NGS in PBST for two hrs. Sections were next washed in PBS (x3, 10mins each).

#### **6.2.2.2. Glutamatergic, GABA-ergic, cholinergic and dopaminergic neuron immunofluorescence staining**

Immediately after the caspase-3 immunofluorescence staining, all sections were incubated in 10% normal donkey serum (NDS, Millipore) diluted in PBST for 1 h. Then sections containing different brain bregma regions were incubated with different primary antibodies (see below). Primary and secondary antibodies were diluted in 1% NDS in PBST. The fluorescent probe: streptavidin Alexa Fluor 488 (1:1000, Invitrogen) was diluted in PBST. Between incubations, sections were washed 3 x 10 min in PBS.

For glutamatergic, GABAergic and dopaminergic neurons, sections were incubated in mouse anti-vesicular glutamate transporter 1 (vGLUT1) (1:100, Millipore), mouse anti-glutamic acid decarboxylase-67 (GAD67) (1:5000, Sigma) and mouse anti-dopamine (1:100, Millipore) monoclonal antibodies overnight respectively. Thereafter, sections were incubated for 1 h in 1:200 biotinylated donkey anti-mouse secondary antibodies (IgG, Millipore) followed by streptavidin Alexa Fluor 488 for 1 h.

For cholinergic neurons, sections were incubated in goat anti-choline acetyltransferase (ChAT) polyclonal antibodies (1:200, Millipore) overnight. Sections were next incubated for 1 h in 1:200 biotinylated donkey anti-goat secondary antibodies (IgG, Millipore) followed by 1 h in streptavidin Alexa Fluor 488.

## 6.3 Results

### 6.3.1 The neuronal types involved in anaesthesia-induced neuroapoptosis

Cingulate cortex: 30 % and 37 % of all apoptotic cells were GABAergic and glutamatergic neurons respectively (Total number of caspase-3 positive cells in the A alone group: 30; GAD67/caspase-3 co-expressing cells: 9; VGLUT1/caspase-3 co-expressing cells: 11) (Figure 6.1). CA1 subarea of hippocampus: 54 % and 14 % of all apoptotic cells were GABAergic and glutamatergic neurons respectively (Total number of caspase-3 positive cells in the A alone group: 56; GAD67/caspase-3 co-expressing cells: 30; VGLUT1/caspase-3 co-expressing cells: 8) (Figure 6.2). Substantia nigra: 22 % of all apoptotic cells were dopaminergic neurons (Total No. of caspase-3 positive cells in the A alone group: 73; dopamine/caspase-3 co-expressing cells: 16) (Figure 6.3). Basal forebrain: none of the apoptotic cells were cholinergic neurons (Total No. of caspase-3 positive cells in the A alone group: 7; ChAT/caspase-3 co-expressing cells: 0) (Figure 6.4).

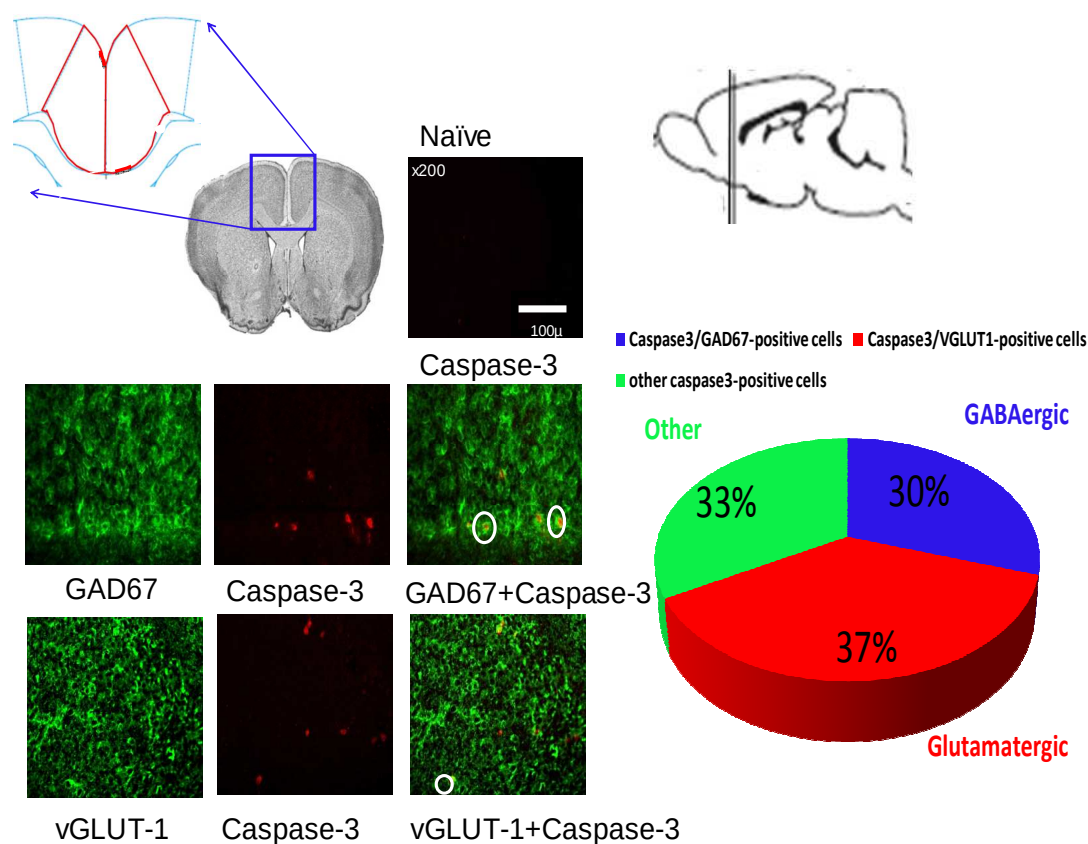


**Figure 6.1 GAD67/caspase-3 co-expressing and VGLUT1/caspase-3 co-expressing cells in cingulate cortex**

Separate cohorts (n= 4-6 per group) of 7-day- old, littermate Sprague-Dawley rat pups were randomly assigned to two groups: Naïve (room air) and Anaesthetics (70% nitrous oxide and 0.75% isoflurane for 6 hrs). The cingulate cortex of hippocampus was sectioned and slices were stained with anti-caspase 3 antibodies together with anti- vesicular glutamate transporter 1 (vGLUT1) antibodies (staining GABAergic neurons or mouse anti-glutamic acid decarboxylase-67 (GAD67) antibodies (staining glutamatergic neurons). The percentage of caspase 3 positive cells in GABAergic neurons, the percentage of caspase 3 positive cells in glutamatergic neurons and the percentage of caspase 3 positive cells in other cells was calculated.

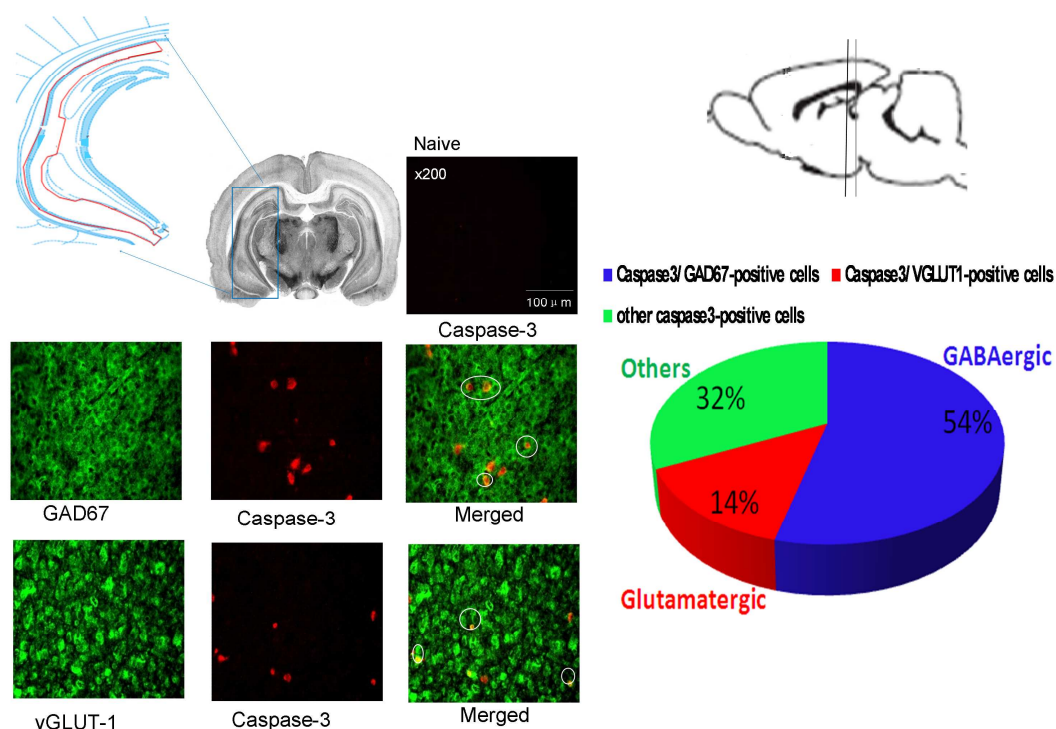


**Figure 6.2 GAD67/caspase-3 co-expressing and VGLUT1/caspase-3 co-expressing cells in hippocampus**

Separate cohorts (n= 4-6 per group) of 7-day- old, littermate Sprague-Dawley rat pups were randomly assigned to two groups: Naïve (room air) and Anaesthetics (70% nitrous oxide and 0.75% isoflurane for 6 hrs). The CA1 subarea of hippocampus was sectioned and slices were stained with anti-caspase 3 antibodies together with anti- vesicular glutamate transporter 1 (vGLUT1) antibodies (staining GABAergic neurons or mouse anti-glutamic acid decarboxylase-67 (GAD67) antibodies (staining glutamatergic neurons). The percentage of caspase 3 positive cells in GABAergic neurons, the percentage of caspase 3 positive cells in glutamatergic neurons and the percentage of caspase 3 positive cells in other cells was calculated.

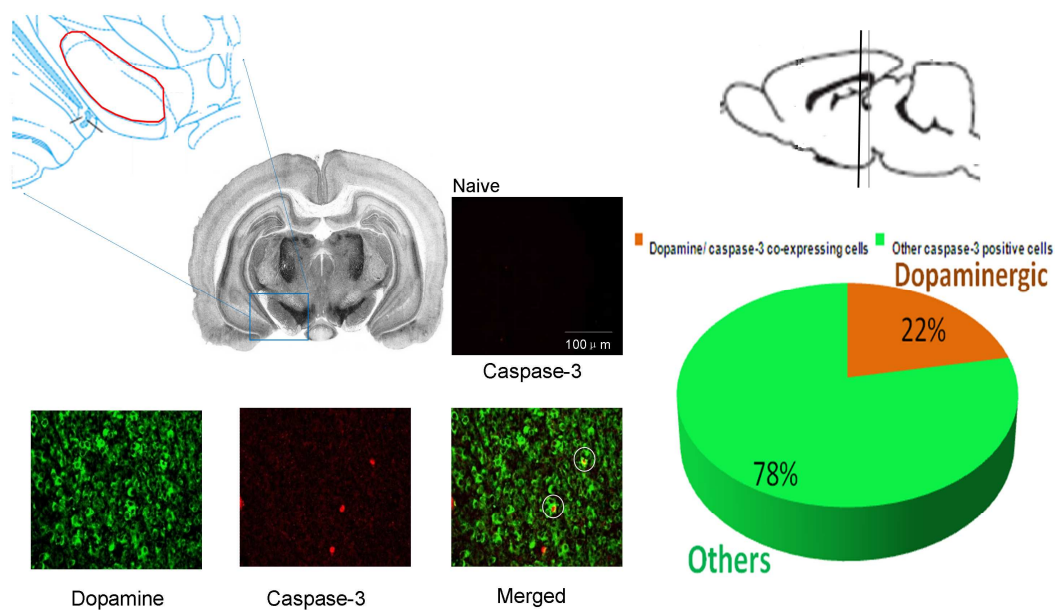


**Figure 6.3 Dopamine/caspase-3 co-expressing cells in substantia nigra.**

Separate cohorts (n= 4-6 per group) of 7-day- old, littermate Sprague-Dawley rat pups were randomly assigned to two groups: Naïve (room air) and Anaesthetics (70% nitrous oxide and 0.75% isoflurane for 6 hrs). The substantia nigra was sectioned and slices were stained with anti-caspase 3 antibodies together with anti-dopamine antibodies (staining dopaminergic neurons). The percentage of caspase 3 positive cells in dopaminergic neurons and the percentage of caspase 3 positive cells in other cells were calculated.

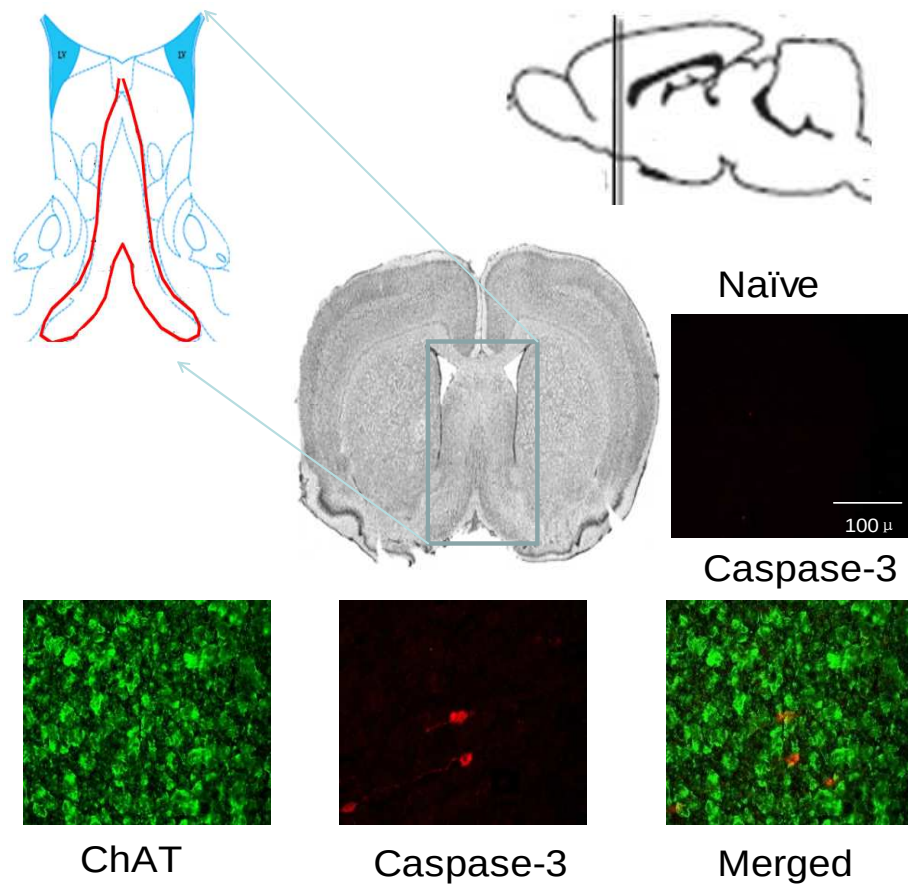


**Figure 6.4 ChAT/caspase-3 co-expressing cells in basal forebrain**

Separate cohorts (n= 4-6 per group) of 7-day- old, littermate Sprague-Dawley rat pups were randomly assigned to two groups: Naïve (room air) and Anaesthetics (70% nitrous oxide and 0.75% isoflurane for 6 hrs). The basal forebrain was sectioned and slices were stained with anti-caspase 3 antibodies together with anti-choline acetyltransferase (ChAT) antibodies (staining cholinergic neurons).

## 6.4 Discussion

The study presented herein demonstrates that clinically commonly-used general anaesthetics administered to rats during the rapid period of synaptogenesis can cause significant increase in cell apoptosis in the cingulate cortex, CA1 subarea of hippocampus and substantia nigra. The cells involved included glutamatergic, GABAergic and dopaminergic neurons. The basal forebrain which contains mainly the cholinergic neurons is spared from such neurotoxicity. Moreover, noxious stimulations can exacerbate anaesthesia-induced cell apoptosis in the cingulate cortex.

### 6.4.1 The mechanisms of activation of apoptosis

The mechanisms of activation of anaesthesia-induced neuroapoptosis remain elusive, though majority of general anaesthetics, i.e. nitrous oxide and isoflurane, are both N-methyl-D-aspartate (NMDA) subtype of the glutamate receptor antagonists and gamma-aminobutyric acid type A (GABA<sub>A</sub>) receptor agonists, so it is postulated that anaesthesia-induced GABA<sub>A</sub> receptor activation and NMDA-receptor blockade during a critical stage in brain development lead to increased cellular stress, which initiates the apoptotic cell death cascade in immature neurons (Jevtovic-Todorovic, 2005; Ikonomidou et al., 2001; Pohl et al., 1999; Ikonomidou, 2009). Moreover, animal studies have demonstrated that anaesthetic exposure is associated with a decrease in brain-derived neurotrophic factor (Lu et al., 2006) and severe depletion of synaptic proteins (Nikizad et al., 2007), which could be responsible, at least in part,

for putting neuronal survival at risk. A recent *in vitro* study has shown that isoflurane activates inositol 1,4,5-trisphosphate (IP3) receptors on the endoplasmic reticulum membrane inside cells, causing excessive calcium release, thus triggering apoptosis (Wei et al., 2008). Moreover, activation of proneurotrophin (proBDNF) signalling pathway *via* p75 neurotrophin receptor has also been shown to contribute to isoflurane-mediated neurotoxicity in mice (Head et al., 2009). Therefore, phenotypical differences among different neuronal cell types (i.e. relative differences in the expression of NMDA and GABA<sub>A</sub> receptors) and the different “niches” in which neurons are found (i.e. relative differences in the level of brain-derived neurotrophic factors in different brain regions) may explain why the glutamatergic, GABAergic and dopaminergic but not the cholinergic neurons are susceptible to anaesthesia-induced neuroapoptosis. Moreover, different parts of the central nervous system mature at different rates (Clancy et al., 2001; Clancy et al., 2007). It is possible that the stage of development of the basal forebrain in neonatal rats is not in the vulnerable period at seven days after birth and thus is spared from anaesthesia-induced neuroapoptosis.

Moreover, noxious stimulations inevitably provoke tissue damage and inflammation. Various pro-inflammatory factors are identified in significant amounts in the blood stream shortly after the onset of tissue injury (Lenz et al., 2007). A recent *in-vivo* study using intravenous anaesthetic, propofol, has demonstrated administration of the drug for greater than four hours was associated with a significant elevation in the level of the pro-inflammatory cytokine, TNF- $\alpha$ , in the brain regions where increased cell apoptosis was observed. TNF- $\alpha$  is one of the activators of the extrinsic

apoptotic pathway (Pesić et al., 2009). Therefore, it is possible that anaesthetic drugs induce the production of pro-inflammatory markers which stimulate the extrinsic apoptotic cascade and that noxious stimulations may further exacerbate this cascade potentiating the apoptotic injury. This could be confirmed by measuring inflammatory factors such as TNF- $\alpha$  and also looking for markers the activation of the extrinsic apoptotic pathway such as caspase-8 in the damaged brain regions. However, this theory does not explain why in the present study, noxious stimulations alone did not induce neuroapoptosis and also why the basal forebrain was spared from such damage. Furthermore, the blood-brain barrier (BBB) in 7-day-old rats is well developed and it can exclude exogenous inflammatory proteins from entering the brain's extracellular space (Saunders et al., 1999). Therefore, I speculate that anaesthetics, somehow, can induce the release of endogenous inflammatory factors from cells present in the CNS, for example from microglial cells or from neurons themselves leading to neuroapoptosis. However, this process only takes place in certain "vulnerable" brain regions such as the cingulate cortex. The endogenous inflammatory factors in the "vulnerable" brain regions lead to conformational changes in the local BBB which becomes permeable and allow extravasations of exogenous inflammatory factors (Abbott, 2000) further exacerbating anaesthesia-induced neuroapoptosis.

The present study demonstrated that noxious stimulations exacerbated anaesthesia-induced neuroapoptosis in the cingulate cortex.

### 6.4.2 Potential anaesthesia-induced long-term effects

The developing brain has a high degree of plasticity (Nudo, 2006). Additional divisions of progenitors in the 7-day-old rat brain should be able to compensate for the loss of cells due to anaesthesia-induced cell death. But a recent study which showed that isoflurane can impair neurogenesis supports the hypothesis that anaesthesia-induced cell death is likely to be irreversible and therefore it can lead to permanent neurocognitive deficits later on in life (Stratmann et al., 2009). The increased neuronal cell death in the cingulate cortex, hippocampus and substantia nigra could be responsible for the observed higher incidences of learning disabilities in children who had multiple general anaesthesia and surgeries (Wilder et al., 2009; Kalkman et al., 2009).

In addition, as the dopaminergic neurons degenerate in Parkinson's disease by processes that are not fully understood (Forno 1996), increased dopaminergic neuronal cell death post 6-hour anaesthetic exposure in rats raises the concern that children having general anaesthesia early in age may be at higher risk of developing Parkinson's disease later on in life. Moreover, the present study showed that the basal forebrain is spared from anaesthesia-induced neuronal cell death suggesting that anaesthetic exposure at an early age may not be a risk factor for developing Alzheimer's disease in older age. Moreover, the sparing of basal forebrain from anaesthesia-induced neuronal cell death also suggests that isoflurane-induced decrease in the release of acetylcholine in the rat cerebral cortex (Shichino et al., 1998) and dorsal hippocampus (Whittington and Virag, 2006) in-vivo may not be

caused by the degeneration of cholinergic neurons in the basal forebrain but rather a direct inhibitory effect of isoflurane on cholinergic neurons.

Cingulate cortex is also in resting and dream function. Emotional features in dreams would be related to the activation of orbito-frontal cortex and anterior cingulate cortex. The relative hypoactivation of the prefrontal cortex would explain the alteration in logical reasoning, working memory, episodic memory and executive functions (Maquet 2004). Anaesthetics may induce psychiatric disorders such as schizophrenia associated with hallucination. Schizophrenia is a complex neuropsychiatric disorder in which symptoms can be classified as either positive, such as delusions and hallucinations in an animal model of schizophrenia an increase in oxidative damage marked by an increase in lipid peroxidation, oxidative protein damage and a decrease in enzymatic defenses (de Oliveiral, 2009).

## **CHAPTER 7**

# **EFFECT OF DIFFERENT PRECONDITIONING ON NEUROAPOPTOSIS IN THE DEVELOPING BRAIN**

## 7.1 Introduction

In most situations, the onset of injury is unpredictable but in those clinical settings in which it can be anticipated, for example, during brain or kidney surgery, pre-emptive measures may be considered. It has been proposed as early as 1943 that rats subjected to small graded amounts of injury, induced by hypoxia, acquired a tolerance enabling them to withstand an otherwise fatal insult (Noble 1943). However, it was not until 1986 that the concept of “preconditioning” was officially introduced into the scientific community, when Murry and colleagues demonstrated through a series of landmark experiments on canine hearts that, contrary to traditional and intuitive thinking, ischaemia is not always harmful (Murry et al., 1986). They showed that pre-treatment of the heart with repeated brief episodes of ischaemia interwoven with reperfusion protected the myocardium from a subsequent, prolonged ischaemic insult and reduced the size of myocardial infarct by as much as 75 % compared to controls (Murry et al., 1986). In the 20 years since its first discovery, Murry’s seminal paper is currently the most cited article published by *Circulation*, and a Pubmed search reveals that there are, to date, in excess of 2000 publications directly attributed to the profound effects of preconditioning. Interestingly, Murry and colleagues's studies overshadowed the work by Schurr, which was published in the same year as theirs, demonstrating the adaptation of adult rat hippocampal slices to anoxia by preconditioning it to hypoxia (Schurr et al., 1986). Since then, the protective effect of preconditioning has been demonstrated in all organs; the first demonstration of cerebral ischaemic preconditioning against ischaemic injury was shown by Kitagawa (Kitagawa et al., 1990).

In rodents, synaptogenesis is predominantly a post-natal event, and is most intense just prior to birth and in the first two weeks postnatal weeks. In humans, synaptogenesis begins at 6 months' gestation and ends a few years after birth. I sought to determine whether the anaesthetic-induced neurotoxicity could be attenuated by preconditioning. I sought to determine whether preconditioning strategies can be effective against anesthetic-induced neurotoxicity. Ischaemia has been used as the stimulus in most studies of preconditioning, however, other interventions can also stimulate preconditioning, for example, hyperoxia, oxidative stress, hyperthermia, heat shock and anaesthetics including xenon.

At mean time cerebral hypoxia or ischemia have shown that neuronal death may occur via necrosis or apoptosis. For example, following hypoxia or ischemia, excitatory amino acid release and alterations in ionic homeostasis contribute to both necrotic and apoptotic neuronal death. However, apoptosis is distinguished from necrosis in that gene activation is the predominant mechanism regulating cell survival. Following hypoxic or ischemic episodes in the brain, genes that promote as well as inhibit apoptosis are activated (Banasiak et al., 2000).

I postulated that preconditioning may protect the brain of neonatal rats from anaesthetic drug induced apoptosis.

## 7.2 Method

### 7.2.1 Animals

Postnatal day 7 (PND7) Sprague-Dawley (SD) rat pups (Harlan UK) were used for *in vivo*, *in vitro* and behavioural 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. The data of the birth was defined as day 0. On PND 7 which is the period of synatogenesis (Clancy et al., 2001), pups were used for experiments.

### 7.2.2 *In vivo*

Seven day old Sprague Dawley rat pups were pre-treated with one of the following: 70% xenon, 70% N<sub>2</sub>O, 8% oxygen (hypoxia). They were then exposed to the anaesthetic combination of 70% N<sub>2</sub>O + 0.75 % isoflurane for 6h (Table 3). The rats were sacrificed after anaesthesia, and their brains processed to assess the severity of apoptosis, caspase 3 as a marker. Brain slices and brain tissue was collected, immunostaining and westernblot was performed to measure the apoptosis.

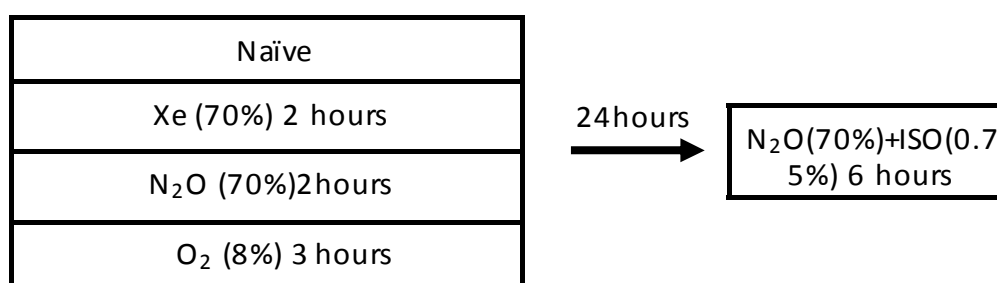


**Table 3. Seven day old Sprague-Dawley rats were exposed to different conditions presented above.**

### 7.2.3 Preconditioning with hypoxic ischaemia

Seven day old Sprague Dawley rat pups were pre-treated with, 8% oxygen (hypoxia) or ischaemia by ligating the right carotid artery. They were then exposed to the anaesthetic combination of 70% N<sub>2</sub>O +0.75% isoflurane for 6h (Table 4). The brains were removed immediately after anaesthetic exposure and sectioned for Cresyl Violet (Nissl) Staining.

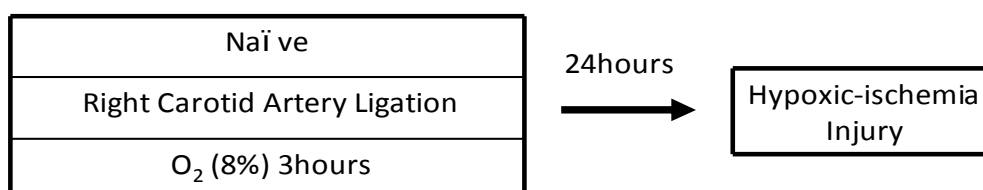


**Table 4. Seven day old Sprague-Dawley rats were exposed to different conditions presented above.**

### 7.2.4 Western blotting

The supernatant was further centrifuged twice, initially at 11,000g for 20minutes at 4°C for western blotting of Bcl-2 and cleaved caspase-3, and a second time at 20,000g for 45 minutes at 4°C to separate cytosolic and mitochondrial material for western blotting of cytosolic cytochrome C. The membrane was probed with primary antibodies for Bcl-2, cleaved caspase-3 (Cell Signaling Technology, Inc, USA), cytochrome C (BD Biosciences Pharmingen, Oxford, UK), P53, and MAPK (Cell Signaling Technology, Inc, USA), followed by relevant species-derived horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. Thereafter, the membrane was re-probed with housekeeping protein  $\alpha$ -tubulin antibody (Sigma, Poole, UK).

### 7.2.5 Caspase-3 Immunohistochemistry

Photomicrographs were taken of the whole hemisphere and hippocampus for *in vivo* and of the hippocampus CA area for *in vitro* experiments with a BX-60 light microscope (Olympus, Southall, United Kingdom) and Axiocam digital camera (Zeiss, Göttingen, Germany). The images were then printed and an investigator blinded to the experimental protocol counted the number of DAB stained (black) cell bodies in the desired areas. Counting was performed on slices from the cortex and the hippocampus for *in vivo* experiments and from the hippocampal CA1-CA3 subregions for *in vitro* experiments.

### 7.2.6 Cognitive function assessment

Cognitive assessment was performed in separate cohort of animals 40 days after anesthetic exposure. The conditioning chamber (30 cm × 24 cm × 21 cm; Med Associates, Inc., St. Albans, VT) had a white opaque back wall, aluminium sidewalls, and a clear polycarbonate front door. The conditioning box had a removable grid floor and waste pan. Between each rat, the box was cleaned with an almond-scented solution and dried thoroughly. The grid floor contained 36 stainless steel rods (diameter, 3 mm) spaced 8 mm center to center, which made contact with a circuit board through which a scrambled shock was delivered. During training and context testing, a standard high efficiency particulate air filter (HEPA) provided a background noise of 65 db. On day 1 (41 days after anesthetic exposure) (Acquisition), all animals received 6 cycles of 214 s (seconds) of trace fear conditioning paradigm. The tone was presented for 16 s (2 kHz) followed by a trace interval of 18 s and subsequent

foot shock (2 s, 0.85 mA). The rats were removed from the conditioning chamber 198 s after the last shock and returned to their home cage. The total time of the acquisition protocol was 26 min, working memory during acquisition was defined as the time spent immobile after a shock divided by the inter-trial interval.

On the next day, (Context Test) trained rats were placed for 8 minutes to the same acquisition environment but without Unconditional Stimulus (the foot shock) or Conditional Stimulus (the tone). The percentage of time an animal froze during the 8-min observation periods was calculated as the number of observations judged to be freezing divided by the total number of observations in 8 min (i.e., 60 observations).

On the third day, the Tone Test was performed. Rats were placed in modified environment (increased lighting; flat floor; and triangular side walls were inserted; along with a cinnamon-scented solution) where they were allowed to acclimatize for 192 seconds before being presented to 3 cycles of a 16 sec. tone followed by a inter-trial interval of 240 sec Freezing time automatically score using VideoFreeze software (Med Associates Inc., Burlington, VT). The overall percentage of freezing time (context results) and the area under curve (tone test) were derived from plots between the percentage of freezing time and trial time in the tone test and were used for statistical comparison.

The overall percentage of freezing time was used to assess hippocampal memory during the context test; while for the Tone Test the area under curve (AUC) after

tone presentation was derived from the graph which plotted the percentage freezing time against trial time.

### 7.2.7 Anaesthetics exposure *in vitro* study

Following hippocampus slice incubation, the slice were assigned to one of eight treatments groups (Table 8) and placed in air-tight chambers (Figure 7.1).

|                                                                               |
|-------------------------------------------------------------------------------|
| Naïve                                                                         |
| Xenon (70%) 2 hours                                                           |
| N <sub>2</sub> O (70%)2hours                                                  |
| O <sub>2</sub> (8%) 3 hours                                                   |
| Normal air after 24hours 70%N <sub>2</sub> O+ 0.75%ISO 6hours                 |
| 70%Xenon 2hours after 24hours 70%N <sub>2</sub> O+0.75%ISO 6hours             |
| 70%N <sub>2</sub> O 2hours after 24hours 70%N <sub>2</sub> O+ 0.75%ISO 6hours |
| 8%O <sub>2</sub> 3hours after 24hours 70%N <sub>2</sub> O+ 0.75%ISO 6hours    |

**Table 4.** Following hippocampus slice incubation, the slices were assigned to one of eight treatments groups presented above.

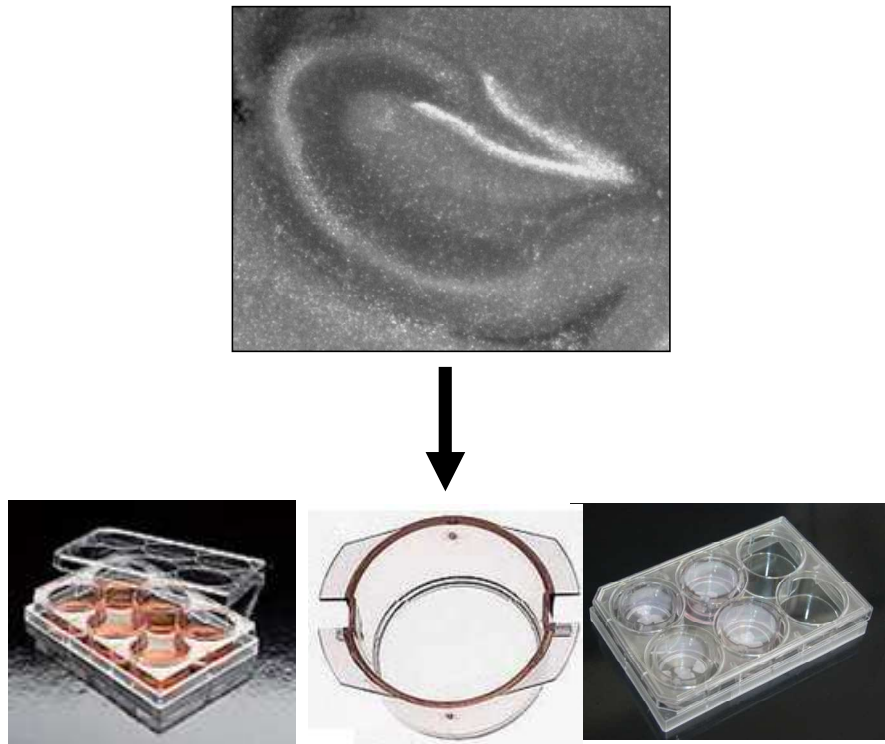


**Figure 7.1 Air-tight chambers used for treatment of hippocampus slice.**

The remaining gaseous constituents were 5% carbon dioxide, 20% oxygen and a variable concentration of nitrogen except during hypoxia treatment (All gases supplies by BOC, UK).

The gases were delivered by a standard anaesthetic machine through the chambers at 2-3L/min. The gases were analysed using an S/5 spirometry module (Datex-Ohmeda, Bradford, UK) and xenon was monitored using a 439XE monitor (Air Products, UK) due to xenon's inert chemical characteristics. After 3 minutes of gas flow the chambers were sealed and placed in a 37°C. (Galaxy R Carbon Dioxide Chamber, Wolf Laboratories)

## 7.3 Results

### 7.3.1 Infarction in neonatal induced by hypoxic-ischaemia *in vivo*

There wasn't any infarction in the brain in naïve control (Figure 7.2 A). Hypoxic-ischemia injury produced infarction in the brain ( $223.8 \pm 20$ ) (Figure 7.2 B). Both hypoxia preconditioning ( $6.2 \pm 1.3$ ;  $p < 0.01$ ) (Figure 7.2 C) and ischemia preconditioning ( $6.8 \pm 0.5$ ;  $p < 0.01$ ) protected the brain from the hypoxic-ischaemic insult and reduced the size of infarction ( $223.8 \pm 20$ ) (Figure 7.2).

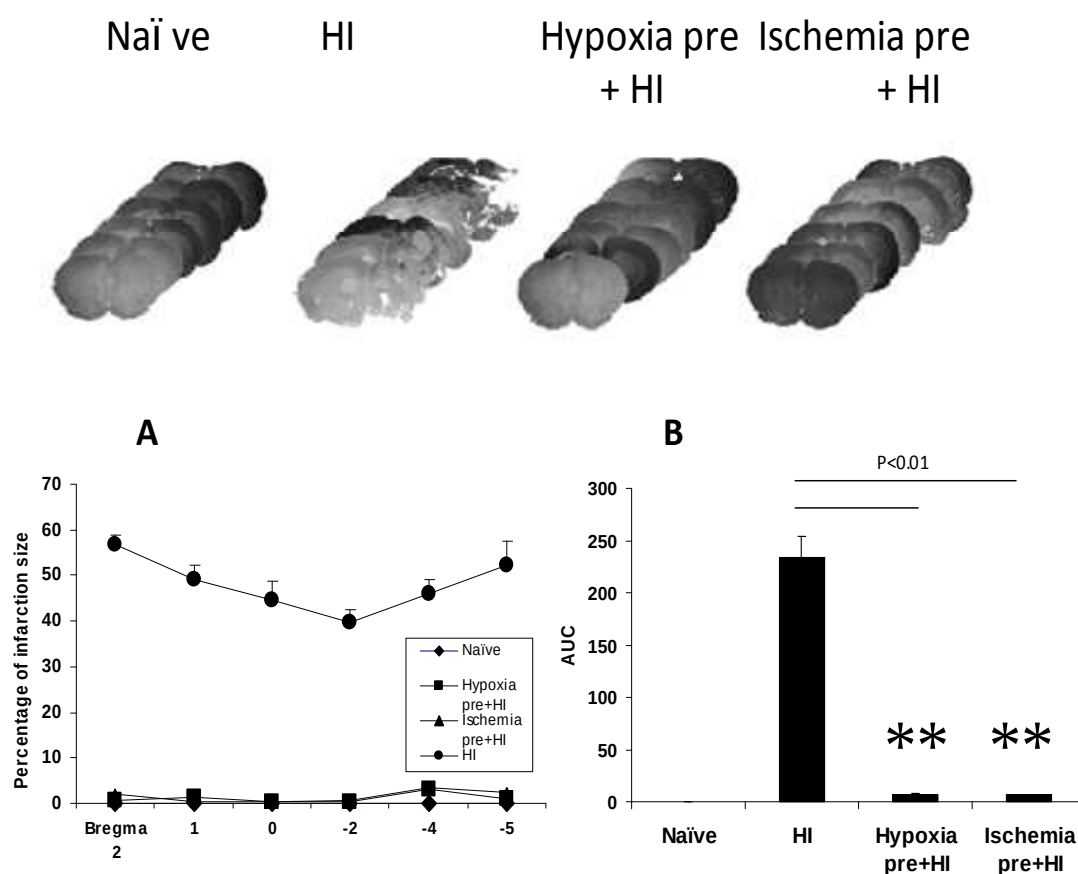


**Figure 7.2 Protection of hypoxia, ischemia preconditioning in hypoxic-ischemia injury in 7 days rat.**

(A) Percentage of infarction size of brain from bregma 2 till -5; (B) Average under curve of derives from (A). Mean  $\pm$  SD,  $n = 3-6$ . \*\* $p < 0.001$  (C) The data of the area under curve (AUC) derived from Figure B. Mean  $\pm$  SD,  $n = 3-6$ . \* $p < 0.05$  vs HI.

### 7.3.2 Neuronal apoptosis induced by anaesthetics *in vivo*

There were some caspase 3 positive cells in the cortex in the naïve control (Figure 7.3 A). However, the combined administration of N<sub>2</sub>O and ISO, (N<sub>2</sub>O + ISO), produced a significant increase in number of caspase 3 positive cells (Figure 7.3 B). Xenon preconditioning significantly decreased the number of caspase 3 cells induced by the administration of the combination of N<sub>2</sub>O + ISO (Figure 7.3 C); N<sub>2</sub>O preconditioning did not have any significant effect (figure 7.3 D). In contrast, hypoxic preconditioning significantly enhanced the cellular apoptosis induced by the combination of N<sub>2</sub>O and ISO (Figure 7.3 E). The preconditioning regimens had no significant effect on baseline cellular apoptosis (Figure 7.3 F). Quantitative data analysis of the immunohistochemically-stained brain slices, showed that hypoxic preconditioning of the rats followed by exposure to N<sub>2</sub>O + ISO produced a significant increase in number of caspase 3 positive cells in the cortex to  $220 \pm 22$  from  $177 \pm 18$  as observed in the group receiving combination of N<sub>2</sub>O + ISO only ( $p < 0.05$ ). Xenon preconditioning significantly decreased it to  $61 \pm 6$  ( $p < 0.01$ ) whilst N<sub>2</sub>O preconditioning produced no significant change. Similar pattern of change in cellular apoptosis was found in the hippocampus (Figure 7.3 G). These findings were also confirmed by western blot analysis (Figure 7.4).

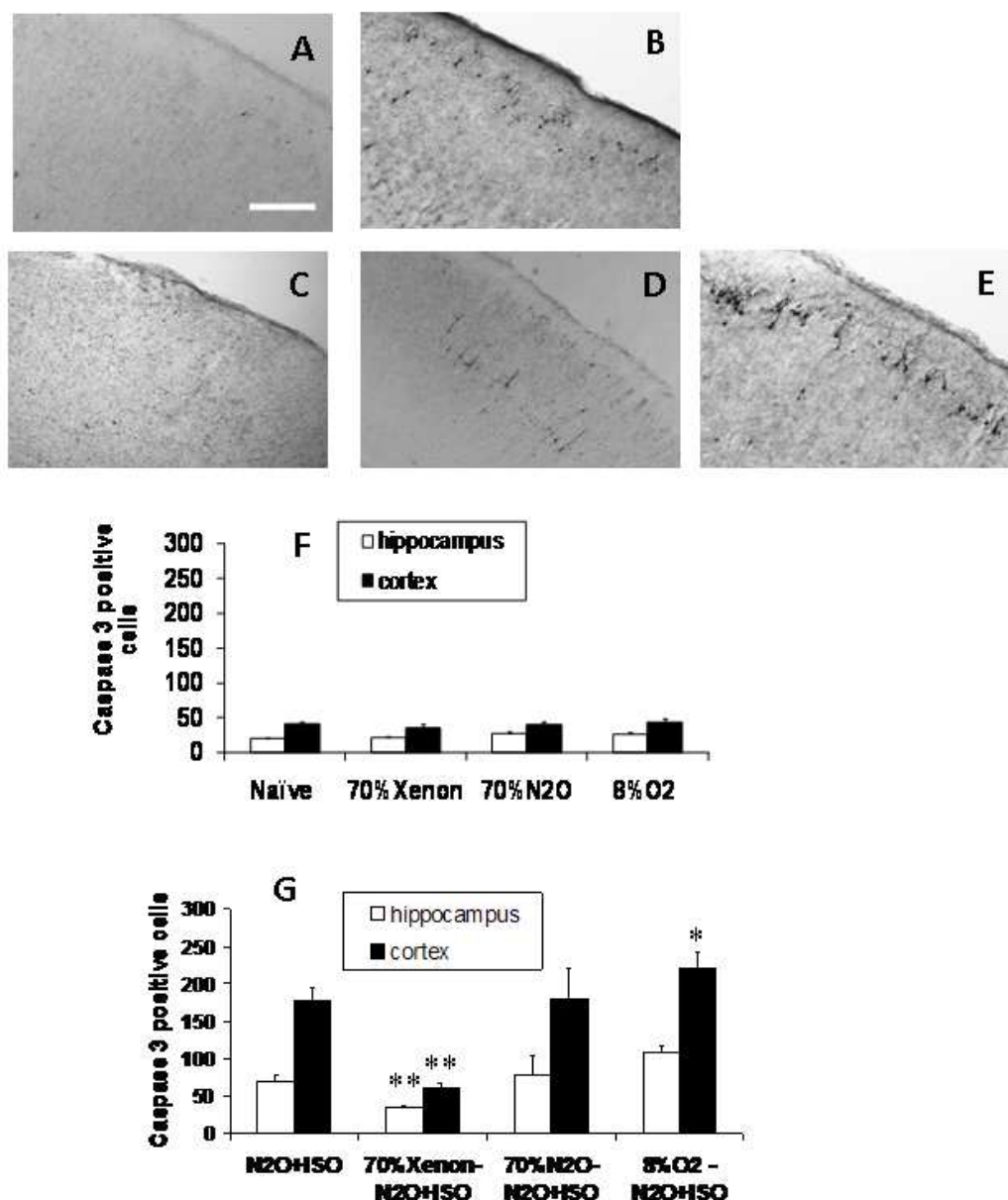


**Figure 7.3. Caspase 3 positive cells in the cortex and hippocampus *in vivo*.**

An example of photomicrograph from cortex of a 7-day old rat pup which received no treatment (naïve control) (A), 70% N<sub>2</sub>O + 0.75% isoflurane (N<sub>2</sub>O+ISO) (B). And pups received 70% xenon (C), 70% N<sub>2</sub>O (D) and 8% hypoxia (E) preconditioning followed by N<sub>2</sub>O+ISO. (F) Mean data of caspase 3 positive cells in both hippocampus and cortex from pups which received no treatment (naïve control), or which were preconditioned with either 70% xenon, 70% N<sub>2</sub>O or 8% hypoxia only. (G) mean data of caspase 3 positive cells in both hippocampus and cortex from 7-day old rat pups which received N<sub>2</sub>O+ISO alone or which were preconditioned with either 70% xenon, 70% N<sub>2</sub>O or 8% hypoxia followed by N<sub>2</sub>O+ISO. Mean  $\pm$  SD, n = 5 - 6. \*p < 0.05, p < 0.01 vs N<sub>2</sub>O+ISO. Bar = 100  $\mu$ m.

### 7.3.3 Molecular Species

Preconditioning with 70% xenon for 2 hours followed 24 hours later by exposure to N<sub>2</sub>O + ISO triggered a relative upregulation in expression of Bcl-2 ( $1.29 \pm 0.05$ ;  $p < 0.05$ ), measured 24 hours after the event, compared to the control which was treated with N<sub>2</sub>O + ISO alone. Pre-treatment with N<sub>2</sub>O ( $1.15 \pm 0.1$ ) followed by N<sub>2</sub>O + ISO caused no significant change in expression of Bcl-2, but preconditioning with 8% hypoxia followed by exposure to anaesthetic gas mixture caused a significant decrease in expression of Bcl-2, ( $0.7 \pm 0.03$ ), ( $p < 0.05$ ) (Figure 7.5 A). Cytochrome C immunoreactivity was analysed by western blotting of the cytosolic fraction of cortical extracts of rats pups subjected to preconditioning. Pre-treatment with 8% hypoxia and 70% N<sub>2</sub>O caused a significant increase in the expression of cytochrome C to  $2.23 \pm 0.2$  and  $1.8 \pm 0.1$  respectively relative to the N<sub>2</sub>O + ISO control ( $p < 0.05$  or  $0.01$ ) (Figure 7.5 B). Xenon preconditioning caused a decrease in expression of p53 to  $0.79 \pm 0.05$  relative to N<sub>2</sub>O + ISO control, while hypoxic preconditioning induced a relative increase to ( $1.37 \pm 0.1$ ) ( $p < 0.05$ ) and N<sub>2</sub>O preconditioning produced no significant change (Figure 7.5 C). There were no significant changes in MAPK phosphorylation following any of the above treatments (Figure 7.5 D).

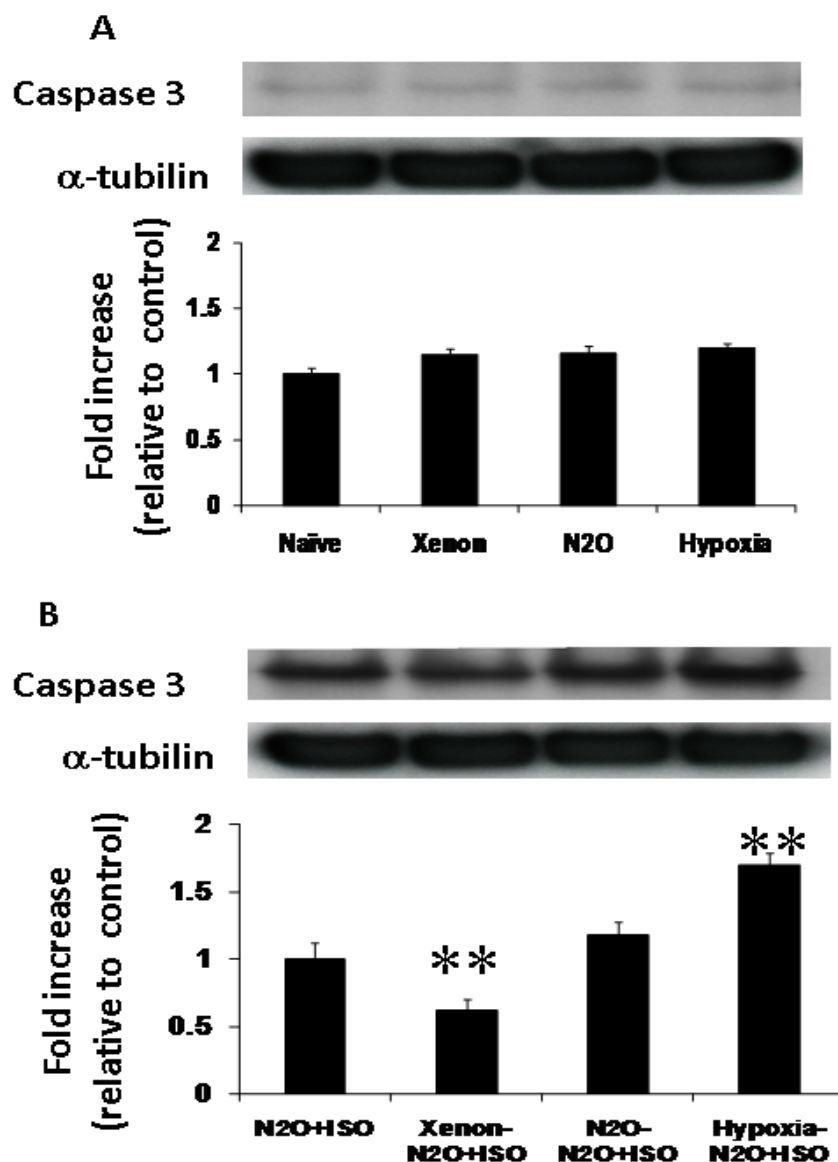


**Figure 7.4 Caspase 3 immunoblotting in cerebral cortex of rat pups after preconditioning or after preconditioning followed by anaesthetic exposure.**

(A) Top panel – example bands from 7-day old rat pups which received no treatment (naïve control), or preconditioning with either 70% xenon, 70% N<sub>2</sub>O or 8% hypoxia only; Bottom panel – mean data of immunoreactivity from pups which received no treatment (naïve control), or preconditioning with either 70% xenon, 70% N<sub>2</sub>O or 8% hypoxia only;

(B) Top panel – example bands from 7-day old rat pups which received N<sub>2</sub>O+ISO alone or preconditioning with either 70% xenon, 70% N<sub>2</sub>O or 8% hypoxia followed by N<sub>2</sub>O+ISO; Bottom panel – mean data of immunoreactivity from pups which received N<sub>2</sub>O+ISO alone or preconditioning with either 70% xenon, 70% N<sub>2</sub>O or 8% hypoxia followed by N<sub>2</sub>O+ISO. Mean  $\pm$  SD, n = 4 -5. \*\*p < 0.01 vs N<sub>2</sub>O+ISO.

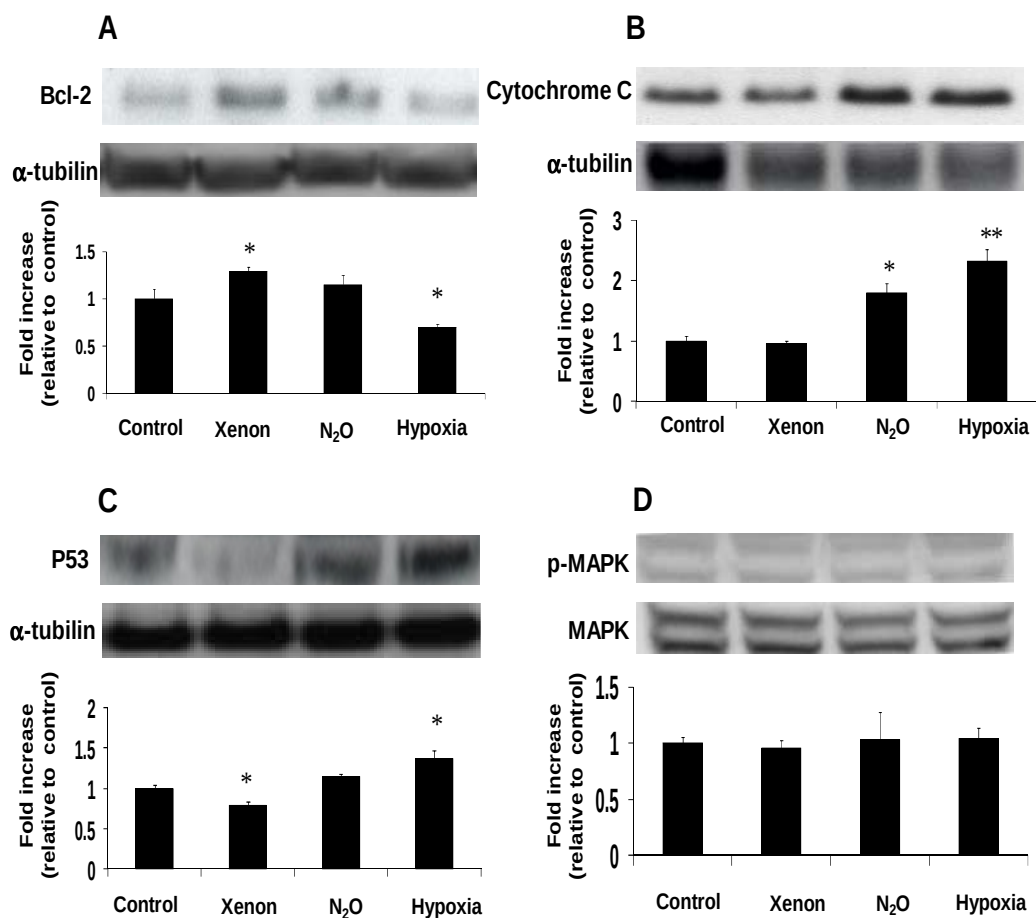


**Figure 7.5 Changes of protein immunoreactivity in pups' cerebral cortex following preconditioning treatments.**

Seven-day old rat pups received N<sub>2</sub>O+ISO alone (control) or preconditioning with either 70% xenon, 70% N<sub>2</sub>O or 8% hypoxia followed by N<sub>2</sub>O+ISO and their cortices were harvested for immunoblotting. (A) Bcl-2; (B) Cytochrome C; (C) P53; (D) pMAPK. In each section, top panel – example bands; bottom panel – mean data  $\pm$  SD (n = 4 - 5). \*p < 0.05, \*\*p < 0.01 vs control.

#### 7.3.4 Neuronal apoptosis induced by anaesthetics *in vitro*

The neuronal apoptosis seen in the *in vivo* experiments could have been due either to the indirect effect of the physiological disturbances or to the inherent effect of anaesthetics or to both. In order to obviate the possible physiological effects, organotypic hippocampal slices was used. The number of caspase 3 cells was significantly increased to  $53 \pm 3$  following  $N_2O$  + ISO exposure from baseline level of  $29 \pm 2$  ( $p < 0.05$ ) (Figure 7.6 A, B and F). The increase in caspase 3 cells was significantly reduced to  $33 \pm 4$  by xenon preconditioning ( $p < 0.05$ ) (Figure 7.6 C and F); apoptosis was unchanged by  $N_2O$  preconditioning. Remarkably, apoptosis produced by exposure to  $N_2O$  + ISO was significantly increased by hypoxic preconditioning to  $78 \pm 7$  ( $p < 0.05$ ) (Figure 7.6 D, E and F).

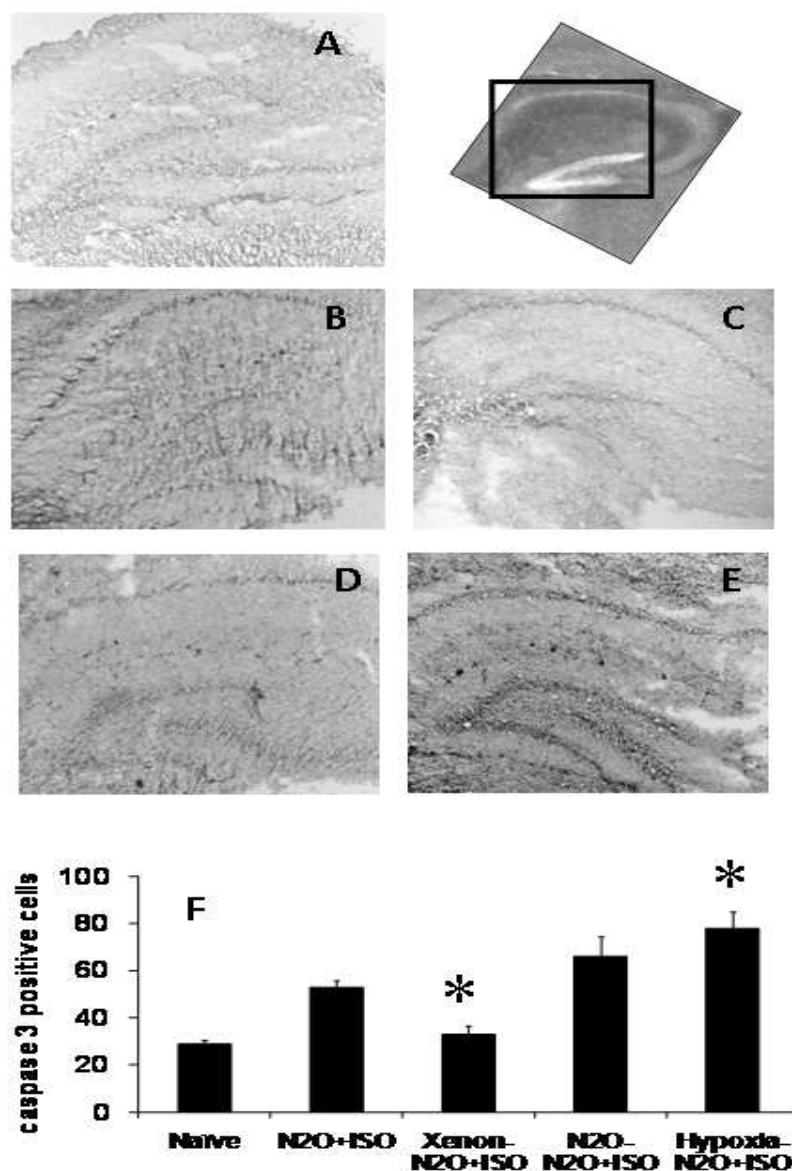


**Figure 7.6 Caspase 3 positive cells in the hippocampus *in vitro*.**

An example of photomicrograph of organotypic hippocampal slice, harvested from post-natal day 8 rat pups, cultured for 5 days prior to no treatment (naïve control) (A) 70% N2O + 0.75% isoflurane (N2O+ISO) (B) pups were treated with 70% xenon (C), 70% N2O (D) and 8% hypoxia (E) precondition and followed by N2O+ISO. (F) mean data of caspase 3 positive cells in the hippocampus from 7 day old rat pups which received no treatment (naïve control), N2O+ISO alone or preconditioning with either 70% xenon, 70% N2O or 8% hypoxia followed by N2O+ISO. Mean  $\pm$  SD,  $n = 5 - 6$ . \* $p < 0.05$ , \*\* $p < 0.01$  vs N2O+ISO. Bar = 100 $\mu$ m. The inserted image - a representative living culture was killed by exposure to glutamate (50  $\mu$ M) and the image was enhanced with propidium iodide staining, acting as a guide to outline the hippocampal area being examined.

### 7.3.5 Cognitive function assessment

Following preconditioning and exposure to anaesthetic gas mixture, cohorts of rats were allowed to live up to the age of 40 days for assessment of cognitive function using trace fear conditioning. Hypoxia preconditioning followed by N<sub>2</sub>O + ISO administration ( $11 \pm 1$ ;  $p < 0.05$ ) and N<sub>2</sub>O + ISO administration alone ( $15 \pm 2$ ;  $p < 0.05$ ) resulted in shorter hippocampal dependent-freezing response time in the Context Test than xenon preconditioning followed by N<sub>2</sub>O + ISO treatment ( $24 \pm 1$ ). The latter was similar to that observed in the naïve controls ( $27 \pm 7$ ;  $p > 0.05$ ) (Figure 7.7 A). The results of the Tone Test, mainly used to assess amygdalar function, showed that the AUC (arbitrary unit) was higher in the xenon preconditioned group exposed to N<sub>2</sub>O + ISO ( $243 \pm 27$ ) than in the hypoxic preconditioned group exposed to the neurotoxic anaesthetic combination ( $157 \pm 25$ ;  $p < 0.05$ ) and the positive control group which received N<sub>2</sub>O+ISO only ( $200 \pm 26$ ;  $p < 0.05$ ). The AUC in the xenon preconditioning followed by N<sub>2</sub>O+ISO group was not significantly different from that of the naïve group ( $261 \pm 26$ ) (Figure 7.7 B and C).

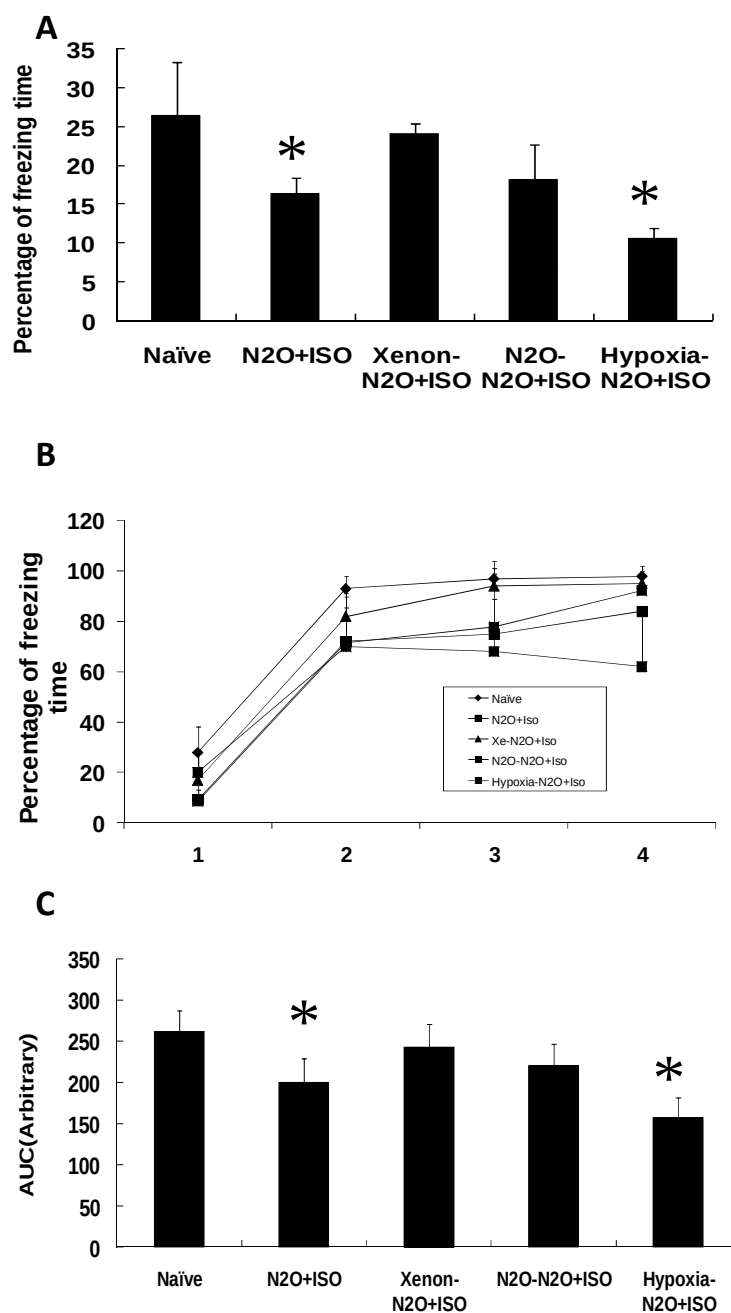


**Figure 7.7 Cognitive function assessed with Trace fear conditioning.**

Seven-day old rat pups which received no treatment (naïve control), 70% N<sub>2</sub>O + 0.75% isoflurane (N<sub>2</sub>O+ISO), and pups which received 70% xenon (C), 70% N<sub>2</sub>O (D) and 8% hypoxia (E) preconditioning followed by N<sub>2</sub>O+ISO were allowed to live up to the age of 40 days for cognitive function test. (A) The mean data of percentage of freezing time after context testing. (B) the mean data of percentage of freezing time after tone testing. (C) The average data of the area under curve (AUC) derived from Figure B. Mean  $\pm$  SD,  $n = 6$ . \* $p < 0.05$  vs N<sub>2</sub>O+ISO.

## 7.4 Discussion

In this study, I observed significant increases in caspase-3 levels, both *in vitro* and *in vivo*, in the hippocampus and cortex of 7 day old rats exposed to 0.7% isoflurane + 70% N<sub>2</sub>O in oxygen for 6 hours at 1 atmospheric ambient pressure compared to naïve control. The observed increase in caspase-3 levels is due to the direct effects of the anaesthetic drugs on neural tissues and not due to altered physiology in the neonatal rats, e.g. hypoperfusion of the brain, hypoglycaemia, because similar results were observed in the organotypic hippocampal slices. In both *in vitro* and *in vivo* studies xenon was effective at suppressing ISO + N<sub>2</sub>O induced apoptosis and subsequent deterioration in cognitive function. Conversely, hypoxic preconditioning, which provided a neuroprotection against ischaemic brain injury demonstrated here, exacerbated apoptotic neurodegeneration and worsened cognitive impairment in the rats when they reached adulthood.

The mechanism responsible for the increase in caspase-3 levels in the developing brain during synaptogenesis following exposure to anaesthetic drugs was not directly addressed. Drugs which block NMDA receptors or promote GABA<sub>A</sub> receptor function have been shown to produce neurodegeneration in neonatal animal models (Ikonomidou et al., 1999; Wang et al., 2006; Olney et al., 2000; Young et al., 2005) GABA<sub>A</sub> and NMDA receptors and their agonists play a fundamental role during synaptogenesis as they are essential for migration of neurons to the appropriate regions of the nervous system, dendritic filopodia stabilisation, synaptic development and stabilisation (Ben-Ari et al., 2007).

In this study, I investigated the therapeutic potential of preconditioning to prevent neuronal apoptosis produced by anaesthetic drugs during synaptogenesis. The protective effect of preconditioning with xenon against ISO + N<sub>2</sub>O induced neuronal apoptosis in neonatal rats could be partly attributed to the drug inhibiting mitochondria-induced activation of caspase-3 pathway as xenon up-regulated expression of anti-apoptotic Bcl-2, and down regulated pro-apoptotic tumour suppressor transcription factor P53. These were associated with no significant change in cytochrome C level. Xenon preconditioning also caused an increase in phosphorylation of CREB transcription factor and BDNF expression. In contrast to xenon preconditioning, hypoxic preconditioning exacerbated ISO + N<sub>2</sub>O induced neuronal apoptosis by decreasing the expression of anti-apoptotic factor Bcl-2 and increasing the expression of pro-apoptotic transcription factor p53 resulting in an increase in cytochrome C release from mitochondria.

It was noted in our study that N<sub>2</sub>O increased both Bcl-2 and cytochrome C. This may be one of the reasons why N<sub>2</sub>O failed to induce a preconditioning state and also failed to protect anaesthetics-induced neurotoxicity although like xenon, N<sub>2</sub>O is also an NMDA receptor antagonist. Interestingly, my result is in keeping with a previous study in rats, which showed that preconditioning with nitrous oxide failed to reduce the extent of myocardial infarction following 25 minutes of coronary occlusion (Weber et al., 2005).

Results from trace fear conditioning testing are in line with previous reports of cognitive impairment in rats after exposure to general anaesthesia during the

neonatal period (Jevtovic-Todorovic et al., 2003; Fredriksson et al., 2007; Robert et al., 2009). While the frontal cortex, hippocampus, periaqueductal gray, and rostral ventral medulla mainly underlies the acquisition of fear memories, hippocampal damage is causally linked to long-term memory deficit (Quinn et al., 2005). Our data showed that rats preconditioned with either nitrous oxide or hypoxia displayed less freezing compared to naïve controls and xenon preconditioned animals, indicating that the rodents in the first two groups suffered from long-term memory deficit compatible with damage to hippocampus. Indeed, this was confirmed in this study where we showed increased apoptosis in the hippocampus of rats which were preconditioned with either hypoxia or N<sub>2</sub>O and then exposed to ISO + N<sub>2</sub>O anaesthetics.

The relevance to the human species of the above experimental findings in neonatal rats, and of those in many other studies conducted in neonatal animals is being questioned (Loepke and Soriano SG, 2008). The main argument is the lack of strong evidence of its toxicity in neonatal humans. However, Wilder and colleagues in a retrospective study of the medical and educational records of children in Olmsted county, showed, using multivariate analysis, that children who had repeated general anaesthetics before the age of 4 years had higher incidence of learning difficulties compared to those who received only one general anaesthetic or no anaesthetic (Wilder et al., 2009). In another retrospective study, Kalman and colleagues showed that children under 24 months undergoing urological procedures under general anaesthesia tended to show more behavioural problems than those operated after 24 months of age under general anaesthesia (Kalkman et al., 2009).

My experimental model showed that firstly hypoxia preconditioning with hypoxic-ischaemia injury protect the hypoxic-ischaemia infarction but period of hypoxia followed soon afterwards by general anaesthesia with an inhalational agent and nitrous oxide during the neonatal period, resulted in neuronal death and subsequent cognitive impairment. This indicates least but not last that hypoxia preconditioning working on hypoxic-ischaemia induced brain infarction and anaesthetics induced apoptosis in different way. This unexpected finding may have important clinical implications. For example, neonates born with transposition of great vessels are hypoxic shortly after birth; they require urgent surgery to allow oxygenated blood from the pulmonary circulation to reach the systemic circulation. Babies born with gastroschisis or severe exomphalos also require urgent surgery to repair the abdominal defect. If they are unfortunate enough to experience foetal distress during child birth, they may be at increased risk of brain injury following surgical correction during anesthesia with the offending agents. If this finding can be extrapolated to clinical practice, then paediatric anaesthesiologists dealing with similar situations need to carefully choose anaesthetic regimen to minimise or even prevent brain injury.

## **CHAPTER 8**

# **NEURONAL APOPTOSIS INDUCED BY ANAESTHETICS IN THE PRESENCE OF NOCICEPTIVE STIMULI**

## 8.1 Introduction

The initial finding that N-methyl-D-Aspartate (NMDA) antagonists induce apoptosis in the neonatal rodent brain (Ikonomidou et al., 1999) has been rapidly succeeded by reports demonstrating that a diverse range of anesthetics may induce similar injury (Sander et al., 2008; Patel et al., 2009). Anaesthetic induced neurodegeneration also occurs in the monkey brain indicating that other primates, including humans, may be vulnerable (Slikker et al., 2007; Sanders et al., 2008; Brambrink et al., 2010). The injury is not due to disruption of systemic physiology though it occurs in a dose and duration dependent manner and combinations of agents produce augmented injury (Sanders et al., 2008). Furthermore the injury is associated with long-term impairments in cognitive function (Jevtovic-Todorovic et al., 2003a; Sanders et al., 2009), however the available evidence suggests that motor and nociceptive systems are not affected (Sanders et al., 2008). Anaesthetics induce widespread neuronal damage occurs in the central nervous system including the cerebral cortex, hippocampus, thalamus, striatum and spinal cord involving both the intrinsic and extrinsic caspase pathways (Jevtovic-Todorovic et al., 2003a; Yon et al., 2005; Lu et al., 2006; Ma et al., 2007; Sanders et al., 2008; Sanders et al., 2008; Sanders et al., 2009a; Sanders et al., 2009b).

Most general anaesthetics, including isoflurane and nitrous oxide, act on both NMDA receptors and gamma-aminobutyric acid type A (GABA<sub>A</sub>) receptors and it has been suggested that these drugs induce neuronal apoptosis *via* these receptors (Ikonomidou et al., 1999; Young et al., 2005; Slikker et al., 2007; Sanders et al.,

2009a; Sanders et al., 2009b; Satomoto et al., 2009). Preliminary evidence suggests that the GABA<sub>A</sub> receptor is not responsible for this injury, at least *in vitro* (Sanders et al 2009b). On the contrary NMDA receptor antagonism may block the neurotrophic effects of synaptic signalling and thus may prevent inactivation of crucial mediators in the intrinsic apoptotic pathway such as BAD (Hardingham et al., 2009; Leveille et al., 2010) or activate death-signaling pathways including that mediated by the p75 neurotrophin receptor (Head et al., 2009).

Recently administration of the intravenous anaesthetic, propofol, to neonatal rats was shown to cause significant elevations in the pro-inflammatory cytokine, tumor necrosis factor (TNF)- $\alpha$ , in the brain regions where increased cellular apoptosis occurred (Pesic et al., 2009). TNF- $\alpha$  triggers the extrinsic apoptotic pathway through Fas activation, therefore it is possible that anaesthetics induce pro-inflammatory cytokines to stimulate the extrinsic apoptotic cascade (Pesic et al., 2009).

Understanding the mechanisms involved in anaesthetic induced neurodegeneration is important (i) to design safe anaesthetic regimens for clinical practice and (ii) to understand the interaction of anaesthetics with other deleterious stimuli. For example I have recently reported that hypoxic preconditioning worsens the anaesthetic injury (Shu et al., 2010). Perhaps an even more important consideration is the effect that surgical stimulation may have on the injury (Sanders et al., 2008; Sanders et al., 2009c). Surgery inevitably activates the pain pathways and also the tissue injury produced can trigger further neurotoxicity through inflammatory mechanisms. Alternatively neuronal stimulation may oppose the apoptotic injury by

preventing synaptic deprivation and loss of neurotrophic effect at the synaptic level. In the present study, the dorsal horn of the spinal cord, thalamus, hippocampus and cerebral cortex of 7-day-old rats were examined after 6-hour exposure to 0.75% isoflurane and 70% nitrous oxide to investigate whether anaesthetic neuroapoptosis is reduced or exacerbated by co-administration of nociceptive stimuli.

## 8.2 Materials and Methods

### 8.2.1 *In vivo* experiment

Separate cohorts (n= 5-6 per group) of 7 day-old, littermate Sprague-Dawley rat pups were randomly assigned to six groups: 1. Naïve (room air); 2. Anaesthetics alone (70% nitrous oxide and 0.75% isoflurane for 6 hrs); 3. Formalin injection alone (subcutaneous injection with 10 µl 5% formalin into the left hind paw); 4. Anaesthetics + formalin injection; 5. Surgical incision alone (1 cm midline cut to the left hind paw); 6. Anaesthetics + surgical incision (Table 5). The remaining gas constituents were 30% oxygen in groups 2-6 and balanced with nitrogen where necessary. Surgical incision and formalin injection were conducted 10 minutes after rats were exposed to 70% nitrous oxide and 0.75% isoflurane and rats in group 3 and group 5 were then exposed to room air rather than anaesthetic reagents for 6 hours. In group 1, naïve rats were placed in exposure chambers for 6 hr without exposure to anaesthetic reagents or noxious stimuli but otherwise identical conditions as animals in groups 2-6.

|                                                                  |
|------------------------------------------------------------------|
| Naïve                                                            |
| N <sub>2</sub> O(70%)+ISO(0.75%) 6 hours                         |
| Formalin Injection (5%)                                          |
| Surgery Incision                                                 |
| N <sub>2</sub> O(70%)+ISO(0.75%) 6 hours+ Formalin Injection(5%) |
| N <sub>2</sub> O(70%)+ISO(0.75%)+Surgery Incision                |

Table 5. Separate cohorts (n= 4 per group) of 7-day- old, littermate Sprague-Dawley rat pups were randomly assigned to six groups presented above.

### 8.2.2 Western blot

The membrane was probed with primary antibodies for IL-beta or TNF- $\alpha$  (Cell Signaling Technology, Inc, USA), others please see Chapter 3.

## 8.3 Results

### 8.3.1 Caspase 3 expression in the cortex, hippocampus, thalamus and spinal cord

Relative to naïve neonatal rats, anaesthesia increased the number of caspase-3 positive neurons in the cortex ( $12 \pm 3$  vs.  $173 \pm 24$ ;  $p < 0.05$ ; Figure 8.1), hippocampus ( $9 \pm 3$  vs.  $51 \pm 7$ ;  $p < 0.05$ ; Figure 8.2), and spinal cord ( $22 \pm 5$  vs.  $5 \pm 1$ ;  $p < 0.05$ ; Figure 8.3). The similar patterns of changes were found in the thalamus (data not shown). Interestingly formalin (cortex:  $45 \pm 5$ , hippocampus  $37 \pm 6$ , spinal cord:  $15 \pm 3$ ;  $p < 0.05$  vs naïve) but not surgical incision (cortex:  $11 \pm 5$ , hippocampus  $8 \pm 2$ , spinal cord:  $5 \pm 2$ ;  $p > 0.05$  vs naïve) increased the number of apoptotic neurons in all three areas of the central nervous system relative to naïve mice. The combination of anaesthesia plus formalin and anaesthesia plus surgical incision both increased the number of caspase-3 positive neurons in the cortex, hippocampus and spinal cord relative to naïve, formalin and incision groups ( $p < 0.05$  –  $p < 0.01$  for all). The number of apoptotic neurons in the anaesthesia plus formalin (cortex:  $282 \pm 25$ , hippocampus  $96 \pm 13$ , spinal cord:  $43 \pm 5$ ) did not differ from anaesthesia plus surgical incision (cortex:  $242 \pm 41$ , hippocampus  $78 \pm 10$ , spinal cord:  $38 \pm 5$ ).

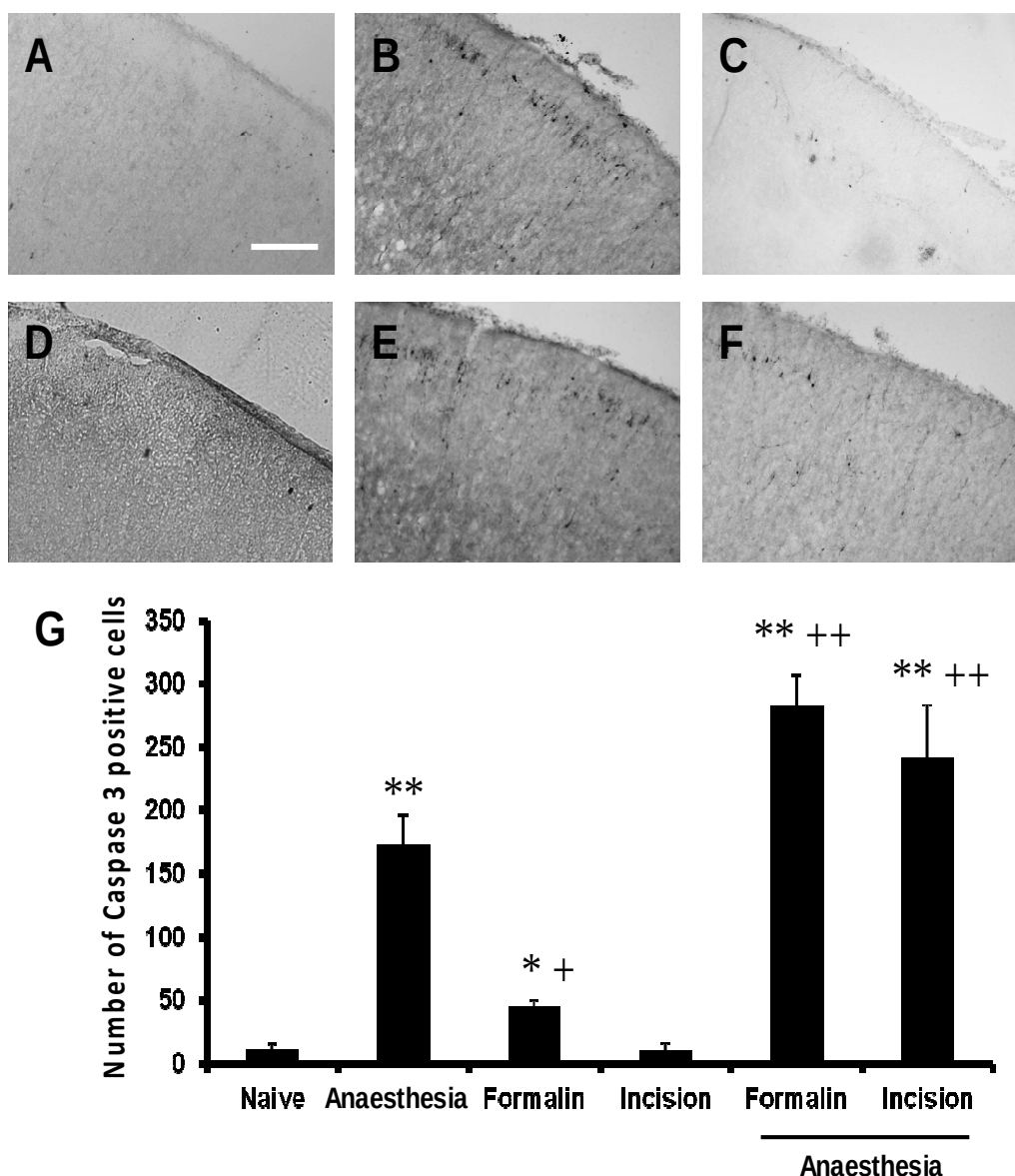


**Figure 8.1 Caspase 3 positive cells in the cortex.**

Seven old rat pups received 70% N<sub>2</sub>O + 0.75% isoflurane anaesthesia with or without the plantar surface of left paw injection with 5% formalin or skin incision for 6 hrs. Other cohorts received six hours of 70% nitrogen balanced with oxygen (naïve control), or combined with left paw plantar surface injection of 5% formalin (formalin group) or skin incision (incision group). An example of photomicrograph from cortex of a 7-day old rat pup which received no treatment (naïve control) (A), 70% N<sub>2</sub>O + 0.75% isoflurane (anaesthesia) (B), formalin injection (C), skin incision (D), formalin injection + anaesthesia (E) and skin incision + anaesthesia (F). (G) Mean data of caspase 3 positive cells in cortex from pups which received the treatments described above. Mean  $\pm$  SD, n = 5 - 6. \*\* p < 0.01 vs Naïve; +p < 0.05, ++ p < 0.01 vs anaesthesia. Bar = 100 $\mu$ m.

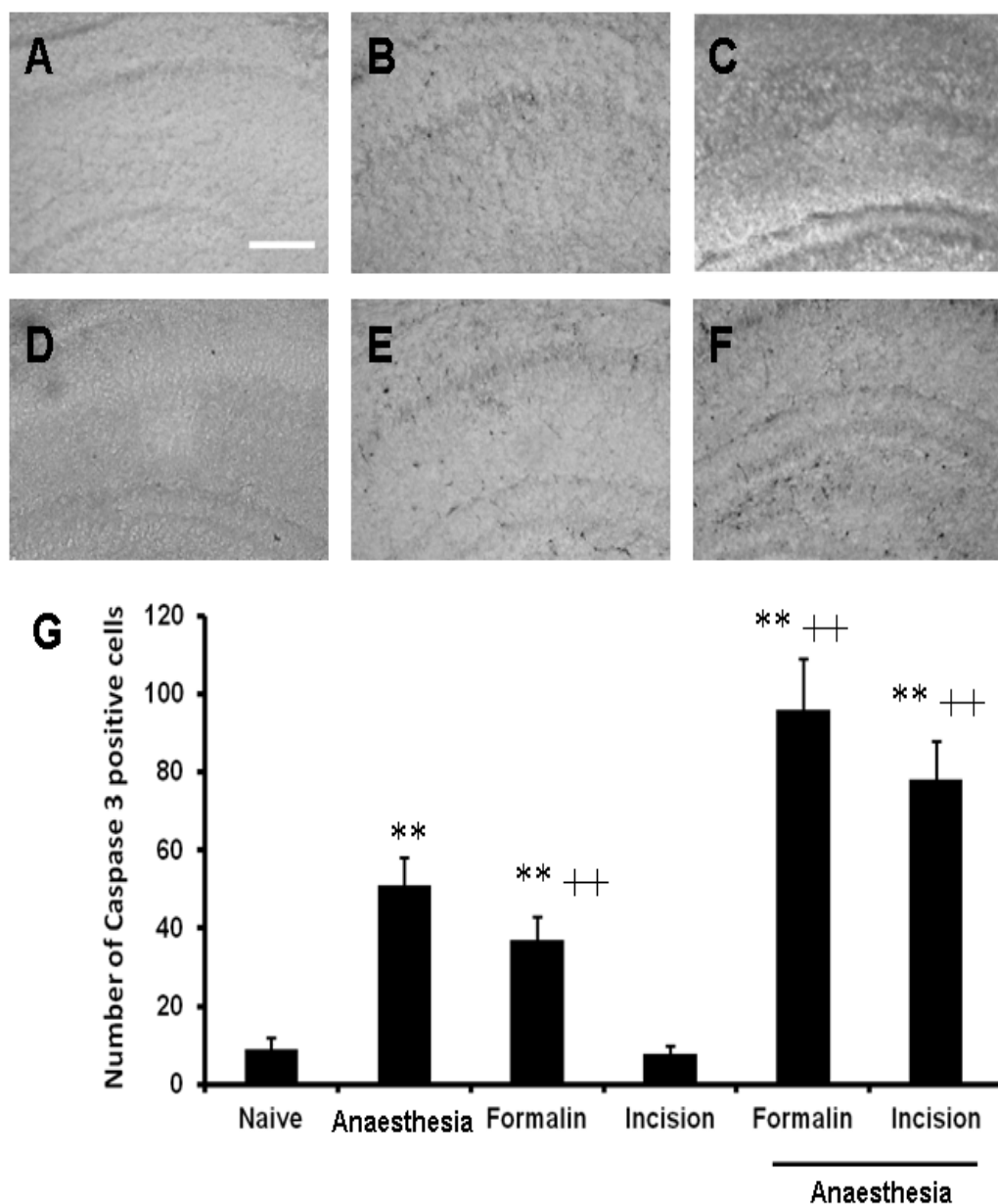


**Figure 8.2 Caspase 3 positive cells in the hippocampus.**

Seven old rat pups received 70% N<sub>2</sub>O + 0.75% isoflurane anaesthesia with or without the plantar surface of left paw injection with 5% formalin or skin incision for 6 hrs. Other cohorts received six hours of 70% nitrogen balanced with oxygen (naïve control), or combined with left paw plantar surface injection of 5% formalin (formalin group) or skin incision (incision group). An example of photomicrograph from cortex of a 7-day old rat pup which received no treatment (naïve control) (A), 70% N<sub>2</sub>O + 0.75% isoflurane (anaesthesia) (B), formalin injection (C), skin incision (D), formalin injection + anaesthesia (E) and skin incision + anaesthesia (F). (G) Mean data of caspase 3 positive cells in cortex from pups which received the treatments described above. Mean  $\pm$  SD, n = 5 - 6. \*\* p < 0.01 vs Naïve; ++ p < 0.01 vs anaesthesia. Bar = 100 $\mu$ m.

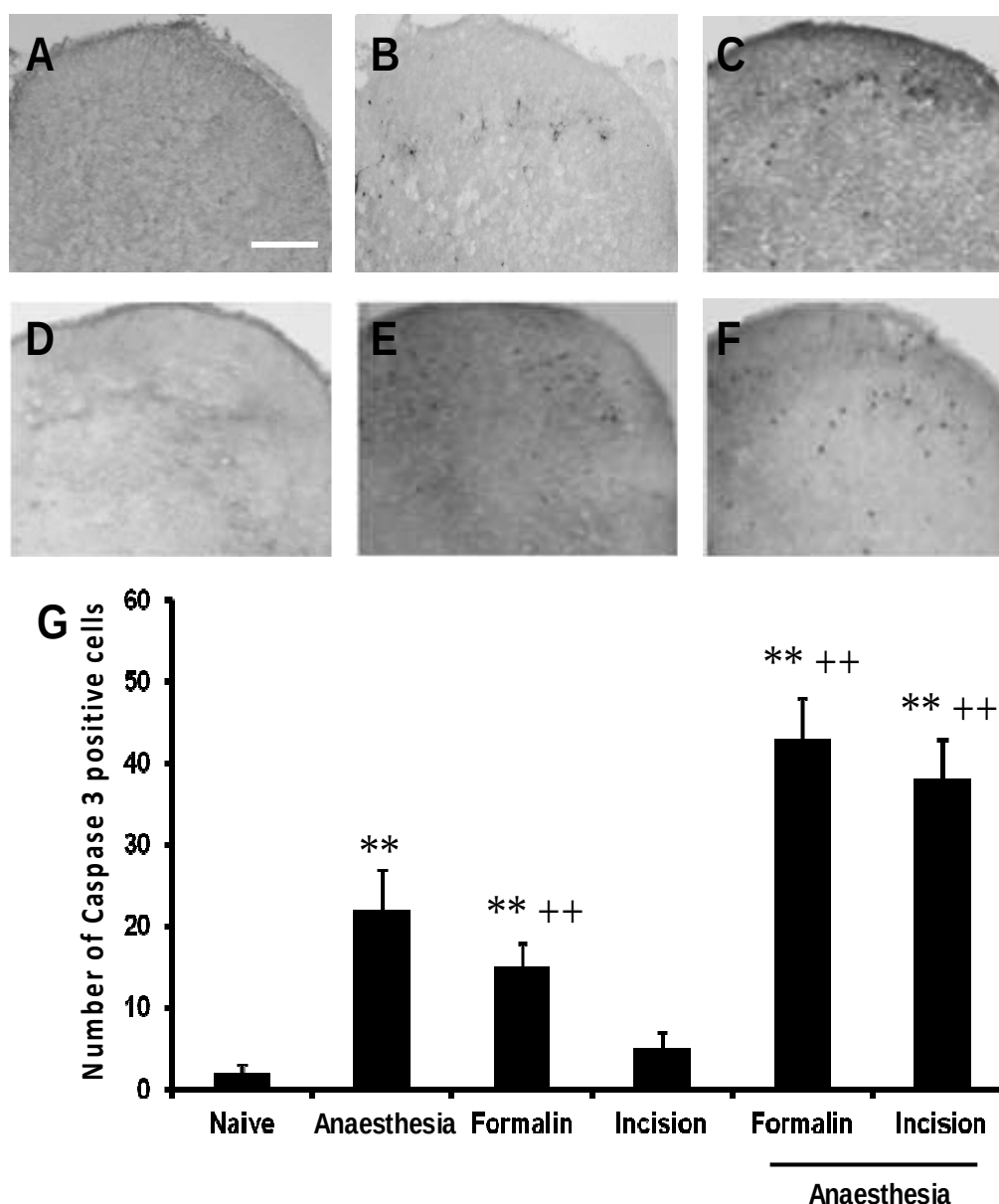


**Figure 8.3 Caspase 3 positive cells in the lumbar level of the spinal cord.**

Seven old rat pups received 70% N<sub>2</sub>O + 0.75% isoflurane anaesthesia with or without the plantar surface of left paw injection with 5% formalin or skin incision for 6 hrs. Other cohorts received six hours of 70% nitrogen balanced with oxygen (naïve control), or combined with left paw plantar surface injection of 5% formalin (formalin group) or skin incision (incision group). An example of photomicrograph from cortex of a 7-day old rat pup which received no treatment (naïve control) (A), 70% N<sub>2</sub>O + 0.75% isoflurane (Anaesthesia) (B), formalin injection (C), skin incision (D), formalin injection + anaesthesia (E) and skin incision + anaesthesia (F). (G) Mean data of caspase 3 positive cells in cortex from pups which received the treatments described above. Mean  $\pm$  SD, n = 5 - 6. \*\* p < 0.01 vs Naïve; ++ p < 0.01 vs anaesthesia. Bar = 100  $\mu$ m.

### 8.3.2 C-Fos expression in the thalamus and the spinal cord

Both nociceptive stimuli increased the number of c-Fos positive neurons in the thalamus (Figure 8.4) and spinal cord (Figures 8.5) relative to naïve neonatal rats ( $p < 0.05$  for both) consistent with activation of the spinothalamic tract. Formalin (thalamus:  $105 \pm 5$ , spinal cord:  $68 \pm 12$ ) and incision (thalamus:  $65 \pm 4$ , spinal cord:  $53 \pm 5$ ) induced c-Fos positive neurons relative to naïve controls (thalamus:  $6 \pm 2$ , spinal cord:  $4 \pm 2$ ). Interestingly anaesthesia plus either of the nociceptive stimuli induced more neuronal activation in the thalamus than in the other groups ( $p < 0.05$ ), anaesthesia plus formalin induced  $201 \pm 12$  and anaesthesia plus incision induced  $144 \pm 15$  c-Fos positive neurons in the thalamus. However anaesthesia co-administered with formalin (spinal cord:  $45 \pm 8$ ) or incision (spinal cord:  $34 \pm 7$ ) reduced c-Fos expression in the spinal cord relative to the nociceptive stimuli alone (Figure 8.5).

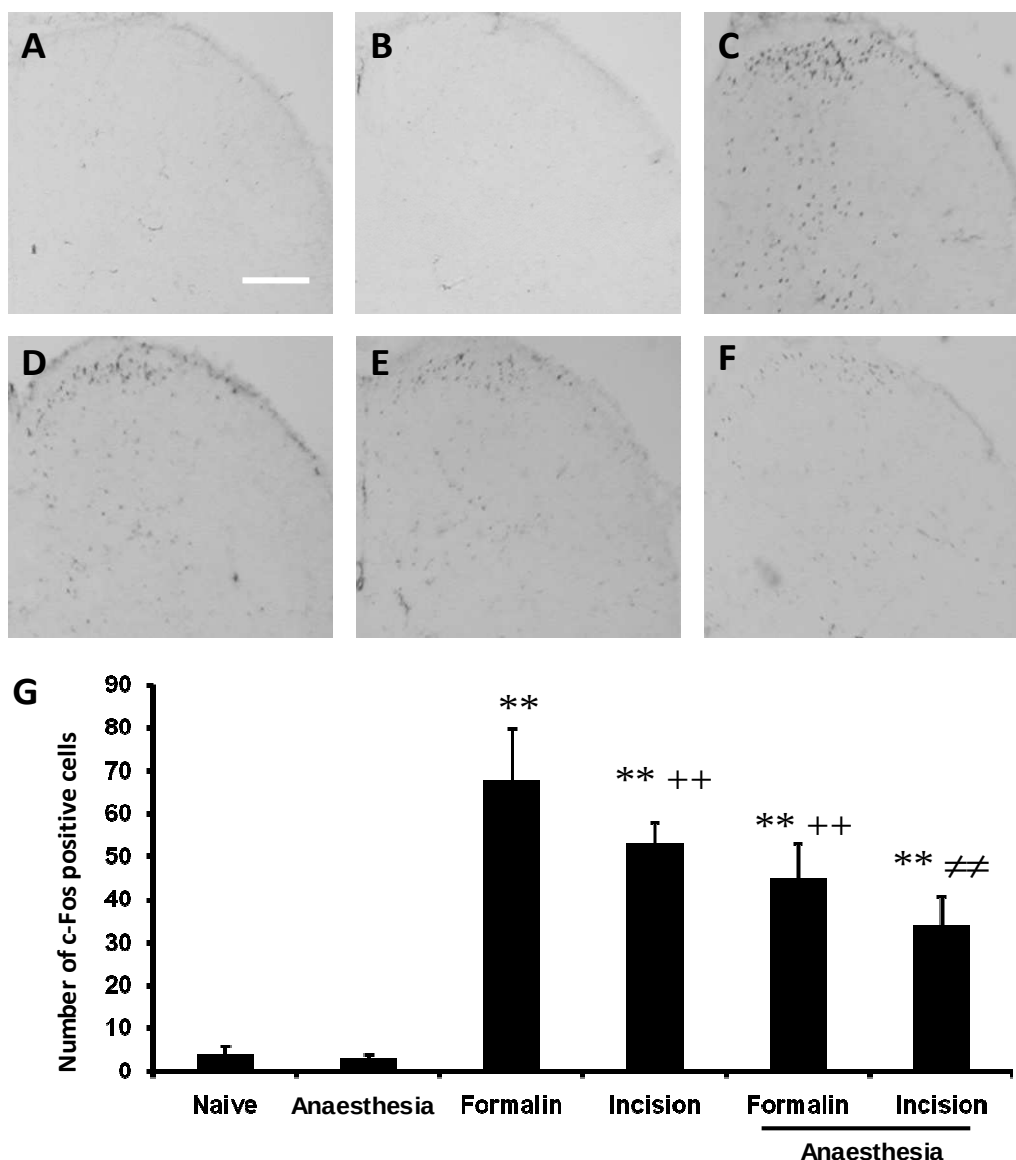


**Figure 8.4 c-FOS positive cells in the lumbar level of the spinal cord.**

Seven old rat pups received 70% N<sub>2</sub>O + 0.75% isoflurane anaesthesia with or without the plantar surface of left paw injection with 5% formalin or skin incision for 6 hrs. Other cohorts received six hours of 70% nitrogen balanced with oxygen (naïve control), or combined with left paw plantar surface injection of 5% formalin (formalin group) or skin incision (incision group). An example of photomicrograph from cortex of a 7-day old rat pup which received no treatment (A) Naïve control (B) 70% N<sub>2</sub>O + 0.75% isoflurane (anaesthesia) (C) Formalin injection (D) Skin incision (E) Formalin injection + anaesthesia (F) Skin incision + anaesthesia (G) Mean data of caspase 3 positive cells in cortex from pups which received the treatments described above. Mean  $\pm$  SD, n = 5 - 6. \*\* p < 0.01 vs Naïve; ++ p < 0.01 vs anaesthesia. ≠ p < 0.01 vs Incision. Bar = 100 $\mu$ m.

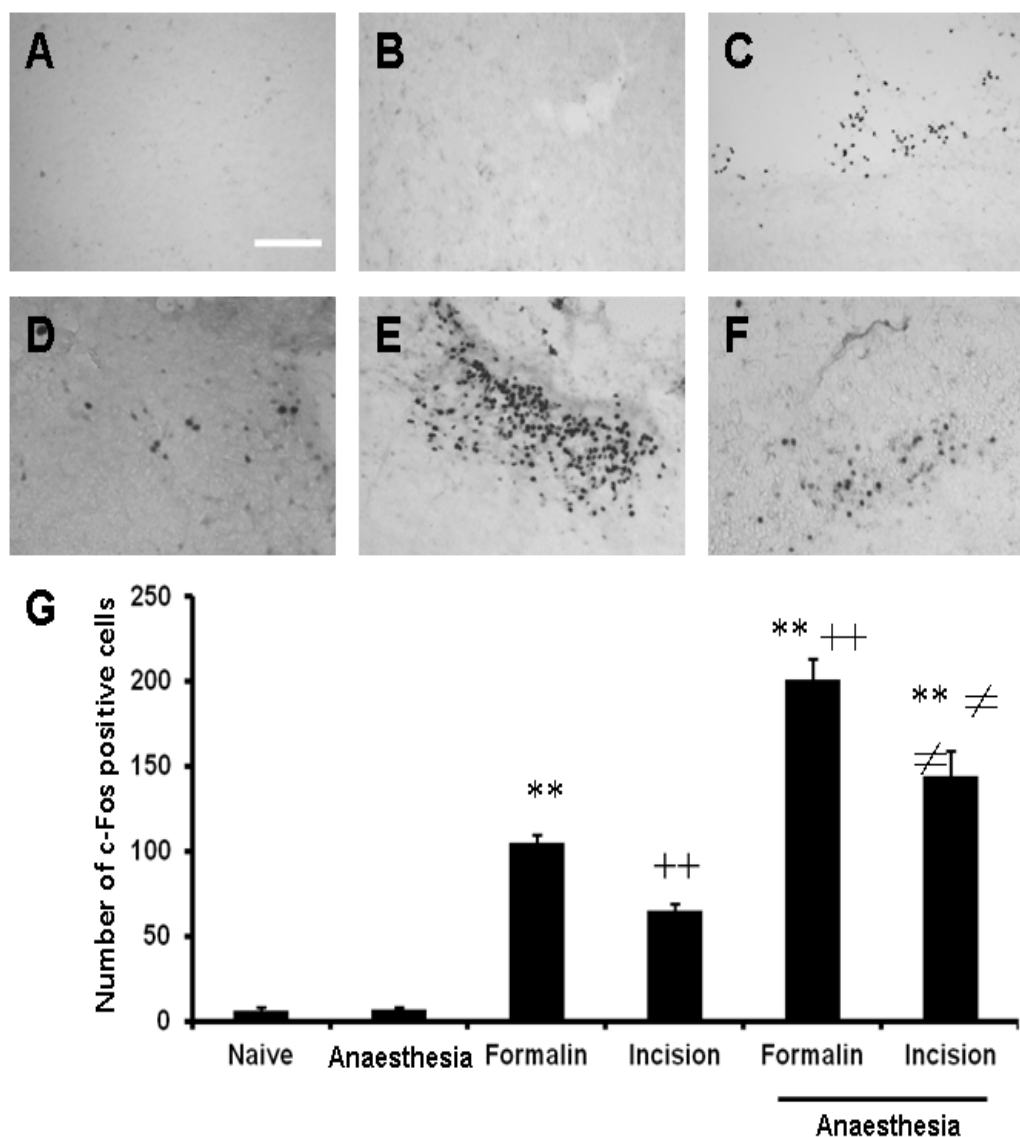


**Figure 8.5 C-Fos positive cells express in thalamas.**

Seven old rat pups received 70% N<sub>2</sub>O + 0.75% isoflurane anaesthesia with or without the plantar surface of left paw injection with 5% formalin or skin incision for 6 hrs. Other cohorts received six hours of 70% nitrogen balanced with oxygen (naïve control), or combined with left paw plantar surface injection of 5% formalin (formalin group) or skin incision (incision group). An example of photomicrograph from cortex of a 7-day old rat pup which received no treatment (A) Naïve control (B) 70% N<sub>2</sub>O + 0.75% isoflurane (anaesthesia) (C) Formalin injection (D) Skin incision (E) Formalin injection + anaesthesia (F) Skin incision + anaesthesia (G) Mean data of caspase 3 positive cells in cortex from pups which received the treatments described above. Mean  $\pm$  SD, n = 5 - 6. \*\* p < 0.01 vs Naïve; ++ p < 0.01 vs anaesthesia. ≠ p < 0.01 vs Incision. Bar = 100  $\mu$ m.

### 8.3.3 Cytokine expression in the cortex

In order to understand whether cortical inflammatory changes correlated with increased cortical apoptosis we performed western blot for IL-1 $\beta$  and TNF- $\alpha$ . Six hours after the start of the treatments anaesthesia plus the nociceptive stimuli increased IL-1 $\beta$  but not TNF-  $\alpha$  level compared to controls (Figure 8.6).

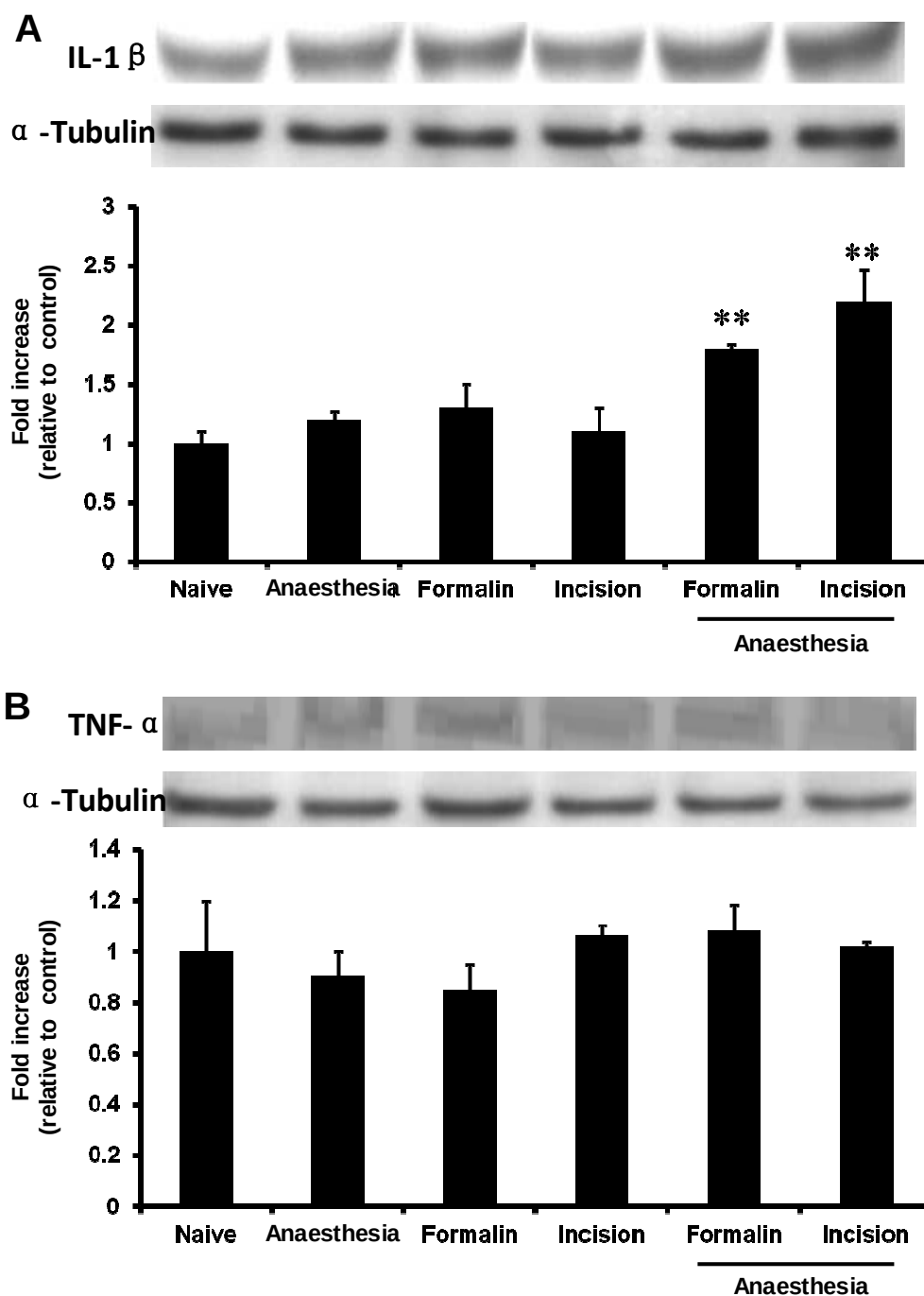


**Figure 8.6 Changes of proinflammatory cytokines of IL-1 $\beta$  or TNF- $\alpha$  protein immunoreactivity in pups' cerebral cortex.**

Seven old rat pups received 70% N<sub>2</sub>O + 0.75% isoflurane anaesthesia with or without the plantar surface of left paw injection with 5% formalin or skin incision for 6 hrs. Other cohorts received six hours of 70% nitrogen balanced with oxygen (naïve control), or combined with left paw plantar surface injection of 5% formalin (formalin group) or skin incision (incision group). (A) IL-1 $\beta$ ; (B) TNF- $\alpha$ . In each section, top panel – example bands; bottom panel. Mean data  $\pm$  SD (n = 5). \*\*p < 0.01 vs Naïve control.

### 8.3.4 Cognitive function changes

Forty days after the initial treatment (on post natal day 7) cognitive function was assessed with Trace fear conditioning (Figure 8.7). Consistent with impaired cognitive function, anaesthesia and anaesthesia plus the nociceptive stimuli both reduced the mean percentage freezing time after context testing compared to naïve rat pups ( $p < 0.05$ ). Tone testing revealed that combining the nociceptive stimuli with anaesthesia induced a long-term detriment in cognitive function that was significantly greater than that induced by anaesthesia alone. The area under the curve data showed that whilst anaesthesia decreased freezing time in the tone test ( $p < 0.05$  vs. naïve) anaesthesia plus formalin and anaesthesia plus incision both decreased freezing even further ( $p < 0.05$  vs. other groups).

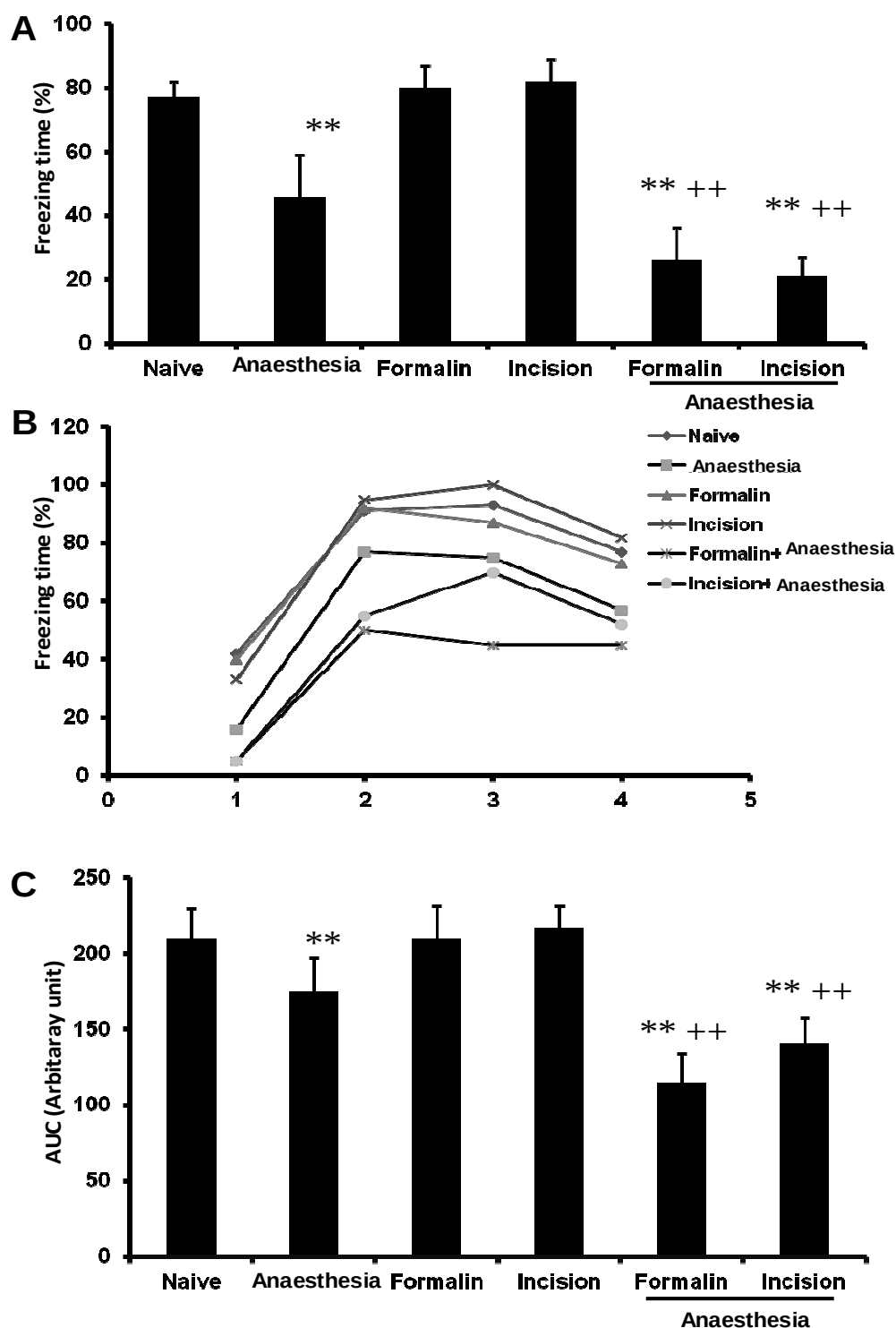


**Figure 8.7 Cognitive function assessed with Trace fear conditioning.**

Seven old rat pups received 70% N<sub>2</sub>O + 0.75% isoflurane anaesthesia with or without the plantar surface of left paw injection with 5% formalin or skin incision for 6 hrs. Other cohorts received six hours of 70% nitrogen balanced with oxygen (naïve control), or combined with left paw plantar surface injection of 5% formalin (formalin group) or skin incision (incision group). They were then allowed to live up to the age of 40 days for cognitive function test. (A) The mean data of percentage of freezing time after context testing. (B) The mean data of percentage of freezing time after tone testing. (C) The average data of the area under curve (AUC) derived from Figure B. Mean  $\pm$  SD,  $n = 6$ . \*\* $p < 0.05$  vs Naïve; ++  $p < 0.01$  vs Anaesthesia.

## 8.4 Discussion

I have shown that administration of nitrous oxide and isoflurane to 7 day old rats produces widespread neuroapoptosis in the cerebral cortex (Figure 8.1), hippocampus (Figure 8.2) and spinal cord (Figure 8.3) consistent with previous studies (Jevtovic-Todorovic et al., 2003a; Ma et al., 2007; Sanders et al., 2008; Sanders et al., 2008; Sanders et al., 2009b; Sanders et al., 2009c; Satomoto et al., 2009) and the production of long-term cognitive impairment (Figure 8.7; (Jevtovic-Todorovic et al., 2003a; Sanders et al., 2009b). Furthermore formalin, but not surgical incision, inflicted after a very short anaesthetic administration (i.e. one that does not produce injury) produced a significant increase in caspase-3 positive cells in all areas of CNS studied compared to naive rats but neither stimulus produced cognitive dysfunction. More importantly, I demonstrated that nociception induced by formalin and surgical incision during anaesthesia augmented the number of caspase-3 positive cells and produced more long-term cognitive impairment compared to anaesthesia alone (Figures 8.1-3 and 8.7). That surgery may exacerbate the injury to the central nervous system produced by anaesthetic drugs during general anaesthesia is significant as it is very relevant to clinical practice and it cannot be ignored.

### 8.4.1 Mechanisms of Anaesthesia-Induced Apoptosis

Several mechanisms have been postulated for anaesthetic drug induced neuronal apoptosis in rodents or primates during the period of synaptogenesis though a role for synaptic deprivation appears to be the most likely explanation (Ma et al., 2007;

Head et al., 2009; Pesic et al., 2009). Recently Head and colleagues showed that isoflurane reduced the release of tissue plasminogen activator (which is dependent on neuronal activity) into the synaptic cleft; this lead to activation of the p75 neurotrophin receptor and neuronal apoptosis (Head et al., 2009). GABA<sub>A</sub> and NMDA receptors and their agonists are essential for synaptogenesis and for control neuronal activation (Ben-Ari et al., 2007). During this period, depolarisation of cells in the developing nervous system is produced primarily by GABA (Ben-Ari et al., 2007). Immature neurons have higher intracellular chloride ion concentration relative to the extracellular environment than in mature neurons; stimulation of GABA<sub>A</sub> receptors, therefore, produces efflux of chloride ions causing neuronal depolarisation (Ben-Ari et al., 2007). As central nervous system mature NMDA receptor population increases and it becoming the predominant excitatory receptor. We suspect that the anaesthetic neurotoxicity is not secondary to activation of GABA<sub>A</sub> receptors as (i) this would not reduce neuronal activation (Ben-Ari et al., 2007) and (ii) the injury is not blocked by the GABA<sub>A</sub> antagonist, gabazine (Sanders et al., 2009b). The most plausible cell surface receptor target for the injury therefore remains blockade of the NMDA receptor and stimulation of the intrinsic apoptotic pathway (Hardingham et al., 2009; Head et al., 2009; Leveille et al., 2010). Other receptor targets including potassium channels, which contribute to neuronal hyperpolarisation and neuronal apoptosis (including TASK channels may also play a role (Patel et al., 2004)).

The description of noxious stimuli inducing apoptosis in the neonatal brain is not new; previous studies administered repeated formalin injections between post-natal

days 1 and 4 and suggested the injury may be excitotoxic in nature with a distinct role for glial cell activation (Anand et al., 2007; Rovnaghi et al., 2008). In the adult, repeated formalin injections induce spinal cord apoptosis (Jalalvand et al., 2008) but a distinct role for pain-induced apoptosis is now noted in the neonate with only one dose of formalin (Figures 8.1-3). Subcutaneous formalin administration into rat's hind paw is a well-established inflammatory pain model (Ma et al., 2004; Anand et al., 2007). It produces a mixed inflammatory, tissue and nerve injury resulting in peripheral and central pain sensitisation; while it is possible that inflammation plays a role in producing the formalin-induced neuronal injury (Rovnaghi et al., 2008), I did observe changes in cortical IL-1 $\beta$  but not TNF- $\alpha$  (Figure 8.6). Surgical incision also produces peripheral and central sensitisation however the duration of the insult is shorter (Zahn et al., 2002) and inflammation plays a less prominent role (Zahn et al., 2004). Indeed both form of nociceptive stimuli induced c-Fos in the dorsal horn of the spinal cord and thalamus (Figure 8.4 and 8.5) indicating activation of the spinothalamic tract.

At present the mechanism of formalin-induced apoptotic injury is unclear though the mechanism of pain clearly differs from that induced by incision due to (i) duration (Zahn et al., 2002), (ii) intensity (Zahn et al., 2002) and (iii) mechanism (Watkins et al., 1997; Zahn et al., 2002). Future studies directed at probing the differences between the ability of formalin and incision to induce apoptosis should focus on the role of the p75 neurotrophin system in producing apoptosis as neurotrophins play an important role in the pain pathway (Pezet and McMahon 2006). Nonetheless the role of inflammation should not be dismissed (Head et al.,

2009). However it is important to note that single administration of either nociceptive stimulus did not induce cognitive dysfunction (Figure 8.7), unlike the administration of multiple doses of formalin (Anand et al., 2007; Rovnaghi et al., 2008).

It is of interest that anaesthesia further activated c-Fos at the level of the thalamus compared to the nociceptive stimuli (Figure 8.4); we suspect that this may relate to nitrous oxide-induced activation of neuronal signalling in supraspinal centers (Sawamura et al., 2000; Sawamura et al., 2003). In the spinal cord, anaesthesia reduced the nociception-induced c-Fos expression (Figure 8.5) consistent with the known antinociceptive effect of isoflurane (Sanders et al., 2005). Importantly both forms of nociceptive stimuli exacerbated the anaesthetic injury.

#### **8.4.2 Mechanisms of Nociception plus Anaesthesia-Induced Apoptosis**

I don't think the mechanisms of nociceptive stimuli can increase apoptosis since animals are anaesthetised by pain memory because though the dose of anaesthetics I used is under 1 MAC, during 6 hours the animals were fully anaesthetic, and the animals were sacrificed immediately after gas exposure. The exacerbation of the anaesthetic injury by noxious stimulation may have occurred secondary to an inflammatory response that stimulated the extrinsic apoptotic pathway via inflammatory cytokine production (Figure 8.6) with downstream caspase-3 activation (Figure 8.1). It is clearly of interest that TNF- $\alpha$  level did not differ from controls and I only observed changes in IL-1 $\beta$  (Figure 8.6). This may be due to the sensitivity of my assay or due to the sampling interval, as I only chose one time point we may have missed the peak

TNF- $\alpha$  level. Nonetheless IL-1 $\beta$  may contribute to apoptosis (Hu et al., 1997; Allan et al., 2001; Allan et al., 2005) and therefore remains a plausible target for neuroprotection (Allan et al., 2001; Allan et al., 2005). Future studies should examine this time course more closely as well as employ cytokine blocking antibodies to probe this suggested association.

Herein we do not consider it likely that the nociceptive stimuli exacerbated anaesthetic injury through an excitotoxic mechanism as we administered a combination of anaesthetics that block the NMDA receptor (Franks et al., 2008) and therefore would be expected to prevent excitotoxicity (Hardingham 2009). A further possibility is that nociceptive signalling increased the availability of neurotrophins at the synapse but simultaneous anesthesia impaired tissue plasminogen activator release, directing the neurotrophin pathway towards p75 neurotrophin activation and cell death (Head et al., 2009). Future mechanistic studies should investigate these possibilities.

### **8.4.3 Caveats**

There are several confounds between extrapolating preclinical results to changes in clinical practice, including species differences, that are well known and necessitate that the clinical implications of a preclinical study be cautiously interpreted (Sanders et al., 2008). Specifically for this study anaesthesia was administered for six hours, a duration that is relatively uncommon in neonatal anaesthesia, although this is an accepted model of anaesthesia-induced neurodegeneration (that also occurs at sub-

anaesthetic doses for administered for one hour (Johnson et al., 2008). Furthermore the pain stimuli that were administered do not last for six hours (Zahn et al., 2002; Ma et al., 2004) and therefore my study does not replicate constant surgical stimulation during anaesthesia however I speculate that if repeated noxious stimuli had been administered a greater exacerbation of anaesthetic injury may have been observed. I administered a level of anaesthesia that is lower than 1 MAC in rodents; in clinical anaesthesia this would represent a subclinical dose. This was chosen to reduce the physiological consequences of anaesthesia though higher doses produce greater neurodegeneration (Jevtovic-Todorovic et al., 2003). Nociceptive stimulation may therefore have greater impact in this paradigm than at deeper levels of anaesthetic though a counter argument that clinical surgery produces greater nociceptive stimulation may also be made. Future studies will concentrate on more closely replicating the clinical scenario with shorter, deeper anaesthesia in a surgically relevant model.

Furthermore, nociception may be deemed to be a similar preconditioning function as hypoxia preconditioning. As discussed in Chapter 7, nociception may have similar mechanisms which are responsible for the hypoxic preconditioning to enhance the neurodegeneration induced by anaesthetics.

#### **8.4.5 Potential Clinical Impact**

Our observation that nociceptive stimulation exacerbates anaesthesia induced apoptosis and cognitive impairment is of concern. Indeed surgical incision itself did not induce apoptosis or cognitive impairment yet augmented the anaesthetic injury on a morphological (Figures 8.1, 8.2 and 8.3) and functional level (Figure 7). The

incision model may be considered analogous to minor or peripheral surgery that occurs without visceral manipulation; it is possible that more major surgery may have a greater deleterious effect. Nonetheless the data suggest that the combination of a surgical incision and anaesthesia may produce more cognitive impairment than anaesthesia alone. The cognitive impairment observed, in both the tone and context fear conditioning tests, indicates involvement of several brain regions including the prefrontal cortex, hippocampus, and amygdala in the injury (Sanders et al., 2003). It is of interest that when isoflurane (0.75%) is administered deficits are only observed in the context test (Sanders et al., 2009b) but herein we show that deeper anaesthesia (isoflurane 0.75% and nitrous oxide 70%) and anaesthesia combined with nociception produces injury in both the tone and context test. This is consistent with a more severe and widespread lesion with the latter stimuli. Indeed the anesthetic injury is known to be dose responsive (Ikonomidou et al., 1999; Jevtovic-Todorovic et al., 2003a; Young et al., 2005; Slikkers et al., 2007; Sanders et al., 2008; Sanders et al., 2009c; Sanders et al., 2009b; Satomoto et al., 2009; Brambrink et al., 2010) and this has also been suggested for nociceptive stimuli (Anand et al., 2007; Rovnaghi et al., 2008).

In conclusion, I have shown that nociceptive stimuli administered during isoflurane and nitrous oxide anesthesia produced significantly more apoptosis in the CNS than anaesthesia alone. This raises serious concern that, should this observation be true to humans also, that surgery may exacerbate an anaesthetic injury causing significant CNS damage to neonates during the synaptogenic period. Potential

therapies to blunt the anesthetic and surgical injuries, identified through further mechanistic investigation, are urgently required.

## **CHAPTER 9**

## **CONCLUSION**

## 9.1 Main findings

Anaesthetics, 0.75% isoflurane and 70% nitrous oxide have been demonstrated to induce apoptotic neurodegeneration when administered during neurodevelopment. It activates the common apoptotic pathway and intrinsic apoptotic pathway.

Anaesthetics provoke spinal cord injury in neonatal rats, increasing caspase-3 within the ventral horn of spinal cord. Caspase 3 expression occurs in cingulate cortex, CA1 subarea of hippocampus, substantia nigra and basal forebrain. Furthermore anaesthetic exposure significantly increased the number of caspase-3 positive cells in the CA1 subarea of hippocampus, cingulate cortex, and substantia nigra, but not in the basal forebrain.

Preconditioning with hypoxia or xenon had been shown to protect against subsequent brain injury induced by hypoxia/ischaemia in rats. These may be potential ways of protecting against anaesthetic drug induced neurotoxicity. However, my data show that xenon preconditioning prevented N<sub>2</sub>O + ISO induced neurotoxicity during neurodevelopment in both *in vivo* and *in vitro*. In contrast, hypoxic preconditioning exacerbated the anaesthetic-induced neurotoxicity whereas N<sub>2</sub>O had no effect on preconditioning. Xenon preconditioning increased Bcl-2 expression and decreased cytochrome C release and P53 expression but the mirror effect on those signals was evident for hypoxic preconditioning. Furthermore, xenon preconditioning attenuated the deterioration in cognitive function produced by anaesthetics, whilst hypoxic preconditioning worsened it. It indicates that

inhalational anaesthesia with nitrous oxide and isoflurane to an asphyxic baby for emergency surgery immediately after birth may result in severe brain damage.

Anaesthetics neurotoxicity in the absence surgical stimuli may be only relevant in the critical care setting. Therefore, the experiments carried out so far do not represent conditions in the operating room where neonates undergo surgery under general anaesthesia where both are occurring at the same time. This study was designed to investigate the impact of nociceptive stimuli on anesthetic induced neuroapoptosis in the rat developing brain. In a model of nociceptive stimulation using formalin injection and surgical incision in the paw in neonatal rats, administration of anaesthesia caused a widespread distribution of caspase 3 positive cells in the brain and spinal cord, and this was significantly enhanced by the nociceptive stimuli. The detrimental effects of nociceptive stimuli further exacerbated the cognitive impairment induced by anaesthetics in these neonatal rats. Activation of the pain pathway as identified using labelled c-Fos was associated with neuroapoptosis enhancement as was the presence of IL-1 $\beta$  cytokine in the brain regions involved. Our data suggest that nociceptive stimuli produced significantly more apoptosis in the CNS than anaesthesia alone. This raises serious concern; should this observation be true to humans also, surgery may exacerbate the anaesthetic induced injury causing significantly more damage to the CNS in neonates during the synaptogenic period.

## 9.2 Clinical Implications

More evidences have been demonstrated that we should choose carefully the anaesthetic drugs we use in critical care. The noble anaesthetic gas xenon displays many of the hallmarks of the ideal anaesthetic agent; its neuroprotective potential, through a preconditioning phenomenon, has been demonstrated in an *in vivo* model of acute neuronal HI injury (Ma et al. 2006) and anaesthetic drugs induced injury as presented in this thesis. This may indicate that this anaesthetic gas may be the ideal anaesthetic drug for paediatric anaesthesia.

Despite intensive research over several decades, there are still no satisfactory clinical strategies to reduce the vulnerability of brain to injury. Mitigating the consequences of HI/R injury with a safe, efficacious and non-toxic agent represents an unmet healthcare need.

Xenon has been used safely in the clinical setting as an anaesthetic in patients of all ages for more than 50 years and its potent preconditioning properties combined with its powerful organ protective capabilities, could be put to a greater use in several fields of medicine, where there is an anticipated risk of HI or IR organ injury. Applications may include the use of xenon intra-operatively for procedures that carry a substantial risk of HI injury, such as cardiopulmonary bypass surgery, renal transplantation surgery or neurosurgery. Xenon may also be pre-emptively administered to expectant mothers in labour who have a high risk of neonatal

hypoxia, such as pre-term labour. In addition, it has a safety profile that is unrivalled (Sanders, Franks & Maze 2003).

Xenon, therefore, represents a promising new therapeutic strategy for reducing the incidence of disorders arising through HI or IR injury. Elucidation of the organ protective mechanism of xenon as a preconditioner is essential if it is to progress and be used safely and efficaciously in the clinical setting.

Until recently, the high cost and scarcity of xenon have prevented its widespread clinical application; with refinements in production of xenon and the advent of xenon-conserving ventilation systems, such as closed-loop or low-flow systems, financial concerns have been alleviated to a large extent. It is still probable at first, however, that its use will have to be reserved for high risk patients.

Clinical translation of my findings will facilitate the use of xenon in preventing hypoxia-related injury. By understanding the mechanisms involved in xenon's preconditioning, it will be possible to prioritise the clinical settings that are most likely to benefit from this safe and efficacious anaesthetic agent.

Moreover, results from these experiments have established that xenon mediates its preconditioning action *via* its effects on the apoptotic pathway; data from these studies will enhance our understanding of the interactions of the putative signalling cascades that are involved.

Even if xenon itself were not to be clinically exploited, data from this study may facilitate the discovery and development of novel chemical entities for action at newly identified therapeutic targets to protect against impending injury, while avoiding those that may promote toxicity.

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## Publication list during the course of my PhD study

Shu, Y., Pac-Soo, C., Zhou, ZW., Sanders, RD., Maze, M., Ma, D., Nociceptive stimuli enhance anesthetics induced neuroapoptosis in the rat developing brain. *Anesthesiology* (submitted).

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