Examining the potential of PGE_2 receptor EP_4 as a neuroprotective target following ischaemic injury

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Abstract

Ischaemic stroke instigates a series of pathological mechanisms which contribute to injury. Despite significant research in this field successful clinical treatment is limited. This highlights the need to identify novel therapeutic targets in order to assess whether clinical investigation is warranted. The enzyme cyclooxygenase-2 (COX-2) is a key contributor to inflammatory injury following stroke. Upregulation of this enzyme results in increased prostanoid synthesis, which mediate many physiological and pathological functions. Prostaglandin E_2 (PGE_2) is a key mediator in inflammation and activation of its receptor subtypes is both neuroprotective and neurotoxic.

COX-2 inhibition in animal models of stroke has demonstrated neuroprotection. However, long term arthritis trials have revealed detrimental effects of COX-2 inhibitors. This highlights the need to identify mediators of the detrimental effects of COX-2 and capitalise those that mediate the beneficial effects. The aim of this study was to investigate the role of the PGE_2 receptor EP_4 following *in vitro* and *in vivo* ischaemia. Organotypic hippocampal sliced cultures (OHSCs) were exposed to oxygen and glucose deprivation (OGD). Treatment with a selective EP_4 agonist following OGD significantly reduced cell death, whereas application of the EP_4 receptor antagonist exacerbated injury. C57/BL6 mice were subjected to focal cerebral ischaemia via middle cerebral artery occlusion (MCAO). Administration of the selective EP_4 agonist significantly reduced infarct volume and prevented the decline in neurological function. COX-2 inhibition and EP_4 receptor stimulation resulted in similar levels of protection both *in vitro* and *in vivo* ischaemia. EP_4 receptor expression was assessed using immunohistochemistry and real time-PCR.

This study provides evidence that selective activation of the EP_4 receptor following ischaemic injury is as neuroprotective as COX-2 inhibition but possibly without the deleterious side effects of COX-2 inhibitors. This supports the concept of targeting protective prostaglandin receptor signalling as a potential therapeutic target for stroke.

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List of Abbreviations

AC	Adenylyl cyclase
APHS	O-(acetoxyphenyl) hept-2-ynyl sulphide
ANOVA	Analysis of variance
BBB	Blood Brain Barrier
Ca^{2+}	Calcium
cAMP	cyclic adenosine monophosphate
CBF	cerebral blood flow
CCA	common carotid artery
cDNA	complimentary DNA
CHO	Chinese hamster ovary
CNS	central nervous system
COX	Cyclooxygenase
cPGES	cytosolic Prostaglandin E synthase
CRF	Corticotrophin-releasing factor
DAB	Diaminobenzamide
DFU	5,5-dimethyl-3-(3-flourophenyl)-4-(4-methylsulphonyl)phenyl-2(5H) furanone
D.I.V.	Days *in vitro*
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DP	Prostaglandin D receptor
DRG	Dorsal root ganglia
ECA	external carotid artery
EGR-1	Early Growth Factor-1
EP	Prostaglandin E receptor
FJ	Fluoro-Jade
FP	Prostaglandin F receptor
GABA	Gamma-aminobutyric acid

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPCRs	G-protein coupled receptors
GSK-3	Glycogen Synthase Kinase-3
HBSS	Hank balanced salt solution
HEK	Human embryonic kidney
HRP	Horseradish peroxidase
IMS	Industrial methylated spirit
IL-1β	Interlukein-1 beta
IP	Prostaglandin I receptor
LDH	Lactate dehydrogenase
LOX	5-lipoxygenase
LPS	Lipopolysaccharide
MCAO	middle cerebral artery occlusion
MCA	middle cerebral artery
MEM	Minimal Essential Medium
$MgCl_2$	magnesium chloride
mPGES-1	microsomal Prostaglandin E synthase-1
mPGES-2	microsomal Prostaglandin E synthase-2
NF-kB	Nuclear factor kappa B
OGD	Oxygen and Glucose Deprivation
OHSC	Organotypic Hippocampal Sliced Cultures
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGs	Prostaglandins
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGES	Prostaglandin E synthases
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$

PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostaglandin I_2
PI	Propidium idodide
PKA	Protein kinase A
PKB/Akt	protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PSD-95	Post synaptic density protein-95
PVN	Paraventricular nucleus
RPL44	Ribosomal protein 44
rt-PA	recombinant tissue plasminogen activator
TNF-α	Tumour necrosis factor alpha
TP	Thromboxane receptor
TTC	Triphenyltetrazolium chloride
TX	Thromboxane
VmPO	Ventromedial preopticnucleus
5-HPETE	5-hydroperoxide

Chapter One

General Introduction

1.1 Stroke Background

Cerebral stroke is the third leading cause of mortality and the primary cause of adult long term neurological disability in the western world having considerable impact on the healthcare system (van der Worp & van Gijn, 2007). Over 70% of patients who experience a stroke are over the age of 65 and as the average life expectancy is on the increase the incidence of stroke is on the rise (Lakhan, Kirchgessner & Hofer, 2009; Durukan & Tatlisumak, 2007). Cerebral stroke can be categorised into ischaemic and haemorrhagic strokes which are both characterised by the loss of blood supply to the brain resulting in cell death but they differ in aetiology. Haemorrhagic stroke occurs due to the rupturing of a weakened blood vessel resulting in accumulation of blood into the surrounding brain tissue. This type of stroke can occur either within the brain, i.e. intracerebral haemorrhage, or in the subarachnoid space. Ischaemic stroke comprises approximately 85% of all stroke cases (Doyle, Simon & Stenzel-Poore, 2008) and occurs due to an obstruction within the blood vessel either caused by an emboli or a thrombus, most commonly affecting the middle cerebral artery (MCA). Cerebral thrombosis is where a thrombus develops causing blockage of the blood vessel, whilst cerebral embolism is the formation of a blood clot in another region of the circulatory system which travels through the circulatory system and becomes blocked in a cerebral vessel. The obstruction in blood flow impedes oxygen and glucose transportation to the brain initiating a cascade of pathological consequences including excitotoxicity, oxidative stress, inflammation and apoptosis resulting in extensive cell death which

continues to progress over time (for review see Doyle et al., 2008; Brouns & Deyn, 2009). The extent of damage which occurs is largely determined by the duration of the occlusion which can last for a number of hours to days (Neumar, 2000). In humans the thrombus or emboli causing the obstruction is broken down which restores blood flow to the ischaemic region, although this is usually incomplete. Reperfusion is critical for salvaging tissue and restoring brain function however, it is also accompanied with secondary injury which can further exacerbate damage (Lakhan et al., 2009). The areas of the brain which are particularly susceptible to ischaemic damage are those which have high metabolic rates such as the hippocampus and cerebellum (Neumar, 2000).

1.2 Clinical problem of stroke

Ischaemic stroke is a major clinical burden and the need for successful therapies is an absolute necessity. The complexity of events following ischaemic injury adds to the difficulty of developing novel therapeutic interventions. However, over the past decade research in this field has increased the understanding of stroke pathology which has enabled identification of potential targets for therapy. Any potential neuroprotectant must undergo rigorous laboratory testing and is recommended to meet the Stroke Therapy Academic Industry Roundtable regulations before being considered for clinical trial (STAIR, 1999). There have been over 1000 compounds which have been tested and found to be effective in experimental models of stroke but unfortunately not all have met the criteria to progress through to clinical trials (O'Collins et al., 2006). The treatments which have progressed on to clinical trials have been eliminated during various stages either due to lack of efficacy or serious side effect.

Potential neuroprotective therapies have been developed to target specific mechanisms involved in the ischaemic cascade including excitotoxicity, inflammation, apoptosis and generation of oxygen free radicals (Ginsberg, 2008; O'Collins et al., 2006; Labiche & Grotta, 2004). These include glutamate receptor antagonists (Grotta et al., 1995; Alber, Atkinson, Kelley & Rosenbaum, 1995), calcium channel antagonists (Grotta, 1991; Gelmers & Hennerici, 1990), sodium channel antagonists (Muir, Holzapfel & Lees, 2000), anti-inflammatory agents (Schneider et al., 1998), thrombolytic therapies (Wardlaw & Warlow, 1992), antioxidants (Yamaguchi et al., 1998; Lees et al., 2001) and anti-apoptotic agents (Bogousslavsky et al., 2002). Other potential therapies which have been tested in clinical trials include hypothermia (Kammersgaard et al., 2000) and modulators of other neurotransmitters such as nitric oxide (Bath, 2002) and gamma-aminobutyric acid (GABA; Lodder et al., 2006). Although many of these therapies have shown promising results using both *in vitro* and *in vivo* models of ischaemia and in early phases of clinical trials, a high risk benefit ratio has resulted in termination of some clinical trials, whilst other agents were eliminated due to lack of efficacy.

Stroke is therefore a devastating disease with largely unmet clinical needs and due to the complexity of events contributing to injury it is difficult to envisage a therapy which only targets a single component of the ischaemic cascade. As a result, targeting several components of the ischaemic cascade via combinational therapy may prove more beneficial. For instance, a combination of neuroprotective agent and thrombolytic therapies confer greater benefits in experimental stroke this could be the result of improved access of the neuroprotective agent to the ischaemic tissue (see review by Fisher et al., 2006). There is a need for basic research to investigate potential neuroprotective compounds in order to determine whether clinical investigation is warranted.

1.3 Current Treatments for ischaemic stroke

The only approved and validated treatment for acute ischaemic stroke available currently is recombinant tissue plasminogen activator (rt-PA). This is a protein involved in the breakdown of blood clots via the conversion of plasminogen to plasmin and consequently restores blood flow to the ischaemic region. Early clinical trials revealed concerns with regard to using thrombolytic treatments due to increased incidence of intracerebral haemorrhage (Hommel et al., 1996). As a result there were initial concerns with the safety of rt-PA and careful evaluation was required in order to determine any potential risks. Major clinical trials using rt-PA have proved beneficial, resulting in neurological improvement in a large number of ischaemic stroke patients (Friedman et al., 1996). However, treatment with rt-PA is most effective when administered within the first 3 hours following ischaemic injury and this narrow therapeutic time window limits the benefits of this treatment to patients who are hospitalised quickly (Elijovich & Chong, 2010). In a clinical setting this is not always the case and < 5% of stroke patients receive rt-PA (Furlan, Katzan & Caplan, 2003). Furthermore this treatment is based on restoring blood flow to the brain region and does not prevent the initiation of the molecular mechanisms which result in cell death. Therefore further research is warranted to identify potential neuroprotective targets not only to reduce ischaemic injury but to extend the time window for successful treatment.

1.4 Cell death in cerebral ischaemia

During ischaemia two distinct forms of cell death occur which are distinguished by the resulting morphological changes and the molecular processes involved (Neumar, 2000). Necrosis is characterised by cellular swelling followed by rupturing of the membrane

and release of the cellular contents into the intracellular space. This exposure of cellular contents can result in subsequent damage by instigating inflammatory processes. This form of cell death is largely confined to the core of the ischaemic injury where the blood supply is most severely restricted, it occurs within minutes and is largely irreversible. The region surrounding the core is referred to as the ischaemic penumbra, where collateral blood flow and sufficient ATP preserve membrane integrity (Ceulemans et al., 2010). Apoptosis is a more common form of cell death in this region (Mehta, Namratta & Raghubir, 2007; Doyle et al., 2008) which differs greatly from necrotic cell morphology. Apoptotic cell death is characterized by a number of morphological features such as shrinkage of the cytoplasm, condensation of chromatin, membrane-blebbing and fragmentation of the cell resulting in apoptotic bodies (Love, 2003). Apoptotic cells are rapidly removed by phagocytosis without initiating an inflammatory reaction. Cells in the penumbra are of particular interest as the slower onset of cell death provides a longer window of opportunity for the administration of potentially beneficial therapeutic measures (Weinberger, 2006).

1.5 Ischaemic cascade

The ischaemic cascade is rapidly initiated following the loss of blood flow to the brain. This subsequently leads to the breakdown of the blood brain barrier (BBB) which permits infiltration of leukocytes and the disruption of the neurovascular structure formed by neurons, astrocytes, endothelium and extracellular matrix, all of which are essential for normal physiological functioning of the brain.

The obstruction of blood flow instigates a series of pathological processes such as excitotoxicity, peri-infarct depolarisations, oxidative stress, inflammation and apoptosis (***Figure 1.1***). These complex pathological mechanisms can occur in sequence or in

parallel, some are initiated within the first few minutes of cerebral ischaemia and others occur over a period of hours and days and ultimately result in the death of neurons, glial cells and endothelial cells (Doyle et al., 2008). The progressive damage that takes place over days provides a broader therapeutic time window for potential treatment.

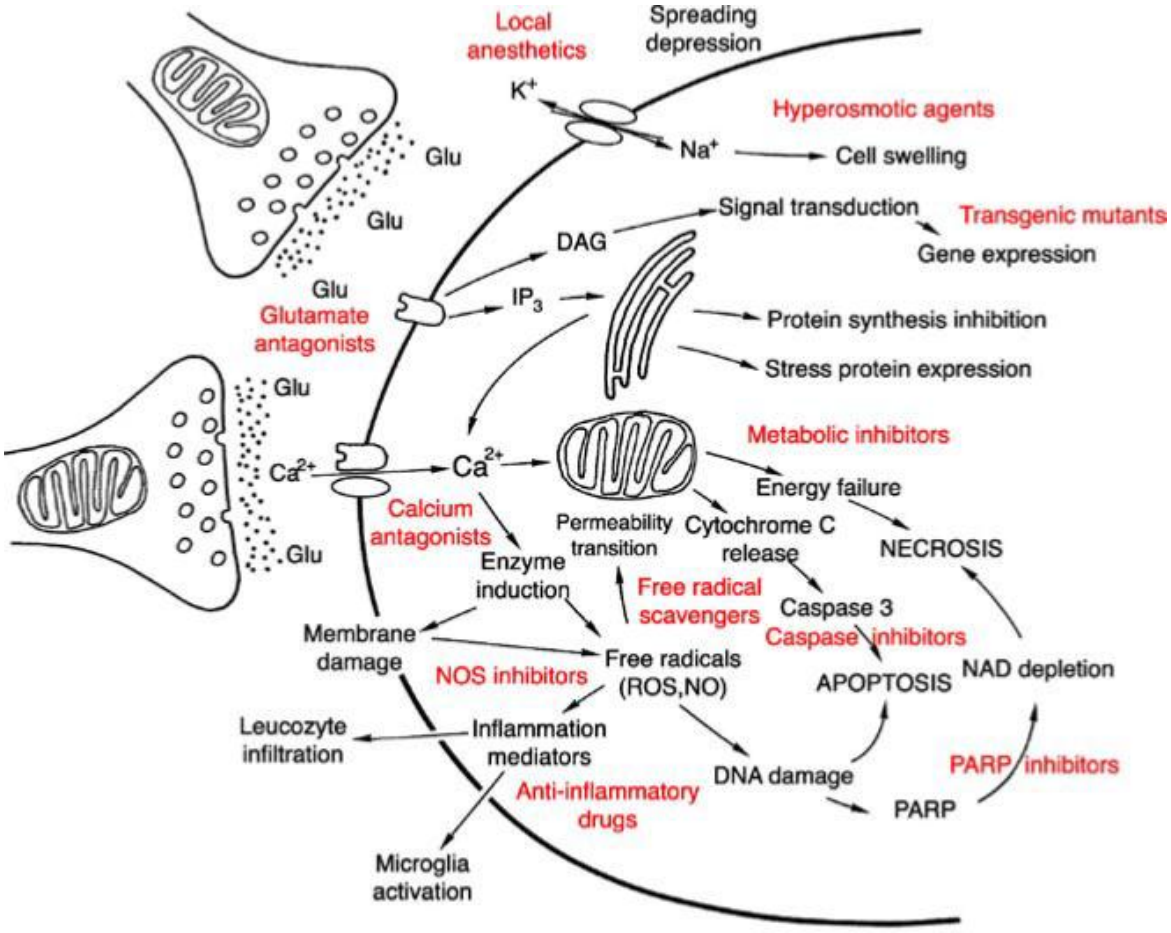


Figure 1.1: Molecular mechanisms contributing to ischaemic injury

A simplistic overview of cellular mechanisms contributing to ischaemic injury and potential therapeutic targets for blocking injurious processes (Hossmann, 2006).

1.5.1 Excitotoxicity

Excitotoxicity occurs when cellular homeostasis of ions is disrupted resulting from energy failure following ischaemia. This leads to insufficient adenosine triphosphate (ATP) which is required in order to maintain ionic homeostasis through sodium/potassium (Na^+/K^+) ATPase and calcium (Ca^{2+}) ATPase. This influx of Ca^{2+} stimulates glutamate release from presynaptic vesicles (White et al., 2000). Depolarisation of the membrane and accumulation of Na^+ inside the cells during ischaemia results in the reversal of glutamate transporters permitting glutamate to exit cells along its concentration gradient (Doyle et al., 2008). Glutamate stimulates the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) receptors which in turn further depolarise the membrane enabling the activation of N-methyl-D aspartate (NMDA) receptors permitting Ca^{2+} entry into cells. Metabotropic glutamate receptors signal through G-protein coupled mechanisms and upon activation they cause Ca^{2+} release from intracellular stores. Collectively, these processes lead to overload of intracellular Ca^{2+} resulting in excitotoxicity and activation of a number of Ca^{2+} dependent enzymes such as proteases, lipases and DNases resulting in necrotic cell death. Under normal physiological conditions the intracellular Ca^{2+} concentrations are maintained around 100 nM compared to 1 mM in the extracellular space (Mehta et al., 2007). During ischaemia the intracellular calcium concentration rises substantially to 50-100 µM (Doyle et al., 2008). These elevated Ca^{2+} levels are sustained during ischaemia and early reperfusion, and contribute to neuronal injury via initiation of processes involved in post-ischaemic inflammation and apoptosis (Neumar, 2000).

Neuroprotective targets for excitotoxicity

The main classes of neuroprotective agents targeting excitotoxicity have largely focused on glutamate receptor antagonists and both Ca^{2+} and Na^{+} channel blockers which have all shown beneficial results in animal models of acute ischaemic stroke (see review Ginsberg, 2008). These agents block the excitotoxic cascade by preventing the accumulation of glutamate and Ca^{2+}.

The use of NMDA antagonists in models of transient and permanent ischaemia have been shown to reduce infarct volume and improve neurological deficits (Mculloch, 1992). A number of NMDA antagonists were discontinued in early clinical trials due to adverse effects including psychometric effects, catatonia and sedation (Muir & Lees, 2003; Davis et al., 1997). Selfotel, a competitive antagonist at the NMDA receptor and aptiganel, a non-competetive antagonist, have been studied in phase III clinical trials. However, these trials were terminated early due to interim analysis which revealed a high risk benefit ratio (Davis et al., 1997; 2000; Albers et al., 2001). Antagonists of the AMPA receptors (YM872) have also demonstrated neuroprotection in animal models of stroke (Håberg et al., 1998; Kawasaki-Yatsugi et al., 1998). For example, two phase II clinical trials investigating the efficacy and safety of YM872, were terminated due to the failing the interim analysis (unpublished, Internet Stroke Center, 2013).

Calcium channel antagonists were one of the first neuroprotective agents investigated in clinical trials (Ovbiagele et al., 2003). Both nimodipine and flunarizine have shown to reduce infarct volume following transient and permanent focal cerebral ischaemia (see review by Barone, Feuerstein & Spera, 1997; Horne et al., 2001). However, an extensive meta-analysis of 22 calcium channel antagonists failed to reveal any beneficial effect of treatment (Mohr et al., 1994). This led to the termination of the Very Early Nimodipine Use in Stroke (VENUS) trials which aimed to determine the efficacy of nimodipine treatment with a time window of 6 h after the onset of stroke (Horn et al.,

2001). An evident side effect of calcium channel antagonists was hypotension resulting from blocking calcium channel activity in vascular smooth muscle cells.

Sodium channel blockers (fosphenytoin and sipatrigine) have both demonstrated neuroprotection in experimental stroke (Calabresi et al., 2003; See review by Hainsworth et al., 2000). However, clinical trials investigating the efficacy of sipatrigine were terminated following a phase II trial which revealed increased incidence of adverse effects such as hallucinations (Muir et al., 2000). Furthermore, phase III clinical trials investigating fosphenytoin in stroke patients were terminated early due to an interim analysis which failed to show any benefit (Pulsinelli et al., 1999).

Clomethiazole, a GABA receptor modulator, enhances neuronal hyperpolaristation by enhancing GABA activity. Treatment with clomethiazole, has shown to promote neuroprotection in both transient and permanent models of focal cerebral ischaemia (Sydserff et al., 2000; Green, 1998). However, in a large phase III clinical trial, treatment with clomethiazole revealed no benefit in ischaemic stroke patients (Lyden et al., 2002).

Preclinical studies in animal stroke models revealed neuroprotection following treatment with Maxipost, an activator of neuronal potassium channels (Gribkoff et al., 2001). Its neuroprotective effect results from inducing neuronal hyperpolarization, counteracting glutamatergic activity. A phase III clinical trial was terminated as it failed to show any benefit (unpublished, see review by Cheng et al., 2004).

Other neurotransmitter modulators including serotonin agonists (repinotan) and opioid antagonists (nalmefene) have also shown neuroprotection in animal models of stroke (Berends, Luiten & Nyakas, 2005; Faden et al., 1990). Repinotan and nalmefene have

both been investigated in phase III clinical trials but were terminated as they revealed no overall benefit with either agent (Teal et al., 2009; Clark et al., 2000).

Although modulators of excitotoxicity present an attractive target for reducing the ischaemic damage they have a limited therapeutic time window. In the clinical setting, treatment is often delayed beyond time points used in experimental models, thus the same neuroprotective effect is not observed. In addition, although these potential therapies have shown promising results in animal models with limited or no side effects, their efficacy in clinical trials has been questioned as some agents have been shown to induce serious psychometric side effects and interfere with respiratory and cardiovascular regulation (Dirnagl et al., 1999). ***Table 1.2*** provides a summary of some of the key compounds which have shown beneficial effects in preclinical studies and have progressed to clinical trials.

Compound	Mechanism	Preclinical models	Clinical trial stage	Outcome	Reference
Aptiganel **Selfotel**	NMDA receptor antagonist	Transient/permanent model of Focal ischaemia	Phase III	Adverse effects High risk benefit ratio	Davis et al. (1997; 2000) Albers et al. (2001)
YM872	AMPA receptor antagonist	Focal/global ischaemia	Terminated at Phase II	Safety issues	Internet Stroke Center (2013)
Nimodopine	Ca^{2+} channel blocker (L-type)	Transient/permanent models of focal ischaemia	Terminated at Phase III	Earlier studies showed benefit. Later studies revealed no benefit.	Horn et al. (2001)
Fosphenytoin	Anticonvulsant	Transient/permanent models of focal ischaemia	Phase III	No overall benefit	Pulsinelli et al. (1999)
Sipatrigine	Anticonvulsant	Focal/global ischaemia Transient/permanent ischaemia	Phase II	Adverse effects	Muir et al. (2001)
Maxipost	K^+ channel activator	Transient/permanent	Phase II	No overall benefit	Internet Stroke Center (2013)
Clomethiazole	GABA receptor agonist	Transient/permanent models of focal ischaemia	Phase III	No overall benefits Adverse effects	Lyden et al. (2002)

Table 1.1: Neuroprotective targets- Excitotoxicity

An overview of some of the key targets aimed to modulate mechanisms of excitotoxicity.

1.5.2 Oxidative stress

The accumulation of intracellular Ca^{2+} and Na^{+} instigate mitochondrial release of reactive oxygen species (ROS). There is considerable evidence indicating that reactive oxygen and nitrogen molecules are important mediators of ischaemic injury (reviewed by Warner, Sheng & Batinic´-Haberle, 2004; Chan, 2001). The two primary classes of free radicals relevant to ischaemia include oxygen free radicals and nitric oxide (Neumar, 2000). Activation of nitric oxide synthase (NOS) and an increase in nitric oxide (NO) and peroxynitrate lead to over activation of DNA repair enzymes, impairing processes such as anaerobic glycolysis and mitochondrial respiration impacting on cell survival (Doyle at al., 2008). However, NO can mediate both beneficial and detrimental effects depending on the type of NOS it was derived from. For example, endothelial derived NO mediates vasodilation, scavenges free radicals and has anti-inflammatory properties, all of which are beneficial following ischaemia (Brouns & Deyn, 2009; Hossmann, 2009). In contrast, neuronal derived NO contributes to glutamate excitotoxicity and generates free oxygen radicals which are detrimental to cells (Hossmann, 2009). NO produced via inducible NOS also has detrimental effects and is derived from infiltrating macrophages, neutrophils, activated microglia and astrocytes and is regulated by glutamate release, oxidative stress and cytokines (Moro et al., 2004; Nakka, Gusain, Mehta & Raghubir, 2008). Oxidative stress also impacts on reperfusion-induced injury where the production of superoxide, NO and peroxynitrite activate matrix metalloproteases (MMPs) which disrupts cerebral vasculature and increases blood brain barrier (BBB) permeability (Doyle et al., 2008). This indicates that ROS are important mediators of ischaemic injury and therefore present an attractive target for neuroprotection.

Neuroprotective targets in oxidative stress

There is extensive preclinical reports demonstrating neuroprotective effects of antioxidant, disodium 4-tert-butylimino methyl benzene-1, 3-disulfonate N- oxide (NXY-059) in both transient and permanent models of focal ischaemia (reviewed by Ginsberg, 2008). Two large double blind clinical trials have been completed. One of the trials showed beneficial results whilst the other trial found no difference between the patient cohorts treated with NXY-059 and placebo (Lees et al., 2006; Shuaib et al., 2007).

Tirilazad is a free radical scavenger and an inhibitor of lipid peroxidation and has shown neuroprotection in experimental stroke (reviewed by Sena, Wheble, Sandercock & Macleod, 2007). Treatment with tirilazad showed maximum efficacy when administered before the onset of ischaemia, the efficacy largely decreased at treatment times greater than 60 minutes. Furthermore, tirilazad was only effective over a narrow dosage range and has limited BBB permeability therefore its beneficial effects are restricted to protecting the integrity of the BBB rather than directly acting at the site of injury. A number of clinical trials testing the efficacy of tirilazad in ischaemic stroke patients have revealed inconsistent findings with one reporting no beneficial effect, one being terminated due to safety concerns and another showing a high risk benefit ratio (van der Worp et al., 2002; Tirilazad International Steering Committee, 2000). These reports led to early termination of any clinical trials investigating the efficacy of tirilazad.

A free radical scavenger and inhibitor of lipid peroxidation, 3-methyl-1-phenyl-2-pyrazolin-5-one (Edaravone) is clinically available in Japan and has demonstrated neuroprotection in experimental stroke (Shichinohe et al., 2004). Currently, there is only

reports of one clinical stroke trial which showed an improvement with a therapeutic time window of 72 h (Endaravone Acute Infarction Study Group, 2003). Further studies designed to determine the clinical efficacy of this agent are currently underway (Internet Stroke Center, 2013).

Citicoline is an exogenous form of choline and is implicated in several mechanisms of the ischaemic cascade including increasing phosphatidylcholine synthesis and inhibition of phospholipase A_2 following ischaemia (Labiche & Grotta, 2004). In experimental stroke treatment with citicoline has been shown to have neuroprotective properties (D'Orlando & Sandage, 1995; Clark et al., 1999). Early clinical trials revealed a significant improvement in ischaemic stroke patients treated with citicoline (Clark et al., 1999). However, a more recent larger trial revealed no significant benefit in patients treated with citicoline and the placebo group (Dávalos et al., 2012).

Lubeluzole down regulates NOS pathway and is neuroprotective in experimental models of focal and global ischaemia (Aronowski, Strong & Grotta, 1996; Haseldonckx et al., 1997). Early clinical stroke trials revealed that treatment with a low dose of lubeluzole was safe, in comparison to higher doses which increased mortality (Diener et al., 1996). However, larger trials failed to show any beneficial effects following treatment with lubeluzole (Diener et al., 2000).

Ebselen has antioxidant and anti-inflammatory properties and is proposed to mediate its beneficial effects by inhibiting lipid peroxidation. Treatment with ebselen has demonstrated neuroprotection in a number of *in vivo* stroke models (reviewed by Parnham & Sies, 2000). Early clinical stroke trials investigating the safety and efficacy of ebselen showed some benefit (Yamaguchi et al., 1998). A large phase III trial was

completed in 2009 (Internet Stroke Center, 2013), however, the results are yet to be published.

Although potential targets for attenuating oxidative stress have shown beneficial effects in preclinical studies, clinical trials have largely revealed no benefit of these agents. This is largely due to low BBB permeability of these compounds. However, further trials determining the efficacy and safety of endaravone are still in progress. ***Table 1.2*** provides an overview of some of the modulators of oxidative stress which have been investigated in clinical trials.

Compound	Mechanism	Preclinical models	Clinical trial stage	Outcome	Reference
disodium 4-tert-butylimino methyl benzene-1, 3-disulfonate N- oxide (NXY-059)	Free radical trapping properties	Transient/ permanent ischaemia	Phase III	Showed some benefit limited time window for treatment	Lees et al. (2006) Shuaib et al. (2007)
Tirilazad mesylate (U-74006F)	Inhibitor of lipid perioxidation Free radical scavenger	Transient/ permanent ischaemia	Phase III	Limited drug efficacy Higher doses raised questions regarding safety	van der Worp et al. (2002) Tirilazad International Steering Committee (2000)
3-methyl-1-phenyl-2-pyrazolin-5-one (Edaravone)	Free radical scavenger	Focal/global ischaemia Temporary/permanent ischaemia	Phase IV	Showed some benefit with a therapeutic time window of 72 h	Endaravone Acute Infarction study (2003)
Citicoline	phosphatidylcholine precursor with additional membrane stabilising properties	Focal/global ischaemia	Phase III	No benefit	Dávalos et al. (2012)
Lubeluzole	Downregulates NO synthase	Transient focal ischaemia	Phase III	No benefit	Diener et al. (2000)
Ebselen	Inhibitor of lipid peroxidation	Focal ischaemia Temporary/permanent	Phase III	Early trials shown some benefits	Yamaguchi et al. (1998)

Table 1.2: Neuroprotective targets- Oxidative stress

An overview of some of the key targets aimed to attenuate processes involved in oxidative stress.

1.5.3 Apoptosis

The apoptotic signalling cascade is initiated by oxidative stress, DNA damage, protease activation and ionic imbalance (Doyle et al., 2008; Durukan & Tatlisumak, 2007). This process involves the complex interplay of the B-cell lymphoma (Bcl) family of proteins which either promote or prevent mitochondrial pore formation (Nakka et al., 2008). Pro-apoptotic proteins such as Bax and Bad mediate the release of cytochrome C from the mitochondria, a key intermediate in apoptosis. Anti-apoptotic proteins such as Bcl-XL and Bcl-2 prevent pore formation and cytochrome C release by activating extracellular kinase pathways. Cytochrome C is involved in the processes which activate downstream caspases. Among the 12 caspases which have been identified, caspase 1, 3 and 9 play a pivotal role in ischaemia mediated apoptosis (Nakka et al., 2007; Dirnagl et al., 1999). These are specific cysteine proteases involved in instigating and terminating processes that cause apoptosis by cleaving a number of proteins including poly (adenosine-diphosphate)- ribose polymerase (PARP), DNA- dependent protein kinase, U1 soluble nuclear RNA polymerase (U1-snRNP), spectrin, lamin A, actin and protein kinase C (Cheng et al., 2004). PARP induces the expression of proinflammatory cytokines and chemokines which contribute to inflammatory injury. Pharmacological modulation and genetic deletion of PARP has been shown to supress proinflammatory gene expression and ameliorate ischaemic damage (see review by Virág & Szabó, 2002).The inhibition of caspases and other pro-apoptotic factors have been shown to promote neuroprotection following ischaemic injury (Akpan & Troy, 2012). The process of apoptosis is energy consuming and therefore occurs in the ischaemic penumbra where sufficient ATP is available or it can be is instigated following reperfusion due to the return of cellular energy stores. Therefore the mechanisms involved in apoptosis

provide a wide array of potential neuroprotective targets for stroke injury and a wider therapeutic time window.

Neuroprotective targets in apoptosis

Neuroprotective targets modulating processes involved in apoptosis selected for clinical development are predominantly mediators promoting anti-apoptotic proteins and those attenuating pro-apoptotic processes. ***Table 1.3*** provides an overview of some of the key agents which have been investigated in clinical trials of ischaemic stroke, their proposed mechanisms and outcome.

Tacrolimus (FK506 Lipid complex-gilead) attenuates apoptotic cell death and also has immunosuppressive properties and has demonstrated neuroprotection in both *in vitro* and *in vivo* models of ischaemia (see review by Macleod et al., 2005). Preliminary studies have demonstrated good tolerance of this compound; however, no further trials have been reported (Labiche & Grotta, 2004).

Erythropoietin (EPO) is growth factor which activates the EPO receptor which is a member of the cytokine-receptor superfamily and has been shown to promote survival and inhibit ischaemic injury in animal models of stroke (Li et al., 2006; Kilic et al., 2005a). As this compound is clinically available, experimental studies rapidly led to testing EPO in clinical trials. Early trials of recombinant human EPO reported that it was well tolerated and showed some benefit in acute ischaemic stroke patients (Ehrenreich et al., 2002). Further trials investigating the efficacy of recombinant human EPO revealed no benefit and also reported an increase in mortality in patients receiving a combination of rt-PA and EPO (Ehrenreich et al 2009).

Trophic factors have emerged as a potential neuroprotective target with particular focus in the recovery phase. The proposed mechanisms of basic fibroblast growth factor (bFGF) is its role in preventing the down-regulation of anti-apoptotic proteins such as Bcl-2 following ischaemia (Ay, Sugimori & Finklestein, 2001). Clinical trials evaluating the efficacy of bFGF have reported that low doses showed no significant benefit whilst higher doses resulted in adverse effects including leucocytosis (Bogousslavsky et al., 2002). Consequently, further trials investigating bFGF in stroke patients were terminated.

Caspase inhibitors are an attractive therapeutic target for stroke and have demonstrated neuroprotection in a number of preclinical studies including focal and global ischaemia models (see review by Akpan & Troy, 2012). However, their clinical development is limited due to their inability to cross the BBB. In order to improve delivery of peptides, current direction is centred around developing delivery vectors with conjugated protein agents.

Compound	Mechanism	Preclinical models	Clinical trial stage	Outcome	Reference
Tacrolimus (FK506 Lipid complex-gilead)	Attenuates apoptotic cell death	Focal/global ischaemia Transient/permanent ischaemia	Early Phase	-	Labiche & Grotta (2004)
Erythropoietin (EPO)	Activation of EPO receptor Anti-apoptotic Antioxidant	Transient focal ischaemia	Phase III	No overall benefit. Raised questions regarding safety	Ehrenreich et al. (2009)
Basic fibroblast growth factor (bFGF)	Prevents down regulation of anti-apoptotic proteins	Transient/permanent focal ischaemia	Phase III	No benefit. Adverse effects-hypotension	Bogousslavsky et al. (2002)

Table 1.3: Neuroprotective targets- Apoptosis

An overview of some of the key targets aimed to modulate apoptotic processes.

1.5.4 Inflammation

Although several molecular mechanisms are involved in the ischaemic cascade contributing to neuronal injury, evidence showing that post-ischaemic inflammation exacerbates injury is accumulating (Ceulemans et al., 2010; Jin, Yang & Li, 2010; Lakhan et al., 2009). The inflammatory response following ischaemic stroke is characterised by the increase in inflammatory cells and inflammatory mediators which invade the ischaemic brain and ultimately exacerbate injury (Denes, Thornton, Rothwell & Allan, 2010; Jin et al., 2010). The key molecules which mediate inflammatory injury are pro-inflammatory cytokines interleukins (IL) IL-1, IL-6 and tumor necrosis factor alpha (TNF-α), chemokines, adhesion molecules (selectins, integrins and immunoglobulins), matrix metalloproteinases (MMP), cyclooxygenase-2 and iNOS (Broussalis et al., 2012; Wang,Tang & Yenari, 2007). The key cellular components responsible for the release of inflammatory mediators are reactive microglia, reactive astrocytes and leukocytes.

Activated microglia are capable of releasing both neurotoxic and neuroprotective substances (Lakhan et al., 2009). Their release in brain derived neurotrophic factor (BDNF) and growth factors such as insulin-like growth factor I (IGF-I) promote neuroprotection. In contrast, microglial release of pro-inflammatory mediators such as cytokines, NO and ROS mediate neurotoxic effects.

Cytokines are glycoproteins which are rapidly induced in response to tissue injury. Many of these cytokines are synthesised as inactive precursors which are subsequently cleaved into the active form. Chemokines are a family of small peptides, which are induced by cytokines following injury and mediate leukocyte infiltration. The cytokines and chemokines which have been extensively investigated in the pathogenesis of

ischaemia include IL-1, TNF- α, growth factors, CXC and CC (Allan & Rothwell, 2001). Anti-inflammatory cytokines promote neuroprotection which include transforming growth factor β (TGF-β) and IL-10 (Ceulemans et al., 2010).

Although the inflammatory response resulting from ischaemia further exacerbates injury, some components of the inflammatory cascade can have beneficial effects depending on whether they activate neuroprotective or neurotoxic mechanisms which consequently direct the outcome of ischaemic damage. One of the major players involved in inflammation is the enzyme cyclooxygenase- 2 (COX-2) which is regulated in its expression by cytokines and mitogens and largely accounts for prostaglandin formation (FitzGerald, 2003). COX-2 is a rate-limiting enzyme for prostanoid synthesis and has been implicated in the basic mechanisms of several CNS disorders, including stroke, multiple sclerosis and numerous neurodegenerative diseases (Minghetti, 2004). The approval of selective COX-2 inhibitors for the treatment of pain and rheumatoid arthritis raised the possibility that these agents could also be used in the treatment of neurological diseases such as stroke (see review by Shi & Klotz, 2008). However, the occurrence of serious cardiovascular complications in patients receiving selective COX-2 inhibitors raised safety concerns and careful evaluation of the therapeutic potential of these drugs was required. Consequently, it is important to identify the deleterious effects of COX-2 enzyme and avoid inhibiting mediators which are potentially neuroprotective. Recently, the focus has turned to modulation of specific pathways downstream of COX-2 such as prostaglandin E_2 (PGE_2) and its EP receptor subtypes (Andreasson, 2010). *In vivo* studies of ischaemic stroke have shown that activation of the EP_1 and EP_3 receptors increase ischaemic injury (Abe et al., 2008 Ahmad et al., 2006, 2007; Kawano et al., 2006) whereas EP_2 receptor activation confers protection (McCullough et al., 2004; Liu et al., 2005). The EP_4 receptor has received the least

attention and could also be a potential therapeutic target in ischaemic stroke. Modulation of PGE_2 receptors provides the opportunity to block the specific receptor pathways mediating COX-2–dependent neurotoxicity without altering the balance between COX-2–derived prostanoids. These novel experimental approaches may offer potential therapeutic targets to ameliorate ischaemic brain injury and warrant further investigation.

Neuroprotective targets for inflammation

Novel anti-inflammatory targets continue to be identified and investigated in preclinical studies and represent an area of potential translational stroke research (O'Neill, Astles, Allan & Anthony, 2004). As these processes occur later in the ischaemic cascade targeting them should provide a wider therapeutic time window.

The complex mechanisms involved in the inflammatory response contribute to ischaemic injury and therefore represent an ideal downstream target for neuroprotection. These include inflammatory cytokines and enzymes, leukocyte infiltration, upregulation of adhesion molecules and factors involved in gene transcription. ***Table 1.4*** provides an overview of some of the molecular targets for inflammation which have reached clinical trials.

Enlimomab is an intracellular adhesion molecule-1 (ICAM-1) antibody that attenuates leukocyte adhesion. Animal models of transient focal cerebral ischaemia have revealed neuroprotective effects of enlimomab by decreasing infarct volume (Zhang et al., 1995). However, the same study demonstrated that this neuroprotection was not observed following permanent occlusion, suggesting that reperfusion is required for the beneficial effects of enlimomab. Unfortunately, a large multi-centre phase III clinical trial revealed

no benefit following treatment with enlimomab and raised questions regarding the safety of this agent (EAST Trial, 2001).

LeukArrest (Hu23F2G) is a humanised monocolonal anti-antibody which recognises CD11/C18 integrins and inhibits leukocytes ability to adhere to endothelial cells. Preclinical studies have a neuroprotective effect following treatment with Hu23F2G in a rabbit model of transient focal ischaemia (Yenari et al., 1998). Early clinical trials established a safety profile of Hu23F2G. However, phase III trials were terminated as early reports revealed no benefits in patients treated with Hu23F2G (Labiche & Grotta, 2004).

Minocycline mediates its anti-inflammatory effects inhibiting microglial activation and the production of other inflammatory mediators (Yrjänheikki et al., 1999). Other proposed neuroprotective mechanisms of minocycline include inhibition of caspases, iNOS and mitogen–activated protein kinases (MAPKs). This compound is an appealing neuroprotectant as it has wide therapeutic effects, a good CNS penetration and an established safety profile (Labiche & Grotta 2004). Early clinical trials of minocycline have shown beneficial results and further trials designed to determine the efficacy of minocycline are under way (Internet Stroke Center, 2013).

Preclinical studies have shown neuroprotection following treatment with IL-1ra (see review by Rothwell, 2003). Recombinant human IL-1 receptor antagonist (rhIL-1ra) presents an attractive target for neuroprotection following ischaemia as it readily crosses the BBB. This compound has been investigated in clinical stroke trials where it has demonstrated some benefit with an established safety profile (Emsley et al., 2005).

Compound	Mechanism	Preclinical models	Clinical trial stage	Outcome	Reference
Enlimomab	Anti-ICAM-1 antibody	Transient focal ischaemia	Phase III	Serious adverse events No benefit	EAST Trial (2001)
LeukArrest (Hu23F2G)	Blocks leukocyte infiltration	Transient focal ischaemia	Phase III	No benefit	Internet Stroke Center (2013)
Minocycline	Anti-inflammatory/anti-apoptotic	Focal/global ischaemia	Early Phase	Significant benefit On-going trials	Internet Stroke Center (2013)
rhIL-1 ra	Inhibition of cytokine	Focal/global ischaemia Transient/permanent ischaemia	Phase II	Some benefit	Emsley et al. (2005)

Table 1.4: Neuroprotective targets- Inflammation

An overview of some of the key targets aimed to decrease inflammatory injury.

1.5.5 Other mechanisms of injury

The BBB is comprised of endothelial cells, basal lamina, astrocytes and extracellular matrix. In normal physiological conditions the integrity of the BBB protects the microvascular environment and ensures normal physiological functioning. Cerebral ischaemia instigates dynamic changes in the BBB integrity resulting from inflammatory molecules and free radicals. These changes are characterised by cell swelling, detachment of astrocytes and rupturing of vessels increasing the permeability of macromolecules into the ischaemic zone (Ceulemans et al., 2010). Following reperfusion vasogenic oedema formation occurs resulting from the breakdown of the BBB. This further aggravates ischaemic injury causing compression and increasing intracranaial pressure.

1.5.6 Other potential therapeutic targets

Other therapies for stroke which are under investigation target multiple aspects of ischaemic injury and these include hypothermia, magnesium sulphate, albumin, caffeinol, antiplatelet agents, benzodiazepines and statins (Sahota & Savitz, 2011; Labiche & Grotta, 2004; O'Collins et al., 2006). From these potential therapies, hypothermia and magnesium sulphate have been extensively investigated and have both demonstrated beneficial effects in animal models and in clinical trials.

Hypothermia has been shown to be neuroprotective in both focal and global models of ischaemia through reducing cerebral metabolism, attenuating apoptosis and inflammation, and preserving BBB integrity (see review by Barone, Feuerstein & White, 1997). The beneficial results observed with hypothermia across multiple laboratories and in different animal models could perhaps be the result of its impact on

multiple pathways, showing a greater overall benefit rather than targeting a single therapeutic target. Early clinical trials have demonstrated feasibility and safety of using hypothermia combined with rt-PA and further trials determining the efficacy of hypothermia are currently underway (Hemmen et al., 2010).

Magnesium sulphate presents an attractive target for therapy. It has many potential mechanisms of neuroprotection which include blocking NMDA activity and Ca^{2+} activity and promoting vasodilation in addition to inhibiting the inflammatory response (Muir, 2002). Further to this, pilot studies using magnesium have demonstrated its safety and tolerability in humans (Muir & Lees, 1995). Further trials are currently under way in order to determine the efficacy of magnesium treatment in stroke therapy (Internet Stroke Center, 2013).

1.6 Summary

Many advances have been made in understanding the pathophysiological consequences of ischaemic stroke; however, this has failed to translate into effective therapeutic strategies. An example is COX-2 in that convincing evidence exists for a key role of COX-2 activation in the ensuing pathology following ischaemic stroke (see review by Iadecola & Gorelick, 2005; Candelario-Jalil & Fiebich, 2008). However, current therapeutic strategies employed to inhibit COX-2 activity have been unsuccessful. This is due to reports of serious adverse effects of selective COX-2 inhibitors following long term clinical trials for arthritis.

Many of the agents discussed above are either limited in efficacy due to their adverse effects or they have a narrow therapeutic time window for treatment. The disappointing results of clinical trials are largely due to methodological issues in both preclinical and

clinical studies. Preclinical research has often resulted in insufficient information regarding therapeutic time window and dosage. In addition, clinical trials require more specific patient selection criteria. The neuroprotective doses used in clinical trials often result in side effects as higher doses are required in order to achieve some benefit.

1.7 Project Aims

A large body of evidence exists indicating a major role of COX-2 activation and subsequent prostaglandin synthesis in cerebral ischaemia. Although COX-2 inhibition *per se* appears to be unfeasible as a therapeutic approach following cerebral ischaemia, it is relevant that the downstream mediators of the benefits of inhibiting COX-2 activity are investigated. In particular, PGE_2 and its designated EP receptors have recently emerged as potential targets for stroke therapy. From the four EP receptor subtypes (EP_1-EP_4), the role of the EP_4 receptor following ischaemia has received the least attention. This project therefore aims to elucidate the role of the EP_4 receptor following ischaemic injury. This will provide a better understanding in the hope to reveal potential therapeutic targets for ischaemic injury.

Both *in vitro* and *in vivo* models of ischaemia will be used to determine the contribution of the EP_4 receptor to ischaemic injury. This will be achieved by application of selective agonists and antagonists of EP_4 receptor. Ultimately this work will provide a better understanding of the consequence of both EP_4 receptor stimulation and inhibition in terms of contributing to ischaemic cell death. If the beneficial effects of PGE_2 activity can be identified and pharmacologically targeted, this has the potential to help patients who have suffered ischaemic injury. In addition, this project aims to determine the expression profile of EP_4 receptors in the brain using immunohistochemistry and real time-PCR techniques.

Chapter Two

Optimizing an *in vitro* model of ischaemia

2.1 Introduction

2.1.1 Different approaches using culture Systems

Several *in vitro* models using cell types from the CNS have been developed to investigate the mechanisms of neuronal degeneration in an attempt to identify potential therapeutic targets. These include primary cell culture models, such as dissociated cultures or sliced cultures, or acute brain slices. Most CNS primary cell cultures are prepared from cortical, hippocampal or cerebellar tissue from young rodents, however, these are then usually restricted to composing largely of one cell type. In contrast, organotypic cultures enable the inclusion of multiple cell types which exist in the CNS such as neurons, astrocytes and microglia. The interaction of these cells is important in modelling not only pathological events but also normal physiological events.

In terms of trying to model ischaemia, the hippocampus is a common target to investigate the consequences of oxygen and glucose deprivation (OGD; Lipski, Wan, Bai, Pi & Li & Donnelly 2007; Sun, Feng, Miki, Seino & French, 2005; Moroni et al., 2001). The hippocampus is an area of the brain most susceptible to ischaemic injury (Neumar, 2000) and due to its characteristic anatomy is an area easy to localise. Each type of culture system has advantages and disadvantages and the method chosen is dependent on the model most appropriate to investigate the proposed research question.

Organotypic hippocampal sliced cultures (OHSCs) have been used in various studies to investigate neurodegeneration and the mechanisms underlying ischaemic cell death

(Chung, Hong, Kang & Chang, 2008; Frantseva, Carlen & El-Beheiry, 1999; Laake, Haug, Wieloch & Ottersen, 1999). OHSCs exposed to OGD result in both necrotic cell death and apoptotic cells which mimic *in vivo* stroke pathology (Cho, Wood & Bowlby, 2007). This model also enables experimental conditions to be examined over long time periods whilst maintaining the neuronal architecture of the hippocampus. In contrast, in most mixed astrocyte and neuronal cultures, neuronal connectivity and cellular organization can be difficult to maintain. However, studies have shown that in OHSCs, cellular integrity is largely preserved (Gogolla, Galimberti, DePaola & Caroni, 2006). Another advantage of using OHSCs compared to dissociated neuronal/glial cultures is that the neuronal-glial interaction is maintained, whilst in dissociated cultures this is destroyed (Stoppini, Buchas & Muller, 1991). Early studies of OHSCs have also confirmed that chemical and synaptic signalling properties are maintained making them suitable for a wide range of studies (De Simoni & Yu, 2006).

OHSCs not only have advantages over other primary cell culture systems but can also offer advantages over using acute brain slices. Firstly, OHSCs have time to recover from the slicing procedure (Adamchik, Frantseva, Weisspapir, Carlen & Velazquez, 2000), which produce microglial activation (McCullough et al., 2004) and changes in metabolic state due to the release of enzymes and ions during slicing (De Simoni & Yu 2006). Recent studies have shown that OHSCs are viable for up to 4 months in culture (Galimberti, Gogolla, Alberi, Santos, Muller & Caroni, 2006) permitting examination of long term consequences of experimental manipulations, giving them an advantage over acute hippocampal slices.

The two well established methods for preparing OHSCs are the roller drum technique pioneered by Gähwiler (1981) and the interface method developed by Stoppini et al. (1991). The roller drum technique is more suitable for experiments where the outcomes

to be analysed involve microscopy as the cultures are grown on glass coverslips, resulting in the flattening of the cultures over time. However, the limitation of this technique is that they are difficult to prepare and the thinning of the tissue to a monolayer results in increased variability between each preparation (Cho, Wood & Bowlby, 2007). Also, it has been reported that this technique can result in degeneration of slices before they are sufficiently developed to use in experiments (Laake et al., 1999). Most studies use various modifications of the interface method, where the cultures are grown on semi porous membrane inserts allowing the tissue to be maintained at the liquid/air interface (Chung et al., 2008). The culture medium provides essential nutrition to the cultures via the semi porous membrane. The interface method is more suitable for testing drug compounds, as the cultures are submerged in medium containing the compound enabling the molecules to diffuse in the slice via the porous membrane.

OHSCs represent a closer comparison to the *in vivo* situation compared to dissociated primary culture systems or acute slices. Also they enable long term investigations compared to other *in vitro* approaches which is important particularly when investigating ischaemic injury as the damage evolves over several days to weeks. In addition, as the cytoarchitecture is largely preserved consisting of a number of different cells this enables broader investigation between the interactions of various cell types. Also, dissociated cultures obtained from hippocampal or cortical neurons have shown glucose deprivation alone resulted in significant neuronal loss (Monyer, Giffard, Hartley, Dugan, Goldberg & Choi, 1992) whereas OHSCs are more resilient to prolonged hypoglycaemic conditions (Pringle, Angunawela, Wilde, Mepham, Sundstrom & Iannotti, 1997). This is likely to be due to the glial-neuronal relationship, where glial cells protect neurons from hypoglycaemia or anoxic insults. This neuron-

glial interaction is severely disrupted in dissociated cultures but largely preserved in OHSCs. Therefore, OHSCs is a suitable platform to investigate mechanisms of neurodegeneration following *in vitro* ischaemia.

2.1.2 *In vitro* models of ischaemia

In vitro models are useful in broadening the understanding of molecular pathways of injury as they can replicate many aspects of the *in vivo* situation. Also culture experiments require fewer animals to obtain reliable data in comparison to *in vivo* models. The most widely used *in vitro* approach to model ischaemic conditions involves a combination of oxygen and glucose deprivation (Rytter, Cronberg, Asztély, Nemali & Wieloch, 2003). However, other models such as glutamate-mediated excitotoxicity (Cimarosti & Henley, 2008), hypoxia (Pringle et al., 1997) and hypoglycaemia (Tasker, Coyle & Vornov, 1992) are also frequently used.

The OGD method is reported to be the most consistent and reproducible method across different labs in inducing ischaemic injury most relevant to the *in vivo* situation in comparison to neuronal toxicity experiments (Cimarosti & Henley, 2008). This is because depriving the cultures from oxygen and glucose simulates *in vivo* ischaemia where the disruption of blood supply ultimately results in oxygen and glucose deprivation initiating the ischaemic cascade. Also, chemical ischaemia results in increased free radical generation in comparison to hypoxia (Woodruff, Thundyil, Tang, Sobey, Taylor & Arumugam, 2011), suggesting that different mechanisms of cell death may predominate in each model. Therefore, models of chemical ischaemia, hypoxia or hypoglycaemia alone may not be a sufficient comparison of *in vivo* ischaemia where there is a depletion of both oxygen and glucose via the disruption in blood supply

(Pringle et al., 1997). This suggests that OGD is the most appropriate method to mimic *in vivo* ischaemic conditions.

2.1.3 Cell Death Assessment

The viability assessment of cells in *in vitro* models include markers of biochemical changes, by measuring lactate dehydrogenase (LDH) release, morphological changes using Fluoro-Jade (FJ) staining, a marker of apoptotic cells, cell loss using cresyl violet staining, or other immunohistochemical analyses or functional endpoints using electrophysiology (Noraberg, Kristensen & Zimmer, 1999). A common approach in studies investigating ischaemia *in vitro* is to use propidium idodide (PI) uptake as a marker of cell death (Chung et al., 2008; Cimarosti, Rodnight, Tavares, Paiva, Valentim, Rocha & Salbego, 2001; Frantseva et al., 1999). PI is non-toxic to cells and enters cells following loss of membrane integrity and stains the cell nuclei to elicit a fluorescent signal when excited by the appropriate wavelength of light. Studies which have used PI labelling usually measure fluorescent intensity as a means for cell death assessment. The fluorescent intensity in the experimental condition is compared with a control sample representing basal cell death, or a sample which represents 100% cell death to obtain a percentage (Cimarosti et al., 2001). PI can also be used in combination with other fluorescent stains such as Hoechst 33342 (Woodruff et al., 2011) which binds to the DNA of living and dead cells enabling the visualisation of all cells. An advantage of using PI staining in comparison to LDH release is that the regions of the hippocampus where cell death has occurred can be directly visualised. Also, PI uptake can be visualised within hours as it enters the cells following loss of membrane integrity, whereas, LDH release occurs following cell lysis. Therefore PI labelling is not only an early marker of cell death but also allows the quantification of neuronal cell

death and is a simpler method of cell death analysis in comparison to using immunohistochemistry.

Studies have either supplemented the medium with PI throughout the experiment, allowing the increase in fluorescent intensity as a measure of cell death to be measured or PI is added to the medium for a brief period (usually 20-30 mins) before visualising the slices (Brana, Benham & Sundstrom, 2002).

2.1.4 Summary & Objectives

OHSCs represent a suitable culture system to investigate *in vitro* ischaemia. The most appropriate method to mimic *in vitro* ischaemic conditions seems to be via deprivation of both oxygen and glucose. A quantitative method for assessing cell viability via PI and Hoechst labelling to obtain a percentage cell death is a suitable protocol for assessing cell death in this model of ischaemia. Therefore, the following experiments aimed to optimize the parameters required to achieve a reliable and reproducible model of *in vitro* ischaemia. According to the literature there are various *in vitro* models which have been developed to investigate the consequences of ischaemia, however, there is no consensus regarding a universal protocol. It is important firstly to produce a consistent *in vitro* model of ischaemia which induces cell death in order to assess potential neuroprotectants. Following optimization, experiments were conducted testing the effects of a known neuroprotectant, i.e. progesterone and its metabolite allopregnanolone (Gibson & Murphy, 2004; Sayeed, Guo, Hoffman & Stein, 2006). These experiments were carried out to ensure the reliability and reproducibility of this model of *in vitro* ischaemia before being used for subsequent experiments.

2.2 Method

2.2.1 Animals

This study was conducted in accordance with the UK Animals Scientific Procedures Act, 1986. *In vitro* cultures were prepared from 4-9 days old C57/BL6 pups.

2.2.2 Dissecting buffer and culture media

All buffers, culture media and supplements were ordered from Sigma unless otherwise stated (see ***table 2.1*** for details about all cell culture reagents that were used). All the reagents in the culture media were combined inside a sterile laminar airflow cabinet under sterile conditions and filtered using a 0.45 µm syringe filter. All media and buffers were refrigerated at ~4°C until use. Hippocampal slices were prepared in ice cold dissecting medium containing HBSS (Hank balanced salt solution), 4.5 mg/ml glucose solution and 3.75 µg/ml amphotericin B. The slices were cultured in growth medium containing 50% MEM (minimal essential medium), 25% heat-inactivated horse serum, 25% HBSS, 0.5 mM glutamine, 4.5 mg/ml glucose and 3.75 µg/ml amphotericin B. The OGD medium contained 75% MEM, 25% HBSS, 1mM glutamine and 3.75 µg/ml amphotericin B and was bubbled for 30 minutes with 5% CO_2 and 95% N_2 before use. Media were never used for longer than two weeks to minimize the chance of contamination.

Name	Description	Catalogue Number
Glucose solution (45%)	Main source of energy	G8769
Amphotericin B	Fungizone	A2942
200mM Glutamine	Essential amino acid, stimulates growth and prolongs the viability of cells	G-7513
Heat-inactivated horse serum	Contains vital vitamins and fatty acids required for growth and repair	26050-088
Hanks Balanced Salt Solution (HBSS)	The basis of buffers, retain osmotic balance	H8264
Minimum Essential Medium Eagle (MEM)	pH indicator	M2279

Table 2.1: Cell Culture Reagents

All from Sigma-Aldrich except for heat-inactivated horse serum (GIBCO/Invitrogen).

2.2.3 Culture Preparation

Cell culture cabinets and all materials were sterilized with 70% Industrial methylated spirit (IMS), and dissecting tools were sterilized in pure ethanol and then heated with a gas flame to ensure sterility. Organotypic hippocampal cultures were prepared according to the methods of Stoppini et al. (1991) with some modifications. Brains were removed from animals and placed in sterile petri dishes containing ice cold dissecting buffer. The hemispheres were separated using a scalpel and the hippocampus dissected and sliced (350µm) using a tissue chopper (McIlwain tissue chopper). The slices were carefully separated using sterile syringe needles and transferred onto Millicell membrane inserts (0.4 µm, MilliPore) using a plastic pasteur pipette. These inserts were placed into six well plates and cultured in growth medium. Cultures were maintained in a humidified incubator with 5% CO_2 at 37°C and culture medium was changed every 3 days. It is important to note that culture medium should be changed on time as any delays resulted in poor survival of cells having an impact on the number of

slices available for subsequent experiments. Cultures were observed under the microscope every few days to monitor growth. Any hippocampal slices showing an increased number of dead or dying cells were not used for experiments.

2.2.4 Oxygen and Glucose Deprivation

Before the start of each experiment the anoxic chamber was placed in the incubator to warm up to 37°C in order that the chamber was at the same temperature as the cultures and the culture media. In addition, a petri dish containing water was placed inside the chamber to maintain humidity. OGD medium was bubbled for 30 minutes with 5% CO_2 and 95% N_2, this time has previously been shown to be sufficient to deoxygenate media by Frantseva et al. (1999). After two washes with the OGD medium, 1ml of OGD medium was placed in the well and the plates were transferred to an anoxic chamber. The chamber was sealed and pumped with 5% CO_2 and 95% N_2 for 10 minutes then placed in an incubator at 37°C for the duration of OGD. The cultures were returned to oxygenated serum-free culture medium and were placed back in the incubator for a further 24 hours.

2.2.5 Assessing neuronal cell death following OGD

In order to assess cell death following exposure to OGD, 30 minutes prior to the end of the 24 hour recovery, the fluorescent cell death marker propidium iodide (PI, 5 µg/ml) and Hoechst (5 µg/ml) were added to the medium. At the end of the 24 hour recovery, slices were fixed with 4% paraformaldehyde at 4°C. Following fixation, the cultures were briefly washed in phosphate buffered saline (PBS). The slices were removed from the membrane inserts and mounted onto glass slides in PBS and glycerol solution and imaged using a Nikon epifluorescence microscope. For each hippocampal slice, images

were taken from two randomly selected regions. For each region, two images were taken, one image allowing visualization of PI-labelled cells and the other showing Hoechst-labelled cells. The number of cells in each image was counted manually. To obtain the percentage cell death, cells labelled with PI (i.e. number of dying cells) were divided by the total number of cells, as indicated by the Hoechst labelling, which labels all cell nuclei.

2.2.6 Progesterone treatment following OGD

Following optimization, experiments were carried out testing the effects of progesterone and allopregnanolone treatment following OGD. After 2 hours of OGD exposure, the cultures were returned to oxygenated serum-free culture medium containing 1 of the following treatments: culture medium only, DMSO, progesterone (0.1, 1.0 and 10 μM) and allopregnanolone (0.1, 1.0 and 10 μM).

2.2.7 Data Analysis

All data are reported as mean ± standard error of the mean and were analysed using GraphPad Prism version 5.0 for windows (GraphPad Software, Sand Diego, USA). Student's t-tests were used for statistical analysis between two groups whereas analysis of variance (ANOVA) with Bonferroni post hoc was used to analyse more than two groups. In the case of non-parametric data, i.e. data which did not show a normal distribution, Kruskal Wallis analysis was used with Dunn's multiple comparisons test. The criterion for statistical significance was $P < .05$. Slice cultures were prepared from 6-10 pups per experiments. Each experimental condition was repeated 4 times to increase the reliability of the results. Two values for percentage cell death were obtained per hippocampal slice. This was done to avoid any possible bias occurring

from focussing on one region of the hippocampal slice. Cell death values for each experimental condition were normalised to the control group.

2.3 Results

2.3.1 Age of animals

Initially postnatal day 9 (P9) C57/BL6 mice were used, however the survival rate of cells was considered insufficient (84.9 ± 1.5 %) therefore for subsequent experiments P4 mice were used. Previous studies have shown that stable cultures can be prepared from younger animals as well as from adult animals (Noraberg et al., 1999). Also, following several culture preparations it became apparent that the time within which the hippocampus is removed and sliced is critical. To maximize cell survival rates, the hippocampus was removed within minutes and the dissecting medium was kept on ice at all times to maintain a low temperature during the dissection and slice preparation. Implementing this practice resulted in optimal survival of cultures.

2.3.2 Establishing a protocol for cell viability

Before conducting any OGD experiments the viability of cells was assessed using PI (GIBCO/Invitrogen). Cell viability was assessed following 7 days *in vitro* and again after 14 days *in vitro* before conducting OGD experiments. To assess cell viability 5µg/ml of PI and 5 µg/ml of Hoechst (this concentration of Hoechst has previously been optimized in the lab) were added to the culture media and the cells were incubated for 30 minutes. After incubation the hippocampal slices were fixed using 4% paraformaldehyde (PFA). Slices were then removed from the membrane inserts and mounted onto glass slides with Citifluor AF1 mountant (UKC Chem. Lab, Kent, UK).

They were visualised using a Nikon epifluorescence microscope under the appropriate filter set (PI - absorbance peak = 535nm, emission peak = 617nm; Hoechst - absorbance peak = 350, emission peak = 461, and photographed. Images were collected in black and white to maximize resolution and cells were counted manually using Image J software. The outcome from these experiments indicated that there were no significant differences in the number of dead cells following either 7 days (16.4 ± 2.0%) or 14 days (17.8 ± 2.8%) *in vitro*.

2.3.3 Optimizing PI concentration

Following several experiments it appeared that background staining with PI was quite intense making it difficult to visualise individual cells. This could be due to the concentration of PI used or time period of fixation in 4% paraformaldehyde. Therefore experiments were conducted to optimize PI concentration. The PI concentrations tested were 3, 4 and 5 µg/ml. The outcome from this experiment suggested that the optimal concentration of PI is 5 µg/ml which was the concentration originally used. At this concentration of PI the dead cells were clearly labelled and therefore 5µg/ml of PI was used for subsequent experiments.

2.3.4 Optimizing the time period of fixation

Previous studies have used a fixation period of 4 hours (Brana et al., 2002). However, longer fixation periods can result in increased background staining making it difficult to visualize individual cells. Therefore to optimize the fixation time, cultures were fixed for 1, 2, and 3 hours. Each concentration of PI (3, 4 and 5µg/ml) was tested with the three fixation times and the slices were viewed under the microscope. These experiments indicated that the optimal fixation period was 2 hours with a PI

concentration of 5µg/ml. This time period of fixation resulted in reduced background staining enabling clear visualisation of dead cells, therefore for all experiments that followed slices were fixed for 2 hours.

2.3.5 Optimizing a method for cell counting

For each hippocampal slice images were taken from two different regions which were randomly selected at X 40 magnification. For each region an image was taken showing PI stained cells and one showing Hoechst labelling. To reduce bias whilst counting the images were coded. Initially, an automated cell counter was used using Image J software; however, the software was not sensitive enough to detect cells which were close together. Cells which were in contact or very close could be seen by eye but not detected using the automated counter, consequently it was decided that manual counting would be more accurate. Initially, all cells from the whole image were counted this was approximately 200 cells for total cell number. This was proving to be significantly time consuming hindering the progress of the project. Therefore a 4 x 3 grid (***Figure 2.1)*** was placed over the image and every third square was counted (4 squares in total). To assess whether the grid method would significantly change the results, data from the images where all cells were counted and the images where 4 squares were counted, using the same set of images were analysed using a Mann-Whitney U test. The results show that there was no significant difference between the two counting methods. In conclusion, the grid method for counting was a reliable method giving a representative of the whole image and this method was subsequently used for all experiments.

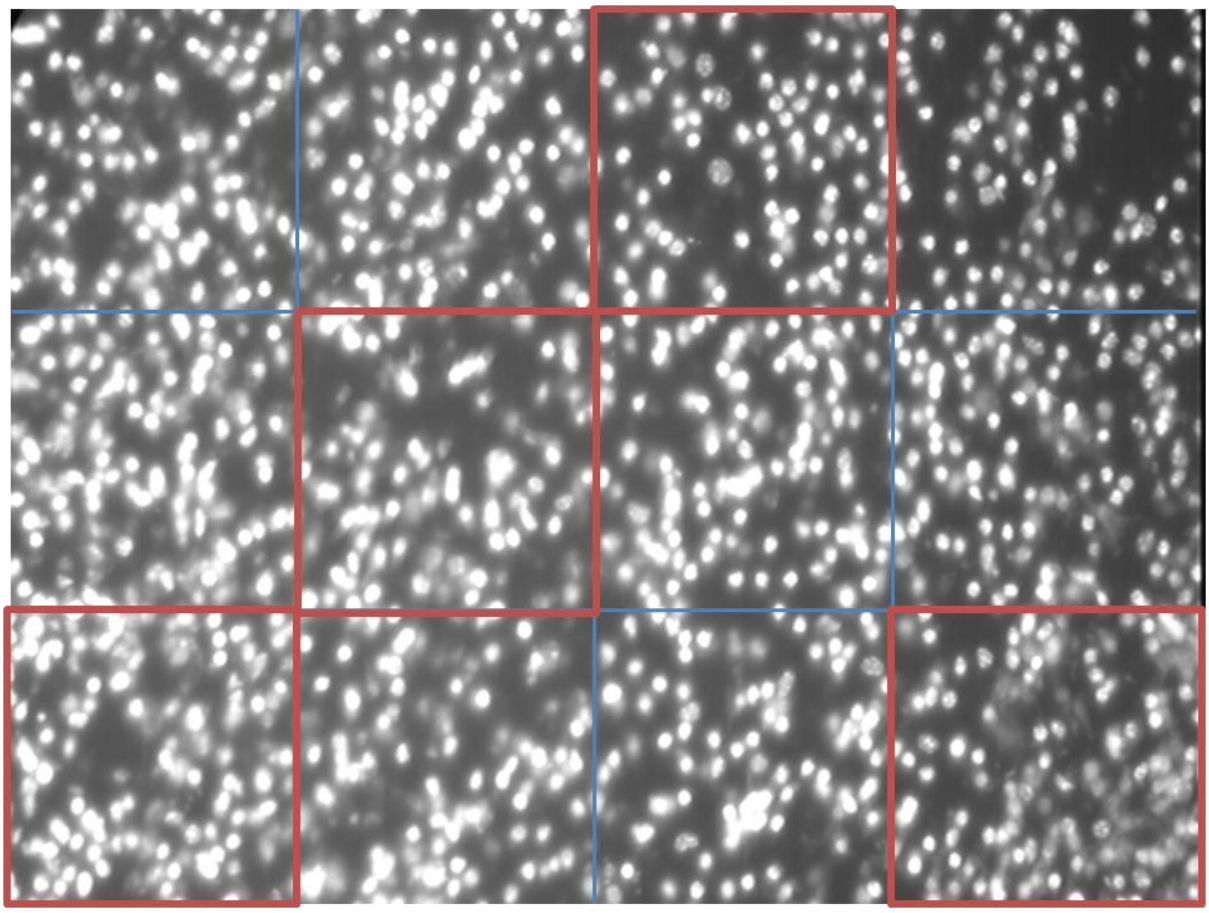
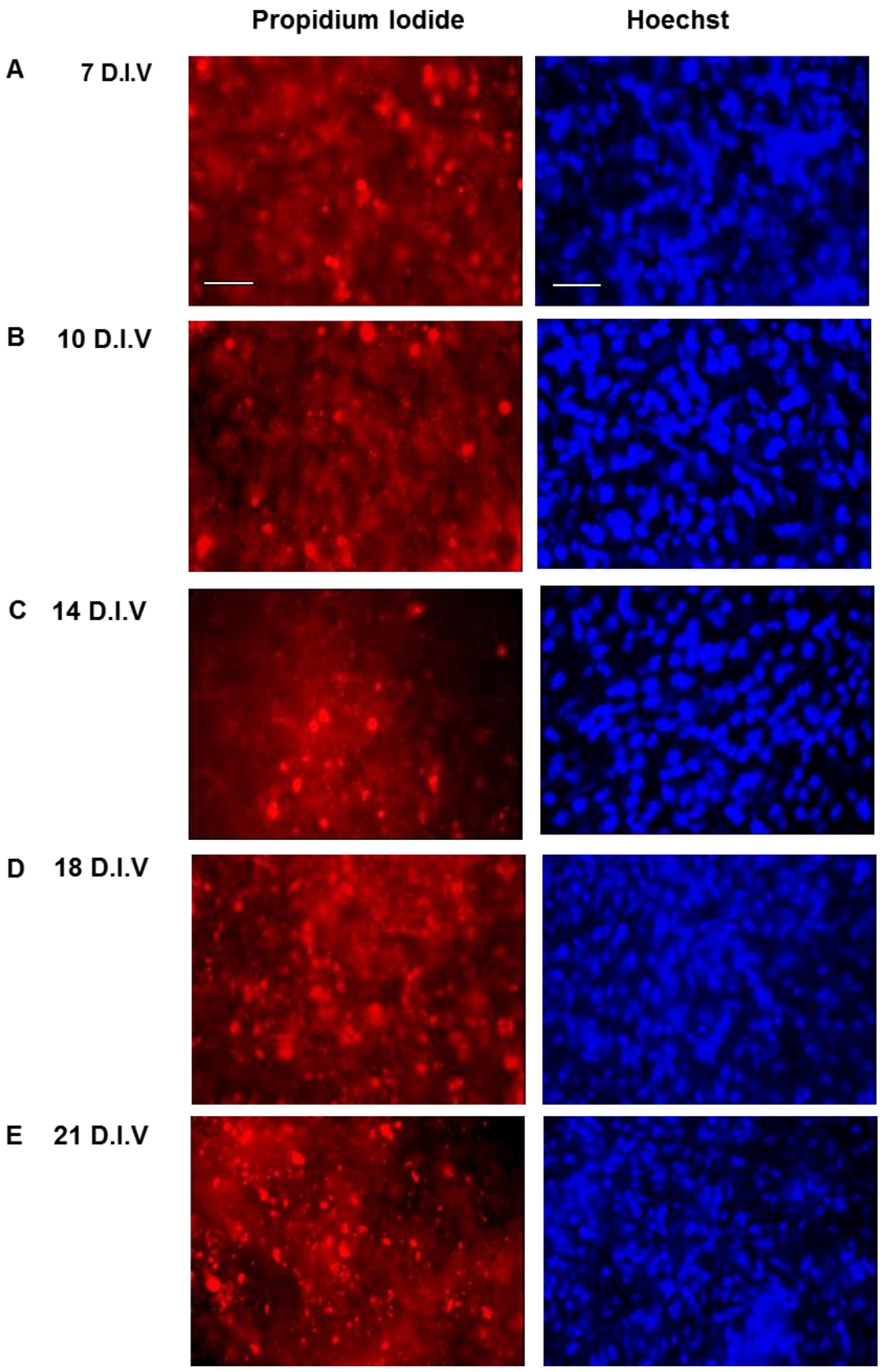


Figure 2.1: Cell counting grid

The grid used for cell counting with an example image showing Hoechst labelled cells. Every third square of the image was counted as indicated by the red square.

2.3.6 Optimizing the time course of culture maintenance

To optimize the time course of culture maintenance before being used for OGD experiments, cell survival of cultures was assessed following 7, 10, 14, 18 and 21 days *in vitro* (D.I.V). Previous reports have used cultures at 14 D.I.V, a time point that has been shown to be sufficient for the slices to have recovered from microglia activation caused by the slicing procedure (McCullough et al., 2004; De Simoni & Yu, 2006). Cell viability was assessed, showing a significant increase in cell death at day 21 ($P < .0001$, 24.7 ± 1.2%, ***Figure 2.2***). There were no significant differences in cell death between days 7 and 18.


Propidium Iodide
Hoechst
A
7 D.I.V
B
10 D.I.V
C
14 D.I.V
D
18 D.I.V
E
21 D.I.V

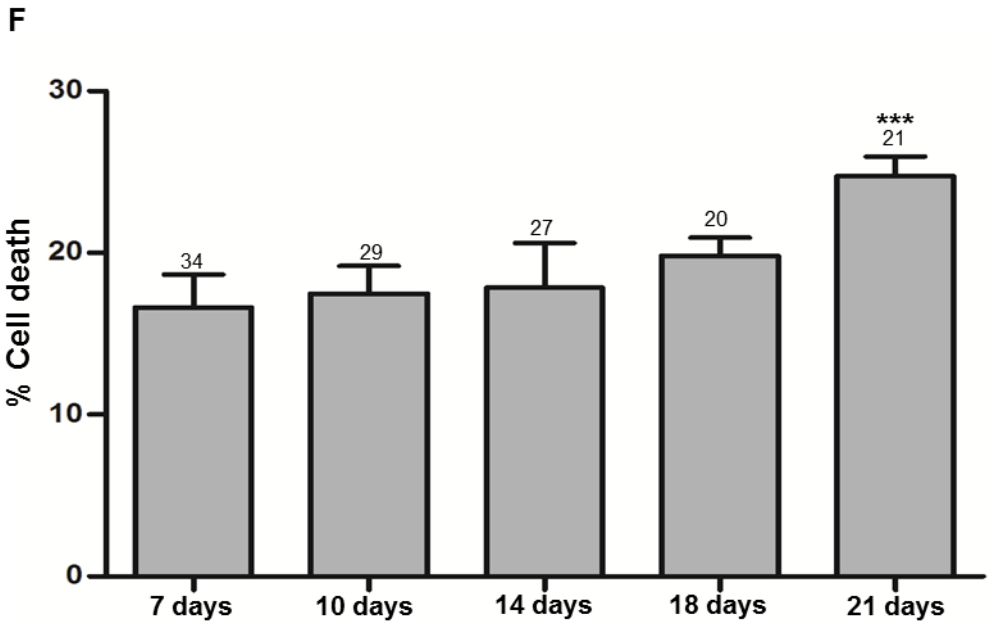


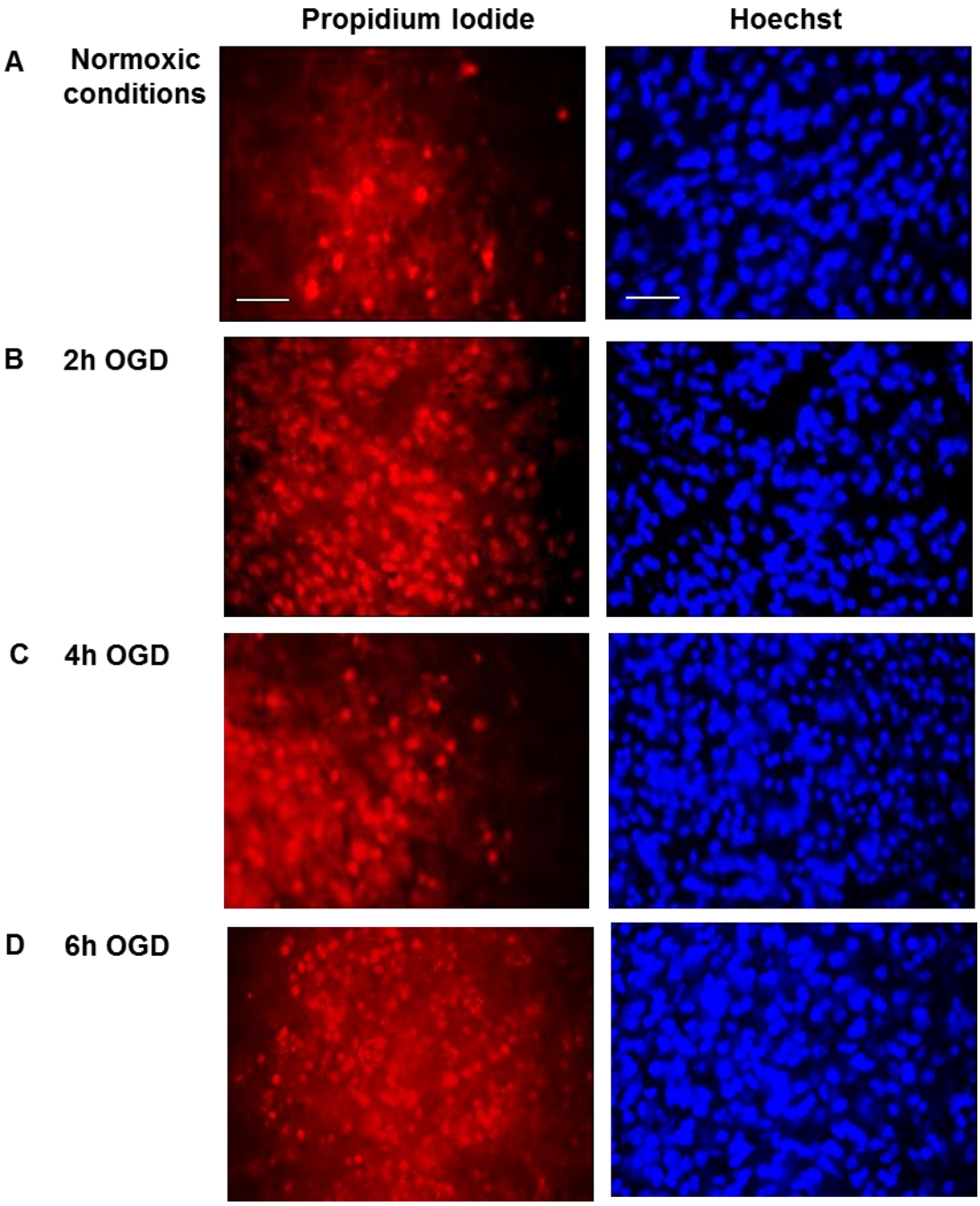
Figure 2.2: Time course of culture maintenance

Representative images of hippocampal slices, cell death is shown in images stained with PI at: 7, 10, 14, 18 and 21 D.I.V. Scale bars represent 50 µM. Hippocampal slices were cultured and maintained for a period of 21 days. Cell death was analysed at different time points. Cell death was significantly increased at day 21 compared to 7, 10 and 14 days *in vitro* (*** $P < .0001$). Data are expressed as mean ± SEM, n=24, number of slices = 20-34 per condition.

2.3.7 Optimizing time period of OGD

Experiments were conducted to establish an optimal time period of OGD that would result in sufficient cell death in order to assess neuroprotection. The time period of OGD which were tested were; 2, 4 and 6 hours. For each experiment two non-OGD controls were used, one with no media change and one with media change. The media change control condition was used to test whether changing the medium can impact on cell survival. There were no significant differences in percentage cell death between the control group with no media change (12.2± 1.7 %) and the control group with media change (17.8 ± 2.8 %). This indicates that changing the media did not significantly increase cell death. Therefore for subsequent experiments the media for the normoxic controls was replaced with normal culture media in order that they received the same treatment as the experimental condition.

Following 2 hours of OGD percentage cell death was significantly increased (34.6 ± 3.1 %) compared to controls (17.8 ± 2.8 %, $P < .0001$, ***Figure 2.3***). Therefore this time period was selected for future experiments. Cell death was significantly increased after 4 hours (60.0 ± 3.2 %) and 6 hours of OGD exposure (71.5 ± 4.0 %) compared to controls, $P < .0001$.


Propidium Iodide
Hoechst
A
Normoxic conditions
B
2h OGD
C
4h OGD
D
6h OGD

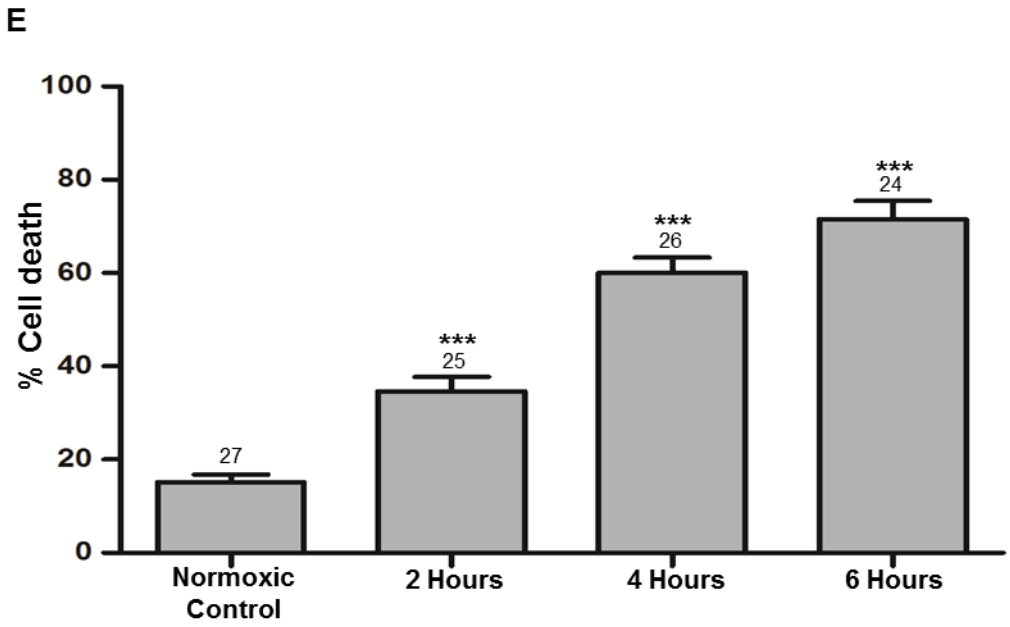
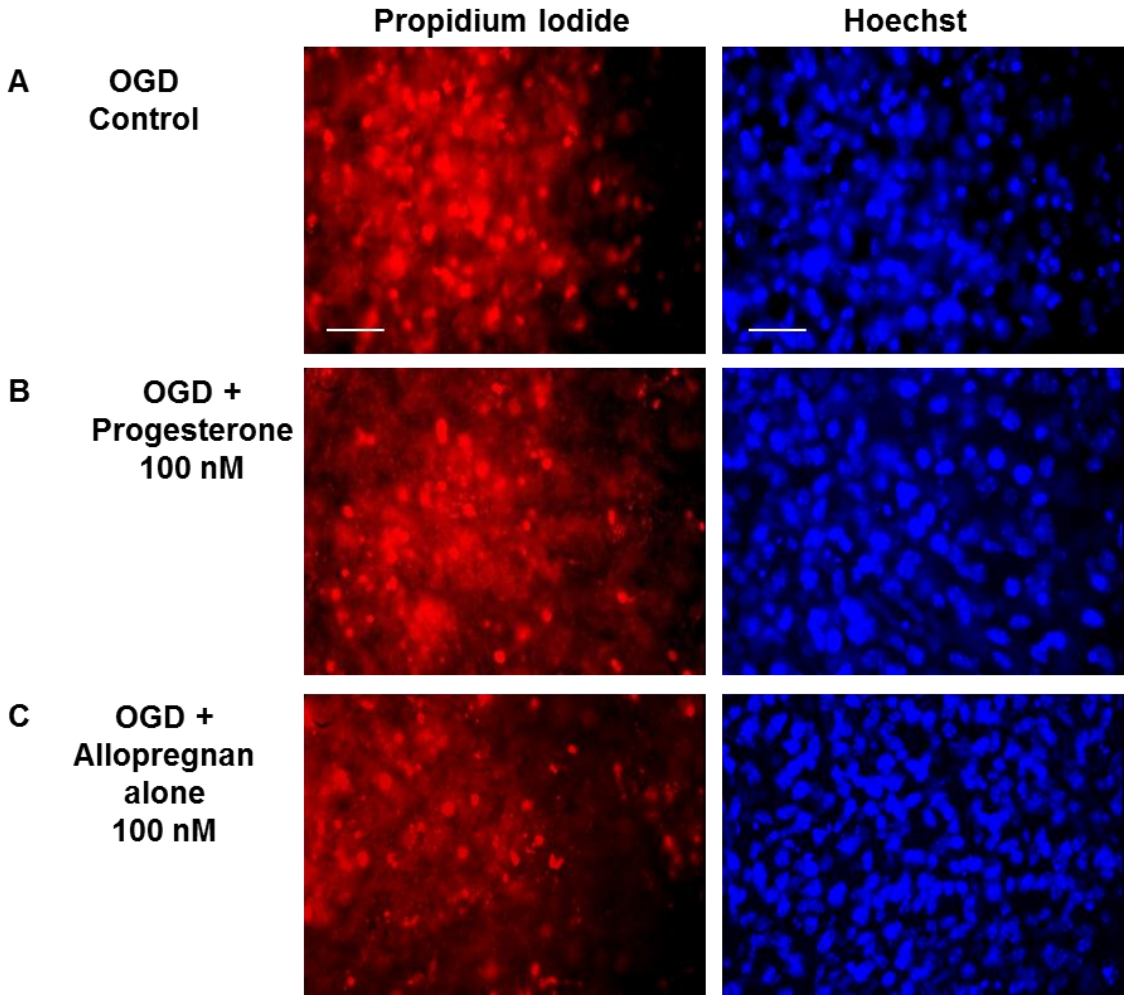


Figure 2.3: Time period of OGD

Representative images of hippocampal slices, cell death is shown in images stained with PI in the following condition: normoxic, 2, 4 and 6 hours of OGD exposure. Scale bars represent 50 µM. Cell death was significantly increased following 2 hours, 4 hours and 6 hours of OGD compared to normoxic conditions (*** $P < .0001$). Data are expressed as mean ± SEM, n= 24, with 24-27 slices per condition.

2.3.8 Progesterone and allopreganolone are neuroprotective following OGD

Treatment with progesterone and allopregnanolone significantly reduced the amount of cell death following 2 hours of OGD compared to OGD control (28.8 ± 3.1%) and OGD with DMSO (30.1 ± 3.1 %, ***Figure 2.4***). A post-hoc analysis revealed that cell death was significantly decreased following treatment with 100 nM progesterone ($P < .05$, 18.3 ± 2.3%) and 100 nM allopregnanolone ($P < .05$, 16.8 ± 2.7%).


Propidium Iodide
Hoechst
A
OGD
Control
B
OGD +
Progesterone
100 nM
C
OGD +
Allopregnan
alone
100 nM

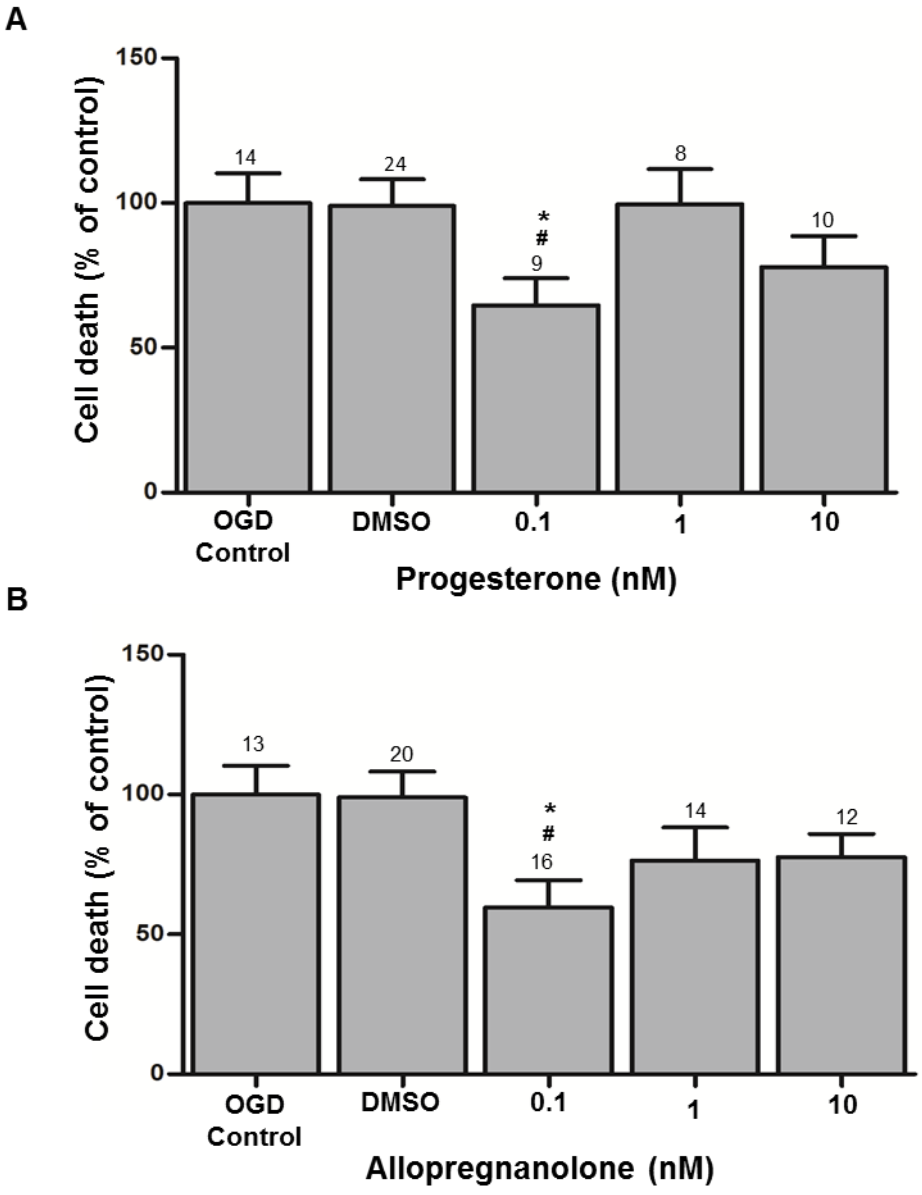


Figure 2.4: Neuroprotection following treatment with progesterone and allopreganolone

Representative images of hippocampal slices treated with progesterone (0.1nM) and allopregnanolone (0.1nM), cell death is shown in images stained with PI 24 hours post OGD. Scale bars represent 50µM. Progesterone and allopregnanolone significantly reduced the amount of cell death following 2 hours of OGD in comparison to OGD controls and OGD with DMSO ($P < .05$). *: values significantly different from OGD

control group. #: values significantly different from DMSO group. Data are expressed as mean ± SEM, n= 24, with 8-27 slices per condition.

2.4 Discussion

2.4.1 Summary of results

The aim of this chapter was to optimize the experimental parameters required to establish a suitable and reliable model of *in vitro* ischaemia. Once the experimental parameters to induce *in vitro* ischaemia had been determined we also examined whether a known neuroprotective agent could induce a protective effect in this model. Thus, we conducted some initial experiments using progesterone and allopregnanlone which have been determined, in the lab, to have neuroprotective properties following *in vivo* ischaemia (Gibson & Murphy, 2004; Sayeed, Guo, Hoffman & Stein, 2006). As a result, this indicates the reliability of this model and therefore was subsequently used for the next set of experiments presented in Chapter 3.

2.4.2 Technical considerations – duration of cultures

Cultures were maintained for 14 D.I.V, as is commonly reported in the literature (Cimarosti & Henley, 2008; Brana et al., 2002), as it allows the cultures to recover from the slicing procedure and also allows synaptic connections to mature. However, the area around the edge of the slices still showed increased cell death in comparison to the rest of the slice. This is likely to be due to the edge of the slice being largely exposed during the dissection and slice preparation process. Therefore, imaging around the edge of the slice was avoided to eliminate any variability in results.

2.4.3 Technical considerations - age of animals used

Initially, the current experiments used animals aged P4 or P9 for slice preparations. Over several preparations, the results indicated that cultures prepared from P4 animals were more stable, in that they showed greater cell survival rates, during the 14 D.I.V. Thus, cultures were prepared from animals on P4 for all subsequent experiments.

Previous studies using a similar approach as described here tend to use organotypic brain slice cultures obtained from neonatal animals (P0 – P10) with substantial evidence showing that viable slices can be prepared from animals anywhere within this age group (for example see Woodruff et al., 2011). Tissue extracted from animals aged between 3-9 days show a greater resistance to mechanical trauma during the slicing procedure, therefore increasing the viability of slices compared to adult animals (Cho et al., 2007). Also, early synaptic connections, particularly in the CA1 region of the hippocampus, have already been formed at this age and during the period *in vitro* synaptic connections continue to develop (De Simoni, Griesinger & Edwards, 2003). Neonatal slices appear to be more suitable for experiments where cultures are maintained for up to 3 weeks compared to slices prepared from older animals aged between 3-4 weeks old. This is because slices prepared from older animals present greater challenges and have been shown to degenerate when maintained over a long period (Cho et al., 2007).

Culture preparations from older animals (3-4 weeks old) may be more suitable for electrophysiological studies as cultures prepared from neonates have shown spontaneous synaptic activity when maintained for over 3 weeks in *in vitro* (Cho et al., 2007). Slices obtained from older animals could also be used for modelling ischaemia as this would be more relevant to clinical stroke.

2.4.4 Protocols for cell death analysis

Although PI may be a crude quantitative measure of cell death several studies have shown a linear relationship between fluorescence intensity of PI and cell death, where an increase in cell death over a period of time has been shown to correspond with an increase in PI uptake (Laake et al., 1999). It was considered relevant to investigate the time period of fixation as previous studies have shown that longer fixation periods can result in significant diffusion of PI throughout the tissue increasing background fluorescence and therefore making it difficult to visualise individual cells (Brana et al., 2002). Therefore, three time periods of fixation were investigated (1, 2 and 3 hours) and following observations of PI staining it was apparent that fixing slices for 3 hours resulted in increased background staining due to the diffusion of PI, which made it difficult to visualise individual cells. It was difficult to count individual cells therefore percentage cell death could not be obtained from these experiments. As a result of these experiments, a 2 hour fixation period was chosen as a valid time period of fixation, a shorter time period of 1 hour could lead to cells not been fixed properly.

This method of assessing viability is a simple method for providing quantitative data. Although other, more complex analyses could have been carried out, for the research aims of this study, measuring the amount of cell death was considered a valid and suitable outcome as we were interested in the changes in cell death following experimental manipulations i.e. drug treatment, in subsequent chapters.

Noraberg et al. (1999) investigated the reliability of using PI as a suitable marker of neurodegeneration by comparing it to the other markers such as LDH release or FJ staining. They reported that the advantages of using PI are that cell death can be quantified and visualised in the same cultures over time. Also distinct regions where

degeneration has occurred can be detected. One limitation which Noraberg et al. reported was that PI is not able to stain dead neurons with denaturation of intracellular nucleic acid. However, this can be overcome by using PI in combination with other fluorescent dyes which stain dead, dying and living cells, such as Hoechst. Also, dual fluorescence labelling with Hoechst and PI both measure membrane integrity i.e. both measure the same end point.

Using PI and Hoechst labelling as a technique for assessing cell viability is a simple but reproducible approach for obtaining quantitative values of percentage cell death which meet the requirements of this study. However, it is acknowledged that there are several limitations to this technique as it does not enable discrimination between the different cell types i.e. neurons or glia. Moreover, although all cells can be visualised by Hoechst labelling the different regions of the hippocampus cannot be clearly discriminated.

2.4.5 Time course for culture maintenance

Studies investigating OGD typically maintain cultures for 14 D.I.V (Brana et al., 2002; Cimarosti et al., 2001; Laake et al., 1999). It is important that cultures are maintained *in vitro* not only to recover from the slice preparation but this also allows time for synapses to recover and develop. During the period of maintenance, cultures undergo reorganization and form new neuronal networks and continue to develop to mimic the developmental processes which take place *in vivo*. It has been reported that cultures maintained for 2-3 weeks are able to replace synapses at a similar level to that observed *in vivo* (De Simoni & Yu, 2006).

The experiments in this chapter assessed cell viability at different time points during culture maintenance. It is important to assess the viability of cells at different time

points *in vitro*, as studies have suggested that it takes up to a week for slices to recover from the mechanical damage during preparation (De Simoni & Yu 2006). It is essential to obtain healthy and viable cultures for their subsequent use in experiments not only to reduce variability between experiments but also to ensure that results obtained are due to the experimental manipulations and not confounded by preparation techniques or the period of maintenance. The findings show that there were no significant differences in cell death between cultures maintained for 7, 10, 14 and 18 D.I.V. However, cultures maintained for 21 D.I.V showed a significant increase in cell death. This finding is consistent with previous protocols which have reported that the optimal time window for which the slices should be used for experiments is between D.I.V 6 and D.I.V 18 (De Simoni & Yu, 2006). This indicates that an appropriate period of maintenance is required in order for the cultures to sufficiently recover and develop for them to be suitable to use for experiments.

2.4.6 Duration of OGD exposure

Studies attempting to model *in vitro* ischaemia have used a variety of lengths of OGD exposure, varying from 30 minutes to 3 hours (Bond, Noraberg, Noer & Zimmer, 2005; Cimorosti et al., 2001). The time point of OGD exposure is largely dependent on the culture system i.e. dissociated or organotypic cultures as this will impact on the severity of injury.

The results obtained from the experiments in this chapter indicated that 2 hours of OGD was a suitable time point to induce cell death which could subsequently be manipulated by potential neuroprotectants. This time point of OGD was selected as the optimal minimum duration of OGD exposure which induced cell death, as previous research has shown that durations as short as 30 minutes resulted in cell death restricted to the CA1

region of the hippocampus (Strasser & Fischer, 1995). Although this is important as these results are consistent with findings from *in vivo* stroke models which have shown that the CA1 region of the hippocampus is more susceptible to damage following stroke injury (Pringle et al., 1997), restricting cell death to one particular region of the slice could results in data which is not representative of the whole slice.

2.4.7 Conclusion

In the current study OHSCs were used to assess cell viability following OGD exposure. Here, cell death was assessed following 24 hours of reperfusion, a time point which has been used previously in *in vitro* studies of ischaemia (Finley et al., 2004; Laake et al., 1999). It has also been reported that maximum cell death is observed at this time point in all hippocampal regions following OGD exposure (Frantseva et al., 1999; Laake et al., 1999). However, other studies have reported that cell death continues to increase at 72 hours post- OGD where the damage extends to CA3 regions (Cho et al., 2004). Futures studies could assess cell death at various time points following the onset of OGD and in specific regions of the hippocampus. This could enable investigations of the expression of specific genes/proteins possibly involved in the injury process. However, as this study was interested in the levels of cell death it was considered appropriate to select one time point and compare cell death between different experimental conditions.

Cell death is usually assessed via morphological or functional changes. Functional recovery is predominantly assessed via electrophysiology (Frantseva et al., 1999), whilst morphological assessment involves staining either with fluorescent dyes or cresyl violet staining. In addition, assessing cell death using morphological and functional analysis can provide both quantitative and qualitative data and provide a more in depth

understanding of both the structural and the functional changes following OGD and whether these occur simultaneously.

Ischaemia-induced changes in organotypic hippocampal cultures have been well characterised in studies investigating neurotransmitter release, modification of proteins and caspase activity (Cho et al., 2004; Finley et al., 2004). These studies indicate that OHSCs present a suitable model to investigate *in vitro* ischaemia.

Although, *in vivo* models represent a closer comparison to humans, *in vitro* models hold several advantages as they permit investigation under a controlled environment avoiding the confounding effects of temperature and vascular components. In addition the lack of blood-brain barrier permits direct access to the cellular compartments. These advantages of *in vitro* model permit the investigation of neuroprotective agents without the influence of other, potentially confounding factors.

Chapter Three

Determining the role of EP_4 activity during *in vitro* ischaemia

3.1 Introduction

3.1.1 Prostaglandin biosynthesis cascade

Prostaglandins (PGs) are potent inflammatory mediators which exert their actions through multiple receptors in a range of physiological and pathological pathways. They have an influence on diverse processes including modulating neurotransmitter release and synaptic transmission. More recently, they have been investigated for their role in neurodegenerative processes (Cimino et al., 2008). The synthesis of PGs involves the action of phospholipase A_2 which catalyses the production of arachidonic acid from membrane phospholipids. The two important pathways for arachindonic metabolism are 5-lipoxygenase (LOX) and cyclooxygenase (COX). The LOX pathway leads to arachidonic acid 5-hydroperoxide (5-HPETE), which ultimately results in the synthesis of leukotrienes. The COX pathway involves the conversion of arachidonic acid to prostaglandin G_2 (PGG_2) and then to prostaglandin H_2 (PGH_2). Following this, prostaglandin synthase enzymes catalyse the conversion of PGH_2 to five biologically active prostanoids which include; prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin D_2 (PGD_2), prostaglandin I_2 (PGI_2) and thromboxane (TX) A_2 (***Figure 3.1***, Rocca, 2006).

The two major isoforms of COX enzymes are COX-1 and COX-2 and each isoform has distinct physiological roles. COX-1 is involved in the maintenance of cellular processes and tissue homeostasis (Rocca & FitzGerald, 2002), whereas, COX-2 is an inducible isoform responsible for mediating inflammatory processes (Simmons, Botting & Hla, 2004). COX-2 is largely responsible for the synthesis of PGE_2 and PGI_2, whilst COX-1 can generate all prostanoids (Bosetti, Langenbach & Weerasinghe, 2004; Brock, McNish & Peters-Golden, 1999). This is due to the functional coupling of these COX isozymes to the corresponding PG synthases. This biosynthesis cascade of PGs is further complicated by the existence of multiple splice variants of some of the terminal synthases and their differential physiological and pathological expression in cells (Candelario-Jalil & Fiebich, 2008).

The family of prostanoids, produced from PGH_2, exert their biological effects via specific downstream receptors of which, PGE_2 has the largest and most recently cloned family of receptors. The receptors which bind PGE_2 and are termed EP receptors (EP_1-EP_4) which are expressed in neurons and glial cells throughout the central nervous system (Cimino et al., 2008). Each EP receptor is associated with a separate gene and marked amino acid sequence homology. However, they each have a distinct pharmacological profile and selective ligands for each receptor subtype have been synthesized and characterized. Furthermore, the EP_3 receptor has multiple splice variants including seven in human, four in rat and three in mouse (Regan et al., 1994; Oldfield, Grubb & Donaldson, 2001). Previous research appears to indicate that PGE_2 production can have opposing functional effects and this is dependent upon which EP receptor and subsequent intracellular signalling pathway is activated (Andreasson, 2010).

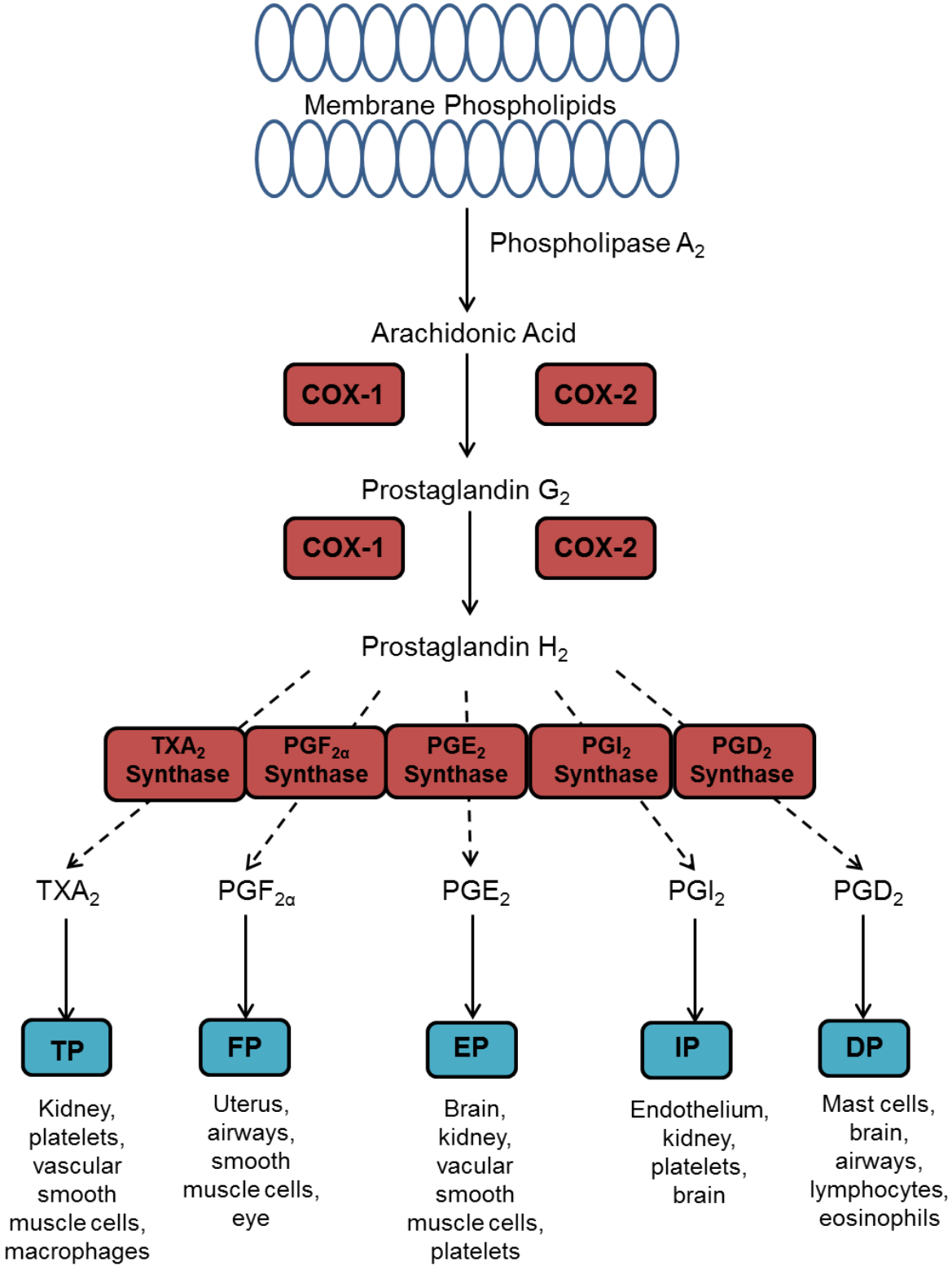


Figure 3.1: Prostanoid biosynthesis cascade

Schematic diagram of the stages in the synthesis of prostanoids and the tissues they act on.

3.1.2 COX activity/expression in the brain

COX-1 is constitutively expressed in all cell types and is responsible for synthesising PGs involved in normal physiological functioning such as cytoprotection in the gastrointestinal mucosa and modulation of platelet aggregation (Rocca & FitzGerald, 2002). Unlike COX-2, COX-1 expression is repeatedly reported as not being significantly upregulated following ischaemic injury (for example see; Nakayama et al., 1998; Nogawa et al., 1997). However, accumulation of COX-1 has been observed in inflammatory cells in the ischaemic region of the brain (Schwab et al., 2000). It may be that this upregulation of COX-1 expression in cells, such as microglia, is involved in cell proliferation and tissue regeneration (Candelario-Jalil & Fiebich, 2008). Although neuroprotective effects of non-selective COX inhibitors have been reported following both global ischaemia (Patel et al., 1993) and focal ischaemia (Cole et al., 1993) it is likely that inhibition of COX-2 activity is accounting for the neuroprotective effect rather than inhibition of COX-1 activity (Candelario-Jalil et al., 2003).

Under normal physiological conditions, COX-2 is expressed in discrete populations of neurons in the brain, particularly in the granule and pyramidal layers of the hippocampus and the cortex, with lower expression detected in the striatum, thalamus and hypothalamus (Yamagata et al., 1993). The expression of COX-2 in the hippocampus is complementary to its physiological role in learning and memory (Rall, Mach & Dash, 2003). COX-2 is colocalised with glutamatergic neurons and its expression is tightly regulated by excitatory synaptic activity. The greatest distinction between the COX enzymes is that COX-2 is rapidly induced by hormones, pro-inflammatory cytokines, tumour promoters and growth factors (Candelario-Jalil & Fiebich, 2008) and therefore has been extensively investigated for its role in

pathological conditions (Strauss, 2008). In addition, an increase in synaptic activity following excitotoxicity and ischaemic injury results in a significant increase in COX-2 expression. Studies have shown that inhibiting NMDA receptor activity prevented the upregulation of COX-2 expression (Yamagata et al., 1993) indicating that COX-2 is regulated by NMDA receptor activity under pathological conditions. This suggests a role of PGs in CNS activity and raises the possibility that selective COX-2 inhibitors could be used to modulate CNS functioning.

Neuroinflammation is a key mediator of the pathological injury resulting from an ischaemic insult. Both *in vitro* and *in vivo* models of ischaemia have shown a marked upregulation of COX-2 expression in neurons, glia and inflammatory cells in response to glutamate release that occurs following ischaemic injury (Ahmad et al., 2009; Govoni et al., 2001; Candelario-Jalil et al., 2003). This increased expression of COX-2, as a result of ischaemia, exacerbates tissue injury which is prevented using selective COX-2 inhibitors (***Table 3.1***). In addition, mice which lack a functional COX-2 gene exhibit less pathological damage, following cerebral ischaemia, compared to mice that possess a functional COX-2 gene (Iadecola et al., 2001). This was accompanied, in the COX-2 deficient mice, with reduced PGE_2 levels indicating that COX-2 activity is directly responsible for the increased PGE_2 synthesis and the subsequent downstream effects. Further to this, administration of a COX-2 inhibitor to COX-2 null mice did not influence ischaemic cell death suggesting that the COX2/PGE_2 pathway is of paramount importance in ischaemic injury. This has been confirmed with further studies showing that mice over-expressing COX-2 have an increased susceptibility to neuronal injury and consequently increased damage following cerebral ischaemia (Doré et al., 2003). This was accompanied by increased levels of $PGE_{2,}$ which were 10-fold higher in the mice over expressing COX-2, and a significant increase in infarct volume compared to

the wild type mice. Studies have also shown that the neuroprotective effect observed following treatment with COX-2 inhibitors is reversed by the application of exogenous PGE_2 (Carlson, 2003). These results add further evidence that PGE_2 is responsible for mediating the downstream effects of COX-2 during ischaemic injury.

Upregulation of COX-2 expression during inflammation, increases the synthesis of PGE_2 however, it was believed that the expression of other prostanoids is not significantly upregulated (Vane & Botting, 1995). Conversely, a study by Govoni et al. (2001) reported an increase in PGD_2 following focal cerebral ischaemia in rats. In addition, recent studies have demonstrated a potential role of other prostanoid receptors in the pathogenesis of ischaemic brain injury. For instance, pharmacological activation of the IP and DP_1 receptors have demonstrated neuroprotection in models of cerebral ischaemia and excitotoxicity (Saleem et al., 2007; Wei et al., 2008; Ahmad et al., 2010; Saleem et al., 2010). Further to this, selective blockade of the FP receptor significantly decreased infarct volume and improved neurological outcome following ischaemia and pre-treatment with a TP receptor antagonist ameliorated brain oedema and cerebral infarction (Kim et al., 2012; Matsuo et al., 1993). This emerging research indicates that prostanoid receptors which positively couple to G_s (IP, DP1, EP_2 & EP_4) and increase intracellular cAMP are neuroprotective following ischaemia. In contrast, receptors which signal through G_q protein and increase intracellular Ca^{2+} (FP, TP, EP_1 & EP_3) mediate toxicity. These findings suggest that although PGE_2 is the predominant prostanoid in inflammation, other prostanoids may also be important under certain pathological conditions.

Although extensive research has implicated COX-2 activity in the pathological mechanisms following ischaemic injury, the precise mechanisms by which COX-2 influences neuronal damage have not been fully established and warrant further

investigation. It is important to note that COX-2 could mediate its direct effects via other mechanisms in addition to downstream prostanoid synthesis. This may include synthesis of free radicals, a reaction product from the conversion of PGG_2 to PGH_2, leading to oxidative stress, a major pathogenic contributor to ischaemic damage. Some studies have demonstrated that the neuroprotective effects of selective COX-2 inhibitors following ischaemia, are partly mediated through the decrease in oxidative stress (Candelario-Jalil et al., 2003). Whereas others have revealed that although COX-2 inhibition resulted in decreased PGE_2 levels it did not influence free radical production (Kunz et al., 2007; Manabe et al., 2004). Therefore it is likely that COX-2 mediates injury by multiple processes, however, the evidence suggests that PGE_2 is likely to be of particular importance as decreased PGE_2 synthesis, following COX-2 inhibition, is neuroprotective. This suggests that PGE_2 is a significant mediator of ischaemic injury and a potential therapeutic target.

Selective COX-2 inhibitor	Ischaemia model	Time of administration	Outcome	Reference
NS-398	Cortical neuronal cultures exposed to NMDA	Pre and post treatment	Decreased neuronal death Inhibition of reactive oxygen species	Carlson, 2003
Rofecoxib	Global ischaemia	Pre-treatment	Decreased neuronal death Decreased PGE_2 levels	Xiang, Thomas & Pasinetti, 2007
O-(acetoxyphenyl)hept-2-ynyl sulfide. (APHS)	Cortical neuronal cultures exposed to NMDA	Pre and post treatment	Decreased neuronal cell death	Carlson, 2003
Naproxen	Excitotoxic neuronal injury *in vivo*	Pre and post treatment	Decreased brain oedema	Silakova, Hewett & Hewett, 2004
5,5-dimethyl-3-(3-flourophenyl)-4-(4-methylsulphonyl)phenyl-2(5H) furanone (DFU)	Transient global ischaemia	Pre and post treatment	Decreased hippocampal neuronal damage	Candelario-Jalil et al., 2002; 2007
Nimesulide	Transient forebrain ischaemia	Pre and post treatment	Reduced brain damage	Candelario-Jalil et al., 2002
SC-58125	*In vitro* OGD	Pre and post treatment	Decreased neuronal death	Gendron et al., 2004
SC-58236	MCAO	Pre-treatment	Decrease in infarct volume	Doré et al., 2003
Valdecoxib	Temporary focal ischaemia	Pre and post treatment	Decrease in infarct volume. Reduced PGE_2 levels	Ahmad et al., 2009

Table 3.1: Selective COX-2 inhibitors in models of ischaemia

An overview of some of the most commonly used selective COX-2 inhibitors in ischaemia.

3.1.2.2 Complications associated with COX-2 inhibition

Although inhibiting COX-2 activity produced clear neuroprotection in experimental stroke studies, long term clinical trials have reported adverse effects following treatment with such agents including an increased risk of myocardial infarction and stroke (Topol, 2004). The main issue is that COX-2 inhibitors (e.g. rofecoxib and celecoxib) altered the balance between pro- and anti-thrombotic components of the clotting system. This resulted in the patient cohort experiencing a 2-4 fold increase in cardiovascular complications as compared to treatment with non-selective COX inhibitors (e.g. aspirin) and such compounds were largely discontinued (Marnett, 2009).

In addition, inhibiting COX-2 can also lead to the accumulation of arachidonic acid which is redirected to the LOX pathway and results in increased leukotriene synthesis. Leukotrienes are established mediators of inflammation and allergy and they have been linked to increased cardiovascular complications (Duffield-Lillico et al., 2009; Folco et al., 2002). This suggests an alternative pathway involved in the cardiovascular side effects with selective COX-2 inhibitors and indicates the importance of identifying novel therapeutic targets. This highlights the importance of targeting downstream mediators of COX-2 activity in the aim to avoid any detrimental side effects of inhibiting overall COX-2 activity. In order to achieve this, it is necessary to determine mediators responsible for the deleterious effects of the COX-2 enzyme and avoid inhibiting those that mediate the beneficial effects. A better strategy for treating ischaemic brain injury might lie in modulating the PGs and PG receptors downstream of the COX pathway.

3.1.3 Downstream mediators of COX-2 activity

PGE_2 is largely responsible for mediating the downstream effects of COX-2 activity via its designated receptors and therefore is a potential therapeutic target for ischaemic injury. Research has indicated that PGE_2 has both neurotoxic and neuroprotective effects depending on which downstream receptor is activated (Andreasson, 2010). Most studies to date investigating the role of PGE_2 in ischaemic injury have used COX-2 inhibitors in order to prevent the metabolism of arachidonic acid, the first stage of prostanoid biosynthesis. However, the synthesis of prostanoids also involves membrane associated proteins known as prostaglandin synthase enzymes. This indicates that the synthesis of PGE_2 can be blocked either via the inhibition of COX-2 or prostaglandin E synthases (PGES).

Three major isoforms of PGES have been identified; cytosolic PGES (cPGES) and microsomal PGES-2 (mPGES-2), which are constitutively expressed in basal conditions in various cell types, and microsomal PGES-1 (mPGES-1; see review by Ikeda-Matsuo, 2010). Like COX-2, mPGES-1, is an inducible enzyme which is upregulated in various models of inflammation (Murakami et al., 2002). COX-2 and mPGES-1 positively couple and are both responsible for the synthesis of PGE_2, indicating that selective modulation of mPGES-1 could be a potential therapeutic target for ischaemic injury. The expression of mPGES-1 is upregulated in microglia, macrophages, neutrophils and endothelial cells in the ischaemic core following experimental stroke (Ikeda-Matsuo, 2010). In the penumbra mPGES-1 expression is restricted to neurons, whereas in the infarct region it is expressed in microglia and endothelial cells, indicating its differential role in ischaemic injury. Models of ischaemia and excitotoxicity have both shown a

marked increase in PGE_2 concentration in the brain which is reversed in mPGES-1 knockout animals (Ikeda-Matsuo et al., 2006).

The upregulation of both COX-2 and mPGES-1 is required for the post ischaemic production of PGE_2 in neurons, microglia, macrophages, neutrophils and endothelial cells (Ikeda-Matsuo, 2010). Models of ischaemia have shown that there are no changes in COX-1, cPGES or mPGES-2 following ischaemic insult (Samuelsson et al., 2007). This increased expression of mPGES-1 together with COX-2 emphasizes the importance of these two enzymes in mediating the brains inflammatory response following ischaemia.

Together this evidence suggests that selectively modulating mPGES-1 results in decreased synthesis of PGE_2 and is potentially neuroprotective. However, selective inhibition of mPGES-1 results in redistribution of the substrate PGH_2, which is then used by other prostanoid synthases, ultimately leading to an imbalance in prostanoids (Yu et al., 2011). This imbalance of prostanoid synthesis may increase the possibility of unexpected side effects, as seen in clinical trials with COX-2 inhibitors. In addition, another potential problem of modulating mPGES-1 is that it is likely to result in blockade of all PGE_2 activity, some of which is neuroprotective. Therefore in order to avoid inhibiting potential protective mechanisms it would be more appealing to target specific pathways downstream of COX-2 and PGE_2 with the aim to limit side effects.

3.1.4 PGE_2 Receptors

G-protein coupled receptors (GPCRs) consist of seven transmembrane spanning helices whose primary function is to link extracellular stimuli into intracellular signalling pathways in a controlled manner. Binding of an endogenous or exogenous ligand,

results in a conformational change within the receptor complex facilitating the G-protein intracellular signalling.

The activation of EP receptors by PGE_2 initiates changes in intracellular cyclic adenosine monophosphate (cAMP) and calcium (Ca^{2+}) levels (***Figure 3.2***). The role of PGE_2 is conflicting as it can exert a diverse range of biological activity which can either be beneficial or toxic depending on which EP receptor type is activated.

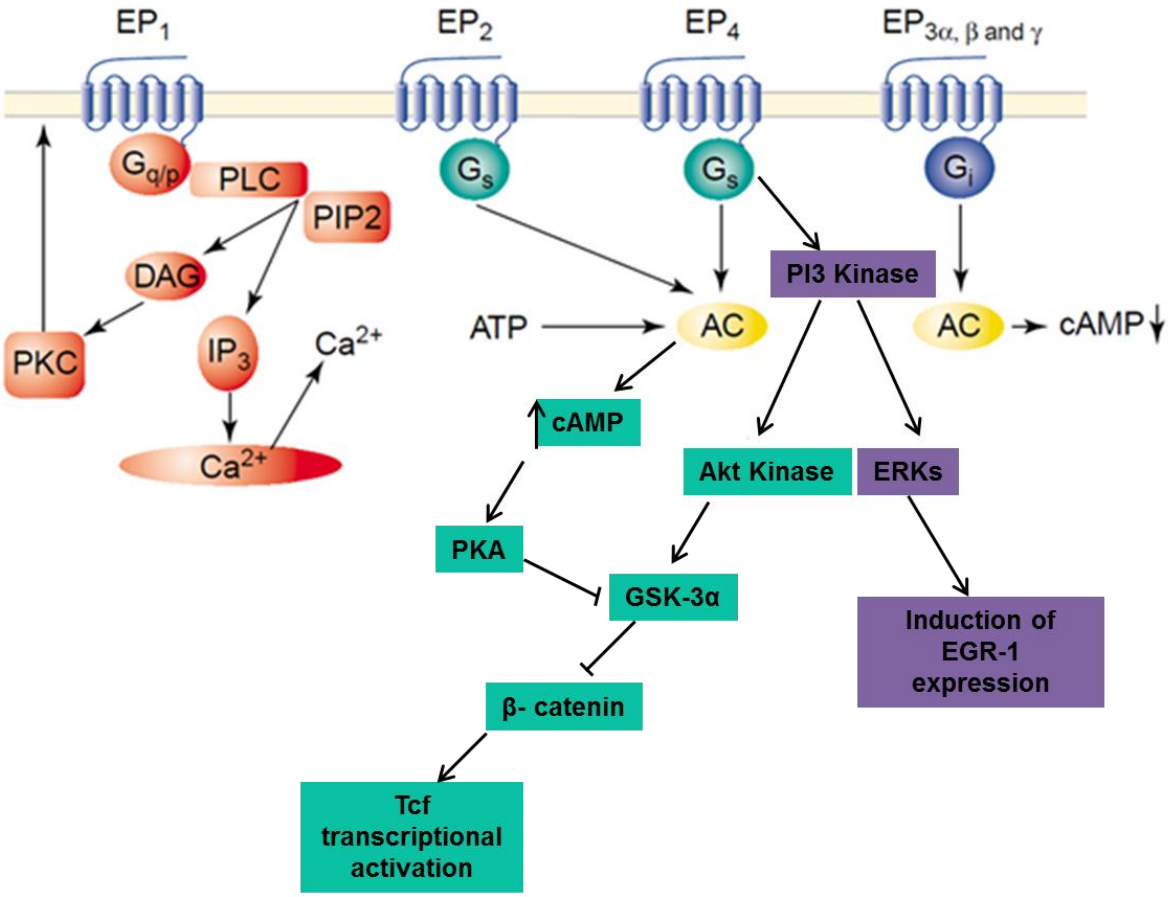


Figure 3.2: EP receptor signalling

An overview of PGE_2 receptor signalling pathways. The four main subtypes of EP receptors (EP_1–EP_4) are coupled to different G proteins and mediate different second messenger signalling pathways (modified from Harris et al., 2002).

There is a wealth of research investigating EP_1 receptor signalling. Coupling to G_q, this receptor disrupts Ca^{2+} homeostasis by impairing Na^{+} - Ca^{2+} exchange by the activation of phospholipase C (PLC) and protein kinase C (PKC) signalling. These findings consistently illustrate the neurotoxic effect of this receptor upon its activation following ischaemic injury (Saleem, Li, Wei & Doré, 2007; Kawano et al., 2006; Doré, 2006; Ahmad, Saleem, Ahmad & Doré, 2006). Moreover, pharmacological inhibition or genetic deletion of this receptor promotes protection following ischaemia by reducing infarct volume and improving neurological deficits (Kawano et al., 2006; Ahmad et al., 2006).

The EP_3 receptor is more complex due to its multiple slice variants arising from alternative splicing of the carboxyl-terminal tail (Sugimoto & Narumiya, 2007). Functional differences exist between the splice forms in regards to the activation of intracellular signalling pathways leading to changes in cAMP levels. The splice forms in mice are referred to as $EP_{3\alpha}$, $EP_{3\beta}$ and $EP_{3\gamma}$. $EP_{3\alpha}$ and $EP_{3\beta}$ have been reported to couple to G_i protein resulting in the inhibition of adenylyl cyclase (AC) and therefore decreasing the levels of cAMP. Interestingly, $EP_{3\gamma}$ can couple to both G_s and G_i proteins resulting in stimulation and inhibition of AC leading to changes in cAMP levels (Irie et al., 1993). The limited availability of selective agonists and antagonists for this receptor poses a problem in determining the exact cellular processes involved.

Both EP_2 and EP_4 receptors positively couple to G_s subunit which elicits increased intracellular cAMP levels and activates protein kinase A (PKA) resulting in phosphorylation of various proteins involved in transcriptional regulation (Regan, 2003). More specifically, PKA phosphorylation of glycogen synthase kinase-3 (GSK-3) results in the translocation of β-catenin to the nucleus where it interacts with transcription factors altering gene expression. It has been shown that accumulation of

nuclear β-catenin and transcriptional activity results in increased COX-2 expression (Araki et al., 2003). McCullough et al. (2004) reported a neuroprotective effect of EP_2 receptor activation following both *in vivo* and *in vitro* ischaemia. However, Takadera, Shiraishi and Ohyashiki (2004) and Takadera and Ohyashiki (2006) conducted a series of experiments to investigate the effects of EP receptors in hippocampal and cortical neuronal cultures and found that EP_2 receptor activation induced apoptosis. These findings indicate that the role of the EP_2 receptor in ischaemic injury remains controversial and the specific processes involved in cAMP signalling and the induction of apoptosis remain to be determined.

The coupling of EP_4 to G_s protein is weaker than that of EP_2 (Fujino, West & Regan, 2002). Another distinction between the two receptors is that although they both couple to G_s and increase cAMP levels, their predominant signalling pathways are different. The differential roles of these receptors may arise from the different lengths of the c-terminal tail, with the EP_4 receptor having a longer C-terminal tail compared to the EP_2 receptor (Bastepe & Ashby, 1999). Stimulation of the EP_4 receptor can activate a phosphatidylinositol 3- kinase (PI3K) and extracellular signal related kinases (ERKs) which results in the expression of functional early growth factor-1 (EGR-1; Fujino, Xu & Regan, 2003). EGR-1 has been shown to up-regulate the expression of mPGES-1 (Naraba et al., 2002). Activation of PI3K also has the ability to increase the expression of COX-2 through GSK-3 and β-catenin mediated signalling. Thus, stimulation of the EP_4 receptor has the potential to induce the expression of both mPGES1 and COX-2 resulting in a positive feedback loop in which the increased synthesis of PGE_2 would further stimulate the receptor. Research investigating the role of EP_4 following ischaemic injury is limited. Studies have shown that EP_4 receptor activation is protective following NMDA-induced excitotoxicity (Ahmad et al., 2005). Research,

published concurrently with ours (Akram et al., 2013), indicates that EP_4 receptor activation results in both neuronal and vascular protection following cerebral ischaemia (Liang et al., 2011). ***Table 3.2*** provides information on some of the most commonly used EP_4 ligands.

The downstream products of COX-2 in particular PGE_2 and its receptors, have recently emerged as important factors associated with inflammatory injury. Further research is required to investigate specific EP receptor pathways and the cellular changes which occur when these pathways are activated.

Compound	**Activity**	**Model**	**Cellular outcome**	**Reference**
CJ-023,423	antagonist	Inflammatory models of pain	antihyperalgesic	Nakao et al., 2007
GW627368X	antagonist	Radioligand binding bioassays	Reduced cAMP levels	Wilson et al., 2006
L-902688	agonist	Isolated vascular preparations	Vasorelaxtion	Foudi et al., 2008
ONO-AE1-39	agonist	Isolated vascular preparations *In vitro* toxicity *In vitro* and *in vivo* ischaemia	Vasorelaxation Decreased neuronal death Decreased infarct volume	Foudi et al., 2008 Ahmad et al., 2005 Liang et al., 2011

Table 3.2: Selective EP_4 receptor ligands

A table showing selective EP_4 agonists/antagonists and their functional cellular effects in various animal models.

3.1.5 Summary & Objectives

COX-2 is a key mediator of the damage produced following cerebral ischaemia. Although COX-2 inhibitors are neuroprotective, they are also associated with increased cardiovascular complications. Thus, there is a need for more specific mediators of COX-2 induced damage to be identified and investigated as potential neuroprotective targets. During inflammation and ischaemic injury, PGE_2 mediates the downstream effects of COX-2 and has been shown to be capable of promoting both detrimental and protective effects on cells. However, the precise mechanisms by which PGE_2 contributes to ischaemic injury remain unclear and warrants further investigation. The experiments in this chapter compare the effects of modulating PGE_2 activity by inhibiting COX-2 activity on cell death using an *in vitro* model of ischaemia (optimized in chapter two). Selective inhibition of COX-2 and overall PGE_2 synthesis via selective modulation of mPGES-1 were investigated. In addition selective activation and inhibition of the PGE_2 EP_4 receptor were also investigated in order to determine the role of this receptor on cell death following *in vitro* ischaemia.

3.2 Method

3.2.1 Culture preparation

Organotypic hippocampal cultures were prepared according to the methods of Stoppini et al. (1991) with some modifications. Brains were removed from animals and placed in sterile petri dishes containing ice cold dissecting buffer. The hemispheres were separated using a scalpel and the hippocampus dissected and sliced (350µm) using a tissue chopper (McIlwain tissue chopper). The slices were carefully separated using sterile syringe needles and transferred onto Millicell membrane inserts (0.4 µm,

MilliPore) using a plastic pasteur pipette. These inserts were placed into six well plates and cultured in growth medium. Cultures were maintained in a humidified incubator with 5% CO_2 at 37°C and culture medium was changed every 3 days. It is important to note that culture medium should be changed on time as any delays resulted in poor survival of cells having an impact on the number of slices available for subsequent experiments. Cultures were observed under the microscope every few days to monitor growth. Any hippocampal slices showing an increased number of dead or dying cells were not used for experiments.

3.2.2 OGD and drug treatment

Hippocampal cultures were exposed to OGD as described previously in chapter two. Following 2 hours of OGD exposure, the cultures were returned to oxygenated serum-free culture medium containing one of the following treatments: culture medium only, DMSO, COX-2 inhibitor i.e. NS-398, PGE_2 synthase inhibitor i.e. CAY10526, EP_4 receptor agonists i.e. L-902688 or CAY10598, EP_4 receptor antagonist i.e. GW627368X or various concentrations of the EP_4 receptor agonist (L-902688) in the presence of antagonist (GW627368X). The cultures were placed back in the incubator for a further 24 hours. The concentration of each drug was determined according to the EC_{50} or IC_{50} value of each agent provided by the manufacturer. The COX-2 inhibitor, PGE_2 synthase inhibitor and EP_4 ligands were all purchased from Cayman Chemicals, USA.

3.2.3 Assessing neuronal cell death following OGD

Following OGD exposure cell death was assessed using PI and Hoechst labelling as described previously in chapter two.

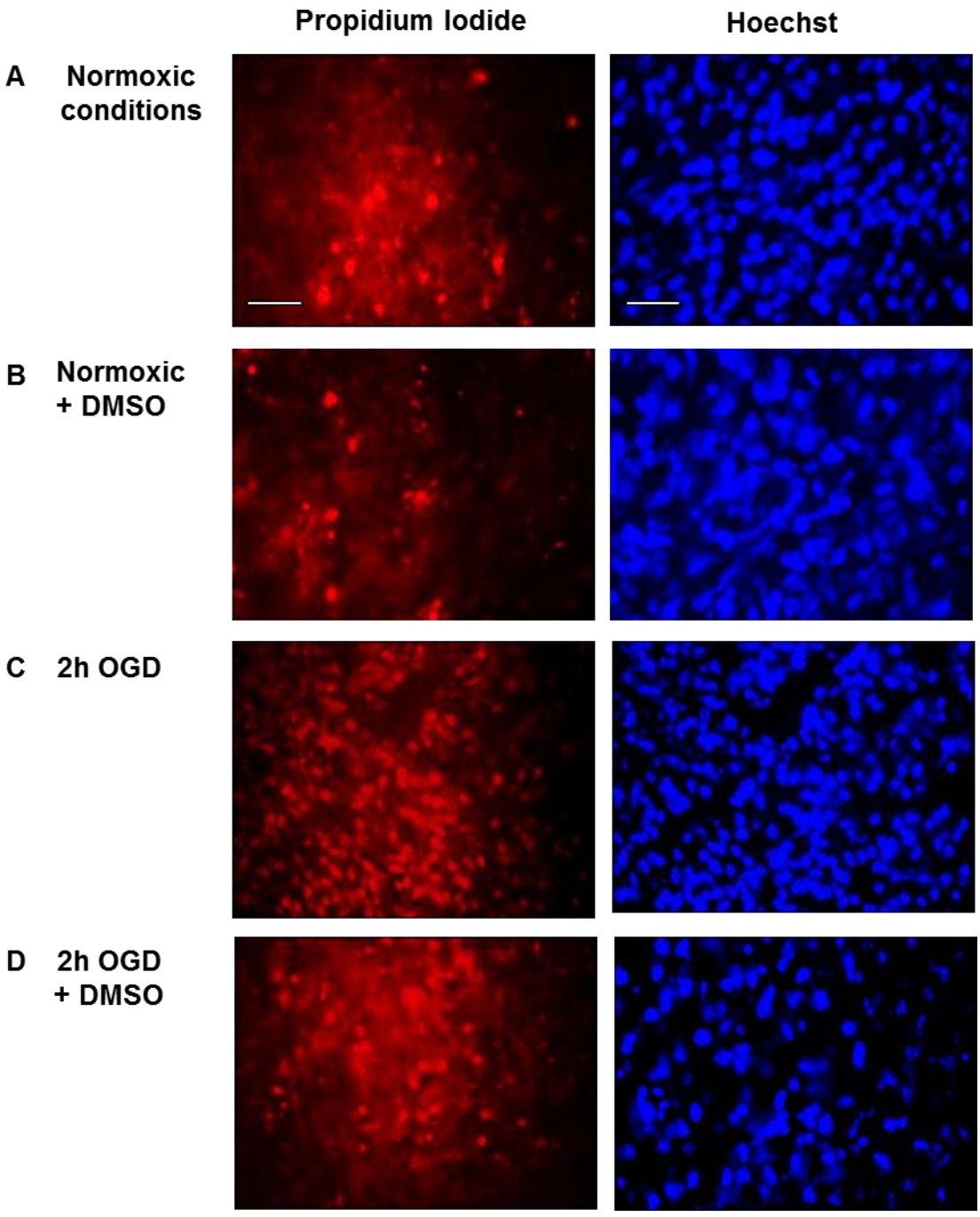
3.2.4 Data analysis

All data are reported as mean ± standard error of the mean and were analysed using GraphPad Prism version 5.0 for windows (GraphPad Software, Sand Diego, USA). Student's t-tests were used for statistical analysis between two groups. Kruskal Wallis analysis was used with Dunn's multiple comparisons test for non-parametric data i.e. data which did not show a normal distribution. The criterion for statistical significance was $P < .05$. Slice cultures were prepared from 6-10 pups per experiments. Each experimental condition was repeated 4 times to increase the reliability of the results. Two values for percentage cell death were obtained per hippocampal slice. This was done to avoid any possible bias occurring from focussing on one region of the hippocampal slice. Cell death values for each experimental condition were normalised to the control group.

3.3 Results

3.3.1 Validation of the *in vitro* model of ischaemia

Exposure of organotypic hippocampal brain slices to 2 hours of OGD significantly increased cell death ($P < .0001$, 36.6 ± 3.1 %) measured from the number of PI stained nuclei, compared to normoxic controls (17.8 ± 2.8 %). Addition of DMSO showed no significant differences in cell death in normoxic conditions (15.8 ± 1.5%) in comparison to normoxic controls without DMSO (17.8 ± 2.8 %). Application of DMSO following OGD (34.0 ± 2.4 %) showed no significant differences in cell death compared to OGD without DMSO (36.6 ± 3.1 %, ***Figure 3.3***). DMSO was used as a control as all drugs were dissolved in DMSO therefore it was essential to eliminate the possibility of DMSO having an impact on cell death.


Propidium Iodide
Hoechst
A Normoxic conditions
B Normoxic + DMSO
C 2h OGD
D 2h OGD + DMSO

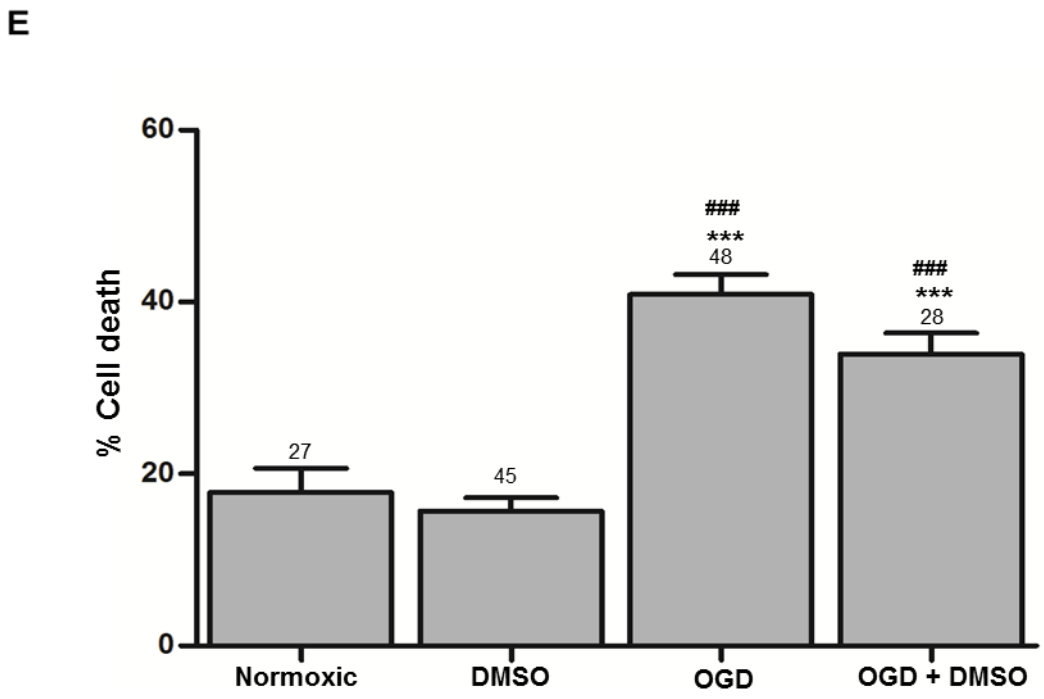
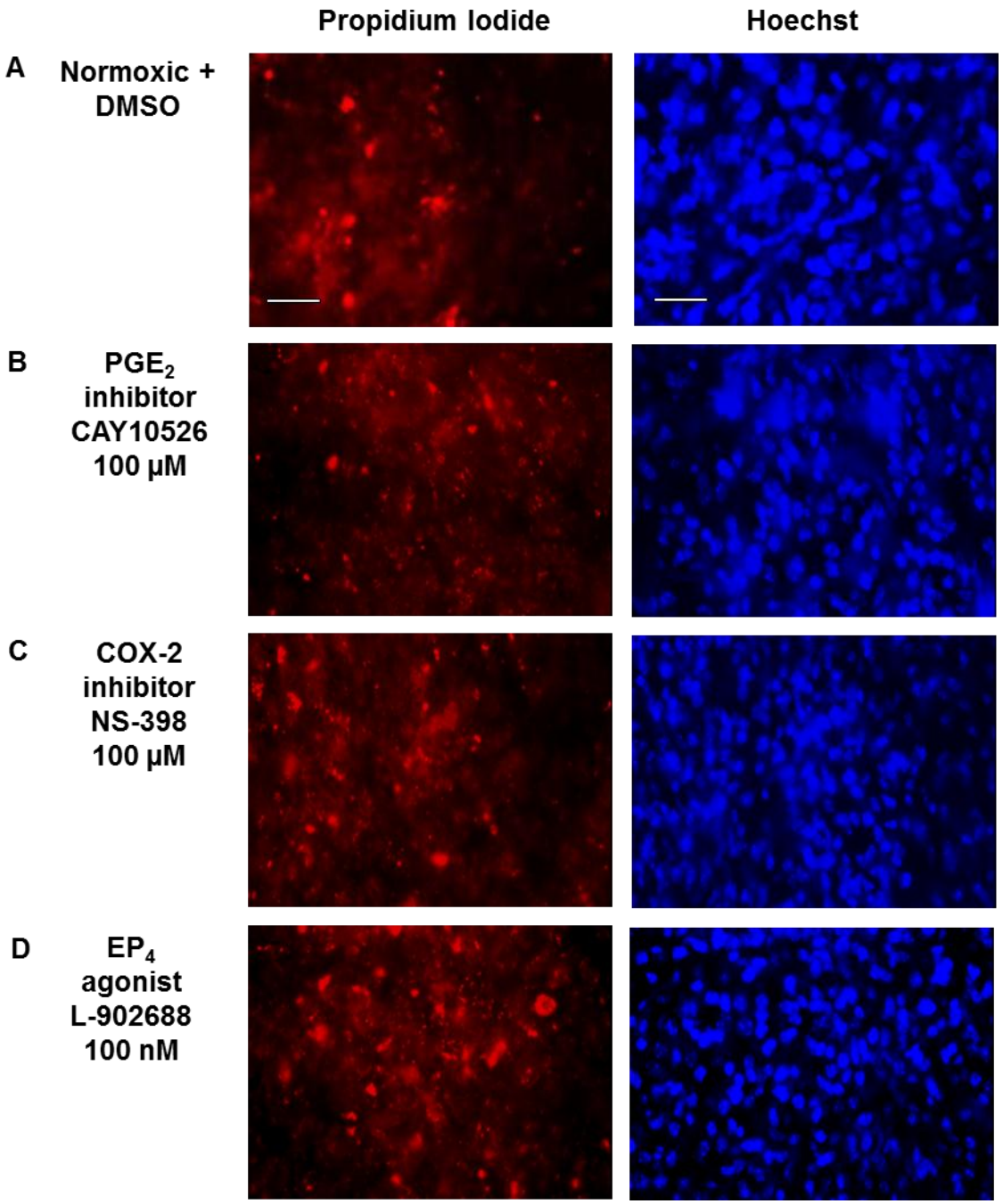


Figure 3.3: Validating an in vitro model of ischaemia

Representative images of hippocampal slices, cell death is shown in images stained with PI in the following conditions: normoxic, normoxic with DMSO, 2h OGD, 2h OGD with DMSO. Scale bars represent 50 µM. Hippocampal slices exposed to 2h OGD showed a significant increase in cell death compared to normoxic conditions (*** $P <$ .0001). There were no significant differences in cell death in slices in the normoxic condition and normoxic with DMSO. There were no significant differences in slices treated with DMSO following OGD compared to OGD only. *: values significantly different from OGD control group, #: values significantly different from DMSO group. Data are expressed as mean ± SEM, n=27 with 27- 48 slices per condition.

3.3.2 Application of drugs in normoxic conditions

In parallel experiments all drug solutions (dissolved in DMSO) used in later OGD experiments were tested in normoxic conditions to determine their effects on cell death. Under normoxic conditions, none of the drug solutions resulted in a significant change in the amount of cell death i.e. (PGE_2 inhibitor, 15.9 ± 1.5%, COX-2 inhibitor 15.5 ± 1.5%, EP_4 agonists, L-902688, 17.9 ± 1.3% and CAY10598, 15.3 ± 1.3%; EP_4 antagonist 13.1 ± 0.8%, ***Figure 3.4***) compared to normoxic controls with DMSO (15.8 ± 1.5%). These experiments were carried out to ensure that the drugs alone had no impact on cell survival/cell death levels. These compounds were subsequently used in OGD experiments.


Propidium Iodide
Hoechst
A
Normoxic + DMSO
B
PGE2 inhibitor CAY10526 100 µM
C
COX-2 inhibitor NS-398 100 µM
D
EP4 agonist L-902688 100 nM

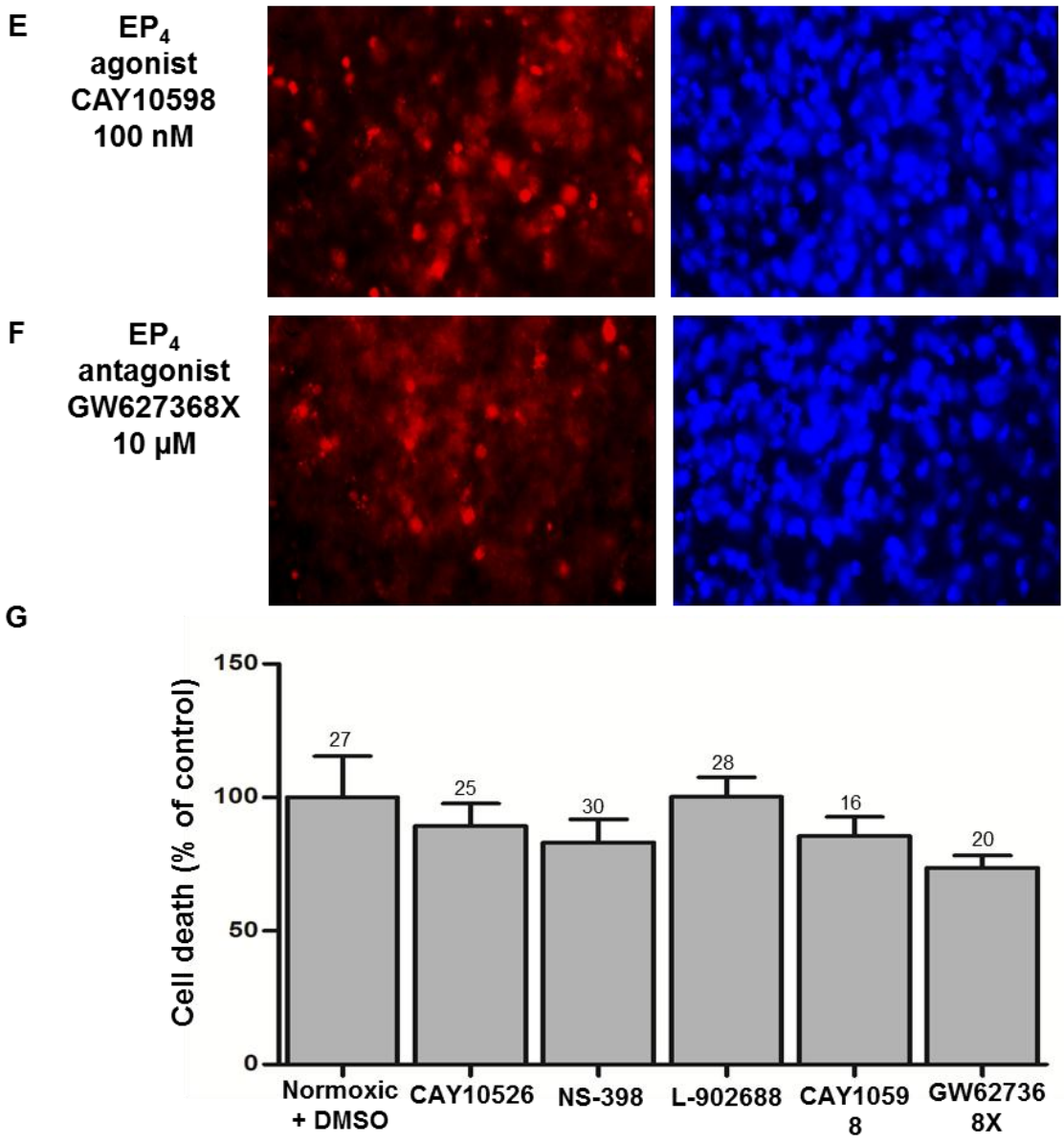


Figure 3.4: Drug application in normoxic conditions

Representative images of hippocampal slices, cell death is shown in images stained with PI in the following conditions: DMSO only, 100 µM CAY10526, 100 µM NS-398, 100 nM L-902688, 100 nM CAY10598 or 10 µM GW627368X in normoxic conditions. Scale bars represent 50 µM. There were no significant differences in cell death levels between the drug treatment groups and the DMSO only 24 h post treatment. Data are expressed as mean ± SEM, n= 30 with 16-30 slices per condition.

3.3.3 COX-2 inhibition is neuroprotective following OGD

The application of a selective COX-2 inhibitor (NS-398) significantly reduced the amount of cell death following 2 hours of OGD ($P < .0001$) compared to OGD controls (36.6 ± 3.1 %). A post-hoc analysis revealed that cell death was significantly decreased when 10 µM ($P < .0001$, 21.7 ± 2.5%) and 100 µM ($P < .001$, 28.4 ± 3.3%) NS-398 was applied (***Figure 3.5***).

3.3.4 Selective inhibition of PGE_2 synthesis promotes neuroprotection

Experiments were conducted to investigate the effect of reducing PGE_2 activity using an mPGES-1 expression inhibitor following OGD. Hippocampal cultures were exposed to OGD and then treated with various concentrations (0.001, 0.01, 0.1, 1, 10 and 100 µM) of the selective mPGES-1 expression inhibitor, CAY10526; cell death was analysed 24 hours post OGD. The results show a significant decrease in cell death compared to OGD control conditions (36.6 ± 3.1 %) at a concentration of 0.01 nM (20.5 ± 1.6%), 0.1 nM (21.9 ± 2.7%), 10 nM (21.6 ± 1.8%) and 100 nM (21.6 ± 1.6%, $P <. 0001$, ***Figure 3.6***).

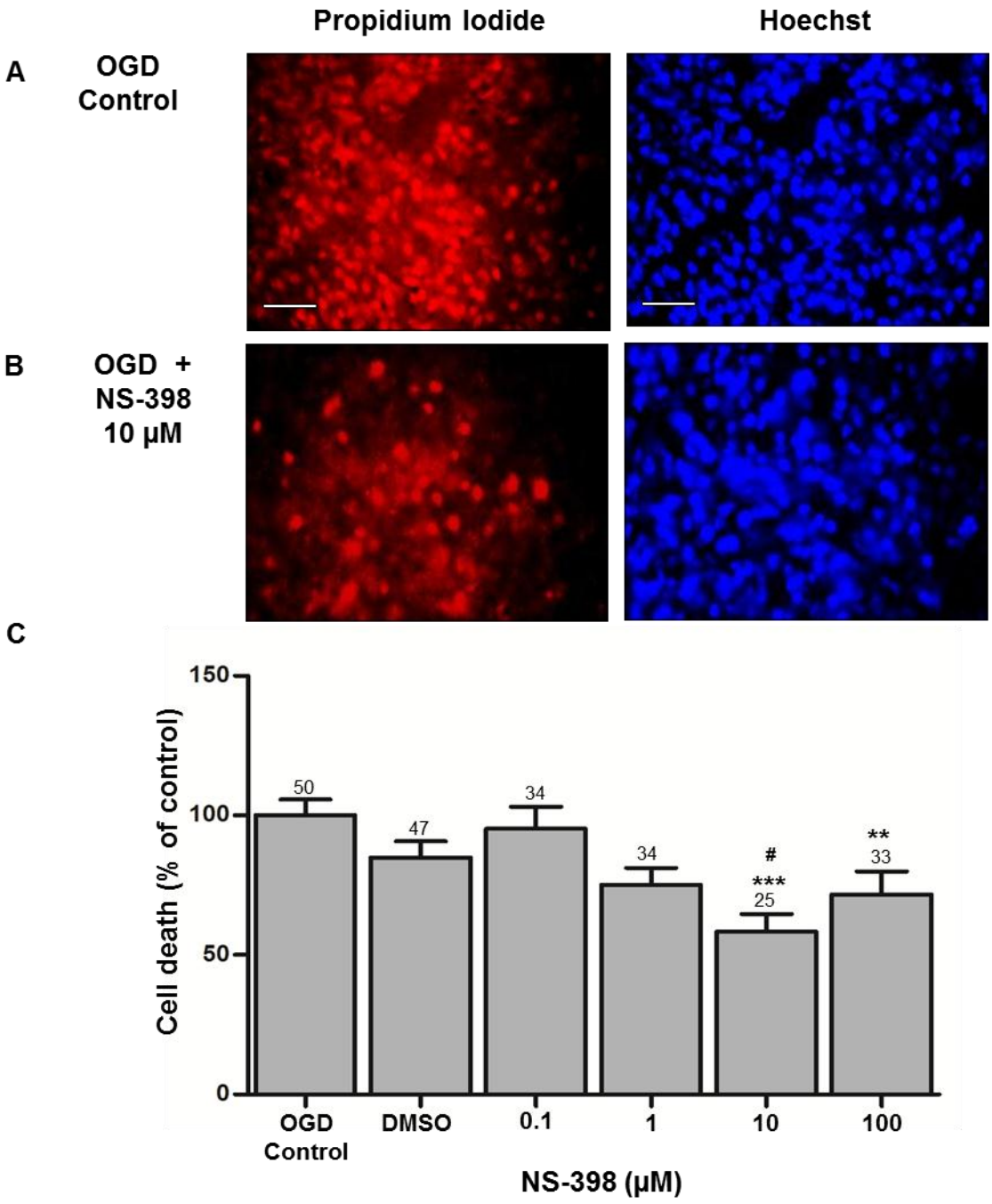


Figure 3.5: Neuroprotection following selective inhibition of COX-2

Representative images of hippocampal slices treated with the selective COX-2 inhibitor (100 µM), cell death is shown in images stained with PI 24 hours post OGD. Scale bars represent 50 µM. Cell death was significantly decreased following treatment with NS-398 (10 µM, *** $P < .0001$, 100 µM, ** $P < .001$). *: values significantly different from OGD control group, #: values significantly different from DMSO group. Data are expressed as mean ± SEM, n=29 with 31-50 slices per condition.

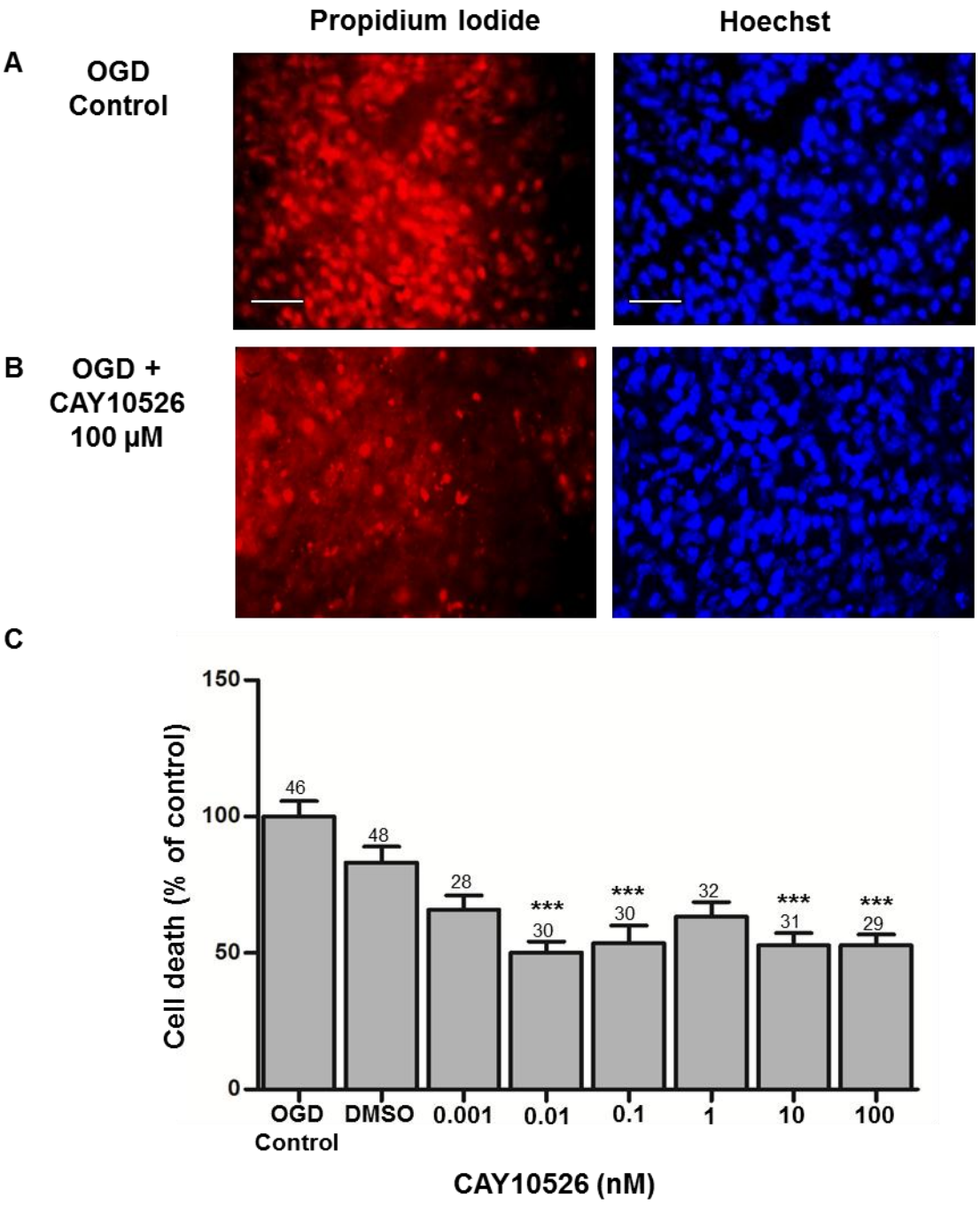
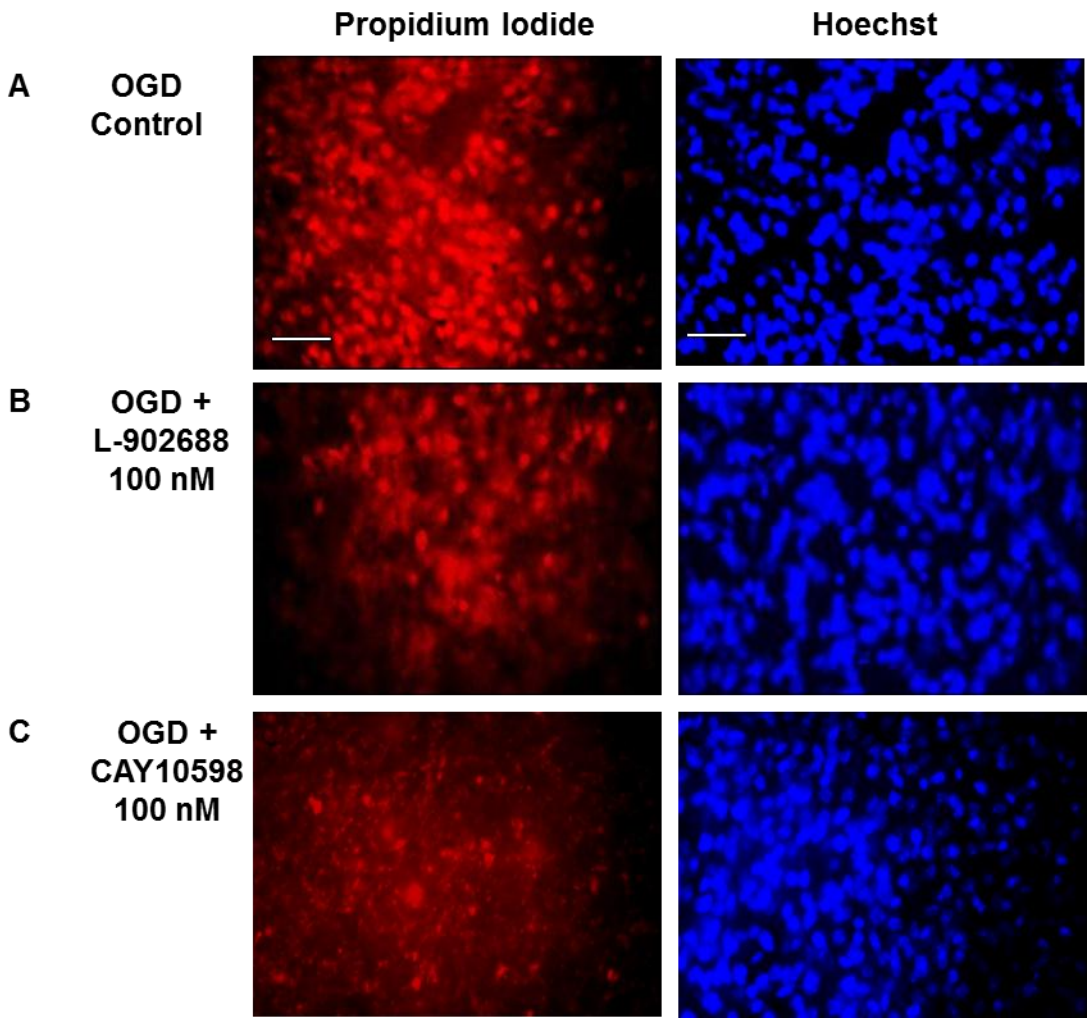


Figure 3.6: Neuroprotection following selective inhibition of mPGES-1

Representative images of hippocampal slices treated with the selective PGE_2 synthase inhibitor (100 µM), cell death is shown in images stained with PI 24 hours post OGD. Scale bars represent 50 µM. Cell death was significantly decreased following treatment with 0.01, 0.1, 10 and 100 µM, ***P < .0001. *: values significantly different from OGD control group. Data are expressed as mean ± SEM, n =30 with 28-48 slices per condition.

3.3.5 Selective activation of the EP_4 receptor shows neuroprotection following OGD

To determine whether EP_4 receptor activity following OGD is protective selective activation of the EP_4 receptor was investigated using a highly selective EP_4 receptor agonist (L-902688). In the presence of the EP_4 agonist cell death was significantly reduced following OGD in a dose-dependent manner compared to OGD controls (36.6 ± 3.1 %, $P < .0001$). A post-hoc analysis revealed that, the minimal concentration required for neuroprotection was 10 nM ($P < .05$, 27.6 ± 2.5%) and the most protective dose of L-902688 was 100 nM ($P < .001$, 25.1 ± 2.4%). To confirm these neuroprotective effects, an alternative EP_4 receptor agonist was also tested. Treatment with CAY10598 (a selective EP_4 receptor agonist) also showed neuroprotection by significantly decreasing cell death in a dose-dependent manner compared to OGD controls (36.6 ± 3.1 %, $P < .0001$). A post-hoc analysis showed that cell death was significantly decreased at the following concentrations; 1 nM ($P < .05$, 19.7 ± 1.5%), 10 nM ($P < .001$, 17.7 ± 3.0%) and 100 nM ($P < .0001$, 13.7 ± 1.1%, ***Figure 3.7***).


Propidium Iodide
Hoechst
A
OGD
Control
B
OGD +
L-902688
100 nM
C
OGD +
CAY10598
100 nM

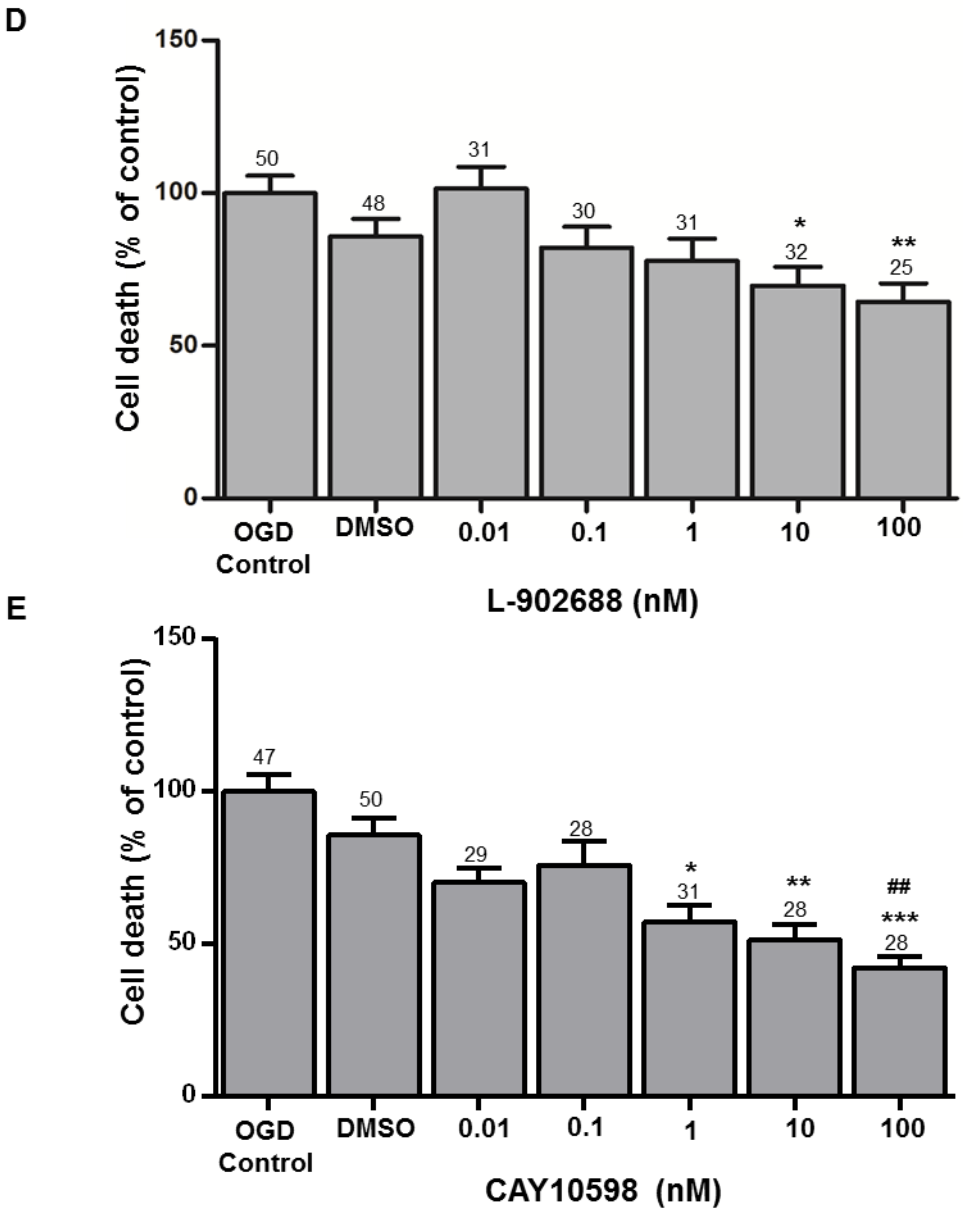


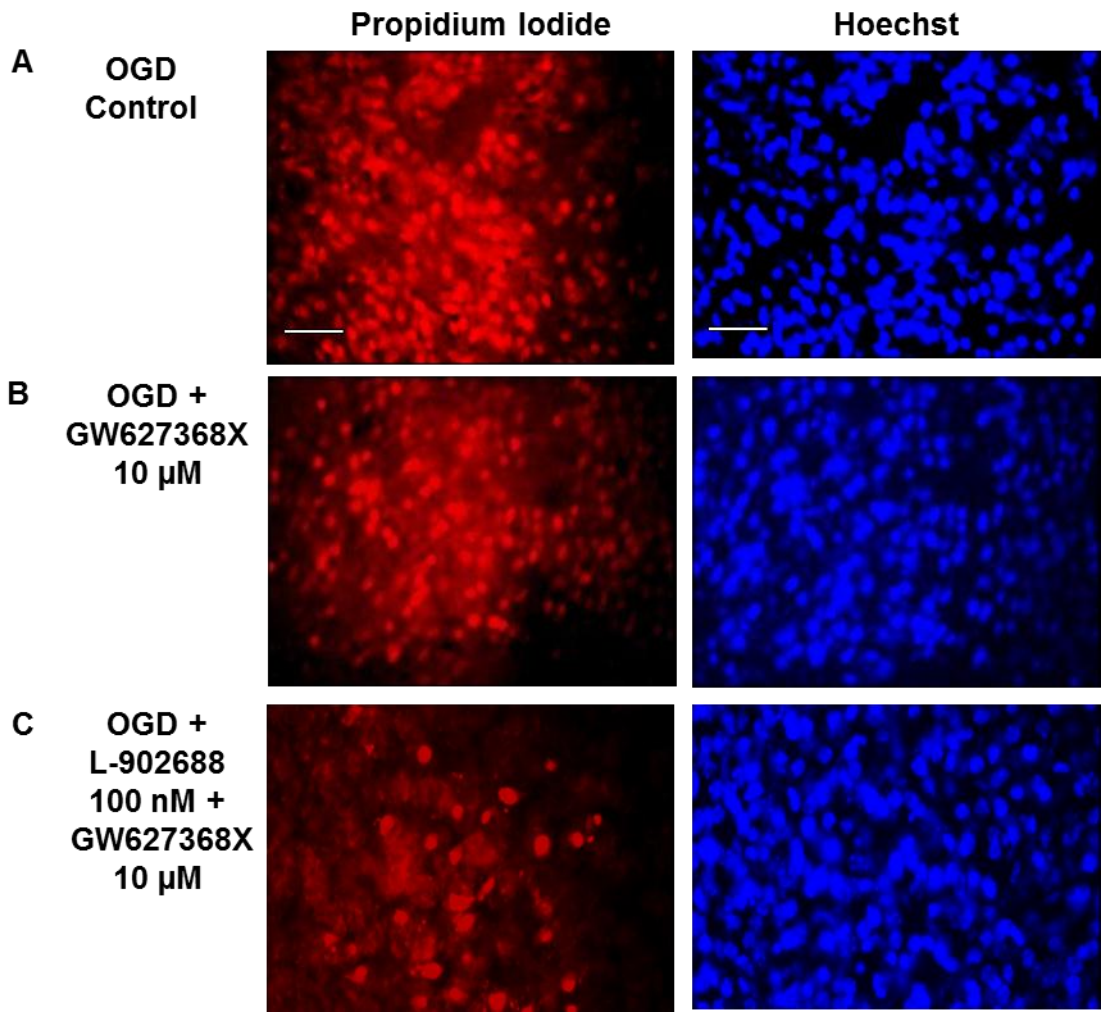
Figure 3.7: Neuroprotection following selective activation of the EP_4 receptor

Representative images of hippocampal slices treated with L-902688 (100 nM) and CAY10598 (100 nM), cell death is shown in images stained with PI 24 hours post OGD. Scale bars represent 50 µM. Cell death was significantly decreased following treatment with EP_4 agonist, L-902688 (10 nM, * $P < .05$, 100 nM, ** $P < .001$), EP_4 agonist, CAY10598 (1 nM, *$P < .05$, 10 nM, **$P < .001$ and 100 nM, ***$P < .0001$). *: values significantly different from OGD control group, #: values significantly different

from DMSO group. Data are expressed as mean ± SEM, n= 31 with 29-50 slices per condition.

3.3.6 EP_4 antagonism is neurotoxic following OGD

To further elucidate the role of EP_4 receptor activity on cell survival following OGD, the effect of blocking EP_4 receptor, using a selective competitive EP_4 antagonist (GW627368X), was investigated. Following OGD, treatment with the EP_4 receptor antagonist significantly increased cell death, in a concentration-dependent manner with the maximum cell death (57.8 ± 3.9%, $P < .0001$) being achieved at a concentration of 10 µM. In order to demonstrate the selectivity of the EP_4 agonist (L-902688), further experiments were carried out in which hippocampal slices were incubated with varying concentrations of L-902688 (10 pM-100 nM) in the presence of 10 µM of the EP_4 receptor antagonist (GW627368X). In the presence of 10 µM of the EP_4 antagonist (GW627368X), the EP_4 agonist (L-902688) was only effective in reducing cell death at the highest concentration (100 nM, $P < .0001$, 21.4 ± 1.7%, ***Figure 3.8***) indicating competitive block of the receptor. Higher concentrations were required to obtain a significant reduction in cells death as both the agonist and antagonist were competing to act on the receptor.


Propidium Iodide
Hoechst
A
OGD
Control
B
OGD +
GW627368X
10 µM
C
OGD +
L-902688
100 nM +
GW627368X
10 µM

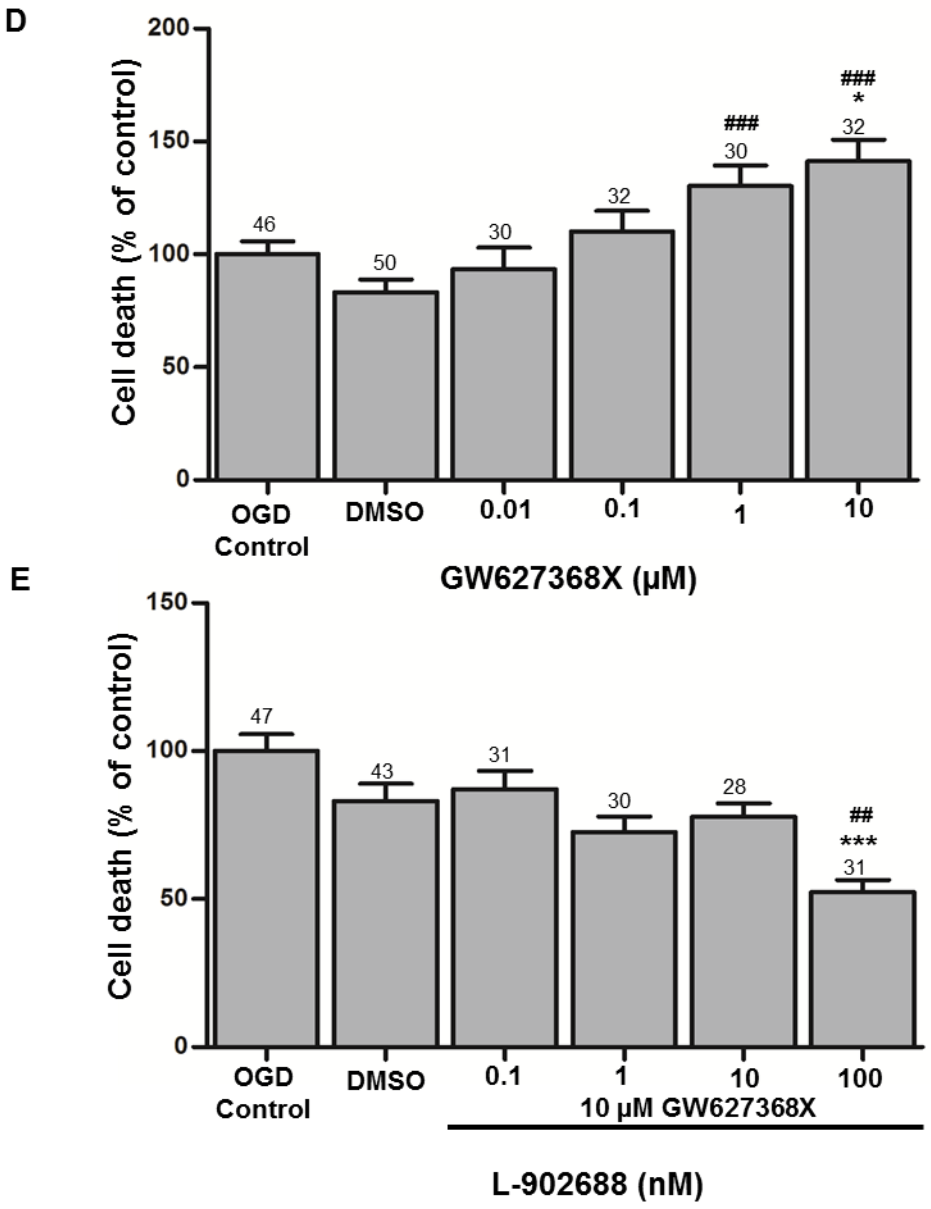


Figure 3.8: Selective EP_4 antagonist increases cell death

Representative images of hippocampal slices treated with a selective EP_4 antagonist (GW627368X, 10 μM) and both the agonist (L-902688, 100 nM) in the presence of the antagonist (GW627368X, 10 μM), cell death is shown in images stained with PI 24 hours post OGD. Scale bars represent 50 μM. Cell death was significantly increased following treatment with GW627368X 24 hours post OGD (10 μM, *P* <. 05). Treatment with L-902688 at concentrations ranging from 10 pM-100 nM in the presence of 10 μM GW627368X showed significant neuroprotection with 100 nM of

the EP_4 agonist ($P < .0001$). *: values significantly different from OGD control group, #: values significantly different from DMSO group. Data are expressed as mean ± SEM, n = 29 with 28-50 slices per condition.

3.4 Discussion

3.4.1 Summary of results

The experiments carried out in this chapter confirmed the findings of previous studies where genetic deletion or selective inhibition of the COX-2 enzyme and inhibition of mPGES-1 promotes neuroprotection in models of ischaemia. The data also show that targeting pathways downstream of COX-2 can provide neuroprotection specifically through activation of the EP_4 receptor. Selective activation of the EP_4 receptor resulted in a significant decrease in cell death, whilst selective antagonism at the EP_4 receptor reversed this neuroprotective effect.

3.4.2 Inhibiting PGE_2 synthesis is neuroprotective

Previous research has shown that PGE_2 is a key mediator of neuroinflammation and its inhibition via mPGES-1 of COX-2 has shown neuroprotection (Ikeda-Matsuo et al., 2010). The experiments reported in this chapter were conducted to show that selectively inhibiting COX-2 or mPGES-1 is neuroprotective in this model of *in vitro* ischaemia.

The competitive, selective COX-2 inhibitor, NS-398 has been widely used in animal models of ischaemic injury and other models of inflammation (Nogawa et al., 1997; Iadecola et al., 2001). It is one of the most selective COX-2 inhibitors which is considered safe compared to traditional NSAIDS with few side effects reported in animal models (Shoup et al., 1998). NS-398 has been shown to block COX-2 activity in

inflammatory cells but not in non-inflamed tissue resulting in fewer gastrointestinal side effects in rodents compared to traditional NSAIDs (Futaki et al., 1994). NS-398 also known as methanesulfonamide, irreversibly binds with COX-2 blocking its activity (Hara, Kong, Sharp & Weinstein, 1998). This compound binds to the cyclooxygenase channel in a conformation that is different to other selective COX-2 inhibitors such a celecoxib, interacting with Arg-120 as oppose to Arg-513, which results in a time-dependent inhibition of COX-2 (Vecchio & Malkowski, 2011). It is a highly selective COX-2 inhibitor with an IC_{50} value of 30 nM and has no effect on COX-1 activity at concentrations as high as 100 µM (Masferrer et al., 1994).

Selectively inhibiting COX-2 produced a significant decrease in cell death following 2 hours of OGD. Cultures were treated with NS-398 at varying concentrations and the largest protective effect was observed at 10 µM. At higher concentrations (100 µM) the protective effect of NS-398 reached a plateau. Interestingly, these results show that the decrease in cell death following treatment with NS-398 was not concentration dependent. This is likely to be due to the opposing effects of downstream PGE_2 activity which indicates that modulating specific pathways downstream of COX-2 activity may be a better approach to identifying novel therapeutic targets.

The majority of studies investigating COX-2 inhibition following ischaemia have reported neuroprotective effects (Nogawa et al., 1997; Iadecola et al., 2001). Interestingly, one study has reported controversial findings, where treatment with NS-398 exacerbated ischaemic injury (Gendron et al., 2004). However, this aggravation of neuronal death was only observed when cortical cultures were treated with NS-398 prior to OGD, treatment post OGD showed neuroprotection. This suggests that the time point at which the treatment takes place is crucial to the outcome of injury.

Studies have shown that positive coupling of mPGES-1 and COX-2 are required for the synthesis of PGE_2 resulting in aggravation of ischaemic injury (Ikeda-Matsuo et al., 2010). CAY10526 is an inhibitor of PGE_2 production through the selective modulation of mPGES-1 expression. As mPGES-1 is involved in PGE_2 synthesis downstream of COX-2 it is possible that targeting this enzyme may avoid the side effects of COX-2 inhibitors. Selective modulation of mPGES-1 selectively inhibits PGE_2 which influences downstream EP receptor activity. Therefore selectively targeting specific downstream receptors of PGE_2 may be a better approach in avoiding any potential side effects. Application of CAY10526 has been reported to have no effect on COX-2 expression (Guerrero et al., 2007).

3.4.3 Activation of the EP_4 receptor is neuroprotective

Cultures treated with a selective EP_4 receptor agonist (L-902688) following 2 hours of OGD showed significant neuroprotection. This neuroprotective effect was observed via a significant decrease in cell death in comparison the OGD control cultures. This decrease in cell death was observed following 24 hours of reperfusion. The protective effect of EP_4 receptor activity was also confirmed using an alternative EP_4 receptor agonist (CAY10598) which showed significant neuroprotection at concentrations as low as 1nM. The most protective concentrations of both agonists were 100 nM. Interestingly, we have shown that activation of the EP_4 receptor following *in vitro* ischaemia, results in the same level of neuroprotection as selective inhibition of the enzyme COX-2 but avoids the detrimental effects of blocking all COX- 2 activity.

Previous studies investigating the role of EP_4 receptor activation using *in vivo* and *in vitro* models of ischaemia are limited. A very recent study by Liang et al (2011) also confirmed the neuroprotection upon selective activation of the EP_4 receptor following

OGD *in vitro* hippocampal cultures using an alternative selective agonist to the one in our study. The selective EP_4 receptor agonist (L-902688) used in this study, is a highly selective and potent agonist, which has been reported as > 4,000-fold selective for EP_4 over other EP and prostanoid receptors (Young et al., 2004). In addition, this EP_4 receptor agonist has a Ki value of 0.38 nM and an EC_{50} of 0.6 nM and therefore is 5-fold more potent for the EP_4 receptor compared to its endogenous ligand, PGE_2. As a result this agonist was subsequently used for *in vivo* experiments presented in chapter 4. Further experiments using an alternative selective EP_4 receptor agonist (CAY10598) were carried out in order to confirm the neuroprotective role of EP_4 following OGD.

Previous research has shown that EP_2 mediates its neuroprotective effects via cAMP/PKA signalling (McCullough et al., 2004). As both EP_2 and EP_4 increase cAMP levels activating PKA, it is possible that the neuroprotective effect observed via EP_4 activation may also involve cAMP dependent mechanisms. However, as the EP_4 receptor signals through an alternative pathway involving PI3-kinase activity it is possible that both pathways may be involved in neuroprotection. The cellular mechanisms by which EP_4 neuroprotection still remain to be determined and further investigations would be of interest.

3.4.4 EP_4 antagonism promotes neurotoxicity

The application of a selective EP_4 receptor antagonist increased cell death in organotypic hippocampal sliced cultures following 2 hours of OGD. This increase in cell death was concentration dependent. Additional experiments were carried out to test whether the selective EP_4 receptor agonist and the competitive selective antagonist (GW627368X) acted at the same receptor. The results from these experiments reveal

that higher concentrations of the agonist were required to achieve neuroprotection, indicating that both ligands were competing to act at the same receptor.

Although GW627368X has not been used in an ischaemic model previously, it has been reported as the most selective EP_4 receptor antagonist to date (Wilson, 2006). Radioligand binding studies have shown that the EP_4 receptor antagonist (GW627368X) used in this study is a 100-fold selective for the EP_4 compared to other prostanoid receptors (Wilson et al., 2006). However, this data is taken from studies using human tissue therefore there may be difference across species. Nevertheless this EP_4 receptor antagonist has also been used in animal models (Fairbrother, Smith, Borman & Cox, 2011; 2012).

3.4.5 Conclusions

The experiments in this chapter investigated the effect of disrupting the PGE_2 synthesis/receptor pathway at different stages. Each catalytic step involved in PGE_2 synthesis influences either other prostanoids, such as PGI_2 and thromboxane, or impacts on other receptors which could potentially be neuroprotective. Blocking the first step of PGE_2 production via inhibition of COX-2 activity, has an impact on the synthesis of other prostanoids, particularly PGI_2, which has a role in maintaining cardiovascular homeostasis. To avoid this, inhibiting m-PGES-1 shows neuroprotection and does not interfere with the synthesis of other prostanoids. However, this has its own limitations since blocking PGE_2 may show overall neuroprotection, possibly through inhibition of EP_1 and EP_3 receptor activity (Abe et al., 2009 Ahmad et al., 2006, 2007; Kawano et al., 2006). However, it should be noted that this also abolishes the potential neuroprotective function of EP_2 and EP_4 receptor activation. A better strategy would be to target specific receptor subtypes, downstream of PGE_2 with the aim of limiting any potential side

effects. This has been demonstrated in the present study by selectively targeting the EP_4 receptor using a selective agonist which showed neuroprotection in this model of ischaemia. Although future studies investigating the mechanisms of EP_4 mediated neuroprotection would be of interest.

It would have been interesting to investigate the cell death in specific regions of the hippocampus. This would provide a more in depth understanding of the damage in different regions of the hippocampus and whether the EP_4 receptor has a greater influence in some regions more than others and in certain cell types compared to others. It would also be useful to investigate cell death at different time points during the 24 hour reperfusion period. This would provide us with a greater understanding of the time course of injury and the therapeutic time window for treatment. For the purpose of these experiments the interest was focused on the impact of each agent on cell survival/death, therefore assessing the sliced culture at the same time point for each agent was more suitable.

Although OGD is one of the most valid *in vitro* models of cerebral ischaemia, it is important to take into account its limitations. *In vitro* models do not simulate all aspects of cerebral ischaemia such the vascular compartment, particularly, the invasion of white blood cells. Therefore it is crucial to investigate potential neuroprotective agents in *in vivo* animal models to uncover their clinical potential. Selective activation of the EP_4 receptor has shown neuroprotection following *in vitro* ischaemia in this model of OGD. To further investigate the understanding of EP_4 mediated neuroprotection, the next chapter goes on to investigate whether this protective effect of EP_4 can be replicated in an *in vivo* model of ischaemia which is a closer representation of human stroke.

Chapter Four

Investigating the role of EP_4 receptor activation following *in vivo* stroke

4.1 Introduction

4.1.1 Targeting COX-2 activity following ischaemic stroke

A key feature of tissue damage and the ensuing inflammatory response is the upregulation of the COX-2 gene and increased prostaglandin synthesis. The deleterious effects of COX-2 activation following stroke have been confirmed using *in vivo* models of stroke where inhibition or genetic deletion of the COX-2 enzyme results in neuroprotection (see review by Candelario-Jalil & Fiebich, 2008). Consequently, COX-2 inhibitors have been developed as potential therapeutic treatments following stroke. Although inhibiting COX-2 activity produced clear neuroprotection in preclinical studies, long term clinical trials have reported adverse effects following treatment with such agents including an increased risk of myocardial infarction and stroke (Topol, 2004). These side effects were due to non-selective inhibition of prostaglandin synthesis. Thus, it is necessary to determine mediators responsible for the deleterious effects of the COX-2 enzyme and avoid inhibiting those that mediate the beneficial effects.

4.1.2 The EP_4 receptor as a potential therapeutic target for ischaemic stroke

A better strategy for treating ischaemic brain injury might lie in modulating the PGs and PG receptors downstream of the COX pathway. PGE_2 receptors have recently emerged as potential therapeutic targets for ischaemic injury. *In vivo* stroke models have been used to investigate the role of EP_1, EP_2 and the EP_3 receptors (Kawano et al., 2006; McCullough et al., 2004; Saleem et al., 2009). These studies have either used genetically modified mice or specific receptor ligands to investigate the role of these receptors following cerebral stroke. At the time this study was conducted there was no literature on the role of EP_4 following *in vivo* stroke. However, our data (chapter 3) shows that EP_4 receptor activation reduced cell death using a model of *in vitro* ischaemia. This justifies the use of *in vivo* approaches to examine the efficacy of EP_4 agonists following ischaemia.

4.1.3 Limitations of *In vitro* models of ischaemia

In vitro models of ischaemia provide a useful platform to study the molecular mechanisms of ischaemic injury in a controlled environment. However, they are limited in terms of their clinical relevance. As a result, *in vivo* animal models have been developed and are used to investigate potential neuroprotective targets for ischaemic injury. Neuroprotection refers to a treatment which intends to reduce cell death, restore blood flow or improve functional outcome (O'Collins et al., 2006) which is evaluated in reference to how well it preserves brain tissue and function. This is assessed using pathological analyses and/or behavioural analyses. Neuroprotective agents which are evaluated for the treatment of stroke are usually done so using focal cerebral ischaemia

models as these models represent a close comparison to ischaemic stroke in humans (Hunter, Mackay & Rogers, 1998). In addition, not only do *in vivo* stroke models enable pathological analysis but they also allow the assessment of behavioural outcome following stroke providing a better understanding of neurological deficits resulting from the ischaemic injury. Experimental manipulations in these models can be assessed over a number of days to weeks providing a broader understanding of the progression of injury. Also, *in vivo* models enable the investigation of the vascular components this aspect is eliminated in *in vitro* models. Therefore, in addition to *in vitro* models, it is crucial to investigate potential neuroprotectants using *in vivo* models of stroke in order provide a broader understanding of the clinical efficacy of the potential therapeutic agent.

4.1.4 *In vivo* stroke models

The limited clinical efficacy of current neuroprotective targets justifies the need to further advance research in order to identify novel targets for neuroprotection. There are a wide variety of animal stroke models which have been reviewed in the literature (for example by Durukan & Tatlisumak , 2007). These various models have been developed over the years to provide optimal models which are reproducible and the most relevant to the clinical situation.

There are many advantages of using rodent models to mimic human stroke. Firstly, their cranial circulatory anatomy is similar to humans and also they are relatively inexpensive and easier to maintain than larger animals. The further advantage of using mice is that genetically modified animals can be easily produced providing a great approach to uncover potential mechanisms of injury and potential neuroprotective pathways. The use of rodent models enables the assessment of ischaemic infarction and pathology

through histological analysis. In addition, external physiological parameters, such as body temperature can easily be controlled resulting in increased reproducibility and reduced variability between animals (Traystman, 2003). Although rodent models of stroke are valuable as their vascular anatomy and neurochemistry is similar to humans (Macrae, 2001), whether they realistically represent the clinical situation is increasingly challenged. This may largely be due to animal models tending to use healthy, young animals whereas clinical stroke patients are associated with comorbidities (e.g. age, hypertension, diabetes). However, a range of rodent models incorporating comorbidities have been developed and are increasingly used in experimental stroke research. Another limitation of using *in vivo* models of stroke is that the potential neuroprotective agent is often administered before the stroke or during the early hours of reperfusion. Such a narrow therapeutic window is often difficult to translate to a clinical situation.

The pathological mechanisms contributing to cerebral ischaemic injury are complex, therefore it is useful to develop and use different models in order to examine different aspects of the ischaemic cascade. The two main classes of *in vivo* stroke models are global ischaemia and focal ischaemia (Woodruff et al., 2011). There are various global ischaemia models, most commonly they result from multiple vessel occlusion. However, these models are considered less relevant to human acute ischaemic stroke and some global ischaemia models result in high mortality rates, and are therefore not used as often as other stroke models (Woodruff et al., 2011). Focal stroke models are widely used to investigate the pathophysiology of ischaemic injury and are claimed to be more relevant to human stroke compared to other stroke models (Woodruff et al., 2011; Hossmann, 1998). A range of middle cerebral artery occlusion (MCAO) models have been developed as the middle cerebral artery is the most common location for stroke in humans (Lui et al., 2009). Broadly speaking, two surgical approaches are used

to give access to the vasculature to allow generation of focal ischaemia in the territory of the middle cerebral artery (MCA). The first approach requires opening of the skull to allow direct access to the cerebral arteries. In most instances, this requires a small craniotomy in order to allow distal branches of the MCA to be ligated, clipped or sealed by photothrombosis or electrocoagulation. However, these approaches are invasive as they require craniotomy and exposure of the brain. The electrocoagulation model induces permanent focal ischaemia and therefore isn't suitable to investigate potential neuroprotective agents which may require reperfusion following ischaemia. Focal ischaemia models which use ligatures or clips to occlude the artery permit control over the duration of ischaemia which is followed by reperfusion. To specifically avoid opening of the skull the second main approach uses intra-arterial approaches to occlude cerebral arteries. The basic process involves introducing an occluding thread into the extracranial internal carotid artery and advancing it until its tip occludes the origin of the MCA (***Figure 4.1***).

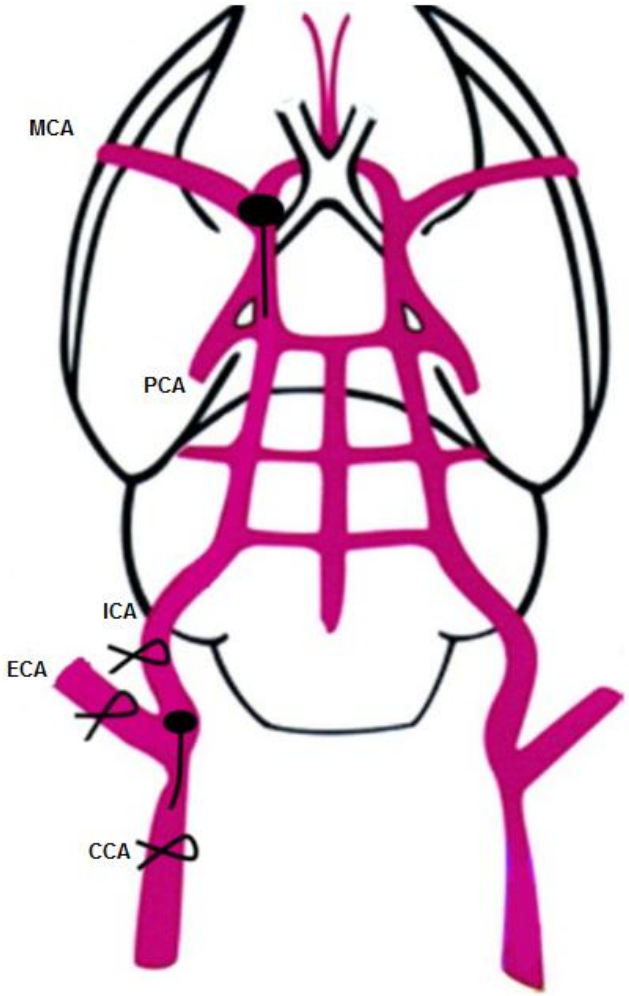


Figure 4.1: Intraluminal suture MCAO technique

Diagram of the intraluminal filament model of MCAO used in this study (modified from Morris et al., 1999).

The intraluminal filament model was first developed in rats (Koizumi, Yoshida, Nakazawa & Ooneda, 1986) and was later modified for mice (Longa, Weinstein, Carlson & Cummins, 1989). The intraluminal filament model of MCAO is the most widely used *in vivo* model of ischaemic stroke (Saleem, Li, Wei & Doré, 2007; Kawano et al., 2006; McCullough et al., 2004) presents a reproducible model of stroke as physiological parameters such as temperature and cerebral blood flow (CBF) can be controlled in order to increase the reproducibility and consistency of the resulting

infarction. This method has several advantages as it avoids the invasive surgical approach of craniotomy and also the occluding filament can easily be withdrawn to permit reperfusion and produce transient focal ischaemia. Laser Doppler flowmetry can be used to measure blood flow in the area of tissue expected to result in infarction. This enables researchers to determine the reduction in blood flow and set a pre-determined criteria by which the blood flow must drop or rise, which helps to increase consistency of infarcts and reducing variability between animals.

However, the intraluminal MCAO model has its own limitations. This model has often reported variability in infarct size between animals of different and same species. This may largely be due to the differences in cerebral vasculature and collateral blood flow to the ischaemic area which has a profound impact on the lesion volume (Majid et al., 2000). However, this limitation can be largely overcome by using different suture lengths and diameters according to the animal's body weight.

4.1.5 Assessment of injury following *in vivo* stroke

Experimental stroke studies use both behavioural and histological end points in order to assess the outcome of injury (Roger, Campbell, Stretton & Mackay, 1997). 2,3,5-Triphenyltetrazolium chloride (TTC) staining has been used since the late 1950s to evaluate infarct size in mammalian tissue and is now one of the commonly used methods to assess infarct volume by researchers in the stroke field. TTC is a measure of metabolic activity and it reacts with dehydrogenases of the mitochondrial membrane, where the tetrazolium salt is reduced by the enzymes into a red lipid-soluble formazan (Joshi, Jain & Murthy, 2004). Viable tissue elicits a pink pigment whilst the infarct region remains unstained indicating a lack of mitochondrial function. This method is useful in detecting infarct volume and allows the infarct area for each brain slice to be

calculated. An alternative method, such as haematoxylin and eosin staining, involves sampling slices and therefore only provides an estimation of infarct size.

During the evolution of the infarct the penumbral region has the ability to recover to an extent and it maintains adequate metabolism due to the nutrients supplied via collateral blood flow. This suggests that the border zone may include a composite of cells, some which are living and some which are in the process of dying which can result in under or over estimation of the infarct size, particularly when the infarct size is small (Holmbom et al., 1993). For instance, the metabolic changes resulting from ischaemia may result in the inability of mitochondrial dehydrogenase enzymes to reduce TTC to formazan despite neurones having well-preserved morphology. This has been shown via histological staining where intact neurons and mitochondria were detected in the region of the infarct detected via a lack of TTC staining (Benedek et al., 2006). This contrasts with the concept that TTC is a marker of irreversible tissue injury following ischaemia, where studies have shown that TTC staining significantly correlated with haemolysin and eosin staining when measuring infarct volume (Hatfield et al., 1991; Isayama, Pitts & Nishimura, 1991). The duration of ischaemia used in the experiments reported in this chapter was chosen based on previous research showing that 60 minutes of ischaemia in mice was sufficient enough to produce a consistent infarct and behavioural deficits (Gibson & Murphy, 2004). Longer periods of ischaemia impact on survival rates and shorter periods may result in less clear infarcts being identified by TTC analysis.

A commonly used method to assess neurological outcome involves the use of a neurological functioning scale (Ahmad et al., 2010; McCullough et al., 2004; Hattori et al., 2000). A variety of scoring techniques are used to assess reflex and sensorimotor functions. Wider scales are generally more sensitive as they permit a more detailed neurological assessment, enabling differences between normal and poor neurological

functioning to be observed more clearly. It is important to analyse both histological and behavioural end points following *in vivo* stroke. This is because although histological analysis provides valuable information of the pathology of the injury, it does not provide any information on how this impacts on functional deficits. In a clinical setting, the assessment of patient outcomes is largely based on neurological assessments, as a result studies investigating potential neuroprotective agents should use both histological and behaviour assessments. In order for a drug to have potential clinical efficacy it should have some role in restoring brain function as well as decreasing the impact of injury following stroke.

4.1.6 Summary & Objectives

The intraluminal suture model of transient focal ischaemia is commonly used to mimic ischaemic stroke, not only is it reproducible, but it is also considered a close representation of this condition in humans. Therefore, this model of stroke was used in the experiments reported in this chapter. Neurological assessment and infarct volume measurement are both widely used measures to assess injury outcome following stroke and were used in these experiments. The experiments in this chapter aim to compare the effects of selective COX-2 inhibitor and EP_4 receptor ligands on ischaemic injury in a model of cerebral stroke. This is important because there is evidence from the literature that non-selective inhibition of prostanoid synthesis elicits a mixture of neuroprotective and deleterious effects due to the wide range of actions of different prostanoid species.

4.2 Methods

4.2.1 Animals

This study was conducted in accordance with the UK Animals Scientific Procedures Act, 1986 (Project Licence 80/2015). These experiments utilised adult male C57/ BL6 mice (10-12 weeks at the time of surgery) all supplied from Charles River (UK).

4.2.2 *In vivo* ischaemia

Anaesthesia was induced by inhalation of 4% isoflurane (in an NO_2/O_2 70/30% mixture) and maintained by inhalation of 1.5% isoflurane. Body temperature was maintained at 37°C ±0.6°C using a heated mat and lubricant was applied to the eyes to prevent drying during surgery. A total of 33 mice underwent transient MCAO; three mice (two vehicle-treated and one EP_4 agonist-treated) died following MCAO and seven mice were excluded due to inadequate occlusion/reperfusion. Focal cerebral ischaemia was induced for 60 min via MCAO and cerebral blood flow was monitored as previously described by Gibson and Murphy (2004). Following a midline neck incision the right common carotid artery (CCA) was isolated. Using a 6.0 silk suture, two ties were placed around the artery and a temporary clip. An additional tie was placed around the external carotid artery (ECA). Once the ties had been placed in the correct positions cerebral blood flow was monitored using laser Doppler flowmetry. A small incision was made to expose the skull where the laser Doppler probe was positioned and secured using vet bond. A 20mm nylon filament coated with thermomelting glue giving a final diameter of 180 µm was inserted into the internal carotid artey (ICA) through the CCA. The filament was advanced 10mm beyond the

origin of the MCA in order to produce an occlusion. Laser doppler flowmetry was used to measure blood flow of animals subjected to MCAO and once the occlusion was in place, blood flow had to be reduced by at least 70% in order for the animals to be included in the study. The MCA was occluded for 60 minutes, during which time the mouse was placed back in the home cage and allowed to recover from the anaesthesia. After 60 minutes the animal was re-anaesthetised and the filament was withdrawn from the MCA back into the CCA to allow reperfusion. The animal was sutured and placed in the recovery chamber where their recovery was monitored. A soft diet was provided for all animals and fluid was administered as necessary in order to prevent dehydration.

4.2.3 Drug treatment

Mice were randomly assigned to receive one of the following: COX-2 inhibitor (NS-398), EP_4 agonist (L-902688), EP_4 antagonist (GW627368X), or vehicle. All drugs were dissolved in 25% DMSO and 75% saline and injected intraperitoneally at the onset of reperfusion and again at 24 hours post MCAO. The COX-2 inhibitor group (n=8) received NS-398 at a dose of 10mg/kg, a dose which has previously shown neurorprotection in *in vivo* stroke (Kunz et al., 2007). The doses for the EP_4 agonist and EP_4 antagonist were determined from the concentrations which significantly reduced cell death in *in vitro* ischaemia. The EP_4 agonist treated animals received 0.75µg/kg of L-902688 (n=7). The EP_4 antagonist group received 0.392mg/kg of GW627368X (n = 7). Mice in the vehicle group (n = 7) received the same volume of 25% DMSO and 75% saline solution. The experimenter was blinded to the treatment the mice had received prior to all subsequent analyses.

4.2.4 Indicators of general well-being

Mice were weighed immediately prior to MCAO and both 24 and 48 hours post-surgery as an indicator of their general well-being. Body weights are presented as a percentage change compared with values recorded immediately prior to undergoing MCAO. Survival rates of animals per experimental group are presented as a percentage compared with the number of animals undergoing surgery.

4.2.5 Focal deficits

At 24 hours and 48 hours post MCAO each animal was assessed for neurological functioning using a standard 6 point neurological scale (0–5, 5 = most severe) and a more detailed 28- point focal deficit scale (***Table 4.1***; Clark et al., 1998).

	0	1	2	3	4
(1) Body symmetry (open bench top)	Normal	Slight asymmetry	Moderate asymmetry	Prominent asymmetry	Extreme asymmetry
(2) Gait (open bench top)	Normal	Stiff, inflexible	Limping	Trembling, drifting, falling	Does not walk
(3) Climbing (gripping surface, 45° angle)	Normal	Climbs with strain, limb weakness present	Holds onto slope, does not slip or climb	Slides down slope, unsuccessful effort to prevent fall	Slides immediately, no effort to prevent fall
(4) Circling behaviour (open bench top)	Not present	Predominantly one-sided turns	Circles to one side (not constantly)	Circles constantly to one side	Pivoting, swaying, or no movement
(5) Front limb symmetry (mouse suspended by its tail)	Normal	Light asymmetry	Marked asymmetry	Prominent asymmetry	Slight asymmetry, no body/limb movement
6) Compulsory circling (front limbs on bench, rear suspended by tail)	Not present	Tendency to turn to one side	Circles to one side	Pivots to one side sluggishly	Does not advance
(7) Whisker response (light touch from behind)	Symmetrical response	Light asymmetry	Prominent asymmetry	Absent response ipsilaterally, diminished contralaterally	Absent proprioceptive response bilaterally

Table 4.1: 28 point focal deficit scale

Focal deficit scale used to assess the neurological functioning of animals following MCAO. Animals can obtain a maximum score of 28.

4.2.6 Quantification of infarct volume

At 48 hours post-MCAO mice were sacrificed by cervical dislocation. Brains were removed and sectioned into 10 x 1mm coronal slices using a mouse brain matrix (ASI Instruments). Slices were stained with 2% TTC in saline for 30 minutes in the dark at room temperature. They were then stored in 4% paraformaldehyde at 4°C until being photographed for analysis using Scion Image Software. Infarct volume was calculated using an indirect method as described by Loihl, Asensio, Campbell & Murphy (1999) which avoids overestimation of infarct area due to oedema formation.

4.2.7 Data Analysis

All data are reported as mean ± standard error of the mean and were analysed using GraphPad Prism version 5.0 for windows (GraphPad Software, Sand Diego, USA). Student's t-tests were used for statistical analysis between two groups whereas analysis of variance (ANOVA) with Bonferroni post hoc was used to analyse more than two groups. The survival data were analysed by applying the Kaplan-Meier curve and Mantel-Haenszel log- rank test to identify differences. The criterion for statistical significance was $P < .05$.

4.3 Results

4.3.1 Cerebral blood flow measurements

The cerebral blood flow of all animals subjected to MCAO was monitored using laser Doppler flowmetry. All animals demonstrated at least 70% reduction in cerebral blood flow during the first 5 minutes following the onset of ischaemia in order to be included

in the study. The results show that there were no significant differences in cerebral blood flow following 5 minutes of occlusion between vehicle (18.7 ± 3.1%), COX-2 inhibitor (18.4 ± 2.2%), EP_4 agonist (17.0 ± 2.1%) and EP_4 antagonist (22.0 ± 4.5%) treated animals.

4.3.2 Indicators of general well-being and neurological score

Survival rate data are displayed using the Kaplan- Meier curve, which showed no significant differences in survival rates of animals treated with the COX-2 inhibitor (χ^2 = 1.89, P = 0.17), EP_4 agonist (χ^2 = 0.31, P = 0.57) or the EP_4 antagonist (χ^2 = 1.44, P = 0.23) compared to the vehicle-treated group (***Figure 5.2***). All COX-2 inhibitor and EP_4 antagonist treated animals survived for 48 hours post MCAO, whereas one EP_4 agonist treated animal and two vehicle treated animals died. All experimental groups showed significant weight loss over the first 48 hours following MCAO (P <. 001, ***Figure 5.3***, ***A***). However, there were no significant differences in weight loss between the different treatment groups, i.e. vehicle, COX-2 inhibitor, and EP_4 agonist, (P = 0.62) following MCAO. All animals were examined for focal deficits at 24 hours and 48 hours post MCAO using a standard 6 point neurological scale and a more detailed 28 point focal deficit scale. Over the 48 period following MCAO, the vehicle treated mice displayed a significant decrease in neurological function (P = 0.03, ***Figure 5.3***, ***C***) demonstrated by an increased neurological score using the 28 point focal deficit scale, which was not seen in the mice that had been treated with either the COX-2 inhibitor, EP_4 agonist or the EP_4 antagonist. There were no significant differences in neurological scores between animals using the 6 point neurological scale (***Figure 5.3, B***).

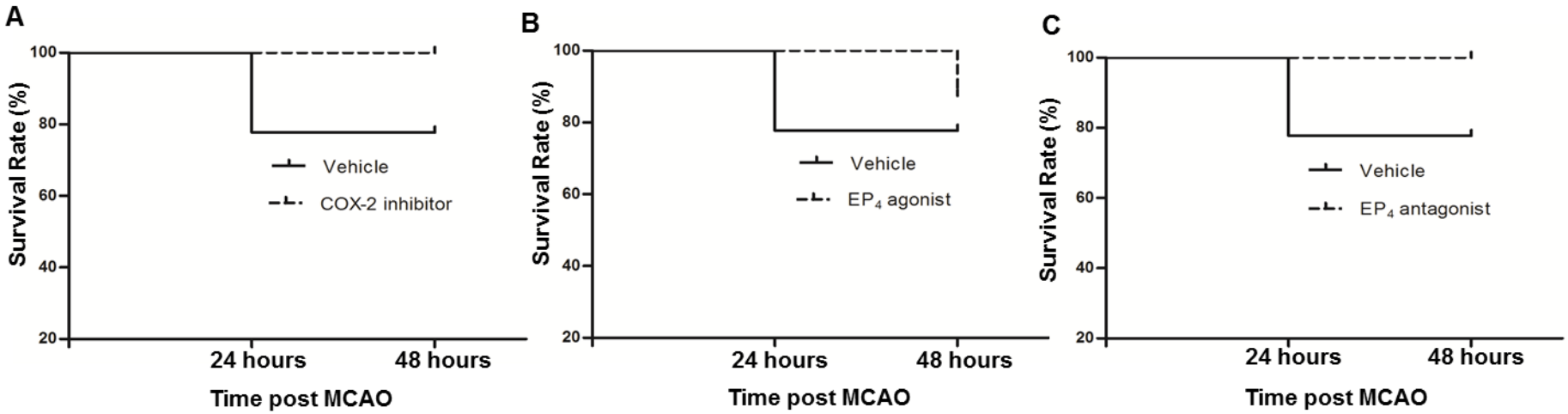
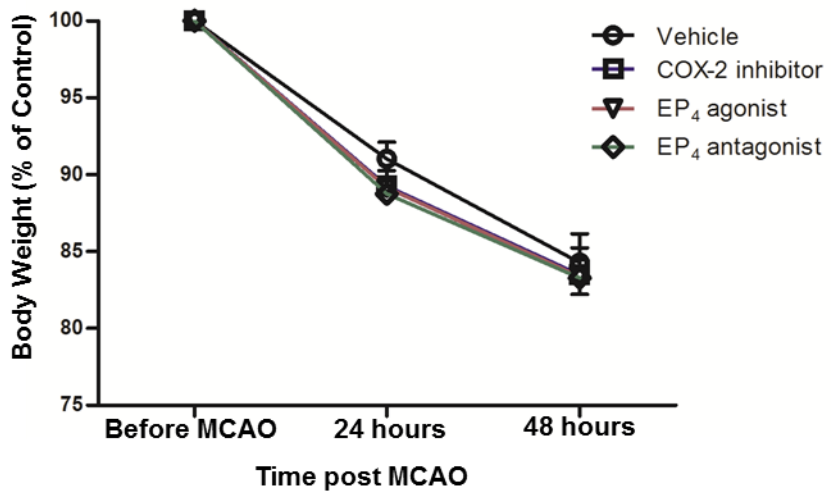


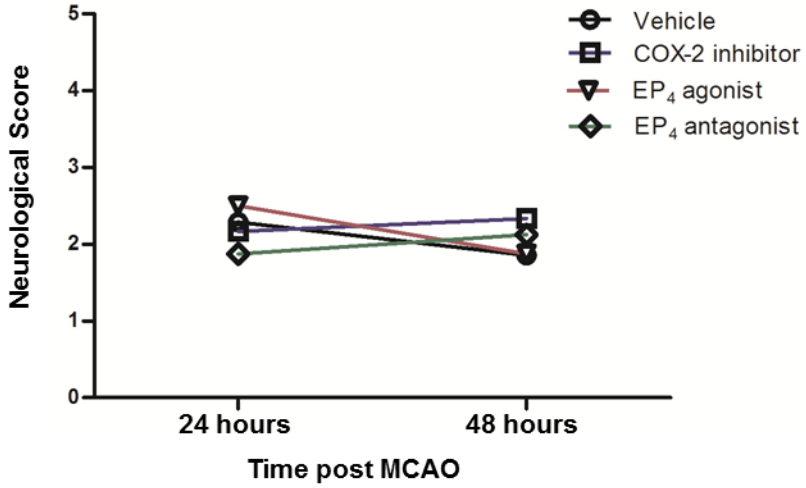
Figure 4.2: Mortality data analysed using the Kaplan-Meier curve

There were no significant differences in survival rates of animals in any of the drug treatment groups (COX-2 inhibitor, EP_4 agonist & EP_4 antagonist) compared to the vehicle-treated animals.

A


Body Weight (% of Control)
100
95
90
85
80
75
Before MCAO
24 hours
48 hours
Time post MCAO
Vehicle
COX-2 inhibitor
EP4 agonist
EP4 antagonist

B


Neurological Score
5
4
3
2
1
0
24 hours
48 hours
Time post MCAO
Vehicle
COX-2 inhibitor
EP4 agonist
EP4 antagonist

C

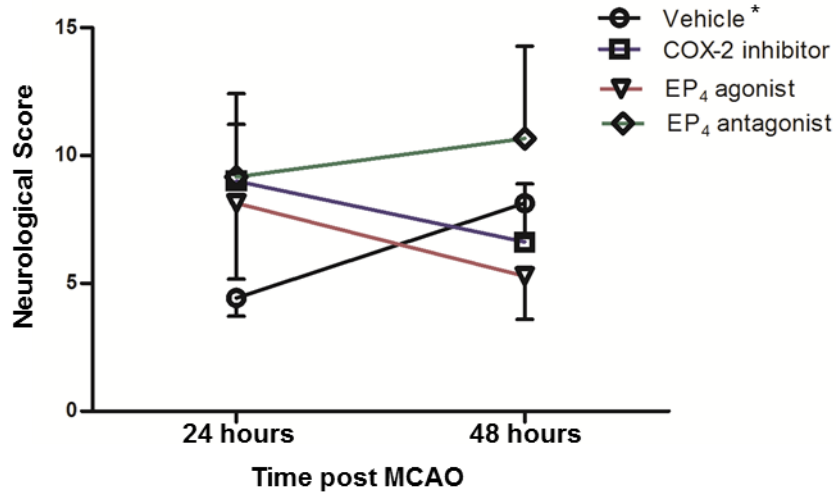

Neurological Score
15
10
5
0
24 hours
48 hours
Time post MCAO
Vehicle *
COX-2 inhibitor
EP4 agonist
EP4 antagonist

Figure 4.3: Body weight and neurological assessment

The general well-being of animals was assessed by measuring body weight and assessing neurological functioning using a standard neurological scale and a 28-point focal deficit scale at 24 hours and 48 hours post MCAO. Treatment with NS-398 (n = 8), L-902688 (n = 7) or GW627368X (n = 7) did not affect body weight compared to vehicle (n = 7) treated animals (**A**). Neurological score was assessed using a 6-point scale (0–5, 5 = most severe), showing no significant differences between any experimental group compared to vehicle (**B**). Neurological scoring was assessed using a 28-point focal deficit scale. Higher scores represent a more severe neurological outcome. Vehicle treated animals showed a significant decrease ($P < .05$) in neurological functioning over the 48 hour period following MCAO (***C***).

4.3.3 EP_4 receptor stimulation reduced ischaemic damage

Lesion volume was calculated by measuring the ischaemic damage, i.e. loss of viable tissue, as indicated by the lack of TTC staining. ***Figure 5.4*** shows representative brain slices stained with TTC 48 hours post-MCAO in vehicle, COX-2 inhibitor, EP_4 agonist and EP_4 antagonist treated animals. Animals treated with COX-2 inhibitor (10.0 ± 1.9 mm^3) showed a significant reduction in lesion volume compared to vehicle treatment (19.6 ± 3.9 mm^3, $P < .05$). In addition, mice treated with the EP_4 agonist (7.8 ±1.9 mm^3) also showed a significant reduction in the amount of ischaemic damage compared to the vehicle treated mice (19.6 ±3.9 mm^3, $P < .05$). There were no significant differences in lesion volume between animals treated with vehicle (19.6 ±3.9 mm^3) or EP_4 antagonist (16.0 ± 4.2 mm^3, $P > .05$).

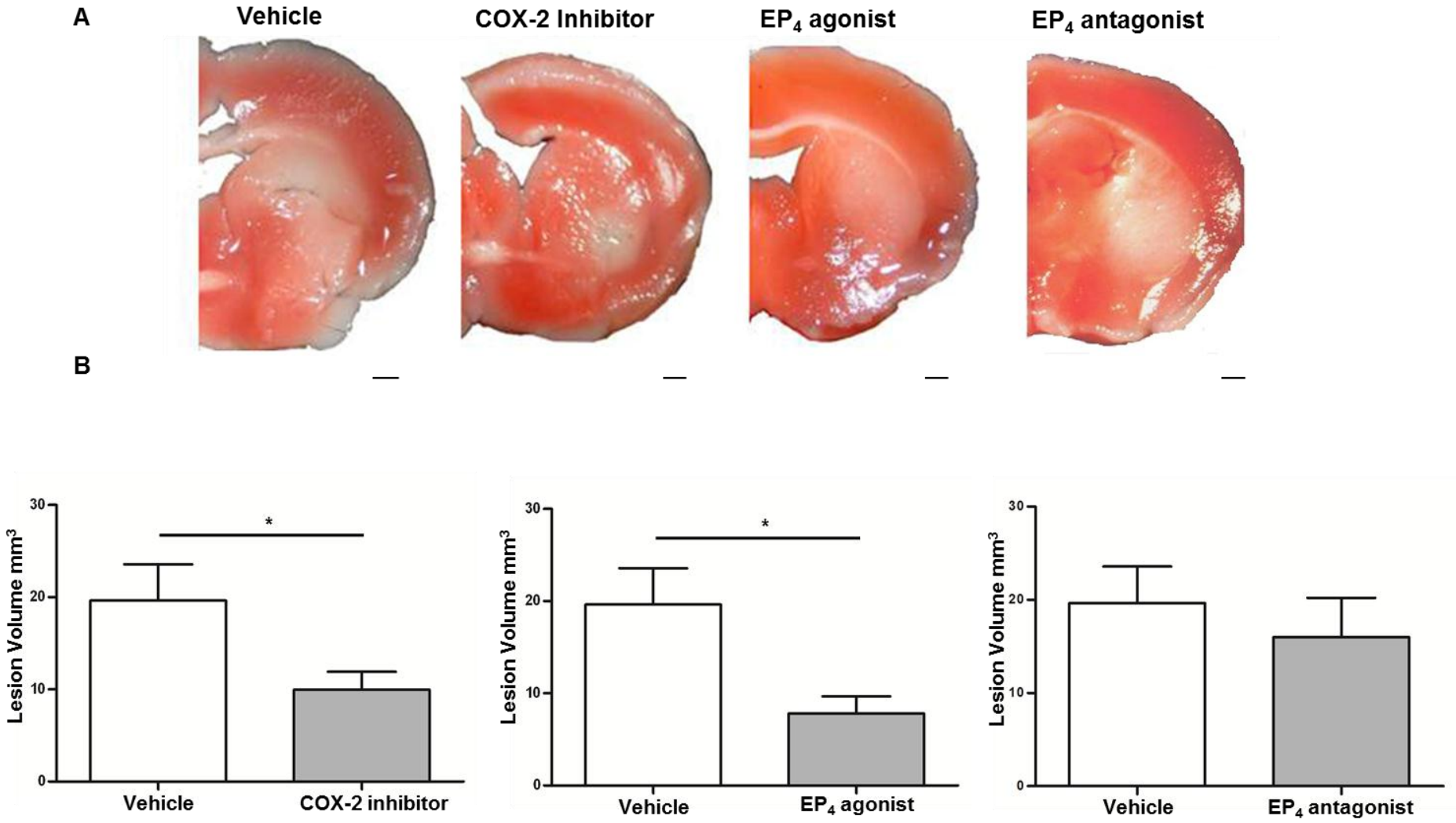

A
Vehicle
COX-2 Inhibitor
EP4 agonist
EP4 antagonist
B
Lesion Volume mm3
30
20
10
0
*
Vehicle
COX-2 inhibitor
Lesion Volume mm3
30
20
10
0
*
Vehicle
EP4 agonist
Lesion Volume mm3
30
20
10
0
Vehicle
EP4 antagonist

Figure 4.4: Lesion volume assessment

The ischaemic infarct was visualized in coronal brain sections stained with 2,3,5-triphenyltetrazolium chloride at 48 hours post-MCAO. Representative coronal brain sections for each experimental group are shown and the ischaemic area is indicated by the lack of staining (***A***). Scale bars represent 1mm. Measurement of lesion volumes indicate a significant reduction in infarct size following treatment with the COX-2 inhibitor (NS-398, n= 8), or the EP_4 agonist (L-902688, n= 7) compared to vehicle (n = 7, $*P < .05$) but not with the EP_4 antagonist (GW627368X, n= 7, ***B***). Data are expressed as mean ± SEM.

4.4 Discussion

4.4.1 Summary of results

The results presented in this chapter show that animals treated with the selective COX-2 inhibitor showed neuroprotection following *in vivo* stroke. These findings are consistent with the literature on neuroprotection via COX-2 inhibition. Treatment with the selective EP_4 agonist following *in vivo* stroke also demonstrated neuroprotection in terms of a decrease in lesion volume and prevented the decline in neurological function. Administration of a selective EP_4 antagonist following MCAO showed no significant effect on ischaemic injury. The results indicate that selective activation of the EP_4 receptor following ischaemic injury could potentially be a novel therapeutic target for the treatment of ischaemic stroke.

4.4.2 Neuroprotection following COX-2 inhibition and EP_4 receptor activation

The findings presented in this chapter further confirmed the protective effects of the EP_4 receptor, using the MCAO model of transient focal ischaemia. Post-ischaemic administration of either a COX-2 inhibitor or EP_4 agonist significantly reduced the amount of ischaemic damage. Importantly, the data reveal that the application of an EP_4 agonist can provide a similar level of protection to that seen following COX-2 inhibition but without the detrimental side effects of COX-2 inhibitors. However, the adverse effects of COX-2 inhibitors were observed following chronic use of such agents, therefore we cannot eliminate the possibility of long term side effects of EP_4 agonists which requires future investigations.

Previous studies investigating the role of EP_4 receptor activation using *in vivo* and *in vitro* models of ischaemia are limited. In terms of cerebral ischaemia, a very recent study by Liang et al (2011), published following the completion of these experiments, also demonstrated neuroprotection upon selective activation of the EP_4 receptor mediated via neuronal and endothelial cells using an alternative selective agonist to the one in our study. Not only is L-902688 a highly selective agonist for EP_4 (as discussed in chapter 3), but it is also well absorbed and exhibits a half-life of 12 hours, giving it an advantage over other EP_4 agonists which have shown a half- life of approximately 2 hours (Young et al., 2004). The side effects reported with the use of this agonist were signs of abdominal discomfort and weight loss in animals but these were only observed at high doses such as 1mg/kg. The present study used a dose of 0.75μg/kg and there were no significant differences in percentage body weight between the EP_4 agonist treated mice and the COX-2 inhibitor or vehicle treated groups.

The structure of endogenous ligands is usually similar to that of exogenous ligands acting at the same receptor. Given that PGE_2 is relatively small in size and has lipophilic properties which enable it to readily cross the blood brain barrier (Kudo & Murakami, 2005) one can postulate that EP_4 ligands are able to enter the brain. Also following stroke injury the blood brain barrier is compromised which may allow molecules to pass through more readily. Nevertheless, further studies will determine the permeability of the EP_4 agonist and the concentration which is able to reach the brain.

The present study found that using the 6 point neurological scale no significant differences in neurological function were observed between treatment groups. However, neurological assessment using the more detailed 28 point focal deficit scale showed that treatment with either the EP_4 agonist or COX-2 inhibitor prevented the decline in neurological function over time compared to the vehicle treated group. This indicates

the importance of using more detailed scoring systems in order to increase the sensitivity of such assessments. These results relate to the reduction in infarct size observed in mice following administration of either the EP_4 agonist or the COX-2 inhibitor. Here, lesion volume was measured at 48 hours and neurological function up to 48 hours following MCAO, thus we cannot discount the possibility that treatment with the EP_4 agonist may have a further delayed effect on functional outcome. Further studies investigating whether treatment with the EP_4 agonist improves neurological function post 48 hours would broaden our understanding of this drug in ischaemic injury. There is great controversy in the literature regarding infarct size and functional outcome where some studies argue that infarct size correlates with certain neurological impairments (Rogers et al., 1997) and others have reported that this is not always the case (Hattori et al., 2000). Additional behavioural testing such as rota-rod, grid walking or water maze test could be used in future studies which would provide knowledge on sensorimotor functioning.

Temporary focal MCAO models usually allow reperfusion after varying lengths of ischaemia, typically ranging from 1h to 3h. The model used in this study permits reperfusion following 60 minutes of MCAO. It is likely that this period of reperfusion permits some recovery of injury. However, in human patients the blood flow rarely resolves after this short period and if it does the clinical symptoms are usually resolved without any intervention (Hossmann, 1998). Therefore it is important to test potential neuroprotective agents in different animal models of stroke in order determine the efficacy of the agent.

Although, treatment with an EP_4 antagonist showed an increase in cell death following *in vitro* ischaemia this effect was not observed *in vivo*, at the dose tested. Treatment with the EP_4 antagonist showed no significant differences in lesion volume or

neurological outcome compared to the vehicle group. These results suggest the blocking EP_4 receptor activity using an antagonist may not necessarily increase toxicity *in vivo* as it did *in vitro*. This difference in results could be due to the complexity of the *in vivo* environment compared to *in vitro*.

4.4.3 Conclusions

These experiments show that selective activation of the EP_4 receptor is neuroprotective following *in vivo* stroke. Interestingly, treatment with the EP_4 agonist showed a similar level of neuroprotection to COX-2 inhibition. This suggests that selective agonists of the EP_4 receptor are a potential therapeutic target for stroke. Given that both the EP_2 and EP_4 receptors couple to G_s protein, increasing cAMP levels and activating PKA, it is possible that these receptors neuroprotective functions are determined by the cAMP-PKA pathway. McCullough et al (2004) demonstrated that inhibiting the PKA pathway reversed the protective effect of EP_2 receptor activation. As discussed in chapter 3, studies investigating the signalling mechanisms of these receptors have reported that the EP_4 receptor is capable of activating additional signalling pathways which are cAMP independent (Regan, 2003). Therefore the cellular mechanisms involved in EP_4 mediated neuroprotection still remain to be determined and warrant further investigation.

Further studies are also required using a range of behavioural tests to provide a more detailed conclusion on the effects of EP_4 on neurological functioning. In addition, experiments using mircodialysis and HPLC techniques would prove useful in determining the concentration of the drug in the brain. All of these are important considerations when assessing the translational properties of this drug for clinical use. In addition, for any agent to reach clinical trials it must show beneficial effects across

different laboratories, in different models of ischaemia and in at least two animal species.

Chapter 5

EP_4 receptor expression and distribution in the brain

5.1 Introduction

5.1.1 EP receptor distribution

EP receptors are expressed throughout the body in almost all organs including the lungs, kidneys and also within the central nervous system (CNS). These receptors regulate multiple functions throughout the body including; inflammation, pain, reproduction, vascular and CNS functions. The EP_3 and EP_4 subtypes are the most widely distributed E-series prostaglandin receptors in the body (Narumiya, Sugimoto & Ushikubi, 1999), whereas EP_1 receptor expression is restricted to several organs such as the kidney and lungs, and EP_2 receptor expression is most abundantly expressed in the uterus, lung, thymus and spleen (Katsuyama et al., 1995).

5.1.2 EP receptor expression and distribution in the brain

The distribution of EP receptors within the brain has not been fully characterised although current research suggests that these receptors are distinctively distributed across the brain. In the rodent brain, all EP receptors have been identified; however, each receptor has a region- and cell-specific expression (Breyer et al., 2008). Immunostaining for the EP receptors has revealed that all four receptor subtypes are expressed in the hippocampus and the cortex, with the EP_3 receptor showing the greatest abundance in its expression followed by EP_2, EP_1 and EP_4 (Zhu et al., 2005). The EP_1 receptor is abundantly expressed in cortical neurons, purkinje cells of the

cerebellum and the hypothalamus (Kawano et al., 2006; Candelario-Jalil., 2005; Båtshake, Nilsson & Sundelin, 1995). EP_2 receptor expression is largely restricted to the hippocampus and nuclei of the hypothalamus and amygdala (Zhu et al., 2005; Zhang & Rivest, 1999). The EP_3 receptor is most widely distributed throughout the brain and its expression has been detected in the cortex, hippocampus, thalamus, hypothalamus, midbrain and lower brain stem (Narumiya et al., 1999). EP receptor expression studies have consistently reported that the EP_4 receptor is expressed less abundantly and is restricted to some hypothalamic nuclei and others have also detected this receptor in the hippocampus (Zhu et al., 2005).

All EP receptors are mainly detected in neurons although some studies have also reported their expression in astrocytes and microglia (Cimino et al., 2008, ***Table 5.1***). All four receptor subtypes, except $EP_{1,}$ are expressed in astrocytes whereas EP_1 and EP_2 receptors have been detected in microglia (Cimino et al., 2008; Caggiano & Kraig, 1999). However, other studies have shown EP_4 receptor expression in primary cultured microglia (Shi et al., 2010). In addition, EP_2 and EP_4 receptors are reported to be expressed in macrophages (Katsuyama et al., 1995) and endothelial cells of blood vessels within the CNS (Li et al., 2008). EP_1 and EP_2 receptors were detected in the cytoplasm and the nucleus, whereas EP_3 showed strong expression the cytoplasm and EP_4 was weakly expressed in a spot like manner (Zhu et al., 2005). Other studies have reported EP_3 and EP_4 expression in the nuclear membrane of the porcine cerebral endothelial cells, where the receptors increased transcription of inducible nitric oxide (Bhattacharya et al., 1999). In addition, studies have shown that perinuclear COX enzymes can synthesize prostanoids that act at the nuclear level (Morita et al., 1995). This suggests that EP receptors may be involved in signalling not only at the level of the plasma membrane but also within the nuclear membrane. Research has demonstrated

that EP_1, EP_2 and EP_4 are co-localised with synaptophysin suggesting that they are mainly present at presynaptic terminals whereas the EP_3 receptor shows co-localisation with post synaptic density protein-95 (PSD-95) indicating its presence at post synaptic sites in hippocampal neurons (Zhu et al., 2005). Furthermore, COX-2 and both mPGES-1 and mPGES-2 are also located at post synaptic sites as they co-localise with PSD-95 (Sang et al., 2005). The localisation of these enzymes and EP receptors at presynaptic and post synaptic terminals supports the theory that PGE_2 mediates synaptic activity. Research by Chen and Bazan (2005) has shown that PGE_2 dynamically regulates membrane excitability and synaptic efficacy in hippocampal pyramidal neurons, an effect which was reversed with the application of a COX-2 inhibitor. Additional research has revealed that PGE_2 modulates synaptic transmission in the hippocampus via presynaptic EP_2 receptors (Sang et al., 2005). These findings suggest that EP receptors play a role in synaptic transmission which is mediated upon binding of PGE_2.

Some studies have reported that EP_4 receptor expression in the brain is restricted to hypothalamic nuclei mediating endocrine function (Cimino et al., 2008; Narumiya et al., 1999) whereas other studies have detected EP_4 receptor expression in the hippocampus, cortex and the striatum (Zhu et al., 2005; Ahmad, Ahmad, Brum-Fernandes & Doré, 2005). This indicates that the expression profile EP receptor distribution in the brain remains uncertain and requires further investigation.

	Neurons	Microglia	Astrocytes	Endothelial	References
EP_1	Cortex, cerebellum, hypothalamus, hippocampus	yes	no	yes	Bhattacharya et al. (1998)
EP_2	Amygdala, hypothalamus, hippocampus	yes	yes	yes	Li et al. (2008) McCullough et al. (2004)
EP_3	Widespread	yes	yes	yes	Ji & Tsirka (2012)
EP_4	Hypothalamus, hippocampus, striatum, cortex	yes/no?	yes	yes	Li et al. (2008) Zhang & Rivest (2000) Ahmad et al. (2005) Shi et al. (2010)

Table 5.1: Cellular expression of EP receptors

Localisation and distribution of EP receptors (modified from Cimino et al., 2008)

5.1.3 Changes in EP receptor expression following a brain inflammatory response

Neuroinflammation has significant impact on neuronal injury which has been demonstrated by various models of neurodegeneration in which the CNS innate immune response is an important element of the disease progression (Nguyen, Julien & Rivest, 2002). The innate immune response is initially protective via clearance of pathogenic constituents but can also contribute to injury because some components of the innate immune response are cytotoxic and contribute to neurodegeneration (Lucas, Rothwell & Gibson, 2006).

Several studies have reported changes in EP receptor expression following an inflammatory stimulus either in response to IL-1β, LPS stimulation or NMDA application. Treatment with IL-1β increased the expression of EP_2 and EP_4 receptors in the hippocampus but did not alter the expression of EP_1 and EP_3 receptors (Zhu et al., 2005; Katsuyama et al., 1998). Upregulation of the gene encoding EP_4 receptors has been detected in neurons of the paraventricular nucleus (PVN) following IL-1β or LPS administration inducing inflammation (Zhang & Rivest, 1999). The PVN of the hypothalamus is involved in modulating a variety of endocrine and autonomic functions including the innate immune response (Hefoc, Olariu, Hefco & Nabeshima, 2004). Circulating IL-1β and administration of exogenous PGE_2 resulted in a significant upregulation of EP_4 expression in the PVN and the caudal ventrolateral medulla (cVLM) indicating the integrative pathways that regulate specific physiological responses during systemic inflammatory processes (Zhang & Rivest, 2000). Interestingly, IL-1β induced EP_4 transcription was abolished following inhibition of PGE_2. These findings provide evidence that EP_4 receptors are significant in mediating

the effects of cytokines on specific physiological responses during systemic inflammation (***Figure 5.1***).

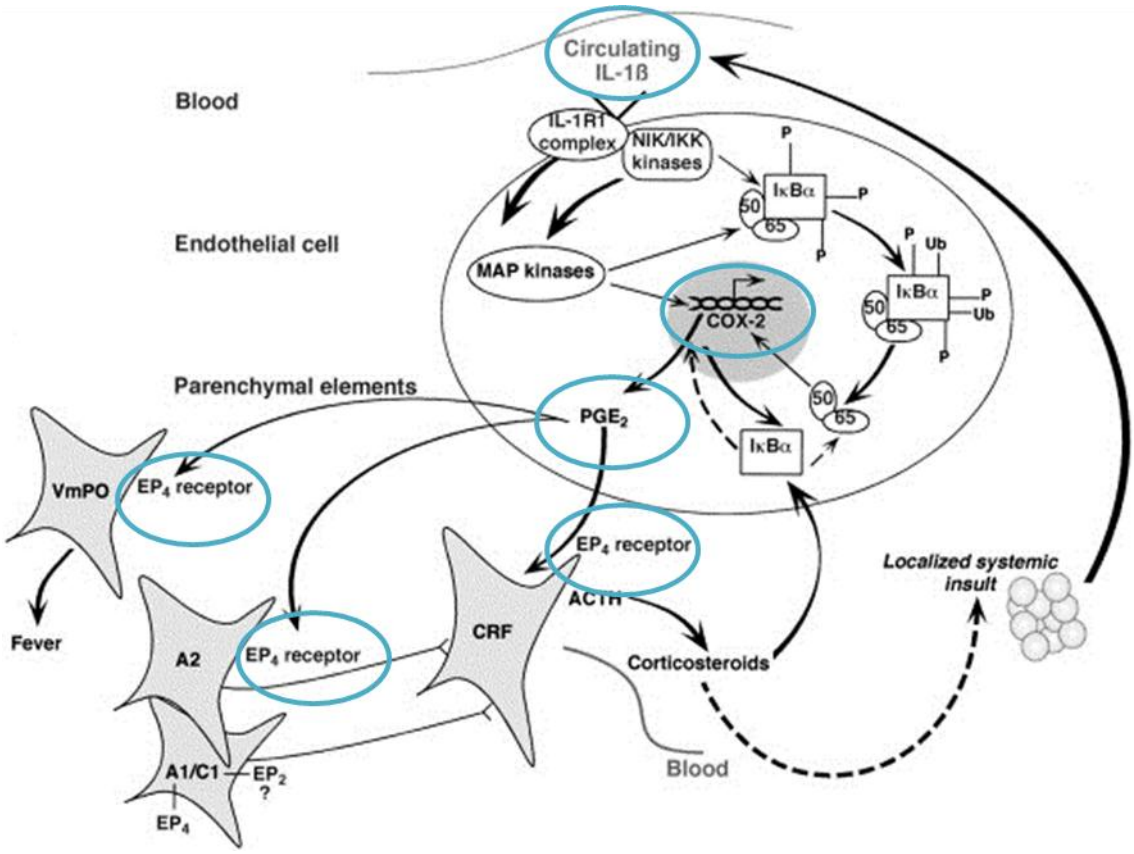


Figure 5.1: Neuroendocrine function in response to systemic inflammation

Systemic inflammatory response results in circulating cytokines such as IL-1β, which target cells of the blood brain barrier (BBB). IL-1β binds to its receptor to activate intracellular signalling cascade which is responsible for initiating COX-2 gene transcription. Upregulation of COX-2 results in increased synthesis of PGE_2 which diffuses into the brain parenchyma and activates EP_4 receptors located on the surface of corticotrophin-releasing factor (CRF) cells, inducing cAMP activation. EP_4 receptors expressed in neurons of the ventromedial preopticnucleus (VmPO) may also be activated by PGE_2 to induce fever. Collectively, these pathways are responsible for mediating neuroendocrine function in response to immune challenge (Figure modified from Rivest (2001).

The inflammatory response induced following LPS treatment resulted in a significant upregulation of both EP_2 and EP_4 mRNA expression in macrophage cell lines (Katsuyama et al., 1998). Further to this, cultured microglial cells treated with LPS showed significant upregulation of pro-inflammatory cytokines (Shi et al., 2010). These cytokines are regulated by transcriptional activity of nuclear factor kappa beta (NF-kB). Pharmacological activation of the EP_4 receptor resulted in significant downregulation of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), IL-1β and IL-6. Activation of the EP_4 receptor increased phosphorylation of protein kinase B (PKB/Akt) reducing nuclear translocation NF-kB and consequently decreasing transcriptional activity (Shi et al., 2010).

Collectively, the findings discussed above suggest that EP_2 and EP_4 receptors are implicated in the response to an inflammatory challenge modelled by IL-1β or LPS application. This suggests that modulating EP_2 and/or EP_4 activity could potentially be viable targets for neuroprotection in CNS disorders which have an inflammatory component.

5.1.4 EP receptor expression following ischaemic injury

Ischaemic stroke initiates a cascade of events including excitotoxicity, peri infarct depolarisations, oxidative stress, inflammation and apoptosis. As discussed above, systemic inflammation results in changes in EP receptor expression, particularly EP_4, which is implicated in the body's neuroendocrine and autonomic response to immunogenic challenges. One study investigated the expression of EP_2 and EP_4 receptors in the rat hippocampus following ischaemia and reported upregulation of these two receptors in response to an ischaemic insult (Choi et al., 2006). The time course of expression between the two receptors differed in that EP_2 and EP_4 expression were

upregulated at 4 h and 12 h post ischaemia, respectively. Research findings reported by Li et al. (2008) and Liang et al. (2011) have reported dynamic regulation of EP_2 and EP_4 receptor expression in neurons and endothelial cells during reperfusion. For instance, during the early hours of reperfusion (4 h) both EP_2 and EP_4 expression was down regulated in neuronal cells in the ischaemic core. However, both these receptors were markedly induced in endothelial cells in the peri-infarct region. At 24 h post MCAO, EP_2 expression persisted in endothelial cells whilst EP_4 expression was absent at this time point. This differential expression of EP_4 in neuronal and endothelial cells reflects its role in cell specific mechanisms of neuroprotection through vascular and neuronal signalling.

The evidence discussed above suggests that EP_4 receptor expression is dynamically regulated following inflammatory and ischaemic exposure. Further work is warranted to investigate the changes in EP_4 receptor expression following ischaemic injury as this would provide a better understanding of the distribution of this receptor in response to ischaemia.

5.1.5 Objectives

Using a selective antibody raised against the EP_4 receptor subtype, immunohistochemistry was carried out using rat dorsal root ganglia (DRG) in order to optimise a suitable staining protocol. The DRG is involved in nociception mediated by PGE_2. Previous research by a former PhD student (Hammond, 2006) has demonstrated positive labelling of the EP_4 receptor in DRG tissue which was localised to small diameter neurons. As a result this work demonstrated that the EP_4 receptor is abundantly expressed in DRG and therefore this tissue was used as a positive control. Subsequent experiments were then conducted in order to establish the expression of EP_4

receptors in the brain. We aimed to establish the distribution of EP_4 receptors in normal brain tissue before going to determine the expression in ischaemic brain tissue. In addition to immunohistochemistry, real-time polymerase chain reaction (PCR) was used to assess EP_4 receptor mRNA levels following OGD in OHSC. The aim of these experiments was to obtain data to form comparisons between the normal and the ischaemic tissue and to assess whether there was a difference in expression and distribution of EP_4 receptors following ischaemic injury.

5.2 Methods

5.2.1 Principles of immunohistochemistry

Immunohistochemistry enables the detection of specific proteins known as antigens in cells and tissue. This process involves the use of antibodies which are highly selective for their specific antigen. There are two types of immunofluorescence protocols known as the direct (primary) and indirect (secondary) methods. The direct technique involves the use of a single antibody which is conjugated to a fluorophore and upon binding to the target antigen it can be directly visualised under a microscope. Advantages of direct detection include the ease of use and also the elimination of non-specific binding of the secondary antibody. The main drawback of direct detection is that it may lack the sensitivity required to visualize lower expression levels. The indirect immunofluorescence technique involves the use of two antibodies. The unlabelled primary antibody is specifically designed to detect the target protein and upon binding forms a complex. This formation of a complex enables the localisation of the antigen in the tissue sample. The secondary antibody is tagged with a fluorescent marker or an

enzyme, these bind to the antigen-antibody complex eliciting a fluorescent signal or a stain when viewed using the appropriate microscope.

5.2.2 Standard solutions

To preserve the morphology of tissue and to prevent degradation, fresh sections were fixed in 4% paraformaldehyde (PFA). This was made fresh on the day of each experiment using 10 ml sodium dihydrogen orthophosphate (solution A), 40 ml di-sodium hydrogen orthophosphate-2-hydrate (solution B), 4g PFA and 50 ml dH_20 to give a final volume of 100 ml.

Phosphate buffered saline (PBS) was made as a stock solution of 1 litre containing 50 ml solution A, 200 ml solution B, 9g NaCl and 750 ml dH20. This was stored at 4°C and was used to wash tissue sections between incubation steps.

The pH for the PFA and PBS were checked and adjusted, if necessary, to give a neutral Ph value 7.4 (neutral).

Sucrose solution was made using 30% sucrose in PBS and stored at 4°C.

Nissl solution was made as a 100 ml stock containing 34 ml acetic acid, 6 ml sodium acetate (1M), 60 ml dH2o, 0.5 g cresyl fast violet. This was filtered each time before use.

5.2.3 Antibodies

Both the primary and secondary antibodies were diluted in PBS containing 10% serum which was specific to the host of the secondary antibody. For instance, the secondary antibody used for fluorescent staining was derived from goat therefore goat serum was

used for these experiments. For non-fluorescent diaminobenzamide (DAB) staining the secondary antibody was derived from pig therefore PBS was supplemented with swine serum for these experiments. All the antibodies used in the experiments are described in ***Table 5.2***.

Antibody	**Host**	**Dilution**	**Supplier**
EP_4 Receptor (N-term)	Rabbit	DRG- 1:500 Brain- 1:200	Cayman Chemical
Anti-rabbit/FITC	Goat	1:200	Jackson ImmunoResearch
Anti-rabbit/Biotin	Swine	1:350	DAKO

Table 5.2: Details of the antibodies used in this study
The table provides information on the primary and secondary antibodies used for the experiments presented in this chapter.

5.2.4 Tissue preparation and fixation

DRG were dissected from adult male Wistar rats (200-300g); the tissue was embedded in OCT compound and rapidly frozen over dry ice and hexane. The tissue was stored at -80°C until future use.

Brain tissue was obtained from adult male C57/BL6 mice subjected to an hour of MCAO and 24 hours of reperfusion (as described in chapter 4). Control brain tissue was obtained from normal male C57/BL6 mice which had not undergone any surgical procedure. All mice were overdosed with pentabarbitone and transcardially perfused with saline (0.9% NaCl in dH_2O) followed by 4% PFA. The brains were removed and placed in 4% PFA for several hours before been transferred to sucrose solution and stored at 4°C. After several days brains were placed in individual tissue holders and embedded in OCT compound. These were frozen at -80°C until usage.

Fresh brains were also obtained from both MCAO mice and normal mice. Animals were killed via a schedule 1 method and brains were rapidly removed. The brains were embedded in tissue holders with OCT compound and rapidly frozen over dry ice and hexane and stored at -80°c.

Frozen tissue sections (10 µm, unless stated otherwise) were obtained from all samples using a cryostat (BRIGHT, model OTF). The sections were mounted onto polylysine coated microscope slides and left to air dry for approximately 30 minutes. Sections which were used for experiments on the same day were briefly washed with PBS whilst the remaining slides were frozen at -80°C until future use.

5.2.5 Immunohistochemistry - Fluorescent Labelling

Slides were immersed in PBS either following air drying straight after sectioning or upon removal from the freezer. Excess PBS was carefully wiped off the slides. The slides were placed in a humidified incubation box and immersed sequentially in the following solutions:

1. 10% goat serum and 0.5% triton- X- 100 (in PBS) for 30 minutes.
2. The primary polycolonal antibody for EP_4 diluted in PBS with 10% goat serum and incubated over night at 4°C.
3. The secondary antibody (goat anti- rabbit IgG conjugated with fluorescein isothiocyanate, FITC) diluted in PBS with 10% goat serum for 2 hours at room temperature.

Between each step sections were washed thoroughly with PBS. The slides were mounted using citifluor. Control experiments were carried out alongside experimental samples where the primary antibody incubation step was omitted in order to determine

any non-specific binding of the secondary antibody. To prevent the control sections from drying out during this step they were incubated in PBS with 10% goat serum.

5.2.6 DAB staining- Non-Fluorescent Labelling

The slides were placed in a humidified incubation box and immersed in the following solutions:

1. 0.2% hydrogen in methanol for 15 minutes.
2. PBS containing 10% swine serum and 0.5% Triton X-100 for 30 minutes at room temperature.
3. Primary polycolonal antibody for EP_4 diluted in PBS with 10% swine serum at 4°C overnight.
4. Swine anti-rabbit biotin conjugated IgG diluted in PBS containing 10% swine serum for 35 minutes at room temperature (DAKO).
5. Streptavadin- horseradish peroxidase (HRP) conjugate diluted in 10% swine serum and PBS for 30 minutes at room temperature.
6. DAB solution using the DAB substrate kit, for 4 minutes at room temperature.
7. 70% IMS for 5 minutes followed by 100% IMS for 5 minutes and 100% xylene twice for 5 minutes.

Between these steps sections were washed thoroughly with PBS. Sections were mounted using DPX. Control sections were carried out alongside experimental samples, excluding the primary antibody incubation step.

5.2.7 Cresyl Violet Staining

Cresyl violet is used to stain Nissl substance in the cytoplasm of neurons and glial cells in tissue samples. This staining enables the visualisation of the cellular architecture of the brain. To accomplish this, tissue sections were incubated sequentially in the following solutions:

1. dH_2O for 5 minutes
2. Nissl solution for 6 minutes
3. dH_2O for 3 minutes
4. 70% alcohol for 5 minutes
5. 90% alcohol for 5 minutes
6. 100% alcohol for 5 minutes
7. Xylene twice for 5 minutes

Slides were mounted in DPX and left to dry.

5.2.8 Microscopy

Fluorescent labelled sections were visualised using an epifluorescent microscope equipped (Labophot 2A, Nikon Instruments) with a monochromatic CCD (Cohu Inc) camera, captured using ImageJ software. Sections were viewed using the appropriate filters for FITC.

For Non-fluorescent labelling (DAB and Nissl staining) slides were visualised using a light microscope (PriorLux, Prior Scientific Instruments Ltd, Cambridge, UK) equipped with a Moticam 2300 digital camera (Motic Instruments, Richmond, Canada) and images were taken using Motic Image Plus 2.0 (Motic Instruments).

5.2.9 Gene expression - Real Time PCR

This technique is used to identify mRNA expression in tissue. It is a sensitive method which allows the detection of changes in mRNA levels. This is accomplished by a two-step process; firstly the mRNA is extracted and reverse transcribed to produce complimentary DNA (cDNA). The second step entails the use of primer pairs which are complimentary to the nucleotide sequence of the target gene. For the initial part of the reaction the temperature is raised to 95°C to enable the double stranded cDNA to denature into single strands. The sample is then cooled to 60°C to allow the primers to anneal to the cDNA sequence complimentary to the site on each strand producing double stranded DNA. The temperature is then extended to 72°C which allows the extension of the DNA from the primers. This part of the reaction allows the exponential copying of the cDNA sequence which is amplified for 40 cycles. These sequential steps of denaturation, annealing and extension are repeated to amplify the reaction product to a level which can be detected.

5.2.9.1 RNA Extraction

Prior to completing the following steps, the work area and equipment were decontaminated using RNAseZap (Invitrogen). RNA was extracted from OHSC exposed to 2 hours of OGD followed 1, 4 and 24 hours of reperfusion (as described in chapter 2 and 3). Total RNA was extracted using the RNeasy mini kit from Qiagen in accordance to the manufacturer's instructions. Briefly, OHSC were homogenised in lysis buffer with the addition of β-mercaptoethanol by passing through a 0.5ml syringe with a 20 gauge needle several times. The sample was passed through a gDNA spin column to remove any contamination of genomic DNA. In order to provide suitable conditions for binding, 70% ethanol was added before the sample was transferred to an

RNeasy mini spin column. Following one wash with Buffer RW1 and two washes with Buffer RPE (containing ethanol), RNA was eluted using 15 µl of RNase free water and stored at -80°C for later use. The quality and quantity of RNA was assessed using a Nanodrop (Thermo Scientific).

5.2.9.2 Reverse Transcription

The ImProm-II Reverse Transcription (Promega) was used to produce cDNA from the resultant RNA. All components were kept on ice throughout the preparation process. The experimental reaction contained 1 µg of RNA and the reverse transcription master mix according to the manufacturer's instructions, with a final reaction of 20 µl. The master mix included the following components; random primer, nuclease free water, reaction buffer, magnesium chloride ($MgCl_2$), dNTP mix, RNAse inhibitor and reverse transcriptase enzyme Alongside these experiments positive controls using nuclease free water (no RNA) and negative controls whereby the reverse transcriptase enzyme was replaced with nuclease free water were added.

5.2.9.3 Real-Time PCR

The reaction set-up was completed in a safety hood decontaminated with RNaseZap. The reaction was set up according to the instructions of the SYBR Green Sensimix kit from Bioline, using 2 µl cDNA as the template. Three controls samples; a positive control; a negative control and a no template control were ran simultaneously in each experiment. Cycling conditions consisted of 40 cycles of 95ºC for 15 s, 60ºC for 15 s and 72ºC for 20 s. Each real time RT-PCR reaction was performed in triplicate using the Qiagen Rotor Gene 6000. The purity of the amplicon in each PCR was assessed through melt curves. Relative quantities of EP_4 mRNA expression in all experimental samples were assessed as comparative cycle thresholds ($\Delta C_t = C_{tEP4} - C_{tRPL44}$). The fold change

in expression between experimental samples were calculated by the formula $\Delta\Delta C_t = \Delta C_{tsample} - \Delta C_{tcontrol.}$

5.2.9.4 Real-Time PCR primers

All primers were designed to cross intron-exon boundaries determined from the mouse gene mRNA sequences (***Table 5.3***). Primer pairs specific for the EP_4 receptor were designed using Primer 3 software. The primers sequences for the house keeping gene ribosomal protein (RPL44) was previously designed and validated by colleagues in the MRC. Endogenous reference genes are expressed constitutively and are not affected by experimental conditions. The expression of the target gene is normalised to the reference gene in order to determine any changes in expression levels.

Primer	Forward Primer 5’3’	Reverse Primer 5’3’	Annealing temperature (°C)	Amplicon Size
EP_4	AAGCCATAGAGAAGATCAAGTGC	AGCTGTCTCTGCCGGAAC	60.9	60
RPL44	GGCCGGTCTCTCGTTCTCA	TTACAGAAAGTCCTTCGGGTTTTT	61.6	80

Table 5.3: PCR Primers

The table provides information on the primers used for this study, designed using Primer 3 Software or previously designed and as stated above.

5.3 Results

5.3.1 Visualising the ischaemic brain

Cresyl violet stains the endoplasmic reticulum of cell bodies and is a useful stain to visualise damaged areas. Following cell injury and axonal degeneration, cellular content disintegrates, resulting in lack of staining in these regions. Sections from ischaemic brain tissue were stained with cresyl violet to enable the visualisation of the cellular architecture in the region of the brain which sustained ischaemic injury. An absent of cells was observed in the striatal and cortical regions of the ischaemic hemisphere, the region of the brain supplied by the MCA (***Figure 5.2***) **.**This pattern of cell loss was not observed in the contralateral hemisphere. The results show the distinction between the ischaemic core identified due to the loss of cell bodies and the surrounding penumbral region where cell bodies appear in greater abundance ***(Figure 5.3***).

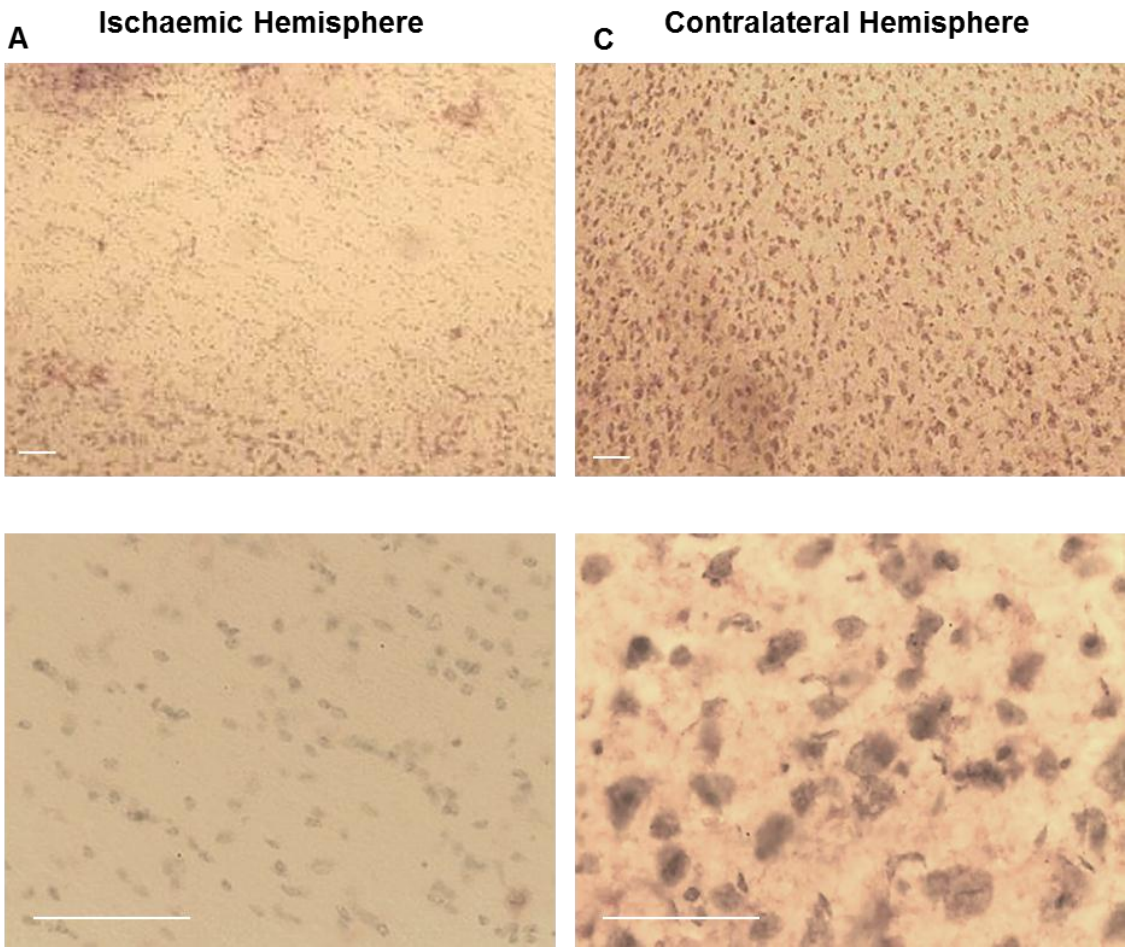


Figure 5.2: Cell loss in ischaemic brain tissue

Micrographs showing representative Nissl stained ischaemic brain sections reveal loss of cell density in the region of the brain which sustained injury (cortical and striatal) following MCAO compared to the contralateral hemisphere. Regions examined include ipsilateral cortex (***A***), ipsilateral striatum (***B***), contralateral cortex (***C***) and contralateral striatum (***D***). Scale bars represent 100 µM**.**

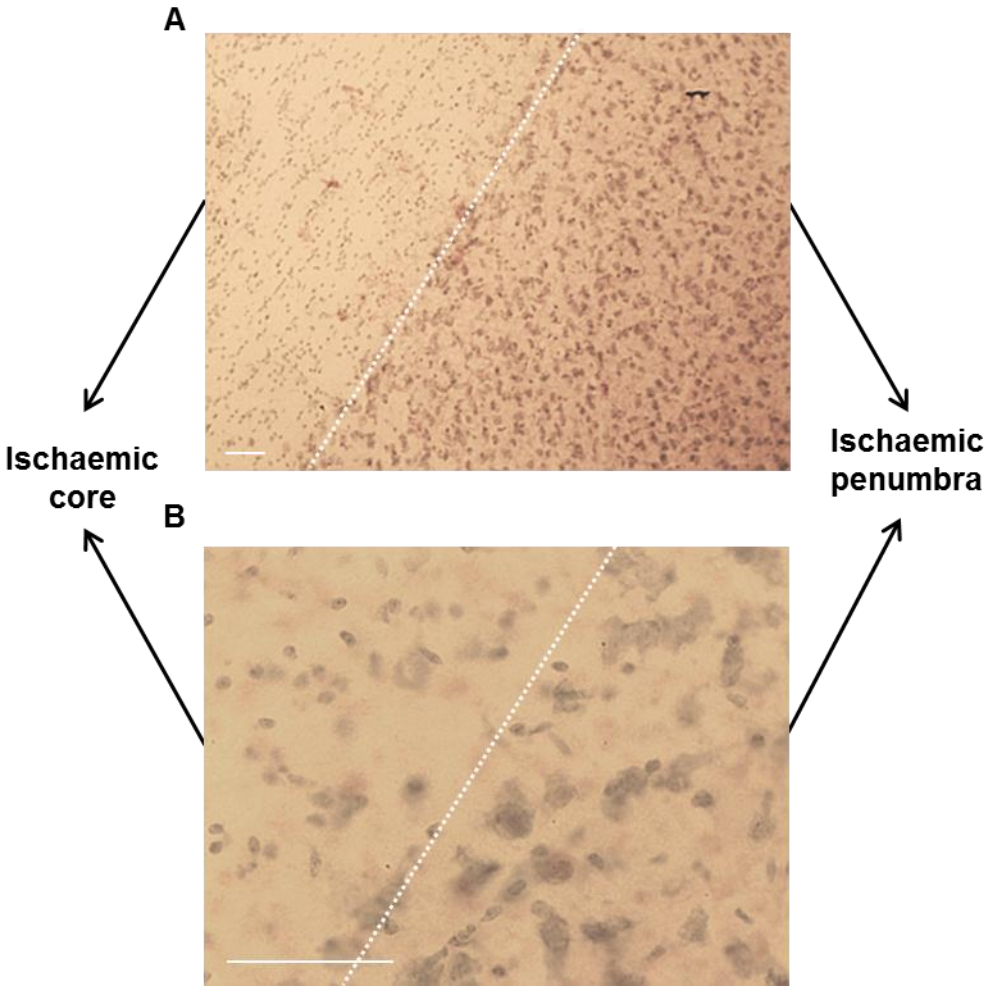


Figure 5.3: Cell loss in the ischaemic core versus penumbra

Micrographs showing representative Nissl stained ischaemic brain sections (ipsilateral cortex) revealing loss of cells in the ischaemic core whilst the surrounding penumbra shows greater number of cells. Scale bars represent 100 μM. Dashed line indicates distinction between the ischaemic core and the surrounding cell bodies in the ischaemic penumbra.

5.3.2 Immunofluorescent labelling of EP_4 receptors

5.3.2.1 Rat DRG

The EP_4 primary antibody was initially tested in the rat DRG as this antibody has previously been tested in the lab in DRG tissue and showed specificity for EP_4 protein. It was also tested with the control peptide which greatly reduced the intensity of labelling, indicating that the EP_4 antibody recognises the peptide against which it was raised. The optimized dilution for the primary antibody was 1:500. The immunofluorescence from the goat anti-rabbit FITC- conjugated secondary antibody was optimized for use at 1:200. The experiments conducted here found that EP_4 fluorescent labelling was observed in the DRG sections emitting a bright green fluorescent signal (***Figure 5.4***). Control sections where the primary antibody was omitted abolished the fluorescent signal indicating that there was no non-specific binding of the secondary antibody.

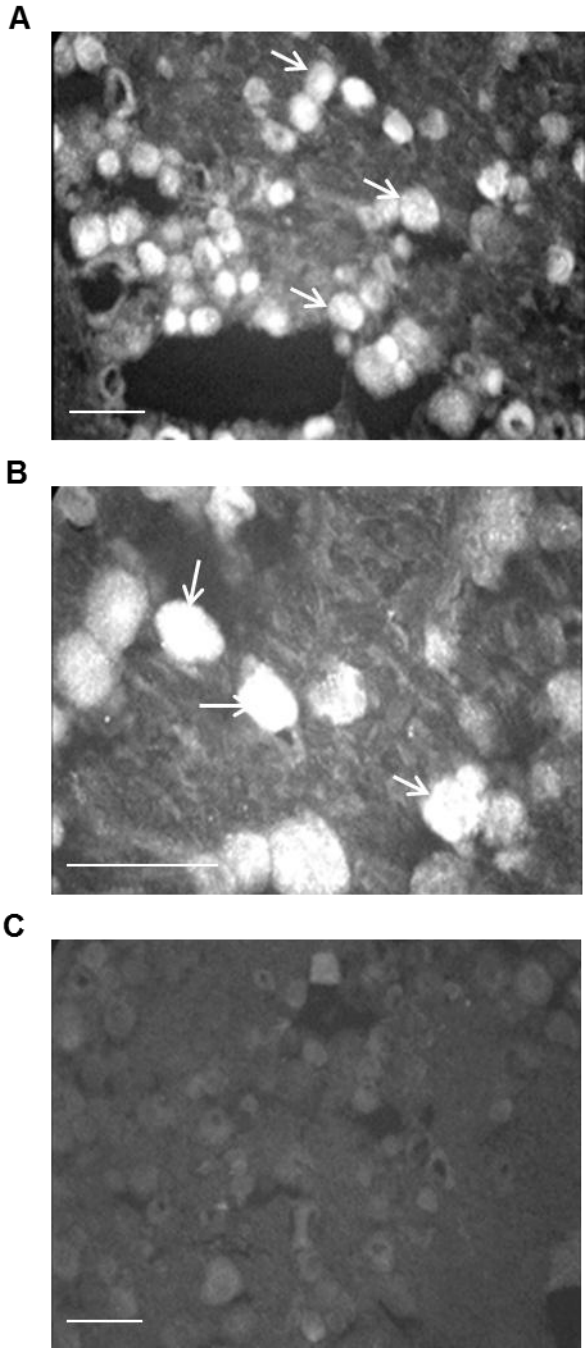


Figure 5.4: EP_4 fluorescent staining in the rat DRG

Micrographs showing representative rat DRG sections incubated in the primary antibody for EP_4 (***A***, ***B***). Small cell bodies are positively stained for EP_4 whilst larger cells remain unstained. This staining pattern is not seen in the absence of the primary antibody (***C***). Scale bars represent 100 µM.

5.3.2.2 Expression of EP_4 receptor in normal brain tissue

Normal brain tissue showed EP_4 positive labelling of cell bodies, however, it was difficult to discriminate between background fluorescence and actual labelling of cell bodies stained for EP_4. In addition, the fluorescence gave a yellowish signal as oppose to a bright green which was previously observed in the lab when staining DRG for EP_4. This difference in signal colour is likely to be due to the storage of tissue fixed with 4% PFA (data not shown).

Therefore control brains which were frozen fresh and fixed in 4% PFA on the day of experiments were used. The results using fresh brain sections showed weak EP_4 labelling throughout the brain with stronger expression in the hippocampus and the hypothalamic regions (***Figure 5.5***). The expression of EP_4 was localised to the cell membrane with some expression observed in axonal tracts, suggesting that the EP_4 receptor maybe transported to other cells and regions within the brain. The fluorescent signal was detectable but intensity of the signal was weak, therefore the primary antibody concentration was increased and used at a lower dilution (1:200). EP_4 expression was detected more clearly with a strong fluorescent signal in the cortex, hippocampus and hypothalamus.

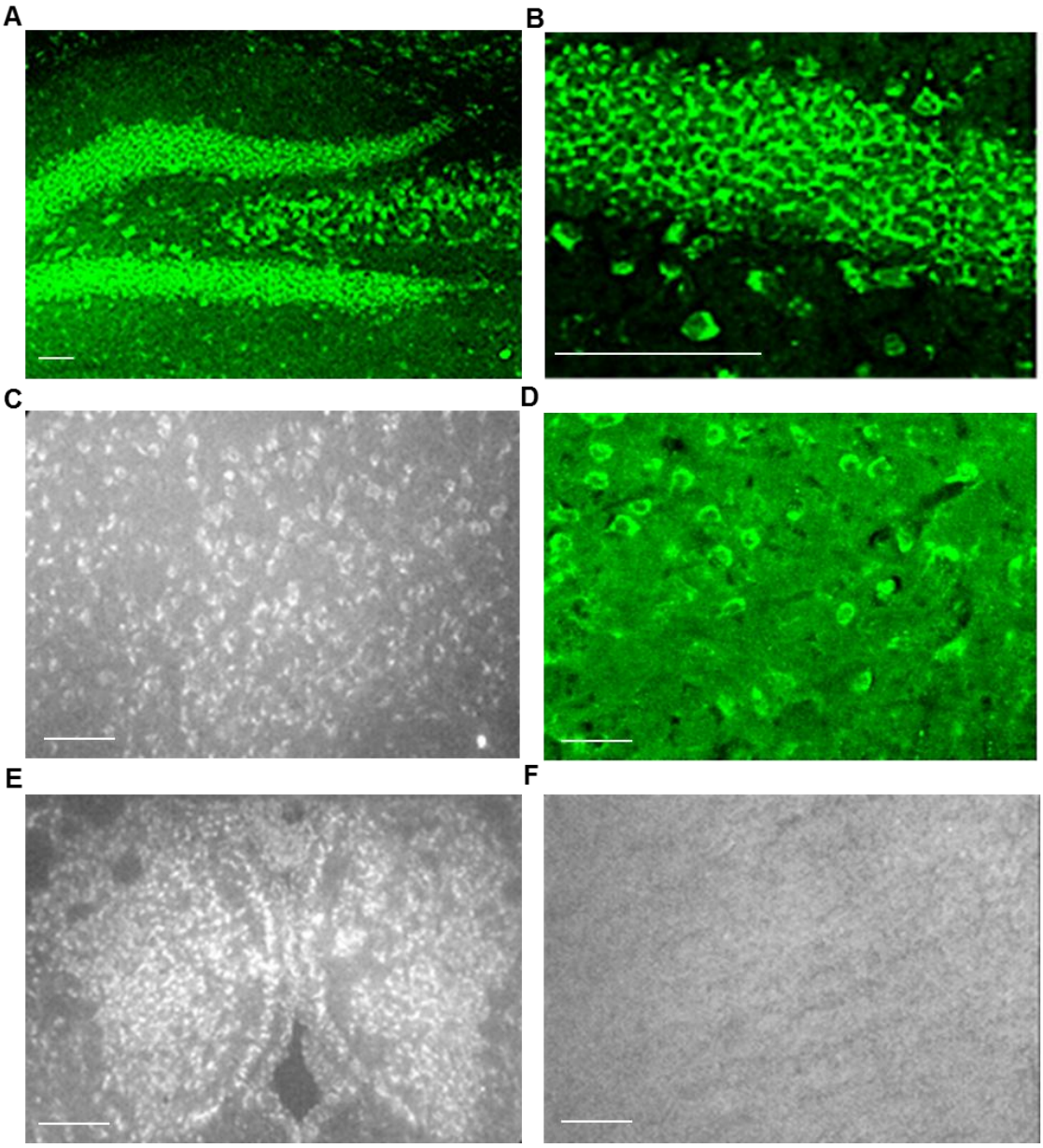


Figure 5.5: EP_4 fluorescent staining in mouse control brain sections

Micrographs showing representative control brain slices incubated in the primary antibody for EP_4. Regions of the brains which show positive staining for EP_4 include the hippocampus (***A*** and ***B***), the cortex (***C*** and ***D***) and the hypothalamus (***E***). Other regions of the brain did not show any positive EP_4 staining. This staining pattern is not observed in the absence of the primary antibody (***F***). Scale bars represent 100 µM.

5.3.2.3 EP_4 receptor expression following ischaemia

The primary antibody for EP_4 which was optimized in the normal brain tissue was used to detect EP_4 expression in the brain following ischaemia (as previously described). Due to the high background seen in the normal brains which had been perfusion-fixed (data not shown) we only examined EP_4 receptor expression in brain which were obtained fresh from mice that had undergone MCAO. In order to overcome problems associated with sectioning fresh tissue, e.g. decreased tissue integrity, the thickness of sections was increased to 20 µM. Although this helped to maintain tissue structure the sections did not adhere to the polylysine slides as well. In order to increase the likelihood of tissue remaining attached to the slides, they were washed carefully by direct application of PBS rather than by placing them on the rocker. However, it was still difficult to maintain tissue morphology and adherence of tissue to the slides in the region of the brain where the ischaemic insult had occurred. It was not possible, using the fluorescent protocol previously established in normal brain tissue to show any positive EP_4 staining in the ischaemic brain (***Figure 5.6***).

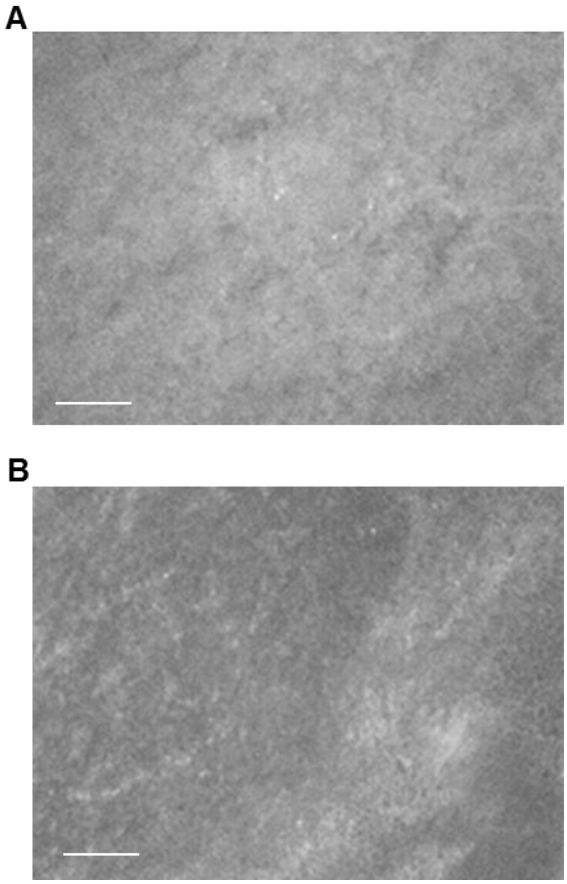


Figure 5.6: EP_4 fluorescent staining in mouse stroke brain sections

Micrographs showing representative ischaemic brain tissue after immunostaining for EP_4 receptor expression. Region of the brain visualised include the penumbra (***A***, ***B***). The section showed no positive staining for EP_4 and did not reveal any difference in comparison to the sections where the primary antibody was omitted (***B***). Scale bars represent 100 µM.

5.3.3 EP_4 staining using non-fluorescent immunostaining

In order to overcome the problems associated with using fluorescent labelling of EP_4 receptors in ischaemic brain tissue, we attempted to determine the expression using non-fluorescent DAB staining. Initially we determined EP_4 receptor expression in DRG, using a protocol previously optimised in the lab by a former PhD student (Alistair Fryatt) before going on to examine expression in brain tissue.

5.3.3.1 DRG

This protocol was first tested in DRG tissue in order to optimise the method in tissue highly expressing EP_4 receptors. The experiments carried out using new reagents, revealed that EP_4 labelled cell bodies were weakly stained, however, the high background staining made it difficult to distinguish specific EP_4 staining from non-specific background staining (***Figure 5.7***). The results show positive staining for EP_4 in smaller cell bodies, whilst larger cell bodies remained unstained. This pattern of staining is typically found in DRG and is consistent with results obtained from previous work in the lab and other studies (Oida, 1995).

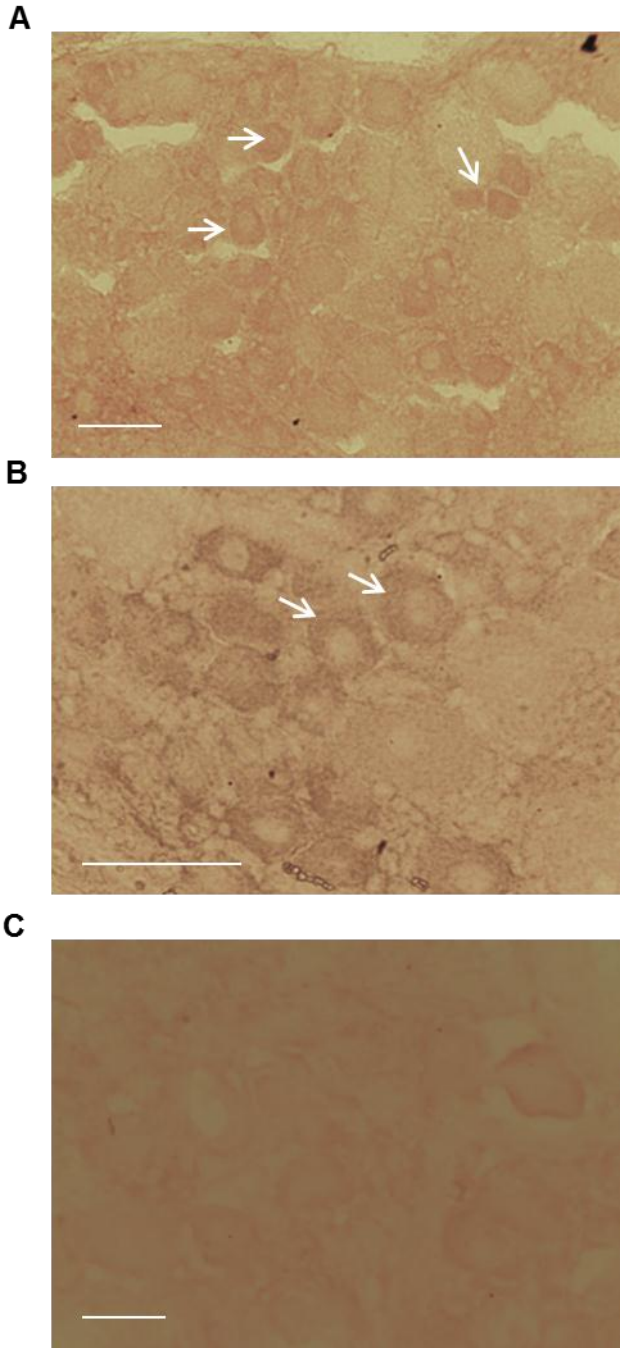


Figure 5.7: EP_4 Non-fluorescent DAB/HRP staining in the rat DRG

Micrographs showing representative rat DRG sections incubated in the primary antibody for EP_4 (***A***, ***B***). Small cell bodies are positively stained for EP_4 whilst larger cells remain unstained. This staining pattern is not seen in the absence of the primary antibody (***C***). Scale bars represent 100 µM.

Due to the EP_4 labelling being relatively weak, further experiments were carried out to optimise the duration for which the sections were incubated in DAB solution. Tissue sections were incubated in DAB solution for 2, 4, 6, 8 and 10 minutes. Unfortunately, increasing the duration of incubating with DAB didn't increase the intensity of the EP_4 labelled cell bodies in the DRG tissue within the duration tested (data not shown).

5.3.3.2 Normal brain tissue

The original time of 4 minutes incubation period in DAB solution was therefore used to detect EP_4 expression in control mouse brain sections. However, no clear labelling was achieved with this non-fluorescent staining method. There was no difference observed between the sections incubated with the primary antibody and those incubated without the primary antibody (***Figure 5.8***). Unfortunately, as no positive labelling for EP_4 could be established in the normal brain tissue using this method of staining it was not possible to further examine EP_4 receptor expression in ischaemic brain tissue.

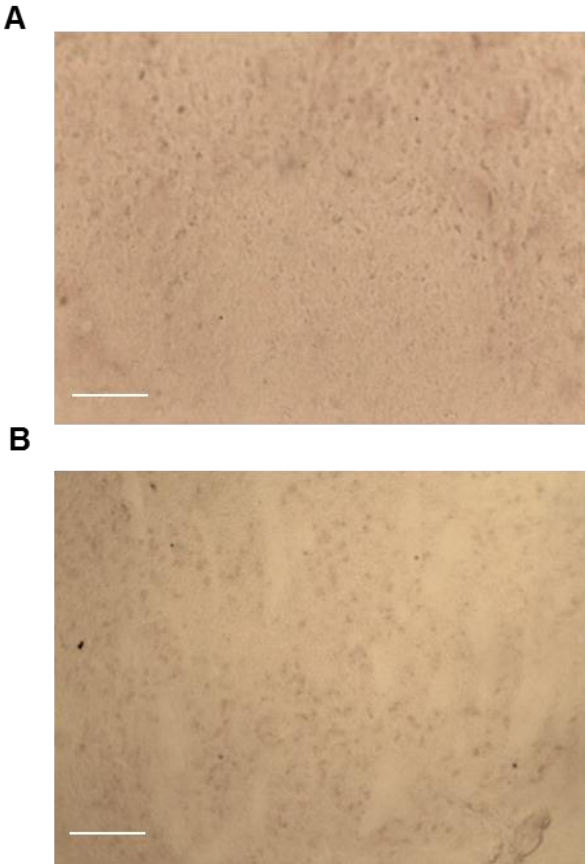


Figure 5.8: EP_4 Non-fluorescent DAB/HRP staining in the mouse control brain
Micrographs showing representative control brain sections incubated in the primary antibody for EP_4. The sections reveal no positive staining for EP_4 and shows no difference in staining compared to the sections where the primary antibody was omitted (***B***). Scale bars represent 100 µM.

5.3.4 EP_4 expression in OHSC using Real Time-PCR- Preliminary evaluation

These experiments were conducted in order to determine EP_4 gene expression in control hippocampal cultures and compare any changes in EP_4 expression following 1, 4 and 24 hours post OGD exposure. OHSC were used due to the ease of reproducibility and increased RNA yield from a single preparation.

5.3.4.1 Optimizing RNA concentration

RNA was extracted from OHSC exposed to OGD. Each sample consisted of 24 slices. Initial extractions resulted in samples with low yields of total RNA. To increase the quantity of RNA, at the final stage of the extraction process, the RNA was eluted twice where the same buffer was passed through the column twice to ensure that the RNA was not causing any blockage in the column. This resulted in improved quantity of the RNA as assessed using a Nanodrop, however, there were great variations between samples. To overcome this variability in RNA concentrations between samples, an additional step was included in the extraction process, where the samples were passed through a Qia shredder column and centrifuged following the lysis step. The addition of this column ensures the cells are completely homogenised which can often be difficult to achieve with sliced tissue samples. This increased the quantity of RNA resulting in consistent concentrations of up to 300 ng/µl per sample.

5.3.4.2 Primer Optimization

Primer efficiency for RPL44 was calculated by constructing a standard curve using the Rotor Gene 6000 from 1 in 10 dilutions of the template (***Figure 5.9***). The reaction efficiency for RPL44 was 88%. The EP_4 primer was specifically designed for this study and therefore it was important to test the specificity of this primer by constructing a

standard curve. Unfortunately, the initial problem was that the melt curve showed 2 peaks. This indicates that the primer is either generating a signal by detecting more than one construct or that primer dimerization is occurring. Furthermore, it was not possible to validate the EP_4 primers by constructing a standard curve, as the neat (undiluted) sample was not detected until after 31 cycles of PCR (***Figure 5.10***).

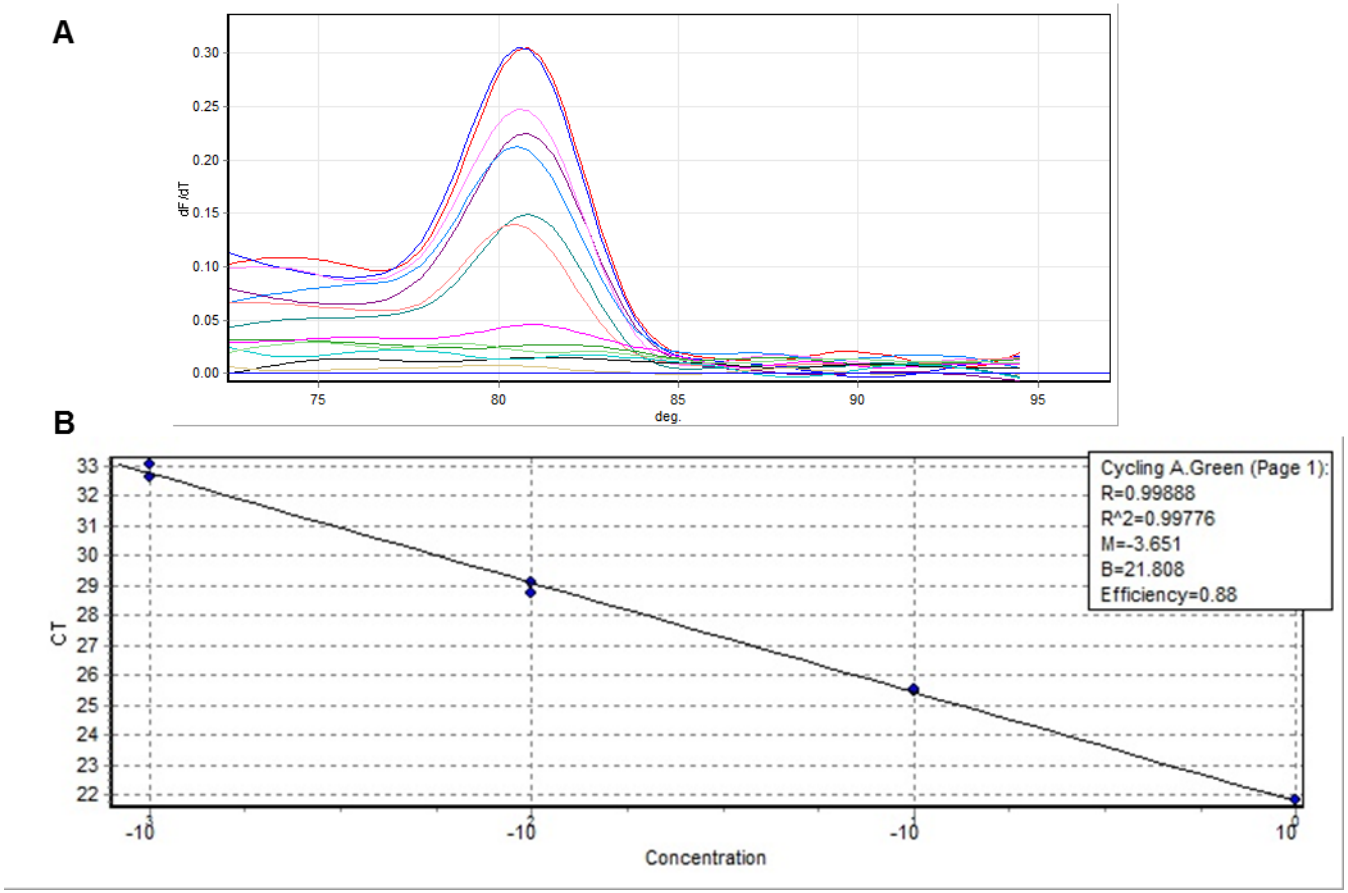


Figure 5.9: Validating RPL44 primers

The melt curve for this reaction reveals a single peak (***A***). A standard curve formed for RPL44 using Rotor Gene 6000 (***B***). The concentration was plotted against the cycle threshold (C_t) and the reaction efficiency is indicated in the inlet.

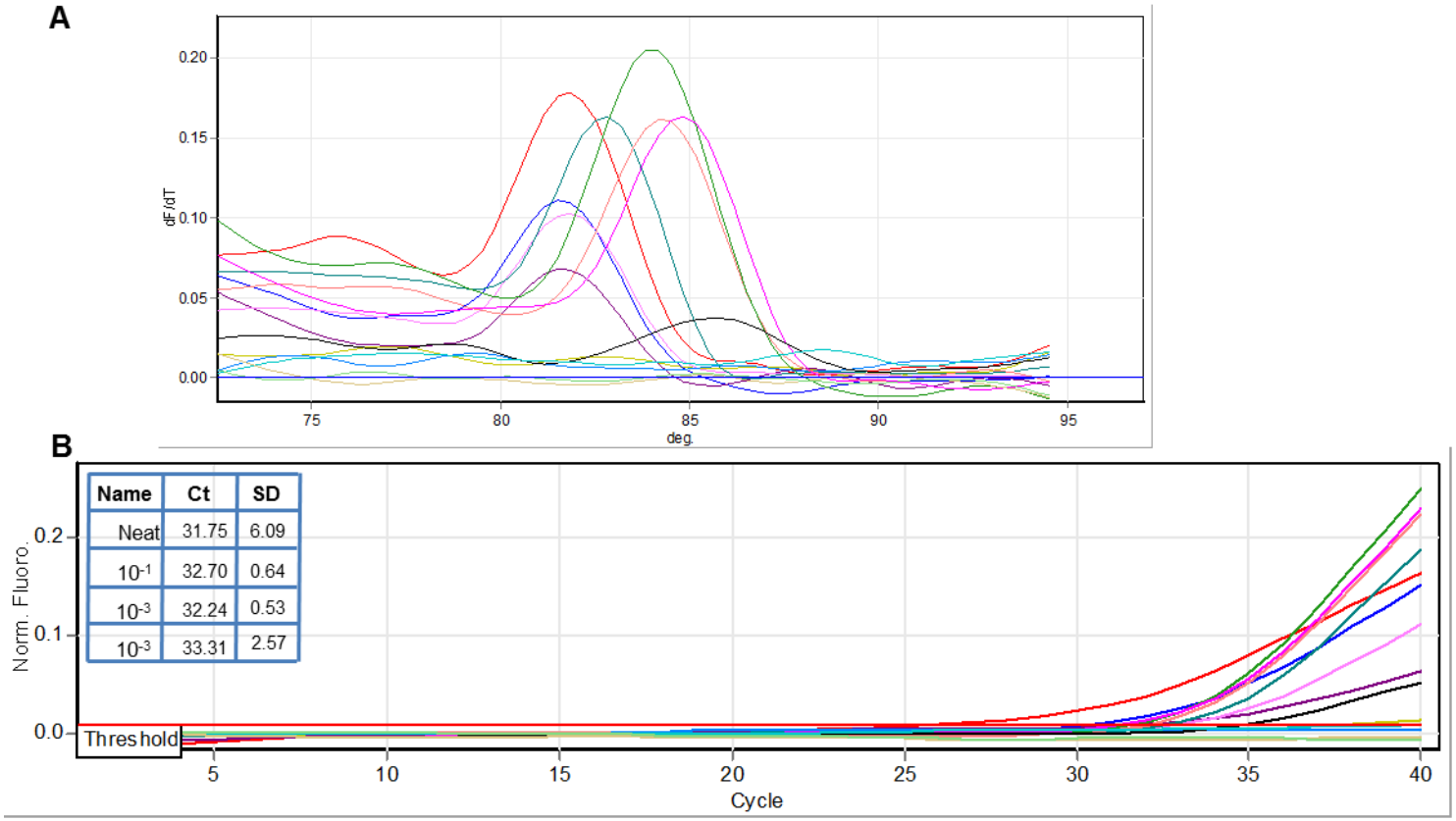


Figure 5.10: EP_4 mRNA Expression in control samples obtained from OHSC

The melt curve for this PCR revealed multiple peaks (***A***). EP_4 mRNA was detected following 31 cycles of PCR for neat samples. Following serial dilution of cDNA EP_4 mRNA was detected between 31-33 cycles (***B***).

Control samples were ran with each PCR reaction these included the following; a positive control i.e. no template, a negative control i.e. no reverse transcriptase. The results showed no amplification indicating that there was no contamination of genomic DNA. These findings suggested that the primer may not be specific for EP_4.

It was noted that the initial primers designed only bound to the cDNA corresponding to one mRNA variant of EP_4. It is possible that this could have resulted in one primer binding to both forms, and resulting in variable PCR products. New primers were designed which spanned an intron-exon region common to both variants. The new primer pairs was tested and revealed consistent melt curves showing specificity of the primer (***Figure 5.11***). The samples were prepared at a lower dilution (1 in 2), however, the cycle threshold (C_t) values for diluted samples were still relatively high (26-29) even though the same mass of template was used for the PCR reaction (***Figure 5.11***). Due To this primer efficieny could be calculated and therefore a standard curve could not be obtained for the EP_4 primers.

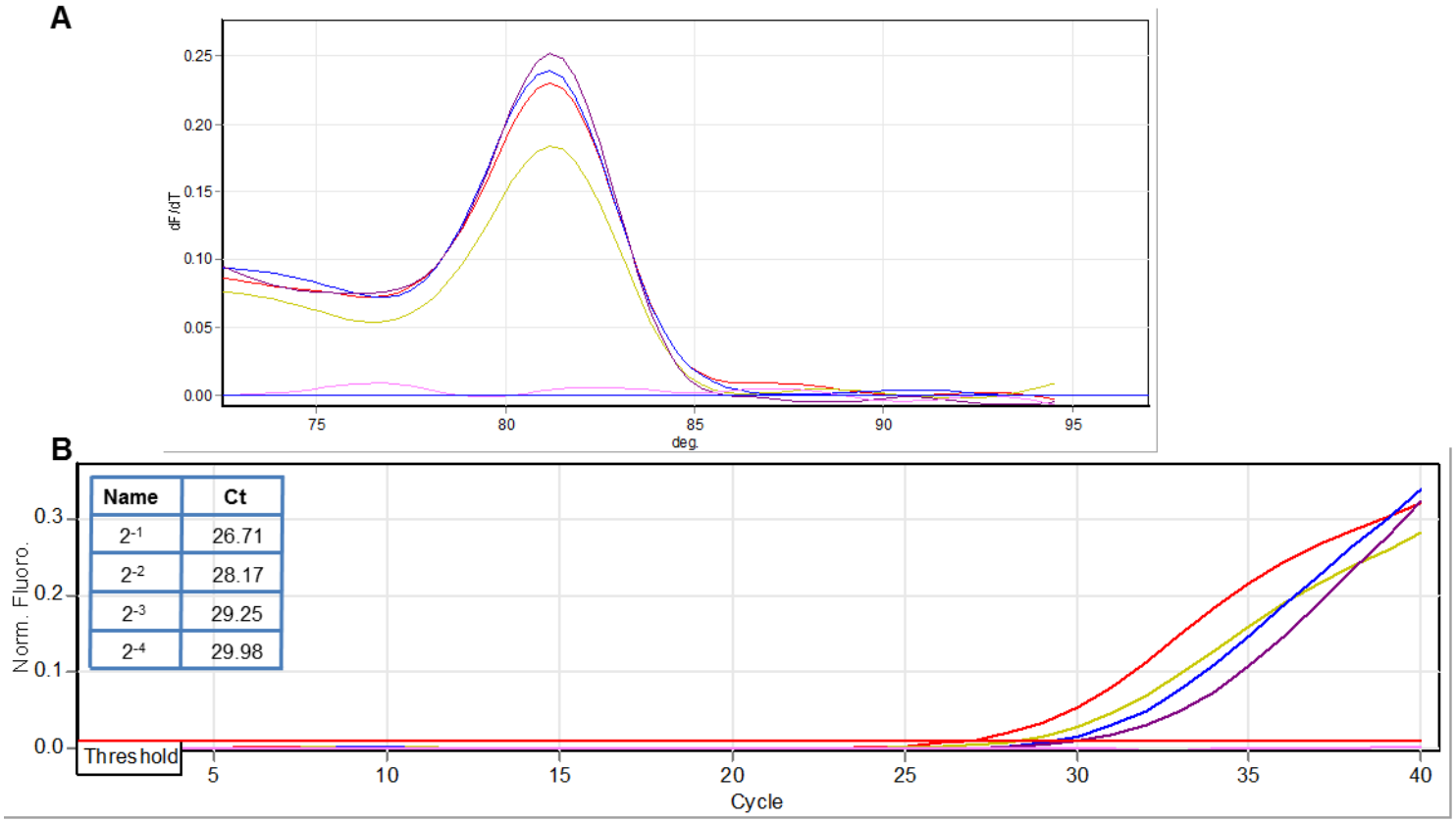


Figure 5.11: EP_4 mRNA Expression in control samples obtained from OHSC

The melt curve for the PCR revealed a single peak indicating specificity of the EP_4 primer (***A***). EP_4 mRNA was detected following 26 cycles of PCR for neat samples. Following serial dilution of cDNA EP_4 mRNA was detected between 26-29 cycles (***B***).

5.3.4.3 EP_4 receptor expression at different time points of reperfusion

EP_4 mRNA expression was assessed using samples from normoxic OHSCs and those collected at 1, 4 and 24 hours of reperfusion following 2 hours of OGD (see chapter 2). The expression of EP_4 was normalised to the reference gene (RPL44). The initial results indicate that EP_4 mRNA expression was upregulated at 1 hour post OGD by approximately 4 folds (***Table 5.4***). Whilst at 4 hours of reperfusion, there appears to be no change in relative EP_4 expression compared to the expression of the reference gene. At 24 hours post OGD there was small upregulation in EP_4 expression by approximately 1 fold.

	Experiment 1			**Experiment 2**			**Average (fold change)**
	Δ Ct	ΔΔ Ct	2^-ΔΔ Ct	Δ Ct	ΔΔ Ct	2^-ΔΔ Ct	
control	7.01			6.01			
1 hour	4.9	-2.1	4.3	4.1	-1.9	3.7	4.02
4 hour	7.8	0.8	0.6	5.9	-0.1	1.08	0.6
24 hour	5.7	-1.3	2.4	6.4	0.4	0.8	1.6

Table 5.4 Relative changes in EP_4 mRNA expression at 1, 4 and 24 hours post OGD.

EP_4 expression is dynamically regulated following OGD in OHSCs. Relative quantification of EP_4 expression was detected in normoxic controls and 1, 4 and 24 hours post OGD. All samples were normalised to RPL44. EP_4 expression was upregulated by 4 folds at 1 hour post OGD. At 4 hours post OGD there were no changes in EP_4 expression and at 24 hours EP_4 expression was upregulated by 1 fold.

5.4 Discussion

5.4.1 Summary of results

Histological staining results show changes in cell density in ischaemic brain tissue, as detected using cresyl violet staining. EP_4 receptors expression was detected in DRG and normal brain tissue using immunofluorescent staining. However, DAB staining revealed weak EP_4 labelling in DRG and an absence of positive labelling for EP_4 in normal brain tissue. Immunofluorescent labelling in ischaemic brain tissue revealed an absence of EP_4 positive labelling. Real time PCR data show that EP_4 gene expression is expressed at low levels due to this a standard curve could not be established. However, EP_4 expression was assessed in normoxic controls and 1, 4 and 24 hours post OGD. The results were normalised to the reference gene and the initial data reveal upregulation of EP_4 expression at 1 hour post OGD.

5.4.2 Changes in cell density following ischaemic injury

Visualization of the cytoarchitecture of the brain is important particularly when investigating cellular loss in infarcted regions resulting from injury. Histopathological assessment using Cresyl violet staining is a commonly used method to assess tissue injury following ischaemia (Vogel, Möbius & Kuschinsky, 1999; Popp, Jaenisch, Witte & Frahm, 2009). Nissl is abundantly expressed in the endoplasmic reticulum of cells particularly neurons but also stains glia (Gittins & Harrison, 2004). Following ischaemia, the Nissl substance disintegrates in injured cells and redistributes around the periphery of the cell body. The experiments presented in this chapter revealed a loss of cell bodies in the region of the brain affected by ischaemia as observed by a lack of

Nissl staining. The MCA supplies blood flow to the cortical and striatal regions of the brains, as a result the severe loss of cell bodies was observed in these regions. This pattern of cellular loss was not evident in the cortex or striatum in the contralateral hemisphere which was not subjected to ischaemic injury.

5.4.3 Specificity of the EP_4 antibody

The EP_4 receptor antibody used in the experiments presented here was previously characterised by a former PhD student (Hammond, 2006). A number of antibodies for EP_4 were tested in human embryonic kidney (HEK) cell lines expressing functional EP_4 receptor. From the four EP_4 antibodies tested, the Cayman antibody showed specificity for EP_4 protein. Western blot of cell lysates prepared from HEK cells expressing EP_4 revealed an immunoreactive band at approximately 53 kDa (molecular weight of the EP_4 receptor is 53kDa). Western blots were also carried out using cell lysate from Chinese hamster ovary (CHO) cells expressing EP_1, EP_2 and EP_3 and also wild type HEK cells. The results revealed no immunoreactive bands in wild-type CHO cells or HEK cells indicating that the antibody showed specificity for EP_4 protein. Immunohistochemistry was carried out in CHO cell line expressing EP_1, EP_2 and EP_3, along with wild type and EP_4 expressing HEK cells. The results showed no EP_4 positive labelling in EP_2 or EP_3 expressing cell lines or wild type HEK cells. In HEK cells expressing EP_4, positive EP_4 labelling was observed which was largely restricted to the plasma membrane of the cells. In addition to western blot and immunohistochemistry analysis, further experiments whereby the EP_4 antibody was pre-incubated with the control peptide reduced the intensity of EP_4 positive staining in HEK cells expressing EP_4. These results indicate that the EP_4 antibody recognises the peptide against which it was raised and not closely related receptor subtypes. Collectively, the above findings

confirm the specificity of the Cayman EP_4 antibody used in the experiments reported in this thesis.

The EP_4 antibody was optimised in DRG tissue which has previously shown positive staining for EP_4 receptor protein. The results in this chapter show positive labelling for EP_4 in rat DRG sections. The staining was observed in small neuronal cell bodies whilst larger cells remained unstained. This is consistent with the findings from previous experiments conducted in the lab which demonstrated that the EP_4 receptor co-localises with markers of nociception in the rat DRG, indicating its role in PGE_2 mediated pain signalling. Further experiments were conducted using non-fluorescent DAB staining. These experiments confirmed EP_4 receptor expression in the rat DRG were localised to small neuronal cell bodies.

5.4.4 EP_4 receptor expression in brain tissue

Immunofluorescent staining in normal brain tissue revealed weak positive staining for EP_4 protein in the cortex, with stronger fluorescent signal observed in the hippocampus and hypothalamus. These results are consistent with findings previously reported by Zhang & Rivest (2000) and Li et al. (2008). The EP_4 immunofluoresecent labelling was predominantly localised at the plasma membrane of neuronal cell bodies, a similar pattern of expression has been reported previously (Li et al., 2008). EP_4 positive labelling was also observed in axonal tracts indicating that these receptors are transported to other neighbouring cells. This pattern of staining was not observed in sections where the primary antibody was omitted indicating no non-specific binding of the secondary antibody.

Ischaemic brain tissue sections were processed for immunofluorescence labelling of EP_4. Unfortunately, it was not possible to observe positive EP_4 expression in the ischaemic tissue, which could be due to a number of technical reasons. It was difficult to maintain tissue integrity in sections from ischaemic brains due to the loss of structural integrity in the core of the ischaemic insult. To overcome this problem the thickness of the sections was increased to 20 µM, unfortunately this didn't show much improvement in integrity. In order to visualise EP_4 receptor expression we attempted to use a non-fluorescent staining protocol for EP_4 staining. Non-fluorescent DAB staining revealed positive staining for EP_4 protein in DRG tissue. However, the intensity of staining was relatively weak and was not strengthened by increasing the incubation time for the DAB reaction. Although we applied the same non-fluorescent staining protocol that was successful in obtaining positive staining in DRG tissue surprisingly, we could not obtain any positive labelling for EP_4 in normal brain tissue. Thus, we did not apply this protocol to ischaemic brain sections. There could be several reasons why EP_4 positive labelling was not observed using the fluorescent staining protocol which showed positive labelling for EP_4 in DRG and normal brain tissue. Firstly, changes in cell density following ischaemic stroke resulted in significant loss of cells in the ischaemic core as observed by the lack of Nissl staining. This suggests that neuronal cells potentially expressing EP_4 receptors are absent as a result of the ischaemic insult. This has previously been reported by Li et al. (2008), where EP_4 receptor expression was absent in the ischaemic zone. Furthermore, the samples from ischaemic brain tissue used to determine EP_4 receptor expression were collected 24 hours post stroke. It is possible that at this time point the expression of EP_4 is absent. In support of this theory, a series of experiments by Li et al. (2008) showed that neuronal expression of EP_4 is lost in the ischaemic core by up to 4 h of reperfusion and by 24 h vascular

expression of EP_4 also disappeared. To address this issue further experiments using samples taken at different time points following stroke may provide a clearer understanding of EP_4 expression following ischaemic injury.

The EP_4 antibody used in these experiments has shown to work well in DRG tissue in both fluorescent and non-fluorescent staining protocols. The previous work conducted in the lab has confirmed the specificity of the antibody using western blot analysis and using the control peptide for EP_4 which confirmed the specificity of the Cayman EP_4 antibody. However, the results obtained from brain tissue were not always reproducible. This could be due to the relatively low expression of EP_4 in the brain, indicating that further experiments testing a number of different EP_4 antibodies would be useful to identify a more sensitive antibody which is successful in detecting low level expression.

5.4.5 EP_4 gene expression using real time-PCR

Real time-PCR is a suitable method to compare mRNA levels of a target gene between different experimental conditions and was considered suitable for assessing the experimental aims of this project. Previous studies using immunohistochemistry have reported dynamic regulation of EP_4 receptor during reperfusion (Li et al., 2008). Therefore the experiments in this chapter aimed to assess the changes in EP_4 gene expression following OGD in OHSC using real time-PCR.

Relative expression of EP_4 was determined and normalised to the expression of RPL44 for each condition. The preliminary results reveal that EP_4 receptor expression is upregulated in OHSCs at 1 hour post OGD. There appear to be no changes in EP_4 expression at 4 and a small upregulation at 24 hours post OGD. Previous studies have shown a downregulation of EP_4 expression in neurons 4 hours post OGD but marked

expression in endothelial cells (Li et al., 2008). This suggests differential EP_4 expression in neurons and endothelial cells. Future studies investigating changes in EP_4 expression in neurons and endothelial cells at different time points of reperfusion would be of interest.

In order to determine the expression of a target gene, validation through internal controls and standard curves is required. In addition, a suitable reference gene which does not change in its expression between different experimental conditions is required in order to determine a ratio of the difference between the target gene and the reference gene. Initially, GAPDH was selected as a reference gene as it has been a routine selection for an endogenous control in many murine expression studies and has previously been used in OGD models (Rawal, Muddeshwar & Biswas, 2004). However, there is some evidence that its expression can be variable under certain experimental conditions such as ischaemia (Gubern et al., 2009). Therefore, for this work RPL44 was selected as a suitable reference gene and has previously demonstrated robust expression in models of neurodegenerative disorders using OHSCs by colleagues at the University. To our knowledge there is no current research indicating that RPL44 expression is variable following OGD, however, this would need to be validated in future experiments.

Due to insufficient expression levels, primer efficiency for EP_4 primers could not be verified. This would need to be validated by the construction of a standard curve in order to calculate primer efficiency. Due to low level of expression in the samples, they could not be diluted to construct a standard curve which requires a minimum of 4 points. In order to increase the concentration of the template, the purified amplicon product could be used to generate dilutions which would yield sufficiently detectable level to create a standard curve. Furthermore experiments using RNA extracted from

ischaemic brain tissue would provide a more accurate representation of EP_4 gene expression following ischaemic injury. However, it could prove difficult to isolate the specific region of the stroke from a whole brain sample. Further studies are required which involve investigating EP_4 gene expression in tissue which highly expresses EP_4 such as the rat ileum in order to determine the efficiency of the reaction obtained by a standard curve.

Other molecular biological techniques used to detect protein of interest include western blot analysis. This method involves the use of gel electrophoresis in order to separate proteins these are then transferred on to a membrane and stained with the antibody specific for the protein of interest. This technique is useful for identifying a protein of interest, however, any differences in expression between experimental conditions is difficult to assess and quantify using this technique. Although the EP_4 receptor has been detected in DRG and spinal cord tissue using northern blot analysis (Donaldson et al., 2001), previous work in the lab was unsuccessful in detecting EP_4 protein using western blot in DRG tissue. In order to circumvent this problem immunopurification of the tissue homogenates may be required in order to enrich the concentration of EP_4 receptor protein. This would also be necessary to detect EP_4 protein in brain tissue as shown by Nakamura et al. 1999 who used this procedure in order to detect EP_3 receptor protein in brain tissue by western blotting.

5.4.6 Conclusions

The results reported in this chapter confirm findings from previous studies which have demonstrated EP_4 receptor expression in the hippocampus, cortex and hypothalamus (Zhang & Rivest, 2000 & Li et al., 2008). Future studies are warranted in order to determine changes in EP_4 receptor expression following ischaemia using optimized

protocols for immunohistochemistry and real time PCR or alternative approaches, as discussed above.

Chapter 6

Conclusions and future work

Cerebral ischaemia results in upregulation of COX-2 expression in neurons, glia, vascular cells and inflammatory cells which invade the ischaemic brain (Nogawa et al., 1997; Iadecola et al., 1999). Pharmacological inhibition and genetic deletion of COX-2 attenuated injury in both *in vitro* and *in vivo* models of ischaemia and excitotoxicity (Candelario-Jalil & Fiebich, 2008; Iadecola & Gorelick, 2005). Although these findings present COX-2 as an appealing target for stroke therapy, long term clinical trials have reported serious cardiovascular complications with selective COX-2 inhibitors (Topol, 2004). These side effects were due to an imbalance between the synthesis of prostanoids, particularly PGI_2 and $TXA_{2,}$ which impaired the balance between pro- and anti-thrombotic components of the clotting system. Consequently, identifying new targets for therapy, associated with fewer unwanted side effects, is imperative for a more successful approach to treating clinical ischaemic stroke. One possible route towards better therapeutic strategies is to target downstream PGE_2 which is well known for its role in inflammatory processes. Therefore the aim of this thesis was to investigate the role of PGE_2 receptor EP_4 and its role in ischaemic injury.

The experiments presented in this thesis have demonstrated that selective activation of the EP_4 receptor confers neuroprotection using both *in vitro* and *in vivo* models of ischaemia. Further to this, the protective effect upon treatment with a selective EP_4 receptor agonist was at a level similar to that observed with a selective COX-2 inhibitor but presumably without the detrimental side effects of COX-2 inhibitors. These findings provide initial proof of principle that EP_4 receptor activation is neuroprotective following ischaemic injury.

The treatment of cerebral ischaemia in clinical settings has been rather disappointing and unsuccessful, suggesting that there is a great need for researchers to evaluate potential neuroprotectants in stroke models which are a closer representation to the clinical situation. The failure to replicate the neuroprotective effects of agents in clinical stroke is likely to be due to incompatibilities of the experimental models. Other reasons which are associated with the failure of neuroprotective targets in stroke is largely due to the design of clinical trials such as the timing of administration, dose of the drug, length of ischaemic exposure, species, gender differences, age and comorbidities (Traystman, 2003). This study used the MCAO model of focal cerebral ischaemia which is considered the closest representation to human stroke and therefore these initial results are promising (Hossmann, 1998). In addition, the EP_4 agonist was administered post stroke and therefore is clinically more relevant, although further experiments designed to determine the therapeutic time window for treatment would be desirable. The work presented in chapter 4 assessed lesion volume at 48 hours and neurological function up to 48 hours following MCAO, thus we cannot discount the possibility that treatment with the EP_4 agonist may have a further delayed effect on functional outcome. Further studies investigating whether treatment with the EP_4 agonist improves neurological function post 48 hours would broaden our understanding of this drug in ischaemic injury.

Additional experiments would prove beneficial in order to determine whether clinical investigation is warranted. This includes an *in vivo* dose response study which would provide information of the optimal dose required for neuroprotection. In addition to this, experiments using HPLC and microdialysis would be useful to determine the concentration of the EP_4 agonist which enters the brain. The results from these experiments will be useful from a clinical perspective, providing information on the

possible route of administration and the optimal dosage required in order to achieve a beneficial effect in potential clinical trials. All of these are important considerations when assessing the translational properties of this drug for clinical use.

The findings reported in this thesis and the study reported by Liang at al. (2011), strengthen the hypothesis that aspects of prostaglandin signalling are beneficial following ischaemic injury. Collectively, these reports have demonstrated neuroprotection following selective activation of the EP_4 receptor in both *in vitro* and *in vivo* ischaemia. Further experiments investigating the potential mechanisms of EP_4 mediated neuroprotection would prove useful in order to take these findings to a new level in the hope for progressing to clinical stroke trials.

The EP_4 antibody which was previously characterised was used to determine the expression of EP_4 in mouse brain slices. Positive labelling of EP_4 in normal brain tissue was detected in the cortex and the hypothalamus, with greater abundance in the hippocampus. These findings are consistent with previous reports (Zhang & Rivest, 2000 & Li et al., 2008). Whilst EP_4 expression was detected in normal brain tissue, it could not be detected in ischaemic tissue. This could be due to the changes in the brain following ischaemia and therefore future experiments testing a number of different EP_4 receptor antibodies may provide some insight. Further work is warranted in order to determine changes in EP_4 receptor expression following ischaemia using optimized protocols for immunohistochemistry and real time PCR. Nevertheless it has been demonstrated that EP_4 receptor expression is significantly upregulated in neuronal and endothelial cells compared to astrocytes and microglia following ischaemia (Liang et al., 2011). This suggests that the neuroprotective effects of EP_4 are likely to be mediated through neuronal and endothelial signalling. Endothelial EP_4 is associated with vascular protection by increasing cerebral blood flow to the ischaemic region. The

neuroprotective mechanisms of neuronal EP_4 remain to be determined; its proposed mechanism of neuroprotection may involve gene regulation. Previous research has demonstrated that the EP_4 receptor exerts its anti-inflammatory effects through down regulation of pro inflammatory gene expression (Shi et al., 2010). Given that both the EP_2 and EP_4 receptors couple to G_s protein, increasing cAMP levels and activating protein kinase A (PKA), it is possible that these receptors' neuroprotective functions are determined by the cAMP-PKA pathway. McCullough et al (2004) demonstrated that inhibiting the PKA pathway reversed the protective effect of EP_2 receptor activation. As discussed in chapter 3, studies investigating the signalling mechanisms of these receptors have reported that the EP_4 receptor is capable of activating additional signalling pathways which are cAMP independent (Regan, 2003). Therefore the cellular mechanisms involved in EP_4 mediated neuroprotection still remain to be determined and warrant further investigation.

Neuroprotection refers to a treatment which intends to reduce cell death, restore blood flow or improve functional outcome which is evaluated in reference to how well it preserves brain tissue and function (O'Collins et al., 2006). Treatment with a selective EP_4 receptor agonist has demonstrated a decrease in cell death in both *in vitro* and *in vivo* models of stroke. Following 1 hour of MCAO, administration of the EP_4 agonist prevented the decline in neurological functioning compared to the vehicle treated group. In addition Laing et al. 2011 reported vasodilation following EP_4 receptor activation which subsequently increased the blood flow to the ischaemic region. Collectively these results indicate that selective activation of the EP_4 receptor meets the complete criteria of neuroprotection by target multiple aspects of damage and therefore is an appealing target for stroke therapy.

Prostaglandin receptors couple to opposing second messenger systems indicating that both neuroprotective and neurotoxic prostaglandin signalling pathways may be active in cerebral ischaemia. A better strategy towards neuroprotection may be to activate receptors which mediate neuroprotection (EP_2/EP_4) and inhibit receptors which mediate the toxic effects (EP_1/EP_3). The work in this thesis focussed on the role of PGE_2 receptor EP_4. However, research is now emerging which indicates that other prostanoids and their receptors may also be involved in the inflammatory injury following ischaemia. Accumulating research is beginning to indicate that prostanoid receptors which cAMP levels mediate neuroprotection (Saleem et al., 2007; Saleem et al., 2010). Whilst, receptor subtypes which increase intracellular Ca^{2+} increase toxicity (Kim et al., 2012; Matsuo et al., 1993). These findings are interesting as they highlight that it is imperative to target specific receptors downstream of COX-2 in order to achieve neuroprotection whilst limiting adverse effects.

The work in this thesis has demonstrated that the EP_4 receptor is involved in mediating neuroprotection following *in vitro* and *in vivo* ischaemia and provides a novel therapeutic target for stroke. Further work is now required in order to determine whether a selective agonist of the EP_4 receptor is a suitable candidate for clinical stroke trials.

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