

**An Investigation of Chemokine
Expression and Modulation Following
Traumatic Brain Injury**

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Declaration

The experiments described in this thesis were the unaided work of the author except where acknowledgement is made by reference. No part of this work has previously been accepted for any other degree nor is any part of it being submitted concurrently in candidature for another degree. All experiments were performed within Astellas CNS Research in Edinburgh, University of Edinburgh.

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Abstract

Over the last 20 years, advances in our understanding of the pathophysiology of severe traumatic brain injury (TBI) and in particular the contribution of secondary injury to poor outcome, has served to improve clinical management and reduce the mortality in these patients. However despite many promising preclinical studies there has been a failure to introduce a specific therapeutic intervention to further improve outcome. Inflammation, with cytokine release and leucocyte infiltration, is a significant secondary injury processes. However the inflammatory response to brain injury, its control and modulation remain incompletely described.

Chemotactic cytokines, known as chemokines, are mediators of leucocyte recruitment and activation. Expression of chemokines and the resultant recruitment of leucocytes into the brain are generally thought to be integral to the enlargement of cerebral contusions which accompany clinical deterioration following severe TBI. Previous studies indicated that the main neutrophil chemokine, macrophage inflammatory protein-2 (MIP-2/CXCL2) and the monocytic chemokine, monocyte chemoattractant protein-1 (MCP-1/CCL2) are derived from glial. However the origin of these chemokines following TBI, has not been established. Furthermore, little is known about the modulation of these chemokines: The relationship of serum levels of pro-inflammatory mediators such as the human neutrophil chemokine, interleukin-8 (IL-8/CXCL8; a functional homologue of MIP-2/CXCL2), MCP-1/CCL2 and soluble interleukin-6 receptor (sIL-6R) to contusion enlargement has not been investigated.

In this thesis, I investigated chemokine expression and modulation both in *in-vitro*, *in-vivo* models and in a clinical study. Initially, I compared chemokine expression in rodent and human glial cell cultures and investigated the modulation of chemokine expression by interleukin-6, the glucocorticoid dexamethasone and the immunosuppressant agent FK506. To investigate chemokine expression *in-vivo* I established the rat lateral fluid percussion injury (LFPI) model of TBI and measured MIP-2/CXCL2 and MCP-1/CCL2 expression in the brain following injury. Inhibition of this expression by dexamethasone and FK506 was then investigated. To identify the cellular source of chemokine expression I developed an immunohistochemical protocol for MIP-2/CXCL2 and MCP-1/CCL2. Finally, in a clinical study of serum chemokine and sIL-6R concentrations after severe TBI, I examined the relationship between these inflammatory mediators and clinical deterioration.

Rat glia (microglia and astrocytes) produce chemokines with a response profile that was qualitatively similar to that of human derived cells. These chemokines were increased in the ipsilateral hemisphere following TBI. Surprisingly, immunohistochemical studies identified marked chemokine expression localised to cells with the morphology of degenerating neurones in contused tissue, rather than in glia. Furthermore, while dexamethasone significantly inhibited both MIP-2/CXCL2 and MCP-1/CCL2 expression in a rat astrocyte derived cell line, only MCP-1/CCL2 expression was reduced by steroid treatment *in-vivo*. Clinically, serum IL-8/CXCL8, MCP-1/CCL2 or sIL-6R were not significantly different in patients that deteriorated due to contusion enlargement from those that remained stable. However these inflammatory mediators were significantly increased in those patients that died. These studies indicate that astrocytes may not be the major source of chemokines following TBI and highlight the need for caution when inferring pathophysiological mechanisms from *in-vitro* studies.

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Dedication

To my wife Deborah
and our children
Verity, Jonathan, Bronte and Christopher.

1. Introduction

1.1. An Overview of Traumatic Brain Injury

1.1.1. The Significance of Traumatic Brain Injury

Injury to the brain is common, associated with high mortality and survivors frequently suffer from significant residual disability. In Britain approximately 1 million head injuries per year present to hospital ($1770/10^5$ of the population). Of these 170,000 are admitted ($300/10^5$ of the population)¹ (Jennett & MacMillan, 1981) and 3,500 are severe requiring management in intensive care (Helmy et al., 2007). Traumatic brain injury (TBI) is the leading cause of death in individuals under the age of 45 years in Europe and the United States (McIntosh et al., 1996). Of the patients with severe traumatic brain injuries approximately 50% of these will be severely disabled, in a vegetative state or will die (Langfitt & Gennarelli, 1982).

The number of cases of TBI presenting to hospital in the United States of America is 1,400,000 annually ($504/10^5$ of the population)² (Langlois et al., 2006). This seems low compared to the British data but probably reflects differences in data collection terms, such as head verses brain injury, access to health care, and a reduction in the incidence of TBI over time. The Jennett study is based on data from the 1970s whilst that of Langlois describes data collected between 1995 and 2001. The introduction of compulsory crash helmet and seat belts wearing, the fitting of airbags and discouraging drink driving have reduced the incidence of head injury due to road traffic accidents. These were the most frequent cause of head injury (Fearnside & Simpson, 2005). None the less, to put the incidence of TBI into context with other pathologies, it is 6 times greater than that of new cases of multiple sclerosis, human immunodeficiency virus infection, spinal cord injury and breast cancer combined (Brain Injury Association of America, 2004). More than 50,000 Americans die each year as a result of TBI with a further 80,000 suffering long term disability (Langlois et al., 2006) This results in an estimated cost of \$60 billion per annum (Brain Injury Association of America, 2004).

¹ Based on a population of 56 million between 1971 and 1981 (Hicks & Allan, 1999).

² From a population of 281 million in 2000 (Evans et al., 2002).

1.1.2. Focal and Diffuse Traumatic Brain Injuries

The biomechanics of TBI are complex and result in specific patterns of pathology. Trauma to the brain occurs when excessive force is applied to it. This mechanical loading can be delivered when an object strikes the skull (impact loading) or when the head moves violently due to impact with another part of the body (impulsive loading). Impact to the skull results in contact and inertial loading (acceleration), whilst impulsive loading results in only inertial effects (Figure 1.1). Contact forces bend and often fracture the skull, deforming the underlying brain and result in focal lesions such as laceration, contusion, intracerebral haematoma, sub and extradural haematomas. Inertial loading can be translational or rotational. In translational acceleration the whole brain moves relative to the inner bony surface of the cranial cavity resulting in contusion, intracerebral haematoma and subdural haematoma. This relative movement probably accounts for the frequency of contusions in the inferior frontal lobes and inferolateral temporal lobes and poles. In these regions, the brain would come into contact with the irregular surface of the anterior and middle cranial fossae. Rotational acceleration produces widespread tissue strains throughout the brain resulting in diffuse injury to axons, gliding contusion and subdural haematomas (McIntosh et al., 1996, Blumbergs, 2005).

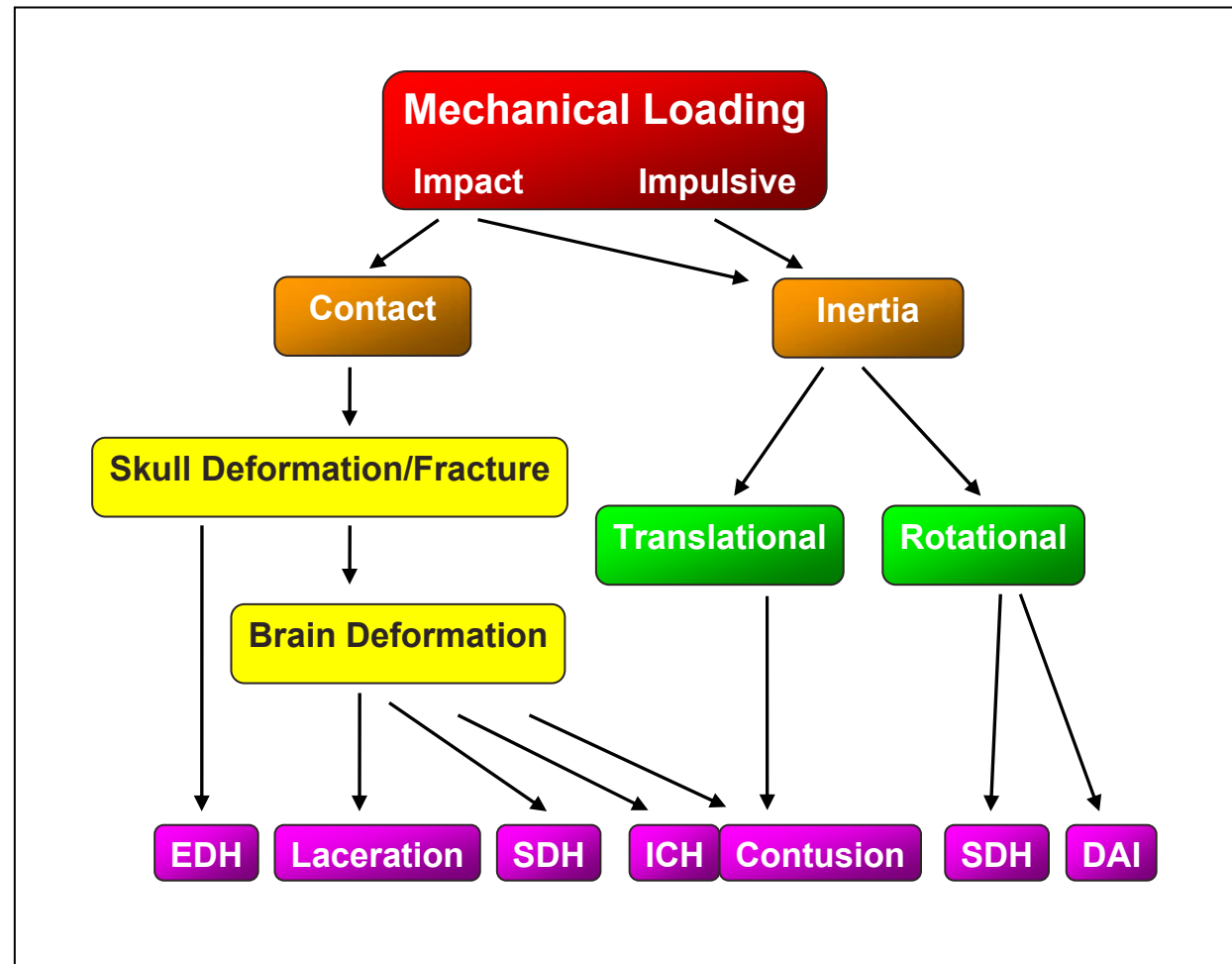


Figure 1.1. Mechanical Loading of the Skull will Result in Contact or Inertial Injuries to the Brain.

Contact forces lead to focal injuries and inertial forces lead to diffuse axonal injury and focal lesions. Extradural haematoma (EDH), subdural haematoma (SDH), intracerebral haematoma (ICH), diffuse axonal injury (DAI). Modified from McIntosh et al., 1996.

1.1.3. Primary vs. Secondary Injury

Trauma to the brain causes an immediate biomechanical disruption of neural pathways and vasculature producing contusions, lacerations, intracranial haematomas and axonal dysfunction. It is now clear that the extent of injury is not limited to that caused by the primary insult but will always be followed by secondary neuronal damage (Doyle & Gupta, 2000), the magnitude of the injury increasing with time (Povlishock & Christman, 1995, MacGregor et al., 2003). This is illustrated clinically by the large numbers of patients that talk, implying preserved higher function and then die as a result of their brain injury (Reilly et al., 1975, Miller, 1992, Reilly, 2001). Common mechanisms for deterioration include contusion enlargement and infarction of cerebral tissue.

1.1.4. Hypoxia and Chemically Mediated Secondary Injury

Trauma to the brain is associated with harmful alterations in neurochemistry occurring either as a direct consequence of trauma or secondary to hypoxia. Indeed cerebral tissue hypoxia is regarded, by many, as central to the progression of brain injury, with the induction of reactive cascades of chemical mediators each capable of adversely prejudicing neuronal survival (Braugher & Hall, 1989, McIntosh et al., 1996, Sharkey et al., 1997). Such chemical insults include excitatory amino acid release, derangement of calcium homeostasis, free radical production and activation of the inflammatory process (Menon & Matta, 2000) (Figure 1.2).

1.1.5. Enlargement of Cerebral Contusions and the Secondary Injury Process

Cerebral contusions are focal injuries occurring when mechanical forces disrupt small blood vessels, neurones and glia. Bleeding from damaged vessels is common and may be macroscopic, microscopic but can also be absent (Blumbergs, 2005). An intracerebral haematoma may be viewed as a more extensive form of cerebral contusion (McIntosh et al., 1996). Interrelated insults within the contused tissue such as hypoxia, the generation of reactive mediators, haemorrhage, leucocyte infiltration and oedema are thought to result in the enlargement of contusions resulting in further compromise of the surrounding brain and often clinical deterioration.

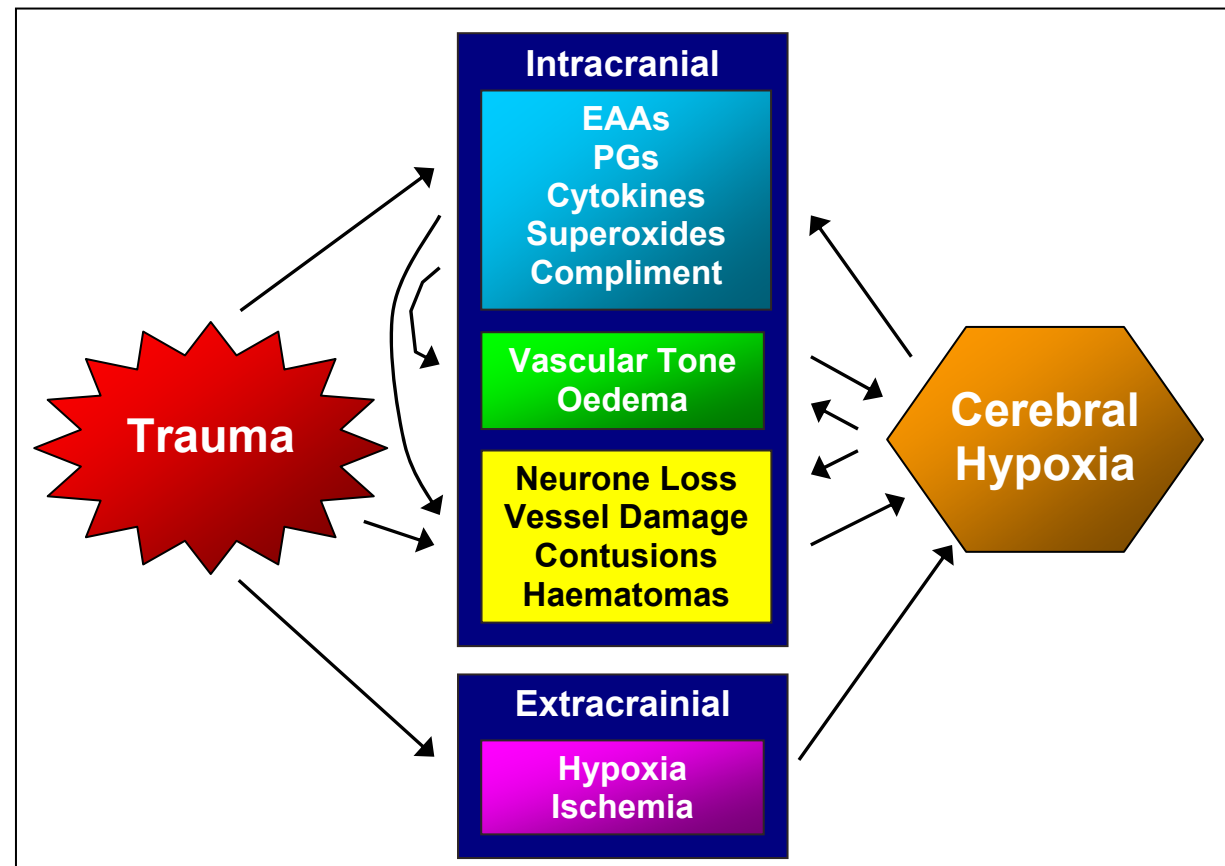


Figure 1.2. Acute Brain Injury Initiates Events Leading to Secondary Neuronal Loss.

Trauma causes early neuronal loss and the derangement of the neurochemical environment. This derangement may occur directly or as a consequence of cerebral hypoxia due to the occurrence of oedema, space occupying lesions, changes in cerebral vascular tone and the effects of extracranial injuries. Hypoxia can cause direct neuronal damage or contribute to secondary neuronal injury via the activation of diverse chemical systems, such as inflammation. Excitatory amino acids (EAA), prostaglandins (PG).

1.2. Hypoxia and Secondary Injury

Hypoxia is the term used to define an inadequate oxygen supply for tissue respiration. It can be subdivided into 4 types. Hypoxic hypoxia or hypoxaemia occurs when there is an inadequate tension of oxygen in the blood. Anaemic hypoxia is the condition of an adequate oxygen tension in the blood but the reduced availability of haemoglobin to carry oxygen and therefore a reduced oxygen content of the blood. Ischaemic or stagnant hypoxia occurs when perfusion of the tissue with blood is insufficient. Finally histotoxic or cytotoxic hypoxia is present when the tissues fail to utilise oxygen despite adequate oxygen tension, carrying capacity (available haemoglobin) and perfusion (Yentis et al., 2000).

Each of these types of hypoxia can be present after trauma to the brain, each with multiple causes. Furthermore tissue hypoxia can also result from the increased utilisation of oxygen within the brain if the supply is relatively impaired.

There are tremendous similarities in the pathophysiology of TBI and primary ischaemic cerebral injury. Therefore many important lessons can be gained from the stroke literature which will be discussed were appropriate throughout this thesis.

1.2.1. Measuring Cerebral Blood Flow and Oxygen Consumption

The rate of cerebral oxygen consumption, more commonly referred to as the cerebral metabolic rate for oxygen (CMRO₂), is the product of cerebral blood flow (CBF) and the difference between the arterial and jugular venous oxygen content of the blood leaving the brain (AVDO₂).

$$\text{CMRO}_2 = \text{CBF} \cdot \text{AVDO}_2 \quad (1).$$

The content of oxygen in the blood is determined by simultaneously sampling from both an arterial catheter and a central venous catheter retrogradely fed into the jugular bulb, which receives blood leaving the brain. The degree to which haemoglobin is saturated with oxygen, as a percentage, is

determined in these samples by a co-oximeter. An estimate of the oxygen content of the samples can then be calculated. Fully saturated haemoglobin binds 1.39ml of oxygen/gram of haemoglobin³. The small amount of dissolved oxygen in the blood is usually ignored⁴ (West, 2005). For example in a normal arterial blood sample with a saturation of 97% and a haemoglobin concentration of 140g/l, the arterial oxygen content (CaO₂) is given by;

$$\text{CaO}_2 = \text{SaO}_2 \times 1.39 \times \text{Hb} = 189\text{ml/l} \quad (2).$$

$$\text{CaO}_2 = 0.97 \times 1.39 \times 140 = 189\text{ml/l}$$

As the haemoglobin concentration (Hb) is the same in arterial and venous blood, equation 1 can be simplified.

$$\text{CMRO}_2 = \text{CBF} \cdot \text{AVDO}_2 \quad (1).$$

$$\text{CMRO}_2 = \text{CBF} \cdot (\text{CaO}_2 - \text{CjO}_2) \quad \text{from (2)}$$

Where CjO₂ is the jugular venous oxygen content.

$$\text{CMRO}_2 = \text{CBF} \cdot (\text{SaO}_2 \cdot 1.39 \cdot \text{Hb}) - (\text{SjO}_2 \cdot 1.39 \cdot \text{Hb})$$

$$\text{CMRO}_2 = \text{CBF} \cdot 1.39 \cdot \text{Hb} \cdot (\text{SaO}_2 - \text{SjO}_2) \quad (3).$$

Where SaO₂ is the oxygen saturation of arterial blood and SjO₂ is the oxygen saturation of jugular venous blood.

The difference in arterial and jugular venous oxygen content can also be expressed as a ratio termed the oxygen extraction fraction (OEF).

$$\text{OEF} = (\text{CaO}_2 - \text{CjO}_2) / \text{CaO}_2 \quad (4).$$

³ In practice this value is closer to 1.34 to 1.36 ml oxygen/gram haemoglobin, as normally some haemoglobin is in the form of methaemoglobin which cannot combine with oxygen.

⁴ 0.0225ml/100ml blood/ kPa of PO₂ (Partial pressure of oxygen in blood).

Whilst cerebral blood flow is not usually determined in clinical practice it can be measured experimentally (Walsh & Matta, 2000). Originally this was performed in a technique described by Ketty and Schmidt in 1945. The principle of the technique was derived from the Fick principle which states that the amount of a substance taken up or eliminated by an organ is equal to the difference in the amount of the substance in the arterial blood and the amount of the substance in the venous blood supplying that organ in the same time period. This can be written as;

$$Q_s \text{ taken up or eliminated} = \text{BF} \cdot (C_s \text{ arterial} - C_s \text{ venous}) \quad (5)$$

Where Q_s =quantity of substance, C_s =concentration of substance and BF=blood flow. This is the same equation as equation 1, but in a more general format. In the Ketty-Schmidt technique inhaled nitrous oxide is used as the substance or tracer. This is inhaled over 10 minutes until the arterial and venous blood concentrations reached equilibrium. At this point the brain content (Q_s taken up) can be calculated from the blood: brain partition co-efficient of nitrous oxide and the C_s venous. By measuring the arterial and jugular bulb concentrations of nitrous oxide during the attainment of this equilibrium an expression for CBF can be derived and a value calculated.

An extension of this technique uses radioactive isotopes such as ^{133}Xe which may be injected or inhaled. The xenon is allowed equilibrate in the brain and CBF calculated from a clearance curve as it is washed out. Detection of the isotope is via scintillation counters placed externally on the scalp.

If CMRO_2 is to be maintained in the face of reduced oxygen supply, due to either an inadequate blood flow (ischaemia) or arterial oxygen content (hypoxic and anaemic hypoxia) without an increase in CBF, an increase in oxygen extraction from the blood must result. A fall in oxygen content or saturation of blood sampled from in the jugular venous bulb (Equation 3) will occur and the ADVO_2 and/or the OEF must increase (Equations 1 and 4).

1.2.2. Local Reduction in Cerebral Blood Flow

At post-mortem evidence of cerebral ischaemia is a common finding in over 80% of fatal traumatic brain injuries (Graham et al., 1978, Graham et al., 1989). Inadequate oxygen delivery occurs locally because of blood vessel disruption by the trauma and due to the pressure effects of haematomas and contusions (Bouma et al., 1992, Coles et al., 2004). Energy failure in the affected tissue and the development of cytotoxic oedema result in further local blood flow impairment. Vasoactive substances released from haematoma may also promote ischaemia (Doyle & Gupta, 2000) whilst extravasated subarachnoid blood can lead to vasospasm (White, 1990).

1.2.3. Global Variation in Cerebral Blood Flow

In addition to local effects, global cerebral blood flow (CBF) varies characteristically after TBI. ¹³³Xeon studies demonstrated that CBF is reduced, in the first 6-24 hours after injury along with CMRO₂ (Bouma et al., 1991, Martin et al., 1997). However ischaemic levels of CBF with increased AVDO₂ are only seen very early after TBI, within 12 hours of injury (Bouma et al., 1991). After 24 hours CBF increases in the face of reduced cerebral metabolism indicating hyperaemia (Bouma et al., 1991, Martin et al., 1997). After 72 hours CBF reduces again. Transcranial doppler study of the middle cerebral artery suggests that this is due to spasm (Martin et al., 1997).

The skull forms a ridged box into which the intracranial contents, consisting of the brain, cerebrospinal fluid and cerebral blood volume, must fit. An increase in the volume of one of these must be balanced by the reduction in the volume of another if an increase in intracranial pressure is to be avoided. This is the basis of the “Munroe (1783)-Kellie (1824) doctrine”. However, only small increases in the volume of the intracranial contents can be accommodated, by such mechanisms as displacement of cerebrospinal fluid (CSF) or reduction of cerebral blood volume. Once the volume of the intracranial contents passes the inflection point of the cranial volume/pressure relationship large increases in intracranial pressure (ICP) are possible and blood flow to the whole brain can be reduced further. Such increases in volume can be due to oedema, haematomas, contusions or cerebral vasodilatation.

1.2.4. Extracranial Failure of Oxygen Delivery

Similarly extracranial events can also be important in impairing cerebral oxygen delivery and may occur either secondary to brain injury or due to concurrent extracranial injuries. Arterial hypoxaemia was found in 9-23% of patients after severe head injury (Gentleman & Jennett, 1981, Wald et al., 1993) and may be due to airway obstruction, aspiration, hypoventilation or chest trauma. Associated hypercapnia will dilate cerebral vessels contributing to a raised ICP and reduced cerebral perfusion pressure (CPP). Hypotension may also occur, often secondary to significant haemorrhage. Hypotension has been identified in 11-34 % of patients either arriving at or being transferred between hospitals (Gentleman & Jennett, 1981, Chesnut et al., 1993, Wald et al., 1993). In the injured brain as blood flow autoregulation is commonly lost (Cold & Jensen, 1978, Lewelt et al., 1980) even modest reductions in CPP will significantly decrease CBF and result in ischaemia. Hypoxaemic and hypotensive episodes can still occur once the patient is admitted to intensive care (Chesnut et al., 1993, Jones et al., 1994). These may happen as a consequence of treatment. For example hypotension resulting from sedation or episodes of hypoxaemia complicating chest physiotherapy. Complications of TBI such as ventilator acquired pneumonia or acute lung injury could also cause hypoxaemia. Whether these insults occur early or late they are strongly associated with a poor outcome (Gentleman & Jennett, 1981, Chesnut et al., 1993, Wald et al., 1993, Jones et al., 1994).

1.2.5. Increased Oxygen Demand

In addition to failures of oxygen delivery to the injured brain, hyperthermia and epileptiform activity can cause tissue hypoxia by increasing the demand for oxygen in the presence of impaired supply (Jones et al., 1994).

1.2.6. Anti-mortem Evidence of Cerebral Ischaemia

Evidence of cerebral ischaemia is a common finding in patients dying as a result of TBI (Graham et al., 1978, Graham et al., 1989) and the multiple potential causes of inadequate cerebral perfusion have been described. However confirmation of progressive anti-mortem cerebral ischaemia has been

difficult to obtain clinically. Following TBI $CMRO_2$ can be highly variable. It may be reduced in association with depressed consciousness or sedation and increased by fighting and hypermetabolism. Reduced CBF values might therefore be adequate in the face of reduced metabolism, whereas normal CBF might be inadequate in conditions where $CMRO_2$ is raised (Coles et al., 2004). Indeed the reduction in global cerebral blood flow after TBI has been associated with a reduced $CMRO_2$ and therefore a normal $AVDO_2$ (Obrist et al., 1984, Bouma et al., 1991, Martin et al., 1997). On the face of it these observations would not support the presence of significant anti-mortem cerebral ischaemia. However a recent study of patients scanned within 24 hours of TBI adds considerably to the understanding of cerebral oxygenation (Coles et al., 2004). Positron emission tomography (PET) scanning of brains after TBI identified areas of abnormally high OEF, indicative of ischaemia, despite both CBF and $CMRO_2$ being significantly reduced. The construction of histograms to describe the frequency of different OEF voxels (volume element on a scan, representing a volume of tissue, analogous to a 3 dimensional pixel) across the brain and the definition of a critically raised OEF, indicative of ischaemia, allowed the calculation of ischaemic brain volume (IBV). Ischaemic areas were commonly observed adjacent to contusions or intracranial haematomas. Jugular bulb oxygen saturation was also measured in this study and correlated with the IBV. However the SjO_2 threshold for ischaemia of 50%, in this study, was only reached when the IBV was 170ml or >10% of brain volume. At a SjO_2 of 60% approximately 70ml of brain tissue was ischaemic. Analysis of voxel OEF values indicated there was an increased spread of values after TBI, with both abnormally high and low OEFs. This suggested that regions of ischaemia and hyperaemia could exist simultaneously. Therefore global measures of CBF could appear appropriate for the $CMRO_2$ with overall oxygen extraction averaging to a normal value, maintaining the SjO_2 , despite the presence of significant ischaemia (Coles et al., 2004).

1.2.7. Neurochemical Derangement Causing Hypoxia

Although a temporal relationship between cerebral hypoxia and chemical injury has been implied, the real time interaction is likely to be complex. As mentioned global cerebral blood flow has been found to decrease early after TBI, in the absence of reduced cerebral perfusion pressure, hyperventilation or

vascular spasm (Martin et al., 1997). Martin and co-workers used transcranial doppler ultrasonography to study the characteristics of cerebral blood flow velocity after severe head trauma. In the first 24 hours after injury the CMRO₂ was depressed and the CBF reduced. The pattern of blood flow velocities in middle cerebral artery (Increased pulsatility index) indicated that the cause of reduced CBF was increased resistance in the microcirculation (Martin et al., 1997). After 24 hours the pulsatility index was significantly reduced indicating a reduction in vascular resistance whilst the CMRO₂ remained low. This would argue against the increased vascular resistance in the first 24 hours resulting purely from a reduction in CBF as a consequence of decreased CMRO₂, known as flow metabolism coupling. Martin discussed the causes of this increased vascular resistance suggesting that microvascular compression due to astrocyte swelling or changes in resistance vessel tone due to trauma induced release of calcium, catecholamines, prostaglandins and neuropeptides might be responsible. Thus biochemical cascades might also result in tissue hypoxia (Huger & Patrick, 1979, Chapleau & White, 1979, Yanagisawa et al., 1988, White, 1990, McIntosh & Ferriero, 1992, Fineman et al., 1993, Shohami et al., 1994). Haemoglobin released from traumatic subarachnoid haemorrhage is also a potent vasoconstrictor (White, 1990) and intravascular thrombosis may also contribute to increased resistance (Hekmatpanah & Hekmatpanah, 1985).

It is also likely that cerebral hypoxia and the induction of chemical mediators can occur independently but interact to exacerbate tissue injury. For example mild mechanical injury to the brain has been shown to potentiate selective neuronal necrosis in ischaemic conditions, which in isolation would not be associated with cell death (Jenkins et al., 1989).

The management of patients with brain injury focuses on maintaining the delivery of oxygenated blood to the brain in an attempt to reduce the occurrence of cerebral hypoxia. Prevention and prompt resolution of episodes of systemic hypoxaemia, hypotension and pyrexia are crucial. The effect of these events has been shown to be cumulative and are significantly related to poor outcome (Gentleman & Jennett, 1981, Chesnut et al., 1993, Wald et al., 1993, Jones et al., 1994). However despite these measures intrinsic reactive mediators once triggered, constitute a significant burden of secondary chemical injury. It is likely that chemical injury occurring both directly and secondary to

further tissue hypoxia are responsible for the inexorable deterioration seen in many patients, despite compliance with guidelines (Bullock et al., 1996, Maas et al., 1997) for the maintenance of cerebral perfusion with oxygenated blood.

1.3. The Diversity of Chemical Injury

Cerebral hypoxia causes cytotoxic oedema and, via both direct and indirect mechanisms, the accumulation of potentially harmful neurochemical mediators. These include excitatory amino acids, free radicals and elements of the inflammatory system.

1.3.1. Acidosis

Tissue ischaemia is associated with anaerobic glycolysis and the development of a lactic acidosis, greatest in the presence of hyperglycaemia. Such acidosis is associated with increased oedema formation, prolongation of the calcium transient and enhanced free radical formation (Siesjo, 1993).

1.3.2. Energy Failure

At its most basic inadequate tissue oxygenation leads to rapid failure of ATP dependant ion pumps that maintain intracellular homeostasis. Potassium is lost from cells and calcium, sodium and chloride ions accumulate within. Depolarisation of plasma membranes follows. Cells swell due to the intracellular accumulation of ions along with water molecules (Siesjo, 1993). This cytotoxic oedema will further compromise cerebral blood flow, either due to local pressure effects or globally as the ICP is increased, and lead to further hypoxia.

1.3.3. Excitatory Amino Acids

Both ischemia and trauma are associated with elevated tissue levels of excitatory amino acids (EAA) such as glutamate and aspartate at the injury site (Benveniste et al., 1984, Faden et al., 1989, Katayama et al., 1990, Nilsson et al., 1990, Choi & Rothman, 1990, Persson & Hillered, 1992, Palmer

et al., 1993). Furthermore injection of the EAA receptor agonists N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) in to the cortex reproduces histological damage (Palmer et al., 1993) and EAA receptor antagonists reduce the extent of the brain injury in animal models of trauma and ischaemia (Simon et al., 1984, Rothman, 1984, Faden et al., 1989, McIntosh et al., 1989, Hicks et al., 1994).

Trauma induced depolarisation of neurones is associated with EAA release (Katayama et al., 1990, Bullock et al., 1998) whilst the traumatic disruption of cell membranes and the vasculature allows leakage of EAAs from the cytosole and blood (Maeda et al., 1998, Bullock et al., 1998, Rose et al., 2002). Hypoxia in turn results in the release of EAAs from neurones (Drejer et al., 1985, Benveniste et al., 1989). When ATP reserves are low enough neuronal depolarisation occurs as ion pumps fail, resulting in glutamate release (Choi, 1994). The osmotic lysis of degenerating cells will also release EAAs from the cytosolic metabolic pool and is likely to account for the increasing EAA concentrations measured in those patients with the most severe injuries (Bullock et al., 1998). Glutamate acts on post-synaptic AMPA and NMDA receptors. AMPA controls a channel permeable to monovalent cations such as sodium, potassium and hydrogen. The NMDA controlled channel is also permeable to calcium but is normally blocked by magnesium unless the membrane is depolarised. Simultaneous activation of AMPA depolarises the membrane allowing calcium influx (Siesjo, 1993). Intracellular calcium can reach lethal levels with excessive activation of proteases leading to structural dissolution and increase membrane permeability.

1.3.4. Free Radicals

Free radicals are generated as part of normal cellular function, such as the use of oxygen by mitochondria to generate energy. Hydrogen peroxide (H_2O_2) is produced as a by-product of the normal activity of enzymes such as monoamine oxidase and tyrosine hydroxylase, and via auto oxidation of ascorbic acid and catecholamines. These compounds become pathological when their generation exceeds their elimination by anti-oxidant protective systems (Gilgun-Sherki et al., 2002).

Owing to their highly reactive nature, free radicals are short lived compounds and *in-vivo* the evidence of their occurrence and role in the pathology of cerebral trauma and ischaemia is largely indirect. Induction of radical generation in normal tissue creates the same necrotic changes seen in trauma and ischaemia, whilst radical reaction products have been reported following the same CNS injuries. Further indirect evidence stems from the measured consumption of tissue anti-oxidants in ischaemic and traumatic injuries to the CNS (Braugher & Hall, 1989). Additionally the use of free radical scavengers such as superoxide dismutase (He et al., 1993, Muizelaar et al., 1993, Truelove et al., 1994, Hamm et al., 1996) and lipid anti-oxidants such as methylprednisolone and tirilazad (Hall, 1985, Hall et al., 1991, Hall et al., 1993) reduce pathological changes and promote recovery in experimental brain injury models (Rhodes, 2003).

Conditions known to generate free radicals in brain tissue are associated with increased release of EAAs (Pellegrini-Giampietro et al., 1990). It is likely that the effects of excess EAAs and the generation of free radicals are interlinked via activation of calcium dependant enzymes such as phospholipase and nitric oxide synthase (Love, 1999, Gilgun-Sherki et al., 2002). Increased intracellular calcium initiates the activity of phospholipase A₂ which leads to the generation of arachidonic acid. The metabolism of arachidonic acid by cyclo-oxygenase and lipoxygenase to eicosanoids is a significant source of superoxide (O_2^-) (Kontos & Povlishock, 1986, Hall & Braugher, 1989, Braugher & Hall, 1989, Hall & Braugher, 1993). This is the most common cellular free radical. The conversion of xanthine dehydrogenase to xanthine oxidase by calcium activated proteolysis might also be important (Braugher & Hall, 1989). During reperfusion/reoxygenation xanthine oxidase can catalyse reactions consuming oxygen and generating O_2^- and H_2O_2 as by-products (Braugher & Hall, 1989, Gilgun-Sherki et al., 2002). During ischaemia O_2^- is also generated by dysfunctional mitochondria, electrons leaking from their usual carriers onto oxygen molecules (Braugher & Hall, 1989). This reaction is also augmented by post-ischaemic reperfusion (Hall et al., 1994). Inflammatory cells such as activated neutrophils are another important source of reactive oxygen radicals (Braugher & Hall, 1989, Roitt, 2001).

Superoxide is not in its self very reactive but under ischaemic conditions lactic acid accumulation can lead to its protonation. This enables it to penetrate membranes and initiate their oxidation. Mitochondria rich in superoxide dismutase (SOD) convert O_2^- to H_2O_2 . Reaction with ferrous iron then generates the highly reactive hydroxyl radical ($\text{OH}^\cdot$), the Haber-Weiss reaction (Gilgun-Sherki et al., 2002). Injury promotes the release of reactive metals such as iron from lysed cells and as haemoglobin following vascular injury (Hall et al., 1994). An alternate route of $\text{OH}^\cdot$ production involves endogenous nitric oxide (NO) formed by nitric oxide synthase. O_2^- reacts with NO to generate peroxynitrite (ONOO^-) forming the nitrosyl radical ($\text{ONOOH}^\cdot$) which then decomposes to $\text{OH}^\cdot$ (Gilgun-Sherki et al., 2002).

An important consequence of free radical generation is lipid peroxidation (LP). In this reaction process free radicals mediate the formation of lipid peroxides. Cell membranes, being comprised predominantly of lipids, are vulnerable to such oxidation, which alters their structure and hence function (Hall et al., 1994). Such alterations include increased membrane permeability, permitting extracellular calcium to enter cells and activation of phospholipases (Clark et al., 1995). Radical damage in the microvasculature carries the risk of vasogenic oedema and the loss of autoregulation (Siesjo, 1993). Once LP is commenced lipid radicals are generated which can then propagate further oxidation reactions (Hall et al., 1994). In addition to lipid membrane effects free radicals can also damage deoxyribonucleic acid (DNA), proteins and induce apoptotic pathways (Royston, 1988, Love, 1999).

1.4. Inflammation

Inflammation is defined as a local reaction at the microvessel interfaces that results in fluid and cell translocation from the intravascular medium into the tissue in order to “wall off” and sequester injurious agents and protect and repair the tissue (Gallin et al., 1988). The word inflammation, derived from the Latin, *inflammatio*, meaning to set on fire, therefore refers to the response of tissues to damage and pathogens. The classical signs of inflammation, seen in peripheral tissues, include swelling, redness, heat, pain and loss of function. Their recognition is attributed to the Roman

encyclopaedist Aulus Celsus (25BC-50) and more recently the German physician Rudolf Virchow (1829-1902). These clinical signs result from the expression of inflammatory cytokines, increased blood flow to the tissue, increased permeability of the capillaries with exudation of plasma proteins and the recruitment of leucocytes.

The central nervous system was traditionally considered an immune privileged site lacking a lymphoid system or antigen presenting dendritic cells (Perry & Gordon, 1988, McKeating & Andrews, 1998). However it has become apparent that injury to the brain, including trauma and ischaemia, is also followed by the expression of inflammatory cytokines, opening of the blood brain barrier with vasogenic oedema, leucocyte migration into areas of damage and activation of resident glia. However many features of this response are atypical when compared to inflammation in the periphery. In the CNS mast cells, required for the rapid release of preformed mediators, are absent. Leucocytes appear reluctant to cross the blood brain barrier (BBB) and the resident macrophages, the microglia, are normally quiescent (Perry et al., 1995). These differences appear to develop in the post-natal period (Lawson & Perry, 1995). To date the induction and function of the inflammatory process remains incompletely understood.

1.4.1. Inflammatory Cytokine Expression

The mediators central to the inflammatory process are the cytokines. Cytokines are defined as soluble, extracellular proteins that regulate innate as well as immunologically regulated inflammatory reactions, cell growth, differentiation, development, and repair processes culminating in the restoration of homeostasis (Oppenheim & Feldmann, 2000). The development of tissue culture techniques in the early 1960s allowed the detection of soluble factors produced by activated lymphocytes which were mitogenic, cytotoxic and more specifically inhibited macrophage migration (due to the induction of adhesion molecules). Dumonde and co-workers used the term lymphokines to describe these lymphocyte derived mediators in 1969. The discovery that activated macrophages also produced factors such as lymphocyte activating factor lead to the use of the term monokines to distinguish this group of molecules. Cohen and co-workers observed that lymphokine

like activity could also be produced by non-macrophage and non-lymphocytic cell lines. They proposed the more inclusive term cytokines in 1974, to refer to the family of small proteins, secreted by a variety of cell types, that engage in immunologically mediated inflammatory reactions (Oppenheim & Feldmann, 2000).

The cytokine family is a diverse group of polypeptides the precise function of which often depends on the organ or species studied. In the systemic circulation the cytokines tumour necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and interleukin (IL-6) are important inducers of the acute defence responses to trauma and infection (Dinarello, 1985, Dinarello, 1991, Woodroffe, 1995, Wang & Shuaib, 2002). These are part of a large grouping termed the pro-inflammatory cytokines, which also includes interferon- γ (IFN- γ), interleukin-12 (IL-12) and granulocyte-macrophage colony stimulating factor (GM-CSF) (Oppenheim & Feldmann, 2000). Cytokines with anti-inflammatory properties also exist which can limit the extent of the response, such as reducing the expression of pro-inflammatory cytokines. This group includes interleukin-4 (IL-4), IL-10 and tissue growth factor- β (TGF- β) (Oppenheim & Feldmann, 2000).

Within the central nervous system some of the inflammatory cytokines are expressed at low levels constitutively. However, following injury expression can increase significantly. Whilst there is evidence to suggest that some cytokines have positive effects on neuronal homeostasis, unchecked release of pro-inflammatory mediators can prejudice cell survival following brain injury (Rothwell & Relton, 1993).

1.4.1.1. Clinical Expression in Serum/Cerebrospinal Fluid

Elevated levels of blood or CSF TNF (Goodman et al., 1990, Ross et al., 1994, Morganti-Kossmann et al., 1997, Csuka et al., 1999, Hayakata et al., 2004, Shiozaki et al., 2005), IL-1 (McClain et al., 1987, Hayakata et al., 2004, Shiozaki et al., 2005) and IL-6 (McClain et al., 1991, Kossmann et al., 1995, Osuka et al., 1996, Heesen et al., 1996, Kossmann et al., 1996, McKeating et al., 1997, Bell et al., 1997, Morganti-Kossmann et al., 1997, Csuka et al., 1999, Hans et al., 1999b, Hayakata et al., 2004) have been reported in patients following both TBI and non-trauma associated craniotomy (Table 1.1).

Levels tend to be either raised on admission or peak within 24 hours of injury. In some studies analysis of both CSF and blood levels were performed simultaneously, finding that TNF- α (Morganti-Kossmann et al., 1997, Csuka et al., 1999, Hayakata et al., 2004, Shiozaki et al., 2005), IL-1 β (Hayakata et al., 2004, Shiozaki et al., 2005) and IL-6 (McClain et al., 1991, Kossmann et al., 1995, Morganti-Kossmann et al., 1997, Csuka et al., 1999, Hans et al., 1999b, Hayakata et al., 2004) were increased in both compartments. The CSF levels were greater than those in blood, suggesting that the injured CNS is the source of the cytokine expression.

Increased concentrations of blood and/or CSF TNF- α , IL-1 β and IL-6 have also been found after ischaemic stroke (Fassbender et al., 1994, Tarkowski et al., 1995, Beamer et al., 1995, Kim et al., 1996, Intiso et al., 2004). Concentrations peaked in the first 1-2 days and as in TBI the CSF concentration of IL-6 was significantly greater than in the serum (Tarkowski et al., 1995)(Table 1.1).

Injury	Compartment	Cytokine	Increased	Peak	Duration of Study	Reference
TBI	Serum	TNF	1 to 5 days	Day 1	5 days	Goodman 1990
TBI	Plasma	TNF	Day 1		24 hours	Ross 1994
	CSF		Day 1			
TBI	CSF	TNF- α	1 to 22 days		22 days	Csuka 1999
TBI	Serum	TNF- α	1 to 4 days	Day 2	4 days	Hayakata 2004
	CSF		1 to 4 days	12 hours		
TBI	CSF	IL-1	On admission		Admission	McClain 1987
TBI	CSF	IL-1 β	1 to 3 days	12 hours	4 days	Hayakata 2004
TBI	Plasma	IL-6	1 to 10 days	Day 1	15 days	McClain 1991
TBI	Serum	IL-6	1 to 14 days	Day 2	14 days	Kossmann 1995
	CSF			Day 2		
TBI	CSF	IL-6	1 to 22 days	Day 2	23 days	Kossmann 1996
TBI	Serum	IL-6	1 to 21 days	Day 2	22 days	Hans 1999
	CSF					
Craniotomy	Plasma	IL-6	3 to 24 hours	24 hours	24 hours	Heesen 1996
Craniotomy	Serum	IL-6	Day 1		7 days	Osuka 1996
TBI	CSF	IL-6	1 to 3 days	Day 1	3 days	Bell 1997
TBI	Serum	IL-6	1 to 22 days	Day 2	22 days	Csuka 1999
	CSF					
TBI	Serum	IL-6	1 to 2 days	6 hours	4 days	Hayakata 2004
	CSF					
Stroke	Serum	TNF- α	4 to 10 days	Day 7	10 days	Intiso 2004
Stroke	CSF	IL-1 β	2 to 90 days	Day 2	90 days	Tarkowski 1995
Stroke	Serum	IL-6	1 to 5 days	Day 1	7 days	Fassbender 1994
Stroke	Serum	IL-6	1 to 90 days	-	90 days	Tarkowski 1995
	CSF		1 to 90 days	Day 2		
Stroke	Serum	IL-6	1-7 days	Day 1	7 days	Kim 1996

Table 1.1. Clinical Pro-inflammatory Cytokine Expression in Blood and/or Cerebrospinal Fluid Following Traumatic Brain Injury or Stroke.

Cerebrospinal fluid (CSF), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), tumour necrosis factor (TNF- α), traumatic brain injury (TBI).

1.4.1.2. Experimental Models

Early increases in tissue pro-inflammatory cytokine mRNAs and proteins have been found in both traumatic and ischaemic experimental models of brain injury.

1.4.1.2.1. Trauma

Experimental trauma to the brain is associated with increased expression of TNF- α , IL-1 β and IL-6 mRNAs in the injured tissue within 1 to 6 hours after injury and which tend to peak within 12 hours (Yan et al., 1992, Taupin et al., 1993, Fan et al., 1995, Fan et al., 1996, Rostworowski et al., 1997, Hans et al., 1999a, Rhodes et al., 2002, Kamm et al., 2006)(Table 1.2). Protein expression is similarly increased within 1-5 hours of injury, peaking by 3 to 8 hours (Yan et al., 1992, Taupin et al., 1993, Shohami et al., 1994, Knoblach et al., 1999, Hans et al., 1999a, Zhu et al., 2004, Kamm et al., 2006). The magnitude and timing of cytokine expression appears to be related to the degree of injury. Following severe lateral fluid percussion injury (LFPI) the increase in TNF- α expression was more prolonged than after a moderate injury. No increase in TNF- α was reported after a mild injury (Knoblach et al., 1999). Another observation is that following experimental penetrating injuries the increase in expression of mRNA and protein are relatively delayed compared to higher energy mechanisms of lesion production such as LFPI, closed head injury (CHI) or the diffuse axonal injury (DAI) models⁵, where mRNA expression is evident by 1 hour (Fan et al., 1995, Fan et al., 1996, Hans et al., 1999a) and that of protein by 1 to 3 hours after injury (Taupin et al., 1993, Shohami et al., 1994, Knoblach et al., 1999, Hans et al., 1999a).

The work of Holmin and colleagues is a noticeable exception to the acute expression of pro-inflammatory mRNAs after TBI. Using a weight drop model and detection of mRNA expression by in-situ hybridisation, TNF- α , IL-1 β and IL-6 were not found to be increased until 4 days after injury (Holmin et al., 1997)(Table 1.2).

⁵ The different models of TBI are discussed in more detail in chapter 3.

Injury	Severity	Species	Cytokine	mRNA/Protein	Method	Increased Cytokine	Peak	Duration of Study	Location	Reference
LFPI	Unspecified	Rat	TNF- α	Protein	Bioassay	3 to 8 hours	3 to 8 hours	3 days	Cortex	Taupin 1993
CHI		Rat	TNF- α	Protein	Bioassay	1 to 8 hours	4 hours	24 hours	Cortex	Shohami 1994
LFPI	Moderate	Rat	TNF- α	mRNA	Northern	1 to 6 hours	1 hour	24 hours	Cortex and hippocampus	Fan 1996
Stab Injury		Mouse	TNF- α	mRNA	RT-PCR	6 hours to 2 days	6 hours	7 days	Cortex	Rostworowski 1997
Weight Drop		Rat	TNF- α	mRNA	ISH	4 to 6 days		16 days	Cortex	Holmin 1997
LFPI	Severe Moderate	Rat	TNF- α	Protein	ELISA	1 to 4 hours and 3 days 1 hour	1 to 4 hours	3 days	Cortex	Knobloch 1999
LFPI	Moderate & Severe	Rat	TNF- α	Protein	Bioassay	8 hours		8 hours	Cortex	Zhu 2004
Penetrating Injury		Rat	IL-1	Protein	Bioassay	2 to 3 days	2 to 3 days	7 days	Striatum microdialysis	Woodroffe 1991
Penetrating Injury		Rat	IL-1 β	mRNA	ISH	From 2 hours	4 hours	24 hours	Striatum	Yan 1992
				Protein	Bioassay	2 to 12 hours		12 hours	Microdialysis	
LFPI	Unspecified	Rat	IL-1 β	Protein	Bioassay	1 to 18 hours	8 hours	3 days	Cortex	Taupin 1993
LFPI	Moderate	Rat	IL-1 β	mRNA	Northern	1 to 6 hours	1 hour	24 hours	Cortex and hippocampus	Fan 1995
Weight Drop		Rat	IL-1 β	mRNA	ISH	4 to 6 days		16 days	Cortex	Holmin 1997
Stab Injury		Mouse	IL-1 β	mRNA	RT-PCR	3 to 12 hours	12 hours	7 days	Cortex	Rostworowski 1997
LFPI	Moderate & Severe	Rat	IL-1 β	Protein	Bioassay	8 hours		8 hours	Cortex	Zhu 2004
DAI		Rat	IL-1 β	mRNA	RT-PCR	2 to 4 hours	2 hours	24 hours	Brain	Kamm 2006
				Protein	ELISA	1 to 8 hours	1 and 8 hours			

Table 1.2. Pro-Inflammatory Cytokine Expression in Traumatic Models of Brain Injury.

Closed head injury (CHI), diffuse axonal injury (DAI), enzyme linked immunosorbent assay (ELISA), in-situ hybridization (ISH), interleukin-1 β (IL-1 β), lateral fluid percussion injury (LFPI), messenger ribonucleic acid (mRNA), reverses transcriptase polymerase chain reaction (RT-PCR), tumour necrosis factor- α (TNF- α).

Injury	Severity	Species	Cytokine	mRNA/Protein	Method	Increased Cytokine	Peak	Duration of Study	Location	Reference
Penetrating Injury		Rat	IL-6	Protein	Bioassay	1 to 2 days	1 to 2 days	7 days	Striatum microdialysis	Woodroffe 1991
Penetrating Injury		Rat	IL-6	mRNA	ISH	From 2 hours	6 hours	24 hours	Striatum	Yan 1992
				Protein	Bioassay	5 to 12 hours		12 hours	Microdialysis	
LFPI	Unspecified	Rat	IL-6	Protein	Bioassay	3 to 18 hours	8 hours	3 days	Cortex	Taupin 1993
						5 hours	5 hours		Hippocampus	
CHI		Rat	IL-6	Protein	Bioassay	4 to 24 hours	8 hours	24 hours	Cortex	Shohami 1994
Weight Drop		Rat	IL-6	mRNA	ISH	6 days		16 days	Cortex	Holmin 1997
DAI		Rat	IL-6	mRNA	ISH	1 to 24 hours		24 hours	Cortex and thalamus	Hans 1999
				Protein	IHC	1 to 24 hours		14 days	Cortex, hippocampus, thalamus	
DAI		Rat	IL-6	mRNA	ISH	6 hours		6 hours	Hippocampal fissure and dentate gyrus	Rhodes 2002
LFPI	Moderate & Severe	Rat	IL-6	Protein	Bioassay	8 hours		8 hours	Cortex	Zhu 2004

Table 1.2 Continued. Pro-Inflammatory Cytokine Expression in Traumatic Models of Brain Injury.

Closed head injury (CHI), diffuse axonal injury (DAI), immunohistochemistry (IHC), in-situ hybridization (ISH), interleukin-6 (IL-6), lateral fluid percussion injury (LFPI), messenger ribonucleic acid (mRNA), reverses transcriptase polymerase chain reaction (RT-PCR).

1.4.1.2.2. Ischaemia and Hypoxia

Following both permanent and transient models of cerebral ischaemia/hypoxia pro-inflammatory cytokine mRNA and proteins are increased acutely. Generally TNF- α , IL-1 β and IL-6 mRNAs increase within 1 to 10 hours of injury and peak within the first day (Minami et al., 1992, Liu et al., 1993, Buttini et al., 1994, Yabuuchi et al., 1994, Liu et al., 1994, Wang et al., 1994b, Szaflarski et al., 1995, Wang et al., 1995c, Buttini et al., 1996, Hagberg et al., 1996, Hill et al., 1999, Ali et al., 2000, Ohtaki et al., 2004, Zawadzka & Kaminska, 2005). Protein expression follows a similar time course tending to lag that of the mRNA. Increased expression is seen from 1 to 6 hours after injury, peak expression also usually occurring within 24 hours (Liu et al., 1994, Buttini et al., 1996, Hagberg et al., 1996, Uno et al., 1997, Botchkina et al., 1997, Bertorelli et al., 1998, Legos et al., 2000, Ohtaki et al., 2004) and prolonged persistent expression for up to 6 days after injury reported in some studies (Liu et al., 1994, Buttini et al., 1996, Block et al., 2000, Legos et al., 2000)(Table 1.3).

Considerable variability in the time to increased cytokine expression can be seen in the data presented. This probably reflects the tremendous differences in the methodology of the studies considered. For example in the study of IL-6 protein production by Block and co-workers the animals were not sacrificed prior to 24 hours post injury (Block et al., 2000). Similarly the methods of detection and their relative sensitivities are likely to be important. Following transient insults increased IL-6 mRNA was first detected by RNase protection assay at 10 hours of reperfusion (Hill et al., 1999) but could be measured by 2 hours using the reverse transcriptase polymerase chain reaction to amplify the signal (Ali et al., 2000). Finally with a very brief transient ischaemic event IL-6 protein was not significantly elevated until 24 hours after injury (Maeda et al., 1994).

Injury	Duration	Species	Cytokine	mRNA/Protein	Method	Increased Cytokine	Peak	Duration of Study	Location	Reference
PMCAO		Rat	TNF- α	mRNA	Northern	3 hours to 5 days	12 hours	5 days	Cortex	Liu 1994
				Protein	IHC	6 hours to 5 days			Cortex and white matter of the corpus callosum and striatum	
PMCAO		Rat	TNF- α	mRNA	RT-PCR	1 to 8 hours	3 hours	4 days	Hemispheres	Buttini 1996
				Protein	Western	6 hours to 4 days	8 to 24 hours	4 days	Hemispheres	
					IHC	30 minutes to 6 days	8 to 24 hours	6 days	Infarct and adjacent tissue	
PMCAO		Rat	TNF- α	Protein	IHC	6 to 24 hours		24 hours	Cortex and choroid plexus	Botchkina 1997
PMCAO		Rat	TNF	Protein	Bioassay	6 to 12 hours	12 hours	24 hours	Cortex	Bertorelli 1998
PCAO + 8% O ₂	3 hours	Neonatal Rat	TNF- α	mRNA	RT-PCR	4 hours	4 hours	2 days	Cortex and Hippocampus	Szaflarski 1995
PMCAO	2 hours	Mouse	TNF- α	mRNA	RPA	4 hours to 4 days post onset	18 hours	4 days	Hemispheres	Hill 1999
TMCAO							10 to 18 hours			
TMCAO	2.6 hours	Rat	TNF- α	mRNA	Northern	1 hour to 24 hours	3 hours	5 days	Cortex	Wang 1994
BTCCAO	30 minutes	Mouse	TNF- α	Protein	IHC	1.5 hours to 3 days	1.5 hours and 3 days	5 days	Hippocampus	Uno 1997
					Western	1.5 hours to 3 days		3 days		
TMCAO	1 hour	Mouse	TNF- α	mRNA	RT-PCR	1 hour ischaemia 6 hours to 2 days reperfusion	1 day	2 days	Cortex	Ohtaki 2004
				Protein	IHC	30 minutes of ischaemia 1 hour to 2 days reperfusion		2 days		
TMCAO	1.5 hours	Rat	TNF- α	mRNA	RT-PCR	6 hours to 2 days	12 hours	2 days	Cortex	Zawadzka 2004

Table 1.3. Pro-inflammatory Cytokine Expression in Ischaemic and Hypoxic Models of Brain Injury.

Bilateral transient common carotid artery occlusion (BTCCAO), immunohistochemistry (IHC), in-situ hybridization (ISH), messenger ribonucleic acid (mRNA) permanent carotid artery occlusion (PCAO), permanent middle cerebral artery occlusion (PMCAO), RNase protection assay (RPA), reverse transcriptase polymerase chain reaction (RT-PCR), tumour necrosis factor- α (TNF- α), transient middle cerebral artery occlusion (TMCAO). For transient models the times represent from reperfusion, unless stated.

Injury	Duration	Species	Cytokine	mRNA/Protein	Method	Increased Cytokine	Peak	Duration of Study	Location	Reference
PMCAO		Rat	IL-1 β	mRNA	Northern	6 hours to 5 days	12 hours	5 days	Cortex	Liu 1993
PMCAO		Rat	IL-1 β	mRNA	ISH	15 minutes to 2 days	3 hours	4 days	Cortex, striatum, hippocampus, thalamus and amygdala	Buttini 1994
PMCAO		Rat	IL-1 β	Protein	ELISA	1 hour to 5 days	3 days	15 days	Cortex	Legos 2000
PCAO + 8% O ₂	3 hours	Neonatal rat	IL-1 β	mRNA	RT-PCR	4 hours	4 hours	2 days	Cortex and Hippocampus	Szaflarski 1995
PCAO + 8% O ₂	1.6 hours	Neonatal rat	IL-1 β	mRNA	RT-PCR	1 and 3 hours	3 hours	14 days	Hemispheres	Hagberg 1996
				Protein	Bioassay	3 hours to 2 days	6 hours			
PMCAO	2 hours	Mouse	IL-1 β	mRNA	RPA	10 to 18 hours post onset	10 hours	4 days	Hemispheres	Hill 1999
TMCAO							18 hours			
Transient Forebrain Ischaemia	15 minutes	Rat	IL-1- β	mRNA	Northern	15 minutes to 7 days	30 minutes and 4 hours	7 days	Cortex, striatum, hippocampus and thalamus	Minami 1992
Transient Forebrain Ischaemia	10 minutes	Rat	IL-1 β	mRNA	ISH	30 minutes		30 minutes	Olfactory bulb, cortex, hippocampus, striatum, thalamus, amygdala	Yabuuchi 1994
TMCAO	2.6 hours	Rat	IL-1 β	mRNA	Northern	1 hour to 2 days	6 hours	5 days	Cortex	Wang 1994
TMCAO	1.5 hours	Rat	IL-1 β	mRNA	RT-PCR	6 hours to 2 days	12 hours	2 days	Cortex	Zawadzka 2004

Table 1.3 Continued. Pro-inflammatory Cytokine Expression in Ischaemic and Hypoxic Models of Brain Injury.

Enzyme linked immunosorbent assay (ELISA), in-situ hybridization (ISH), interleukin-1 β (IL-1 β), messenger ribonucleic acid (mRNA) permanent carotid artery occlusion (PCAO), permanent middle cerebral artery occlusion (PMCAO), RNase protection assay (RPA), reverses transcriptase polymerase chain reaction (RT-PCR), transient middle cerebral artery occlusion (TMCAO). For transient models the times represent from reperfusion, unless stated.

Injury	Duration	Species	Cytokine	mRNA/Protein	Method	Increased Cytokine	Peak	Duration of Study	Location	Reference
PMCAO		Rat	IL-6	mRNA	Northern	3 hours to 1 day	12 hours	5 days	Cortex	Wang 1995
PMCAO		Rat	IL-6	Protein	ELISA	2 hours to 1 day	1 day	15 days	Cortex	Legos 2000
PCAO + 8% O ₂	1.6 hours	Neonatal Rat	IL-6	mRNA	RT-PCR	1 to 20 hours	1 to 3 hours	14 days	Hemispheres	Hagberg 1996
				Protein	Bioassay	6 hours	6 hours			
PMCAO	2 hours	Mouse	IL-6	mRNA	RPA	10 to 18 hours post onset	18 hours	4 days	Hemispheres	Hill 1999
TMCAO							18 hours			
PMCAO	1.5 hours	Rat	IL-6	mRNA	RT-PCR	2 hours to 1 day post onset	1 day	3 days	Cortex	Ali 2000
TMCAO						6 hours to 1 day post onset	1 day	3 days		
BTCCAO	5 minutes	Gerbil	IL-6	Protein	Bioassay	1 to 4 days		4 days	Cortex	Maeda 1994
						Day 4		4 days	Hippocampus	
TMCAO	3 hours	Rat	IL-6	Protein	IHC	1 to 14 days	3 to 7 days	14 days	Predominantly penumbra	Block 2000
TMCAO	1.5 hours	Rat	IL-6	mRNA	RT-PCR	6 to 2 days	12 hours	2 days	Cortex	Zawadzka 2004

Table 1.3 Continued. Pro-inflammatory Cytokine Expression in Ischaemic and Hypoxic Models of Brain Injury.

Bilateral transient common carotid artery occlusion (BTCCAO), enzyme linked immunosorbent assay (ELISA), immunohistochemistry (IHC), interleukin-6 (IL-6), messenger ribonucleic acid (mRNA) permanent carotid artery occlusion (PCAO), permanent middle cerebral artery occlusion (PMCAO), RNase protection assay (RPA), reverse transcriptase polymerase chain reaction (RT-PCR), transient middle cerebral artery occlusion (TMCAO). For transient models the times represent from reperfusion, unless stated.

1.4.2. Inflammation and Leucocyte Function

1.4.2.1. CNS Injury, Cytokines and Leucocyte Recruitment

In a peripheral tissue such as skin, TNF- α and IL-1 β produce a similar accumulation of neutrophils and monocytes within hours of administration (Andersson et al., 1992a, Andersson et al., 1992b). However whilst the inflammatory response of the meninges and choroid plexus resembles that of the periphery, the brain parenchyma appears resistant to leucocyte recruitment and microglial activation. Neuronal degeneration induced by hippocampal injection of kainic acid (Andersson et al., 1991) or pro-inflammatory stimuli such as LPS (Andersson et al., 1992b), IL-1 β and TNF- α (Andersson et al., 1992a) failed to induce an acute inflammatory response in the parenchyma. Accumulation of leucocytes in blood vessels and localisation to the meninges, choroid plexus and ventricles occurred within 24 hours. In the parenchyma there was either minimal leucocyte accumulation (Andersson et al., 1992a) or leucocyte accumulation slowly 48 hours after the stimulus was applied (Andersson et al., 1991, Andersson et al., 1992b). However the microinjection of IL- β in to the striatum was associated with a marked recruitment of neutrophils into the parenchyma whereas TNF- α injection lead to the recruitment of monocytes (Blond et al., 2002). Again this leucocyte recruitment developed slowly over 24 hours (Schnell et al., 1999). In contrast the microinjection of TNF- α and IL-1 β into the spinal cord induced a brisker and much more intense leucocyte accumulation. IL-1 β again preferentially induced neutrophil recruitment whereas TNF- α was associated with a mixed PML/monocytic infiltrate (Schnell et al., 1999).

Although derived from the same origins as monocytes and macrophages, microglia are distinct in several ways. Mature microglia ramify long processes not seen on monocytes and macrophages. In the presence of neurones and astrocytes they enter a quiescent state, with reduced expression of surface receptors, secretory activity and migratory behaviour. Signals such as components of infective organisms and tissue debris induce quiescent microglia to become reactive. This is associated with retraction of the processes and a more amoeboid morphology (Giulian, 1999).

Taken together these studies illustrate that the CNS response to inflammation is highly modified in comparison to the stereotypical peripheral immune response and specific to the region examined.

1.4.2.2. Leucocyte Recruitment in Traumatic and Ischaemic Brain Injury

Both traumatic and ischaemic lesions in the brain lead to characteristic patterns of leucocyte infiltration and glial activation which can be very similar.

1.4.2.2.1. Focal Traumatic Injury

Following trauma leucocyte recruitment is much more pronounced in focal injuries when compared to diffuse ones (Soares et al., 1995, Csuka et al., 2000). Focal injuries are characterised by a marked invasion by neutrophils and mononuclear phagocytes combined with the activation of resident microglia and astrocytes. In diffuse injury leukocyte invasion of the parenchyma is much less significant, the dominant reactive cells being the microglia and astrocytes.

In focal brain injury with contusion neutrophils have been reported lining the subarachnoid/subdural spaces and large blood vessels at 4 hours after injury (Soares et al., 1995). Thereafter they enter the parenchyma and accumulation peaks at around 2 days (Biagas et al., 1992, Clark et al., 1994b, Soares et al., 1995, Holmin et al., 1995). Monocytes also line the vasculature at 12 hours (Soares et al., 1995) accumulating in the contused tissue and predominating in the lesion by 3-6 days (Soares et al., 1995, Holmin et al., 1995, Hausmann et al., 1999). T lymphocytes and natural killer (NK) cells are also reported in lower numbers but with a similar temporal profile to monocytes (Holmin et al., 1995). Reactive astrocytes are seen from 1 day onwards (Holmin et al., 1995, Hill et al., 1996). Whilst macrophages appear to accumulate in the area of focal injury, activated microglia become more widespread over the days following experimental injury and are distributed throughout the injured hemisphere (Aihara et al., 1995).

1.4.2.2.2. Diffuse Traumatic Injury

In diffuse injuries the pattern of cellular response is distinct, with little parenchymal invasion by circulating neutrophils or monocytes (Soares et al., 1995) but widespread up regulation of microglial activity (Csuka et al., 1997). In the Marmarou weight drop model round cells with monocyte/macrophage morphology were present principally in the meninges, basal subarachnoid space, Virchow-Robin's space, ventricles and choroid plexus within 24 hours and persisted for up to 2 weeks after trauma. Only in the basal cortex were monocyte/macrophage like cells observed. These were thought to be migrating from the subarachnoid space into the underlying tissue. After trauma activated microglia became apparent in the white matter tracts (corpus callosum, internal capsule and optic tract), cortex and hippocampus. Reactive astrocytes were visible within 4 hours of injury with maximal GFAP staining between 48 hours and 2 weeks. Astrogliosis was found mainly in the cortex, thalamus, hypothalamus and perivascularly. Lymphocytes and neutrophils remained confined, in low numbers, to vessels, mainly in the meninges and choroid plexus (Csuka et al., 1997).

1.4.2.2.3. Ischaemic Injury

In animal ischaemia models maximal neutrophil infiltration occurs within 72 hours of ischaemia onset (Garcia & Kamijyo, 1974, Garcia et al., 1994, Clark et al., 1994a). Neutrophils appear on the endothelial surface of microvessels after 1 hour, peaking in number by 12 hours, with migration into the parenchyma then occurring. Monocytes are seen in the vessels by 4 hours but widespread infiltration does not occur until 72 hours and peaks around 7 days (Garcia & Kamijyo, 1974, Garcia et al., 1994, Clark et al., 1994a, Jander et al., 1995). Significant T cell infiltration is also apparent from 2 to 7 days after infarction (Jander et al., 1995).

The recruitment and activation of immune cells appears to differ between transient and permanent insults. In transient ischaemia the accumulating neutrophils have been found to be more numerous and parenchymal infiltration occurring earlier at 6 hours than when compared to a permanent injury (Clark et al., 1994a). Similarly following transient ischaemia microglia activation has been reported as early as 3 hours post-insult. Subsequently the ramified processes of the cells retract and an

amoeboid, macrophage like morphology is assumed (Korematsu et al., 1994). Ischaemia leads to increased expression of immune molecules such as MHC class I and II (Kato et al., 1995) as well as scavenger receptors (Giulian & Vaca, 1993). In contrast following permanent MCAO activated microglia did not become abundant in the cortex of affected tissue until 24 hours after injury (Morioka et al., 1993). This difference in the microglial response time between transient and permanent ischaemia has been attributed to the differing severity of insult (Korematsu et al., 1994).

1.4.2.3. Regulation of Leucocyte Recruitment

The movement of leucocytes in to tissues requires their activation and adherence to the vascular endothelium prior to migration in to the parenchyma. This process is highly orchestrated through the interplay of adhesion molecules and signalling proteins known as chemokines, which guide the leucocytes to the site of inflammation.

1.4.2.3.1. Adhesion Molecules

The recruitment of leukocytes from the blood into inflamed parenchyma requires the interaction of leucocytes with the vascular endothelium. Three major protein families have been identified which play a role in these leucocyte–endothelial interactions; the selectins, $\beta 2$ integrins, and the intracellular adhesion molecules (ICAMS) reviewed by Furie & Randolph, 1995 and Wahl and colleagues, 1996.

The selectin family includes E-selectin, P-selectin and L-selectin. These mediate adhesion by binding to carbohydrate residues on glycoproteins and glycolipids. P and E-selectins are expressed on activated endothelium, whilst L-selectin is constitutively expressed on leucocytes. The $\beta 2$ integrins are heterodimeric proteins consisting of a common $\beta 2$ chain, CD18, and differing α chains, CD11 a, b or c. Members of this family include lymphocyte function-associated antigen (LFA: CD11a/CD18), macrophage antigen-1 (Mac-1, CD11b/CD18) and p150.95 (CD11c/CD18). These integrins, expressed by leucocytes, bind to endothelial surface proteins of the immunoglobulin super family (ICAM-1, ICAM-2, ICAM-3). $\beta 1$ integrins are also described which consist of a similar heterodimer arrangement. For example $\alpha 4\beta 1$ (Very late antigen-4 (VLA-4) or CD49d/CD29) binds to vascular

cell adhesion molecule-1 (VCAM-1) and the matrix component fibronectin (Furie & Randolph, 1995, Wahl et al., 1996).

During recruitment to sites of inflammation leucocytes initially tether “loosely” to the endothelium, rolling along it with the haemodynamic shear forces. This interaction is mediated by the selectin family. Leucocyte activation by chemokines then leads to integrin-ICAM interaction and firm adherence to the endothelium, prior to migration across the vessel wall (Johnston & Butcher, 2002)(Figure 1.3).

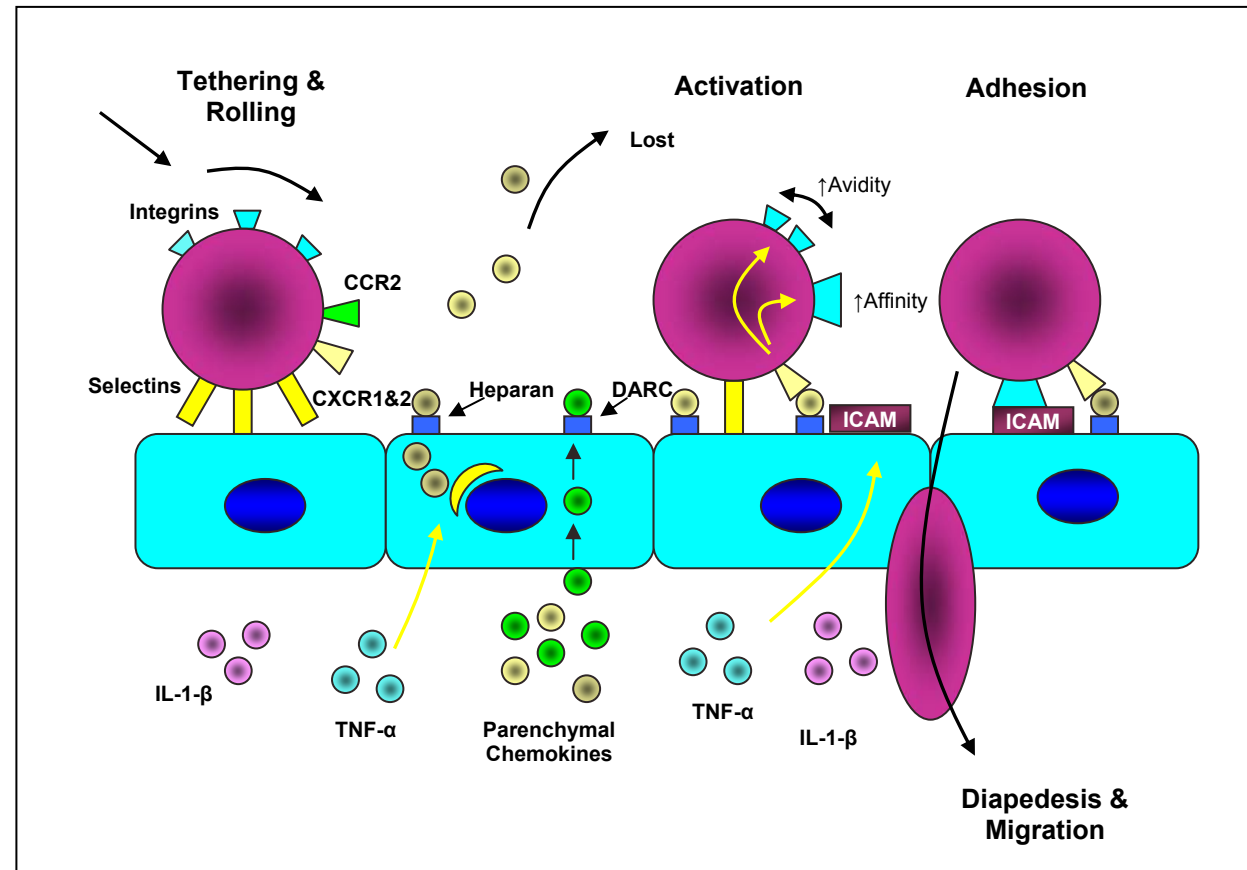


Figure 1.3. Model of the Interaction of Adhesion Molecules and Leucocyte Recruitment in Inflammation.

Leucocytes rolling along the vascular endothelium initially tether loosely to its surface, mediated by selectins. The local expression of chemokine proteins in inflamed tissue, which are held on the surface of the endothelium, allows interaction with their respective receptors on the leucocytes. This induces conformational changes in leucocyte surface integrins. These changes in integrin avidity, and affinity for intercellular adhesion molecules (ICAM), also expressed on the endothelial surface, result in the firm adherence of integrin to ICAM. This adherence holds the leucocyte in place prior to migration into the inflamed tissue. Chemokine receptors (CXCR1& 2, CCR2), Duffy antigen receptor for chemokines (DARC), interleukin-1 β (IL-1 β), tumour necrosis factor- α (TNF- α). Adapted from Johnston & Butcher 2000.

1.4.2.3.1.1. In-vivo Expression of Adhesion Molecules

In rats constitutive expression of ICAM-1 on the choroid plexus and vascular endothelia has been identified immunohistochemically (Carlos et al., 1997, Isaksson et al., 1997). Following cortical weight drop the number of positively staining vessels in the ipsilateral cortex, hippocampus and thalamus increased by 4 hours after injury and remained elevated until 72 hours post injury (Carlos et al., 1997, Isaksson et al., 1997). Following LFPI in rats, increased ICAM-1 expression in the vessels of the ipsilateral cortex and hippocampus has also been reported (Knoblauch & Faden, 2002). Similarly in mice 24 hours following, controlled cortical impact (CCI), ICAM-1 expression was localised immunohistochemically to the choroid plexus and vascular endothelium in injured brain tissue (Whalen et al., 1999a). E-selectin, although not identified constitutively, was also induced on the vascular endothelium in the traumatised hemisphere between 4 and 72 hours after cortical weight drop (Carlos et al., 1997). Following experimental diffuse axonal injury ICAM-1 expression was also increased but delayed relative to the focal injuries described. The number of ICAM-1 positive vessels increased in a biphasic manner. Expression was not increased until 24 hours after injury, declining to baseline at 48 hours and then peaking at 96 hours post injury. Increased endothelial ICAM-1 was seen in the cortex, thalamus and corpus callosum. Expression in the choroid plexus increased from 24 hours after injury (Rancan et al., 2001).

Increased expression of ICAM-1 has also been observed in several studies of cerebral ischaemia in animals. ICAM-1 mRNA is induced in the ischaemic cortex following both PMCAO and TMCAO. Following 160 minutes of temporary occlusion, mRNA was significantly increased after 1 hour of reperfusion. With PMCAO the increase occurred later at 3 hours. In both cases peak expression had occurred by 12 hours (Wang et al., 1994a). ICAM-1 was localized to the endothelial cells of blood vessels of the ischaemic tissue, increasing as early as 1 hour after TMCAO (Okada et al., 1994, Zhang et al., 1994, Matsuo et al., 1994b, Connolly, Jr. et al., 1996). In a photothrombosis model of cortical ischaemia ICAM-1 was localized to the vessels of the infarcted area in the first 48 hours. In the days thereafter ICAM-1 positive leucocytes were seen infiltrating the lesion. Infiltrating leucocytes also expressed LFA-1, a ligand for ICAM-1 (Jander et al., 1995). ICAM-1 positive vessels have also been identified in human cerebral cortical infarcts along with LFA positive cells (Sobel et al., 1990).

Similarly increased E-selectin mRNA has also been described in ischaemic cortex after 12 hours of PMCAO (Wang et al., 1995a). Prolonged TMCAO occlusion, up to 3 hours, induced endothelial immunoreactivity for P-selectin. This was observed after 2 hours of ischaemia and the number of positive vessels remained elevated over 24 hours of reperfusion (Okada et al., 1994).

1.4.2.3.1.2. In-vitro Expression of Adhesion Molecules

Under the action of pro-inflammatory mediators endothelial cells upregulate factors associated with leucocyte transmigration (Soares et al., 1995). For instance, in cultures of CNS endothelial cells, TNF- α , IL-1 β and interferon- γ (INF- γ) increase ICAM-1 expression (Wong & Dorovini-Zis, 1992, Gourin & Shackford, 1996) whilst TNF- α also induces expression of VCAM-1 and E-selectin (Dore-Duffy et al., 1994).

1.4.2.3.2. Chemokines

A substance which has the property of cause cells to migrate along a concentration gradient is said to be chemotactic. In the 1960's the media of activated T lymphocytes was shown to contain lymphokines which were chemotactic for neutrophils and monocytes (Ward et al., 1969, Ward et al., 1970), reviewed by Oppenheim and colleagues (Oppenheim et al., 2000). The term chemokine, a compression of chemotactic cytokine, was adopted following the 3rd Symposium on Chemotactic Cytokines, Baden, Austria in 1992 (Kunkel et al., 1995), to describe this subgroup of the cytokine family with chemotactic effects on leucocytes. In addition, within the central nervous system a diverse range of functions have been ascribed to chemokines including the migration of multipotent progenitor cells and cell proliferation during CNS development, modulation of synaptic activity, neurotrophic effects, induction of the febrile response and anorexia (Bajetto et al., 2001, Cartier et al., 2005). Chemokine expression has been reported, in association with inflammation, in both acute and chronic CNS disorders including trauma, ischaemia, multiple sclerosis and Alzheimer's disease (Cartier et al., 2005).

1.4.2.3.2.1. Chemokines Families, Naming and Function

The chemokines are low molecular weight (8-10 kDa) basic heparin binding proteins, the family consisting of over 50 ligands which act on multiple receptors (Bajetto et al., 2001). All members contain a conserved sequence of 2 to 4 cysteine units. The position of the first 2 cysteines of this grouping has been used to divide the chemokines into subgroups, the CXC, CC, C and CX3C chemokines (Figure 1.4). The largest and most comprehensively described subgroups are the CXC and CC families. In the CXC chemokines the cysteines are separated by another amino acid whereas in the CC chemokines they are adjacent (Bajetto et al., 2001). In humans the CXC chemokines tend to be grouped on chromosome 4 and the CC chemokines on chromosome 17 (Zlotnik & Yoshie, 2000). The CXC chemokines are further sub-classified based on the presence of a glutamic acid-leucine-arginine tripeptide motif (ELR). The CXC-ELR positive chemokines, interleukin-8 (IL-8/CXCL8), growth regulated oncogenes (GRO α /CXCL1, GRO β /CXCL2, GRO γ /CXCL3) and macrophage inflammatory protein-2 (MIP-2/CXCL2) are predominantly, neutrophil chemoattractants. The CXC-ELR negative chemokines such as interferon inducible protein-10 (IP-10/CXCL10) attract lymphocytes and monocytes. The CC chemokines monocyte chemoattractant protein-1 (MCP-1/CCL2), macrophage inflammatory proteins-1 α and β (MIP-1 α /CCL3, MIP-1 β /CCL4) and regulated on activation normal T cells expressed and secreted (RANTES/CCL5) principally attract monocytes, lymphocytes, eosinophils and basophils (Bell et al., 1996, Hausmann et al., 1998, Bajetto et al., 2001).

The classification and understanding of the chemokine family is further complicated by a lack of a common naming system, resulting in some members being named after the cells that expressed them, some named after their function and others more arbitrarily (Thorpe, 2002). Secondly the same molecule has often acquired several names, varying across species. For example, the human chemokine GRO- α (P09341)⁶ is often referred to as keratinocyte cytokine (KC) (P12850)⁶ in mice and cytokine induced neutrophil chemoattractant-1 (CINC-1) (P14095)⁶ in rats (Laing & Secombes, 2004). Many of these issues have been addressed by the adoption of the standardised naming system devised by Yoshie and Zlotnik in which the chemokines members are grouped by the conserved cysteines (Figure 1.4), as mentioned and assigned individual numbers to distinguish them apart

⁶ Swissprot accession number

(Zlotnik & Yoshie, 2000). This is complimentary to the naming system for chemokine receptors and although individual receptors may bind multiple chemokines, specificity is maintained within the chemokine sub-families (Murphy et al., 2000, Thorpe, 2002). Thirdly the chemokines identified to date are not necessarily represented by “one to one” equivalent proteins between different species. For example MIP-2, identified in mice macrophages by Wolpe and co-workers in 1989 (Wolpe et al., 1989), is represented in humans by 3 homologs, GRO- α , GRO- β and GRO- γ (Tekamp-Olson et al., 1990, O'Neill & Converse, 2005). Finally, further difficulty can also arise in some cases from the lack of homology between species. In the case of the prototypical human neutrophil chemokine IL-8/CXCL8 (P10145)⁶ there is no known structural homologue in mice or rats (Tekamp-Olson et al., 1990, Zlotnik & Yoshie, 2000, Zwijnenburg et al., 2003). However, MIP-2/CXCL2 and KC/CINC/CXCL1 behave as functional homologues acting via the same 7 trans-membrane domain G-protein coupled CXCR2 receptor as IL-8/CXCL8, inducing chemotaxis, adhesion molecule expression (CD11b/CD18) and respiratory burst activity (Baggiolini et al., 1989, Smith et al., 1991, Bozic et al., 1994, Bozic et al., 1995, Frevert et al., 1995a, Frevert et al., 1995b, Shanley et al., 1997, Bajetto et al., 2001).

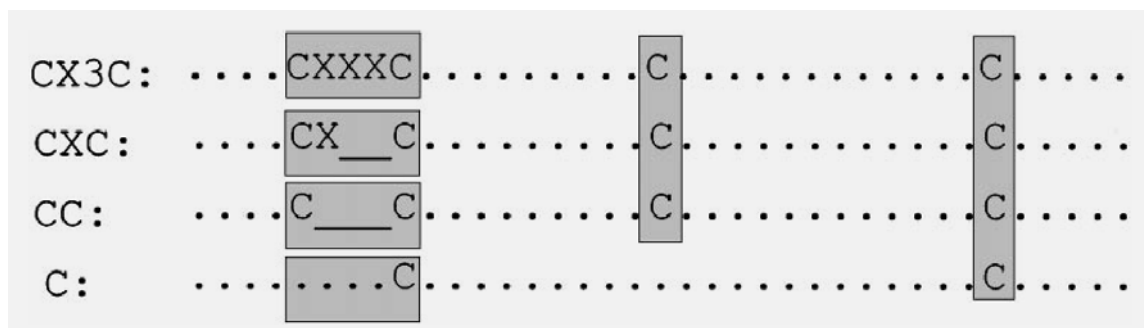


Figure 1.4. Structural Classification of the Chemokine Family.

Cysteine (C), an amino acid other than cysteine (X), dots indicate other amino acids. Underlining indicates gaps in the alignment. From Bajetto 2001.

It has been proposed that chemokines probable arose from a single ancestral gene by duplication and thus the major families became clustered in specific chromosomal locations. Human CXC chemokines are clustered on chromosome 4 and many CC chemokines are clustered on chromosome 17. Despite the duplication of genes and resultant cluster formation, the functions of the evolved products have remained related, explaining why they share receptors and act on the same cell type (Zlotnik & Yoshie, 2000). In humans the principle neutrophil chemokine is IL-8/CXCL8 with GRO- α /CXCL1, GRO- β /CXCL2 and GRO- γ /CXCL3 also being described. However in the rat the major neutrophil chemokine is MIP-2/CXCL2 with KC/CINC-1/CXCL1 also possessing significant chemotactic activity (Shanley et al., 1997, Zwijsen et al., 2003).

The classification of the CC chemokines is rather more straightforward and MCP-1/CCL2 is described in humans (P13500)⁷, rats (P14844)⁷ and mice (P10148)⁷. However it has been known by a variety of names including monocyte chemotactic and activating factor (MCAF), glioma derived chemotactic factor (GDCF) and monocyte secretory protein JE. MCP-1/CCL2 is chemotactic for monocytic cells (Furutani et al., 1989, Matsushima et al., 1989, Robinson et al., 1989, Yoshimura et al., 1989, Zachariae et al., 1990, Cross et al., 1997), microglia (Cross & Woodroffe, 1999), T lymphocytes (Carr et al., 1994) and basophils (Luster & Rothenberg, 1997).

In common with the other chemokine families the CC chemokines act via their own sub-group of 7 trans-membrane domain G protein coupled receptors. MCP-1 receptors include CCR2, CCR4, CCR9 and CCR11 (Bajetto et al., 2001). The chemotactic action of MCP-1/CCL2 on monocytes appears to be mediated via the CCR2 (Kurihara et al., 1997, Kuziel et al., 1997). *In-vitro* studies have suggested that MCP-1/CCL2 is more potent in this function than other CC chemokines such as MCP-2/CCL8, MCP-3/CCL7, MIP-1 α /CCL3, MIP-1 β /CCL4 or RANTES/CCL5 (Ugucioni et al., 1995, Cross et al., 1997), and through the use of MCP-1/CCL2 knockout mice it has been shown it to be essential for monocyte recruitment *in-vivo* (Lu et al., 1998).

⁷ Swissprot accession number

MCP-1/CCL2 also induces superoxide production (Zachariae et al., 1990), and the expression of p150 (CD11c), Mac-1 (CD11b) and CD18 integrins in monocytes (Jiang et al., 1992, Vaddi & Newton, 1994).

1.4.2.3.2.2. Central Nervous System Chemotaxis In-vivo

The largely *in-vitro* observation of a chemotactic response to the CXC and CC chemokines have also been demonstrated by several workers *in-vivo* as part of the CNS inflammatory response. Intracisternal injection of MIP-2/CXCL2 is associated with a significant CSF pleocytosis, predominantly consisting of neutrophils and the addition of CINC-1/CXCL1 increased the leucocyte count in a synergistic manner (Zwijnenburg et al., 2003). Similarly intra-hippocampal microinjection of either MIP-2/CXCL2 or IL-8/CXCL8 in mice induces a predominantly neutrophil rich recruitment by 24 hours after injection, but with a greater response to MIP-2/CXCL2 than IL-8/CXCL8. This would be consistent with species differences in the dominant CXC-chemokine (Bell et al., 1996). In keeping with observations that acute inflammatory stimuli provoke a diminished response in the CNS, the effect of the CXC chemokines on neutrophil recruitment was delayed in this study compared to the inflammatory response reported peripherally (Andersson et al., 1991, Andersson et al., 1992a, Andersson et al., 1992b). Whereas intra-dermal injection of IL-8/CXCL8 induces maximal recruitment of neutrophils into the skin of rabbits after 30 minutes (Colditz et al., 1989), neutrophil recruitment was not evident until at least 6 hours after intra-hippocampal injection (Bell et al., 1996).

Similarly the intra-hippocampal injection of MCP-1/CCL2 induces potent monocyte recruitment into the CNS (Bell et al., 1996) and mice over expressing MCP-1/CCL2 demonstrated increased numbers of perivascular mononuclear cells in the brain (Fuentes et al., 1995). Furthermore, macrophage and T cell recruitment into the denervated hippocampus was prevented in CCR2 deficient mice (Babcock et al., 2003) and monocyte recruitment in experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis, is impaired in MCP-1/CCL2 or CCR2 deficient mice (Izikson et al., 2000, Huang et al., 2001). Taken together these studies suggest that in the CNS MIP-2/CXCL2, IL-8/CXCL8 and MCP-1/CCL2 are chemotactic for their respective cell targets, *in-vivo*.

1.4.2.3.2.3. *Chemokines in CNS Pathology*

A significant and fundamental role in the CNS inflammatory response is indicated by the identification of these chemokines, or their receptors, in diverse disease processes including traumatic brain injury (Glabinski et al., 1996, Berman et al., 1996, Hausmann et al., 1998, Grzybicki et al., 1998, Muessel et al., 2000, Otto et al., 2001, Rancan et al., 2001, Babcock et al., 2003, Valles et al., 2006), cerebral ischaemia (Kim et al., 1995, Wang et al., 1995b, Matsumoto et al., 1997, Gourmala et al., 1997, Yamagami et al., 1999, Che et al., 2001, Minami & Satoh, 2003, Popivanova et al., 2003) (Table 1.4), multiple sclerosis (Ransohoff et al., 1993, Berman et al., 1996, Glabinski et al., 1997, McManus et al., 1998, Simpson et al., 1998, Kennedy et al., 1998, Van DerVoom et al., 1999, Nygardas et al., 2000) and Alzheimer's disease (Ishizuka et al., 1997, Horuk et al., 1997, Xia et al., 1997). Generally chemokines such as MIP-2/CXCL2 and MCP-1/CCL2 are undetectable or only present in low levels in uninjured tissue and increase transiently after injury (Kim et al., 1995, Wang et al., 1995b, Glabinski et al., 1996, Gourmala et al., 1997, Hausmann et al., 1998, Yamagami et al., 1999, Muessel et al., 2000, Otto et al., 2001, Che et al., 2001, Rancan et al., 2001, Babcock et al., 2003, Valles et al., 2006).

In-vivo the intracerebral expression of many CXC and CC chemokines, including MIP-2/CXCL2 (Nygardas et al., 2000), IL-8/CXCL8 (Popivanova et al., 2003) and MCP-1/CCL2 (Ransohoff et al., 1993, Kim et al., 1995, Glabinski et al., 1996, Berman et al., 1996, Ishizuka et al., 1997, Glabinski et al., 1997, Gourmala et al., 1997, McManus et al., 1998, Simpson et al., 1998, Van DerVoom et al., 1999, Che et al., 2001, Minami & Satoh, 2003) have frequently been described in astrocytes and microglia (Table 1.4).

Injury	Duration	Species	Chemokine	mRNA/Protein	Method	Increased Chemokine	Peak	Study Length	Location	Cells	Reference
CHI		Mouse	MIP-2	Protein	ELISA	4hours	4hours	7 days	Hemisphere	-	Otto 2001
Cortical Impact		Rat	MIP-2	mRNA	RT-PCR	2 to 24hours	8hours	24 hours	Cortex	-	Vallès 2006
						2 hours	2 hours		Hippocampus	-	
				Protein	Western	8 to 24hours	8hours	24 hours	Cortex	-	
Entorhinodentate lesion		Mouse	MIP-2	mRNA	RPA	24 hours		24 hours	Entorhinal Cortex & Hippocampus		Babcock 2003
Stab Injury		Mouse	MCP-1	mRNA	RT-PCR	3 to 24hours	3 to 12 hours	24 hours	Cortex		Glabinski 1996
				Protein	ELISA	12 to 24hours	12 hours	48 hours			
				mRNA	ISH	6 hours				Astrocytes	
Stab Injury		Rat	MCP-1	Protein	IHC	6 to 72 hours			Cortex	Monocytes & Endothelial Cells	Berman 1996
				mRNA	Northern	3 to 72 hours	6 to 24 hours	3 days	Hemispheres		
Cortical Resection		Mouse	MCP-1	mRNA	Northern	2-96h	12h	7 days	Hemispheres	-	Hausmann 1998
Cortical Cryoinjury		Mouse	MCP-1	mRNA	RT-PCR	6-24h	6h	14 days	Hemispheres	-	Grzybicki 1998
Cortical Resection		Mouse	MCP-1	mRNA	Northern	1 hour to 14 days	3 to 24 hours	14 days	Cortex		Muessel 2000
						1 to 24 hours	12 hours		Thalamus		
				Protein	ELISA	6 to 72 hours	12 hours	7 days	Cortex		
						6-72h	12 hours		Thalamus	Suggested Glia at 12 hours	
DAI		Rat	MCP-1	Protein	ELISA	4 to 16 hours	16 hours	14 days	Brain Homogenates	-	Rancan 2001
Entorhinodentate lesion		Mouse	MCP-1	mRNA	RT-PCR	3 to 48 hours	24 hours	2 days	Entorhinal Cortex & Hippocampus		Babcock 2003
				mRNA	ISH	24 hours				Microglia & Astrocytes	

Table 1.4. *In-vivo* Experimental Chemokine Expression Following Trauma and Ischaemia.

Closed head injury (CHI), diffuse axonal injury (DAI) enzyme linked immunosorbent assay (ELISA), immunohistochemistry (IHC), in-situ hybridization (ISH), macrophage inflammatory protein-2 (MIP-2), messenger ribonucleic acid (mRNA), monocyte chemoattractant protein-1 (MCP-1), RNase protection assay (RPA), revers transcriptase polymerase chain reaction (RT-PCR).

Injury	Duration	Species	Chemokine	mRNA/Protein	Method	Increased Chemokine	Peak	Study Length	Location	Cells	Reference
TICAO, TACAO, TMCAO	250 minutes	Rabbit	IL-8	Protein	ELISA IHC	6 to 12 hours 6 hours	12 hours	12 hours	Cortex, Striatum	Vessels and Neutrophils	Matsumoto 1997
TBISAO	20 minutes	Monkey	IL-8	Protein	IHC	30 to 60 minutes		4 days	Hippocampus	Microglia	Popivanova 2003
PMCAO TMCAO	2.6 hours	Rat	MCP-1	mRNA	Northern	6 hours to 5 days 3 hours to <5 days	12 to 48 hours	5 days	Cortex	-	Wang 1995
PMCAO		Rat	MCP-1	mRNA	IHC Northern Northern	6 hours to 7 days 6 to 48 hours post onset	48 hours 24 to 48 hours post onset	7 days 4 days	Striatum, Preoptic Area, Cortex Hemispheric Sample	Monocytes & Endothelial Cells -	Kim 1995
PMCAO		Rat	MCP-1	mRNA	ISH	6 hours to 6 days	24 hours	6 days	Penumbra Infarct	6 hours to 2 days Astrocytes 4 to 6 days Microglia/Monocytes	Gourmala 1997

Figure 1.4 Continued. *In-vivo* Experimental Chemokine Expression Following Trauma and Ischaemia.

Enzyme linked immunosorbent assay (ELISA), immunohistochemistry (IHC), in-situ hybridization (ISH), interleukin-8 (IL-8), messenger ribonucleic acid (mRNA), monocyte chemoattractant protein-1 (MCP-1, permanent middle cerebral artery occlusion (PMCAO), transient middle cerebral artery occlusion (TMCAO), transient bilateral innominate/subclavian artery occlusion (TBISAO). For transient models the times represent from reperfusion, unless stated.

Injury	Duration	Species	Chemokine	mRNA/Protein	Method	Increased Chemokine	Peak	Study Length	Location	Cells	Reference
PMCAO		Mouse	MCP-1	Protein	IHC	12 hours to 3 days			Basel Ganglia (Core)	Neuronal	Che 2001
						12 hours to 5 days		5 days	Cortex		
						> 3 days			Striatum, Corpus Callosum, Hippocampus	Astrocytes	
						ELISA	1 to 3 days	24 hours	Hemispheres		
TMCAO	1 hour	Rat	MCP-1	Protein	ELISA	6 hours to 7 days	48 hours	7 days	Hemispheric Sample	-	Yamagami 1999
TMCAO	2 hours	Rat	MCP-1	mRNA	ISH	2 to 24 hours	2 & 24 hours	24 hours	Mainly Penumbra	Astrocytes & Microglia/Macrophages	Minami 2003

Figure 1.4 Continued. *In-vivo* Experimental Chemokine Expression Following Trauma and Ischaemia.

Enzyme linked immunosorbent assay (ELISA), immunohistochemistry (IHC), in-situ hybridization (ISH), messenger ribonucleic acid (mRNA), monocyte chemoattractant protein-1 (MCP-1, permanent middle cerebral artery occlusion (PMCAO), transient middle cerebral artery occlusion (TMCAO). For transient models the times represent from reperfusion, unless stated.

1.4.2.4. Pathological Effects of Leucocyte Accumulation

Accumulation of circulating leukocytes and activation of microglia after injury is generally considered to be a significant contributing factor to secondary brain injury. Toxic mediator release and neuronal death have been demonstrated *in-vitro* (Colton & Gilbert, 1987, Chao et al., 1992, Giulian et al., 1993b, Dinkel et al., 2004) (Table 1.5). In experimental models of brain injury, perfusion is impaired by the intra-vascular accumulation of leucocytes whilst the reduction in leucocyte number or inhibition of recruitment is frequently associated with a reduction in pathology (Table 1.6).

1.4.2.4.1. Neutrophils

Neutrophils contain numerous potentially toxic agents such as myeloperoxidase and elastase. *In-vitro* co-culture of neutrophils with hippocampal neurones is associated with increased cell death (Dinkel et al., 2004) (Table 1.5). Neutrophils are particularly potent sources of free radicals. Activation of the hexose-monophosphate shunt generates reduced nicotinamide adenine dinucleotide phosphate (NADPH). Electrons from this pass via a flavine adenine dinucleotide (FAD)/cytochrome b₅₅₈ to reduce oxygen directly to $\cdot\text{O}_2^-$. Neutrophils and macrophages also contain inducible nitric oxide synthase (iNOS). The nitric oxide radical ($\cdot\text{NO}$) generated can react with $\cdot\text{O}_2^-$ to form toxic $\cdot\text{ONOO}$ radicals (Roitt, 2001) (Section 1.3.4).

1.4.2.4.2. Microglia and Macrophages

Microglia function as the resident monocytes of the brain phagocytosing damaged cells and scavenging debris (Giulian & Vaca, 1993). Monocytic cells have been implicated in free radical generation and EAA like toxin production. Microglia are a source of $\cdot\text{O}_2^-$ (Colton & Gilbert, 1987) and $\cdot\text{NO}$ (Chao et al., 1992). Microglia co-cultured with neurons secrete potent, small (<500D), heat and protease stable neurotoxic factors which although neutralized by NMDA receptor antagonists can be distinguished from brain derived NMDA agonists (Piani et al., 1992, Giulian et al., 1993b) (Table 1.5) Although microglia can respond within hours of ischaemic injury, peak neurotoxic secretory activity of tissue biopsies from traumatic and ischaemic lesions only occurred after 2 days. This

coincided with peak mononuclear cell activation/accumulation (Giulian & Vaca, 1993, Giulian et al., 1993a) and implies that the neurotoxic potential of microglia develops over time.

Injury	Species	Cell Culture	Timing	Result	Reference
Opsonised Zymosan	Rat	Microglial Astrocytes	0-4 hours	Superoxide production No superoxide	Colton 1987
-	Rat	Neuronal/microglial co-cultures	0-72 hours	Reduced neurone survival. Neurite retraction, necrosis and phagocytosis by microglia Increased generation of superoxide radical	Théry 1991
		Neuronal/astrocyte co-cultures		No neurotoxicity	
IFN- γ +LPS	Mouse	Neuronal/microglial co-cultures	72 hours	Neuronal death/injury, associated with an NO metabolite and both blocked by an L-arginine antagonist	Chao 1992
		Neuron/astrocyte co-cultures		No neuronal injury	
Zymosan	Rat	Microglial products	48 hours of indirect co-culture with neurones	Reduced neurone survival. Effect blocked by NMDA receptor antagonists but toxin distinct from glutamate or aspartate	Giulian 1993
		Astrocyte products		Increased neurone survival	
-	Rat	Hippocampal neuron/glia co-cultures with neutrophils	3 day co-culture	Neutrophil induced neuronal death and exacerbated the effect of kainic acid and oxygen glucose deprivation	Dinkel 2004

Table 1.5. *In-vitro* Effects of Immune Cells on Neurone Survival.

Interferon-gamma (IFN- γ), lipopolysaccharide (LPS), nitric oxide (NO), N-methyl-D-aspartate (NMDA).

1.4.2.4.3. Inhibition of Leucocyte Accumulation in Acute Brain Injury

1.4.2.4.3.1. Neutrophil Depletion and Anti-Adhesion Molecules

Following transient ischaemic insults experimental results suggest that the inhibition of leucocyte recruitment into the brain can have protective effects (Table 1.6). Neutrophils and monocytes have been observed plugging capillaries and venules after both transient and permanent ischaemic insults in animals (del Zoppo et al., 1991, Garcia et al., 1994). Deficient microvascular perfusion, “no-reflow”, was seen in > 60% of capillaries within 1 hour of reperfusion following middle cerebral artery occlusion (del Zoppo et al., 1991). Following transient ischaemia neutropenia, anti-neutrophil antibody or inhibition of adhesion improved cerebral blood flow (Dutka et al., 1989, Helps & Gorman, 1991, Mori et al., 1992, Connolly, Jr. et al., 1996). The intra-cerebral accumulation of neutrophils has also been associated with BBB breakdown. Induction of neutropenia prevented PML accumulation and associated BBB dysfunction after intraparenchymal injection of CXC chemokines (Bell et al., 1996).

Transient ischaemia is associated with a marked increase in the extracellular free radical concentration over the first hour of reperfusion, returning to baseline before increasing again at 24 hours. Depletion of circulating neutrophils with an anti-neutrophil antibody prevented this increase in extracellular oxygen radical concentration after reperfusion (Matsuo et al., 1995). Similarly depletion of neutrophils or inhibition of adhesion was associated with reduced oedema formation after TMCAO (Shiga et al., 1991, Matsuo et al., 1994a, Matsuo et al., 1994b).

Neutrophil depletion, inhibition of neutrophil function or the inhibition of adhesion molecules after transient ischaemia is also associated with reduced lesion size (Chen et al., 1994, Chopp et al., 1994, Zhang et al., 1994, Matsuo et al., 1994a, Matsuo et al., 1994b, Jiang et al., 1995, Zhang et al., 1995b, Connolly, Jr. et al., 1996) and improved functional outcome (Chen et al., 1994, Connolly, Jr. et al., 1996). In adhesion molecule knockouts, lesion size (Connolly, Jr. et al., 1996, Soriano et al., 1999) and neurological deficit are also reduced (Connolly, Jr. et al., 1996).

Contrary to the evidence presented an anti-CD18 antibody administered during 90 minutes of transient ischaemia in cats did not reduce injury volume after 3 hours of reperfusion. This could imply that the specific epitope of the CD11/CD18 molecule is important in affording protection. The choice of species could also be important, as the cat does not have an internal carotid artery. Unlike the predominantly rodent studies where the MCAO was occluded via the internal carotid artery, in this cat model, ischaemia was produced by the occlusion of both common carotids and a middle cerebral artery. Alternatively assessment at 3 hours of reperfusion could have been too early to detect a benefit (Takeshima et al., 1992) (Table 1.6).

In permanent ischaemic lesions the protective effects of leucocyte inhibition are lost. Lesion volume is not reduced (Zhang et al., 1995a, Garcia et al., 1996, Zhang et al., 2003). A reduction in neurological deficit was only found in one study and this effect was lost when administration was delayed (Zhang et al., 2003). In 2 other studies no reduction in neurological deficit was seen (Clark et al., 1991, Garcia et al., 1996). Therefore the inhibition of leucocyte recruitment can benefit vulnerable tissue but will make no difference if the cell death is inevitable as occurs in a permanent ischaemic injury.

In experimental TBI the inhibition of leucocyte adherence by treatment with anti-CD11b or anti-ICAM-1 antibody reduced cortical myeloperoxidase activity, a marker of neutrophil infiltration (Carlos et al., 1997, Weaver et al., 2000, Knoblich & Faden, 2002). However, whereas in experimental transient ischaemic injuries neutropenia or the inhibition of leucocyte adherence to the endothelium has repeatedly been shown to reduce neutrophil infiltration, lesion size and neurological deficit (Table 1.6) the results for traumatic injuries are much less convincing. Only one study has reported a modest reduction in neurological deficit after administration of a mouse anti-rat ICAM-1 antibody. Curiously improved function was also seen in controls receiving a non-specific mouse IgG, when compared to saline treated controls. Neutrophil recruitment was reduced in both groups but only significantly in the anti-ICAM-1 treated animals. This suggests the possibility that some of the observed effect might not be due to ICAM-1 interaction but could also be a consequence of the administration of mouse IgG to rats (Knoblich & Faden, 2002). As the authors pointed out, in

previous studies either saline or IgG had been used as controls, but not both, preventing such an observation. In ICAM-1 deficient mice there was no reduction in neutrophil accumulation, lesion volume or neurological dysfunction after controlled cortical impact (Whalen et al., 1999a). In a companion paper by the same group, similar results was reported for mice deficient in both ICAM-1 and P-selectin, although in these animals oedema was reduced in the knockout animals (Whalen et al., 2000). These findings contrast with the reduction in lesion volume and improved function reported in ICAM-1 deficient mice after TMCAO (Connolly, Jr. et al., 1996). Although direct comparison between these different studies is complicated by variations in the selection of control groups, the different end points and times post injury when assessments were made, the data suggests that inhibition of adhesion molecule function is less effective in traumatic injuries compared to primary ischaemia. Although neutrophil infiltration was reduced by antibody treatment (37 to 66% reduction in the TBI studies) (Carlos et al., 1997, Weaver et al., 2000, Knoblich & Faden, 2002), it was not prevented. This approach may therefore be insufficient, on its own, to consistently improve outcomes after TBI.

Injury	Duration/Severity	Species	Antagonist	Control	Route	Timing	Result	Assessed	Reference
Weight Drop		Rat	Anti-ICAM-1 antibody	IgG	IV	30 minutes pre	Reduced MPO	24 hours	Carlos 1997
CCI		Mouse	ICAM-1 KO				No reduction in neutrophil number No reduction in lesion volume No reduction in motor deficit No reduction in memory dysfunction	24 hours 21 days 1 to 5 days 14 to 18 days	Whalen 1999
CCI		Rat	Anti-CD11b antibody	IgG F(ab)2'	IV	30 minutes & 24 hours post	No reduction in neurological deficit Reduced MPO	1, 12 and 24 hours 24 hours	Weaver 2000
CCI		Rat	Anti-ICAM-1 antibody	Saline	IV	Immediately, 1, 2 & 3 days post injury	Preserved memory, day 10 only No reduction in lesion area	10 to 13 days 15 days	Isaksson 2001
LFPI	Moderate	Rat	Anti-ICAM-1 antibody	IgG vs. Saline	IV	1,10, 24 hrs post	Reduced neurological deficit (Also reduced with non-specific IgG) Reduced MPO	14 days 26 hours	Knobloch 2002
Hippocampal MIP-2/IL-8		Mouse	Leucocyte depletion by irradiation				Less BBB disruption	24 hours	Bell 1996
ICA Air Embolism (repeated)	1 hour of depressed evoked responses	Dog	Mechlorethamine granuloctopenia			4 days prior	Improved cortical somatosensory evoked responses Increased CBF	1 hour	Dutka 1989
ICA Air Embolism	Transient ischaemia	Rabbit	Mechlorethamine granuloctopenia			3 days prior	Preserved cortical somatosensory evoked responses Preserved CBF	Over 3 hours	Helps 1991
TMCAO	1 hours	Rat	Anti-neutrophil antibody	Saline	IP	24 hours pre & at reperfusion	Reduced oedema	24 hours	Shiga 1991
TMCAO	1 hour	Rat	Anti-neutrophil antibody	PBS	IP	24 hours pre & at reperfusion	Prevents MPO increase Reduced oedema Reduced lesion area	24 hours	Matsuo 1994

Table 1.6. The Effect of the Inhibition of Leucocyte Accumulation on the Pathology of Acute Brain Injury.

Blood brain barrier (BBB), cerebral blood flow (CBF), controlled cortical impact (CCI), immunoglobulin (IgG), interleukin-8 (IL-8), internal carotid artery (ICA), intercellular adhesion molecule (ICAM), intraperitoneal (IP), intravenous (IV), lateral fluid percussion injury (LFPI), macrophage inflammatory protein-2, myeloperoxidase (MPO), phosphate buffered saline (PBS), transient middle cerebral artery occlusion (TMCAO).

Injury	Duration/Severity	Species	Antagonist	Control	Route	Timing	Result	Assessed	Reference
TMCAO	1 hour	Rat	Anti-neutrophil antibody	PBS	IP	24 hours pre & at reperfusion	Prevented oxygen radical increase	Over 24 hrs	Matsuo 1995
TMCAO	45 minutes	Mouse	Anti-neutrophil antibody	Not specified	IP	Daily for 3 days prior	Reduced lesion volume Increased CBF Reduced neurological deficit	24 hours	Connolly 1996
TMCAO	2 hours	Rat	Neutrophil inhibitory factor CD11b/18 antagonist	Saline	IV	Reperfusion & continued as infusion	Reduced lesion volume Reduced neutrophils	48 hours	Jiang 1995
TMCAO	3 hours	Baboon	Anti-CD18 antibody	Not specified	IV	15 minutes prior to reperfusion	Increased reflow	1 hour	Mori 1992
TMCAO	2 hours	Rat	Anti-CD11b antibody 1mg/kg	IgG	IV	At reperfusion & 22 hours	Reduced neutrophils	48 hours	Chen 1994
			Anti-CD11b antibody 2mg/kg			Reperfusion	Reduced neurological deficit Reduced lesion volume Reduced neutrophils	22 hours 48 hours	
			Anti-CD11b antibody 2 & 1 mg/kg			1 & 22 hours of reperfusion	Reduced lesion volume Reduced MPO	48 hours	
TMCAO +TBCCAO	45 minutes	Cat	Anti-CD18 antibody	PBS	IV	45 minutes post onset	No reduction in lesion volume No improvement in CBF No recovery of electrophysiology	3 hours	Takeshima 1992
TMCAO	1 hour	Rat	Anti-CD11a antibody Anti-CD18 antibody Anti-ICAM antibody	PBS	IP	15 minutes prior to ischaemia & at reperfusion	Reduced oedema Reduced lesion size Reduced MPO	24 hours	Matsuo 1994

Table 1.6 Continued. The Effect of the Inhibition of Leucocyte Accumulation on the Pathology of Acute Brain Injury.

Cerebral blood flow (CBF), immunoglobulin (IgG), intercellular adhesion molecule (ICAM), intraperitoneal (IP), intravenous (IV), knockout (KO), lateral fluid percussion injury (LFPI), myeloperoxidase (MPO), permanent middle cerebral artery occlusion (PMCAO), phosphate buffered saline (PBS), transient bilateral common carotid artery occlusion (TBCCAO), transient middle cerebral artery occlusion (TMCAO).

Injury	Duration/Severity	Species	Antagonist	Control	Route	Timing	Result	Assessed	Reference
TMCAO	2 hours	Rat	Anti CD11b antibody	IgG	IV	Reperfusion	Reduced MPO	48 hours	Zhang 1995
			Anti-CD11b or CD18 antibody			0 ± 22 hours of reperfusion	Reduced lesion volume	7 days	
			Anti-CD11b antibody			2 & 22 hours of reperfusion			
TMCAO	2 hours	Rat	Anti-ICAM-1 antibody	IgG	IV	2 & 22 hours of reperfusion	Reduced lesion volume	7 days	Zhang 1995
TMCAO	3 hours	Mouse	CD11b/18 KO				Reduced lesion volume Reduced neutrophils	24 hours	Soriano 1999
TMCAO	2 hours	Rat	Anti-ICAM-1 antibody	IgG	IV	Reperfusion & 22 hours	Reduced lesion volume Reduced MPO	48 hours	Zhang 1994
TMCAO	45 minutes	Mouse	ICAM-1 KO				Reduced lesion volume Increased CBF Reduced neurological deficit Reduced mortality	24 hours	Connolly 1996
Embolic PMCAO		Rat	Neutrophil Inhibitory Factor CD11b/18 antagonist	Saline	IV	2 hours	Decreased neutrophils Decreased neurological deficit	7 days	Zhang 2003
						4 hours	No effect on lesion volume No reduction in neutrophils No reduction in neurological deficit No reduction in lesion volume		
Multiple Embolism	Permanent ischaemia	Rabbit	Anti-CD18 antibody	Saline	IV	30 minutes prior to ischaemia	No reduction in neurological deficit	18 hours	Clark 1991
PMCAO		Rat	Anti-ICAM-1 antibody	IgG	IV	2 hours and 24 hours	No reduction in lesion volume	7 days	Zhang 1995
PMCAO		Rat	Anti-CD11b antibody	IgG vs. PBS	IV	1 hour	Less necrotic neurones No reduction in neutrophils	12 hours	Garcia 1996
							No reduction in necrotic neurones		
							No reduction in neurological deficit		
							No reduction in neutrophils No decrease in lesion volume	4 days	

Table 1.6 Continued. The Effect of the Inhibition of Leucocyte Accumulation on the Pathology of Acute Brain Injury.

Cerebral blood flow (CBF), immunoglobulin (IgG), intercellular adhesion molecule (ICAM), intravenous (IV), knockout (KO), myeloperoxidase (MPO), permanent middle cerebral artery occlusion (PMCAO), phosphate buffered saline (PBS), transient middle cerebral artery occlusion (TMCAO).

1.4.2.4.3.2. Anti-Chemokines

Limited evidence for the potential benefit of the inhibition of chemokine function also exists. Following 60 minutes of TMCAO in the rat anti-CINC/CXCL1 antibody, given at reperfusion, significantly reduced neutrophil accumulation and oedema formation after 24 hours of reperfusion and infarct area at 7 days (Yamasaki et al., 1997). Similarly in rabbits subjected to 2.5 hours of transient anterior and middle cerebral artery ischaemia anti-IL-8/CXCL8 antibody given at reperfusion also reduced neutrophil accumulation, BBB opening and infarct volume after 12 hours of reperfusion (Matsumoto et al., 1997).

Viral macrophage inflammatory protein-2 (vMIP-2) is a chemokine like peptide with a broad spectrum of antagonistic activity against mainly CC chemokine receptors (Kledal et al., 1997). ICV administration of vMIP-2 1 hour prior to 1 hour of TMCAO and again after 1 hour of reperfusion results in a dose dependant reduction in infarct volume at 2 days post-ischaemia. The number of activated microglia/macrophages, but not neutrophils, were significantly reduced in the ischaemic penumbra after 48 hours of reperfusion in the vMIP-II treated group (Minami & Satoh, 2003).

1.5. Thesis Objectives

1.5.1. The Standard of Care in Traumatic Brain Injury

To date the mainstay of TBI therapy is aimed at the prevention of secondary injury due to tissue hypoxia. Guidelines from both Europe and North American suggest optimal management based on the best available evidence (Maas et al., 1997, The Brain Trauma Foundation, 2007). Surgical intervention to decompress the brain by removal of space occupying lesions, protection of the airway, control of arterial blood gas tensions, maintenance of cerebral perfusion with vasoactive drugs, the manipulation of ICP and avoidance of hyperpyrexia form the basis of routine management and serve to reduce the incidence of cerebral hypoxia. There are no randomised controlled trials demonstrating that such an approach is beneficial and it is unlikely there will be due to the widespread acceptance of this approach based on data from observational studies and concerns about the ethical recruitment of a control group (Citerio, 2006). However analysis of mortality data from patients in the traumatic coma data bank (TCDB) and control arms of clinical studies suggests there has been a reduction in deaths associated with TBI. In 1984 mortality was 39% and by 1996 it was down to 27%. The mortality in the period 1984 to 1987 was significantly greater than from 1988 to 1996 (Lu et al., 2005). This reduction coincides with the greater awareness of the importance of improving early stabilisation, transport and specialist management of brain trauma patients to minimise secondary brain injury.

Despite many years of clinical trials an effective pharmaceutical agent capable of protecting the brain from the multiple derangements in neurochemistry, including inflammation, which occur after brain injury is lacking. Preclinical evidence supports the antagonism of leucocyte recruitment as an appropriate potential neuroprotective strategy (Section 1.4.2.4.3). Clinically, the induction of neutropenia is impracticably as the resultant sepsis would most likely offset any benefit of the treatment. Based on promising results of experimental studies, clinical trials of anti-adhesion molecules have been conducted in patients with ischaemic stroke. The largest of these examined the effect of a murine anti-ICAM-1 antibody in a randomised placebo controlled trial of over 600 patients. Treatment was commenced within 6 hours of symptoms. Neurological outcome was significantly poorer and there were more deaths in the treatment group at 90 days. Fever and infection, particularly

pneumonia, were also more common with the anti-ICAM-1 treatment (E.A.S.T, 2001). The possible reasons for the failure of this study were many. Contrary to the laboratory data, only 12% of the patients were treated within 3 hours of symptoms. Furthermore whereas anti-adhesion molecule treatment has been found to be effective experimentally only when the ischaemic insult is transient this important discriminator was not required for entry into this study. Latter consideration of the effect of giving a murine antibody to humans suggested that this foreign protein could in fact lead to neutrophil activation via the compliment system (Becker, 2002). The glycoprotein UK-279,276 which binds to the CD11b region of the CD11b/CD18 integrin, predominantly expressed on neutrophils, has been examined in a safety and tolerability study of acute stroke patients. In this case there was no increase in fever or infection in the study patients and the drug was well tolerated (Lees et al., 2003). However the clinical trial of this agent involving over 900 patients treated within 6 hours of symptom onset found no improvement in outcome and was terminated early due to futility. In a subset of patients treated with thrombolysis as well as study drug there was a small non-significant additional improvement. The study was not designed or powered to investigate the interaction of thrombolysis and UK-279,276 (Krams et al., 2003). The phase III trial of Hu23F2G, an antibody against CD18, was also stopped after interim analysis also suggested futility. The results have not been published (Becker, 2002). The pre-clinical data for TBI and the use of anti-adhesion molecules is less convincing than it is for transient ischaemia models. As discussed in section 1.4.2.4.3.1 it could be that this approach is not be sufficient, on its own, to consistently improve outcomes after TBI. A greater understanding of chemokine expression after TBI and exploration of the inhibition of these cytokines potentially offers an alternative approach to controlling leucocyte recruitment. Such an inhibitory strategy may also have to be confined to the CNS to avoid systemic side effects.

1.5.2. Scientific Issues to be Addressed

Considerable evidence suggests that the recruitment of leucocytes into the brain after acute injury represents a significant secondary injury process (Section 1.4.2.4) and chemokines appear to be important communication molecules regulating this recruitment into CNS (Sections 1.4.2.3.2.2 & 1.4.2.3.2.3).

At the start of this degree only one paper describing chemokine expression following experimental focal TBI had been published (Otto et al., 2001). The expression of the major chemokine families after TBI was incompletely described. Furthermore, the types of cells expressing these proteins were only speculated upon, extrapolating data from *in-vitro* studies. Comparisons of chemokine expression between rodent and human cells are also limited. Therefore the relevance of chemokine expression in animals models to human disease is uncertain.

The modulation of chemokine expression after TBI may prove to be a useful additional means of regulating the inflammatory response and reducing secondary injury. However, very little is published on the inhibition of chemokine expression after acute brain injury.

Finally the development of cerebral contusions containing large numbers of inflammatory cells is often associated with the neurological deterioration of patients who have sustained TBI. Increased chemokine concentrations have been reported in the blood and CSF of patients with acute brain injury (Section 4.2.1.1.). However it is not clear if the concentration of chemokines detected systemically are more elevated in those patients with enlarging contusions compared with those who remained stable. Such a biochemical marker might, by initiating therapeutic interventions, prevent clinical deterioration and/or further secondary brain injury.

1.5.3. Hypotheses to be Tested

1. That the expression of chemokines by rodent and human glial cells are comparable between the species and that the study of chemokine expression in a rat model of TBI is pertinent to human pathology.
2. That the chemokines MIP-2/CXCL2 and MCP-1/CCL2 are increased in the contused cortex of rats after fluid percussion injury and that microglia and/or astrocytes are the cells producing these chemokines *in-vivo*.
3. That chemokine expression can be inhibited pharmacologically. Specifically that IL-6 acting via its soluble receptor will inhibit MIP-2/CXCL2 expression. The anti-inflammatory steroid dexamethasone and the immunosuppressant FK506 will also be evaluated.
4. That in patients with cerebral contusions IL-8/CXCL8, MCP-1/CCL2 and sIL-6R can be detected in the serum after injury. In patients with enlarging contusions the concentrations of IL-8/CXCL8 and MCP-1/CCL2 will be increased and the concentration of sIL-6R will be reduced compared to stable patients.

1.5.4. Research Aims

In chapter 2 I will;

- Describe the expression of MIP-2/CXCL2, IL-8/CXCL8 and MCP-1/CCL-2 by rat and human glia treated with pro-inflammatory cytokines.
- Compare this expression between the species.
- Investigate the inhibition of chemokine expression by glia using:
 - IL-6 acting via its soluble receptor (sIL-6R).
 - The anti-inflammatory agent dexamethasone.
 - Immunosuppressant agent FK506.

Both dexamethasone and FK506 have anti-inflammatory properties and their administration is associated with decreased pathology in models of acute brain injury.

In chapter 3 I will;

- Establish and validate the fluid percussion model of focal TBI in Edinburgh.
- Describe the cortical expression of MIP-2/CXCL2 and MCP-1/CCL2 after fluid percussion injury.
- Confirm the cellular source of MIP-2/CXCL2 and MCP-1/CCL2 expression.
- Investigate the effect of immunomodulatory strategies on chemokine expression.

In chapter 4 I will:

- Determine the expression of IL-8/CXCL8, MCP-1/CCL2 and sIL-6R in the serum of patients with cerebral contusions admitted to intensive care after TBI.
- Determine if a relationship exists between serum chemokine or sIL-6R concentrations in patients with contusional brain injury and the enlargement of contusions over time.

2. The Comparative Expression of Chemokines by Glia and the Investigation of Possible Inhibitory Strategies

2.1. **Chapter Aims**

- To describe the expression of MIP-2/CXCL2, IL-8/CXCL8 and MCP-1/CCL-2 by rat and human glia treated with pro-inflammatory cytokines.
- Compare this expression between the species.
- Investigate the inhibition of chemokine expression by glia using:
 - Il-6 acting via its soluble receptor (sIL-6R).
 - The anti-inflammatory agent dexamethasone.
 - Immunosuppressant agent FK506.

Both dexamethasone and FK506 have anti-inflammatory properties and their administration is associated with decreased pathology in models of acute brain injury.

2.2. Background

2.2.1. Chemokine Expression Induced by Pro-inflammatory Cytokines

The expression of the pro-inflammatory mediators TNF- α and IL-1 β are often described, both clinically and in experimental studies, in similar CNS pathologies to those in which chemokine expression has been identified. These include trauma, ischaemia/hypoxia (Tables 1.1, 1.2 and 1.3), multiple sclerosis (Hofman et al., 1989) and Alzheimer's disease (Griffin et al., 1989, Tarkowski et al., 1999). *In-vivo* the microinjection of TNF- α or IL-1 β in to the striatum of rodents increased α and β chemokine mRNA expression in the affected hemisphere within hours of injection (Anthony et al., 1998, Glabinski et al., 2003). The role of pro-inflammatory mediators stimulating chemokine expression is also supported in some disease models. Following focal traumatic brain injury cerebral MIP-2/CXCL2 expression was significantly reduced in TNF/Lymphotoxin- α knockout mice compared to wild types, implicating TNF- α in the expression of this CXC chemokine (Otto et al., 2001). Similarly, in TNF- α deficient mice, the early induction of MCP-1/CCL2 seen in EAE was delayed, as was the onset of disease (Murphy et al., 2002).

In-vivo the intracerebral expression of CXC and CC chemokines have frequently been described in astrocytes and microglia (Table 1.4). Consequently many groups have studied chemokine expression by glial cell cultures in response to cytokine stimulation. Human astrocytes have been shown to produce IL-8/CXCL8 (Aloisi et al., 1992, Ehrlich et al., 1998, Oh et al., 1999, Janabi et al., 1999, Hua & Lee, 2000, Croitoru-Lamoury et al., 2003) or MCP-1/CCL2 (Barna et al., 1994, Hurwitz et al., 1995, Peterson et al., 1997, Oh et al., 1999, Croitoru-Lamoury et al., 2003) when incubated with pro-inflammatory cytokines such as TNF- α or IL- β . Similarly human microglial cultures express IL-8/CXCL8 (Ehrlich et al., 1998, Janabi et al., 1999, Hua & Lee, 2000) following stimulation with pro-inflammatory mediators.

In rodent cells, MIP-2/CXCL2 (Otto et al., 2000) and MCP-1/CCL2 (Hayashi et al., 1995) protein production by mouse astrocyte cultures has also been demonstrated. MCP-1/CCL2 mRNA expression has been reported in rat microglial cultures (Calvo et al., 1996) (Table 2.1). However, the production

of MIP-2/CXCL2 and MCP-1/CCL2 protein by rat astrocyte or microglial cultures and the comparison of this expression with human cells have not been described.

Therefore in this thesis I have compared the expression of chemokines in rat glia with that of human cells. This comparison will also serve to support the relevance to human pathology of the *in-vivo* study of chemokine expression in the rat following experimental TBI, as described in chapter 3. To make this comparison of chemokine expression a combination of primary and immortalised cell lines were studied. For the rat these were primary neonatal microglia and astrocytes, the highly aggressive proliferating (HAPI) microglial cell line and an immortalised astrocyte cell line, CTX-TNA2. For human cells the immortalised microglial cell line, CHME-5, and the immortalised U373 astrocyte line were studied. In addition ethical permission was granted to obtain human cerebral temporal lobe tissue, resected for the treatment of intractable epilepsy, with the aim of using this material as a source of glial cells for primary culture.

Chemokine	Cell Type	Species	Line	Agonist	mRNA/Protein	Reference
MIP-2	Astrocyte	Mouse	Neonatal Primary	TNF- α	Protein	Otto 2000
IL-8	Astrocyte	Human	Embryonic Primary	TNF- α IL-1 β	Protein	Aloisi 1992
IL-8	Astrocyte	Human	Foetal Primary	TNF- α IL-1 β	- Protein	Ehrlich 1998
IL-8	Astrocyte	Human	U251 astroglioma	TNF- α IL-1 β	mRNA+Protein	Oh 1999
				IL-6+sIL-6R	- mRNA	
			U373 astroglioma	TNF- α IL-1 β	mRNA+Protein	
				IL-6+sIL-6R	- mRNA	
			Adult Primary	TNF- α IL-1 β	mRNA+Protein	
				IL-6+sIL-6R	- mRNA	
IL-8	Astrocyte	Human	Embryonic Primary	TNF- α + IL-1 β	Protein	Janabi 1999
IL-8	Astrocyte	Human	Foetal Primary	TNF- α IL-1 β	mRNA	Hua 2000
IL-8	Astrocyte	Human	Foetal Primary	TNF- α IL-1 β	Protein	Croitoru 2003
				IL-6	-	
		Monkey	Adult Primary	TNF- α IL-1 β	Protein	
				IL-6	-	
IL-8	Microglia	Human	Foetal Primary	TNF- α IL-1 β	mRNA+Protein	Ehrlich 1998
			Adult Primary	TNF- α IL-1 β	- Protein	
				IL-1 β	Protein	
IL-8	Microglia	Human	Embryonic Primary	TNF- α + IL-1 β	Protein	Janabi 1999
IL-8	Microglia	Human	Foetal Primary	TNF- α IL-1 β	mRNA	Hua 2000

Table 2.1. *In-vitro* Chemokine Expression by Astrocytes and Microglia Treated with Pro-Inflammatory Cytokines.

Interleukin-1 β (IL-1 β), interleukin-6 (IL-6), interleukin-8 (IL-8), macrophage inflammatory protein-2 (MIP-2), monocyte chemoattractant protein-1 (MCP-1), no expression (-), soluble interleukin-6 receptor (sIL-6R), tumour necrosis factor- α (TNF- α).

Chemokine	Cell Type	Species	Line	Agonist	mRNA/Protein	Reference
MCP-1	Astrocyte	Human	P1N-non neoplastic W3N - non neoplastic	TNF- α	mRNA	Barna 1994
MCP-1	Astrocyte	Human	Foetal Primary	TNF- α	mRNA+Protein	Hurwitz 1995
MCP-1	Astrocyte	Mouse	Neonatal Primary	TNF- α IL-1 β IL-6	Protein -	Hayashi 1995
MCP-1	Astrocyte	Human	Foetal Primary	TNF- α IL-1 β	Protein	Peterson 1997
MCP-1	Astrocyte	Human	U251 astroglioma	TNF- α IL-1 β	mRNA+Protein	Oh 1999
				IL-6+sIL-6R	- mRNA	
				TNF- α IL-1 β	mRNA+Protein	
			U373 astroglioma	IL-6+sIL-6R	- mRNA	
				TNF- α IL-1 β	mRNA+Protein	
				IL-6+sIL-6R	- mRNA	
MCP-1	Astrocyte	Human	Adult Primary	TNF- α IL-1 β	mRNA+Protein	Croitoru 2003
				IL-6+sIL-6R	+/- mRNA	
				TNF- α IL-1 β	Protein	
			Foetal Primary	IL-6	-	
				TNF- α IL-1 β	Protein	
				IL-6	-	
MCP-1	Microglia	Mouse	Neonatal Primary	TNF- α IL-1 β	-	Hayashi 1995
MCP-1	Microglia	Rat	Embryonic Primary	TNF- α IL-1 β IL-6	mRNA	Calvo 1996
MCP-1	Microglia	Human	Foetal Primary	TNF- α IL-1 β	-	Peterson 1997

Table 2.1 Continued. *In-vitro* Chemokine Expression by Astrocytes and Microglia Treated with Pro-Inflammatory Cytokines.

Interleukin-1 β (IL-1 β), interleukin-6 (IL-6), interleukin-8 (IL-8), macrophage inflammatory protein-2 (MIP-2), monocyte chemoattractant protein-1 (MCP-1), no expression (-), soluble interleukin-6 receptor (sIL-6R), tumour necrosis factor- α (TNF- α).

2.2.2. Inhibition of Glial Chemokine Expression

Although the expression of chemokine family members by glial cultures has been described, little is known concerning their modulation in the CNS. In this chapter, I investigated the influence of the IL-6, dexamethasone and FK506 on pro-inflammatory cytokine induced chemokine expression in rat derived glial cell lines.

2.2.2.1. Interleukin-6

IL-6 is expressed constitutively by neurones (Schobitz et al., 1993, Gadiant & Otten, 1994, Hans et al., 1999a) and mRNA and/or protein concentrations are increased after both traumatic and ischaemic injury to the brain (Table 1.2 & 1.3). This expression has been localised to neurones (Hans et al., 1999a, Block et al., 2000), monocytes (Holmin et al., 1997, Block et al., 2000) and astrocytes (Maeda et al., 1994) (Appendix table 7.1). Pro-inflammatory mediators such as IL-1 β or TNF- α stimulate cultured neurones to express IL-6 (Ringheim et al., 1995). Similarly IL-1 β and TNF- α or an injurious processes such as TBI or ischaemia have been shown to stimulate production of IL-6 by astrocytes cultures (Benveniste et al., 1990, Aloisi et al., 1992, Sawada et al., 1992, Hariri et al., 1994, Maeda et al., 1994, Cadman et al., 1994). Following acute injury to the brain the role of elevated tissue IL-6 is not yet clear (Appendix section 7.2.3). Neurotrophic, neuroprotective and both pro and anti-inflammatory actions have been described.

2.2.2.1.1. Soluble Interleukin-6 Receptor

The signal transduction of IL-6 involves the interaction of three proteins, IL-6, IL-6 receptor (IL-6R, also known as gp80) and gp130 (reviewed in Kishimoto et al., 1995 and Heinrich et al., 2003). The binding of IL-6 to its receptor leads to the association of IL-6R with gp130, molecules of which form a homodimer. Gp130 is a ubiquitously expressed (Hibi et al., 1990, Saito et al., 1992), non ligand binding, signal transducing, transmembrane glycoprotein. This homodimerisation activates the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) and/or the mitogen-activated protein kinase (MAPK) pathways leading to transcription of IL-6 responsive genes (Kishimoto et al., 1995, Heinrich et al., 2003) (Figure 2.1).

Although gp130 is ubiquitously expressed, the sensitivity of cells to IL-6 is potentially restricted by the limited expression of IL-6R to the spleen, thymus, lung, liver and brain (Sugita et al., 1990, Saito et al., 1992, Schobitz et al., 1993). Soluble extracellular portions of several transmembrane receptors have been identified in biological material (Novick et al., 1989, Honda et al., 1992). The soluble forms of IL-1 and TNF- α receptors are antagonistic, competing for their ligands with membrane bound receptor. However soluble IL-6R (sIL-6R) transduces a signal, via gp130, conferring IL-6 sensitivity to tissues lacking membrane anchored IL-6R (Jones & Rose-John, 2002). This phenomenon is known as trans-signalling. Soluble forms of cytokine receptors may be produced by limited proteolysis of membrane receptors, known as shedding, or the translation of alternatively spliced mRNA.

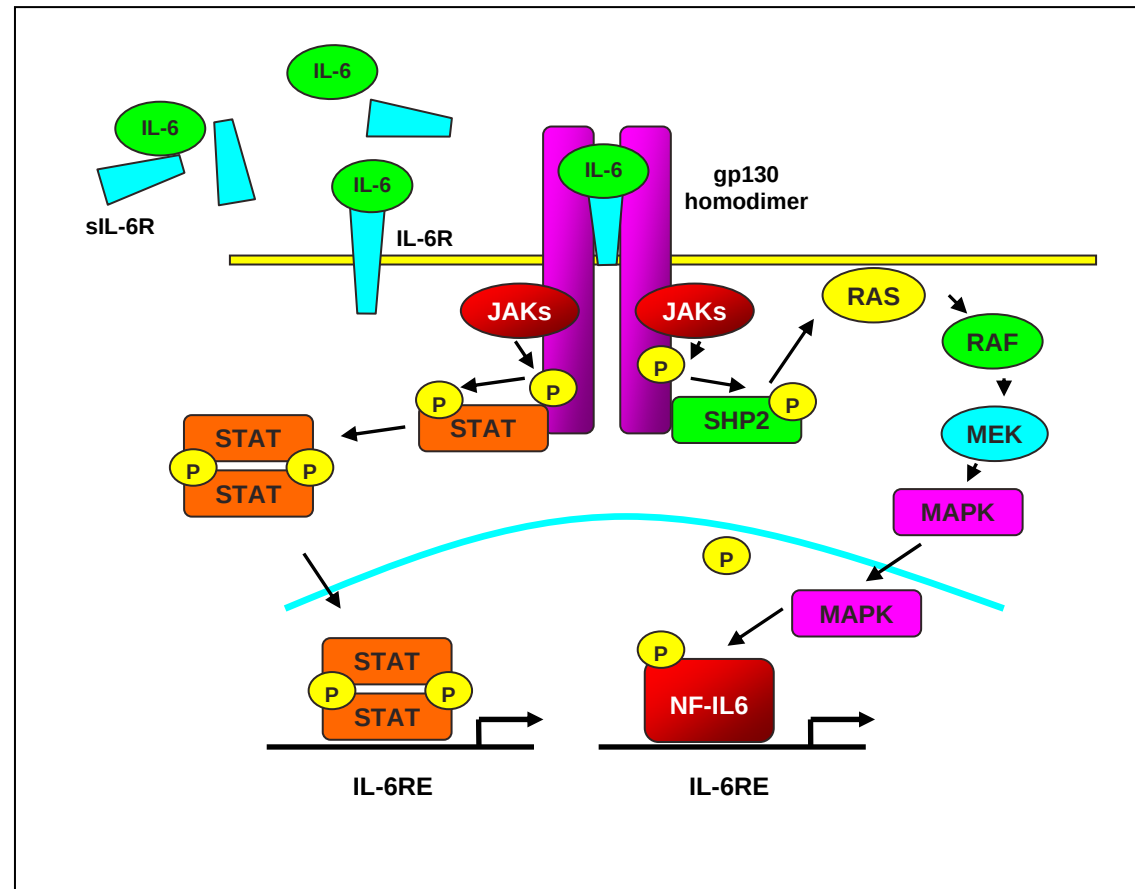


Figure 2.1. Representation of Interleukin-6 Receptor Mediated Signalling.

Interleukin-6 (IL-6) binding with either its soluble (sIL-6R) or membrane bound receptor (IL-6R) leads to the dimerisation of gp130. This activates Janus Kinases (JAKs) associated with gp130. Tyrosine phosphorylation of the gp130 molecule then occurs which attracts the signal transducer and activator of transcription (STAT) protein, which is then also tyrosine phosphorylated. This allows dimerisation of STAT and translocation in to the nucleus to initiate gene transcription. Alternatively JAK phosphorylation of gp130 associates SH2-domain containing tyrosine phosphatase (SHP2) which is in turn phosphorylated. Indirectly the Ras-Raf-mitogen activated protein kinase (MAPK) cascade is activated. Ultimately nuclear factor IL-6 (NF-IL6) undergoes tyrosine phosphorylation leading to gene transcription. Adapted from Kishimoto et al., 1995 and Heinrich et al., 2003.

2.2.2.1.2. Effects of Soluble Interleukin-6 Signalling

IL-6, acting via a soluble receptor has been shown to induce significant biological effects on the nervous system both *in-vitro* and *in-vivo*. The addition of human IL-6 and sIL-6R promotes neurite growth or neuron survival in cultured rodent dorsal root ganglion neurons (Hirota et al., 1996, Thier et al., 1999), induces morphological change and neurotrophin expression in rat primary astrocytes (Marz et al., 1999) and inhibits the expression of VCAM-1 in a human astroglioma line (Oh et al., 1998). The significance of sIL-6R mediated signalling is suggested *in-vivo* by the parietal administration of human IL-6 and sIL-6R together, but not individually, delaying the progression of a mouse model of motor neuron disease (Ikeda et al., 1996).

Evidence is emerging that IL-6 and its soluble receptor may contribute to the control of leucocyte recruitment. In 2001, Hurst and colleagues demonstrated that *in-vitro* the combination of IL-6 and sIL-6R could inhibit TNF- α or IL-1 β mediated IL-8/CXCL8 and GRO α /CXCL1 production and increase MCP-1/CCL2 expression, by peritoneal mesothelial cells. Furthermore following the induction of peritoneal inflammation in IL-6 deficient mice, the restoration of IL-6/sIL-6R signalling reduced MIP-2/CXCL2 and KC/CXCL1 chemokine levels and neutrophil numbers. MCP-1/CCL-2 production and monocyte accumulation were also increased. Hurst proposed that sIL-6R was released from the infiltrating neutrophils by CXC chemokines, orchestrating the switch from a pro-inflammatory neutrophil rich infiltrate to an anti-inflammatory/healing, predominantly monocytic, infiltrate (Hurst et al., 2001). This is in keeping with the increase in systemic MIP-2/CXCL2 seen in IL-6 deficient animals exposed to endotoxin (Xing et al., 1998) or the IL-6 induced expression of MCP-1/CCL2 by cultured human monocytes and rat microglia (Calvo et al., 1996, Biswas et al., 1998).

Conversely in vascular endothelial cell cultures, in which IL-6R is not expressed, the addition of sIL-6R induced the expression of IL-8/CXCL8 and MCP-1/CCL2 (Romano et al., 1997, Modur et al., 1997).

The temporal pattern of white cell recruitment described by Hurst, in the peritoneal inflammatory response is very similar to that seen in the brain after acute injury. Following trauma or ischaemia neutrophils accumulate in the vasculature prior to becoming the predominant leucocytes to infiltrate the parenchyma initially. Maximal neutrophil infiltration occurs between 24 and 72 hours after insult. Monocytic cells also accumulate in the vasculature and infiltrate the lesion becoming the predominant leucocytes by 3-7 days after injury (Section 1.4.2.2). Constitutively expressed IL-6R mRNA has been localised to neurones in the cortex, hippocampus, thalamus, hypothalamus and cerebellum. Increased CSF sIL-6R has been suggested in patients with TBI (Hans et al., 1999b) and activated neutrophils have been demonstrated to shed sIL-6R (Modur et al., 1997, Hurst et al., 2001). Increased expression of both CXC and CC chemokines has also been identified in the brain after traumatic injury (Section 1.4.2.3.2.3, Table 1.4). Since there is similar leucocyte recruitment and expression of inflammatory mediators in the brain and peritoneum after injury I have investigated the role of sIL-6R in modulating the expression of MIP-2/CXCL2 by astrocytes and microglia to determine if a similar pathway could regulate cerebral chemokine expression.

2.2.2.2. Dexamethasone

In clinical practice corticosteroids have been used widely to treat cerebral oedema associated with brain tumours and postoperative brain swelling (GALICICH & FRENCH, 1961). This lead to their administration to manage raised intracranial pressure generally (Alderson & Roberts, 1997). However this clinical use of corticosteroids in acute brain injury remains controversial.

2.2.2.2.1. Corticosteroids in Experimental Brain Injury

Corticosteroid treatment is frequently associated with reduced lesion volume or cerebral oedema, improvement of function or reduced mortality in animal models of CNS injury (Table 2.2). Beneficial effects are associated with pre-insult administration (Pappius & McCann, 1969, Barks et al., 1991, Tuor & Del Bigio, 1996, Limbourg et al., 2002, Felszeghy et al., 2004) or early treatment within 1 hour of injury (Harrison et al., 1973, Kobrine & Kempe, 1973, Hoppe et al., 1974, Hall, 1985, Bertorelli et al., 1998, Slivka & Murphy, 2001). This would suggest that the positive effects of

corticosteroid administration arise through the modulation of processes that occur very early after injury. In those studies where corticosteroids have not been beneficial insufficient dosage or greater injury severity may well account for the inconsistency ((Siegel et al., 1972, Donley & Sundt, Jr., 1973, Spillert et al., 1990, Slivka & Murphy, 2001).

Injury	Species	Drug	Dose	Route	Timing	Result	Assessed	Reference
Moving sled CHI	Monkey	Dexamethasone	1mg	IV	1 hour post then 6 hourly for 72 hours	Reduced mortality Reduced ICP Reduced BBB permeability	72 hours	Kobrine 1973
Weight Drop CHI	Mice	Methylprednisolone	30mg/kg	IV	Within 3 to 5 minutes	Improved neurological function	1 hour	Hall 1985
		Prednisolone	60mg/kg					
Weight Drop CHI	Mice	Methylprednisolone	30mg/kg	IP	30 minutes post, then 24 and 48 hours	All mice dead at 7 days. No improvement in steroid group	Up to 7 days	Spillert 1989
Diffuse Axonal Injury	Rat	Methylprednisolone	30mg/kg then 15mg/kg 4 hourly	IP	1 or 4 hours post then maintenance	Significant reduction in brain water content.	Up to 48 hours	Park 1998
Dural Freezing	Cat	Cortisone	12.5mg/kg	IP	48 hours pre then 12 hourly up to 72 hours post lesion	No effect. Cortisone poorly absorbed from the peritoneum	48 hours	Pappius 1969
		Dexamethasone	0.125mg/kg or 1.25mg/kg			Reduced cerebral oedema		

Table 2.2. The Effect of Corticosteroid Administration in Experimental Acute Brain Injury.

Blood brain barrier (BBB), closed head injury (CHI), intracranial pressure (ICP), intraperitoneal (IP), intravenous (IV).

Injury	Duration	Species	Drug	Dose	Route	Timing	Result	Assessed	Reference
PCCAO + 8% O ₂	3.5 hours	Neonatal Rat	Dexamethasone	4mg/kg or 40mg/kg	SC	Onset of hypoxia	No reduction in cerebral oedema Increased in mortality with dexamethasone, particularly the high dose group.	30 minutes 5 days	Altman 1984
PCCAO + 8% O ₂	3 hours	Neonatal Rat	Dexamethasone	0.01 to 0.5mg/kg 0.1 or 0.5mg/kg 0.1mg/kg	IP	48, 24 hours & immediately pre Immediately, 24 & 48 post 24 hours pre	Reduced lesion area. Post hypoxia administration did not prevent infarction. No reduction in infarction Prevented infarction. Effect lost when pre hypoxia time reduced	7 days	Barks 1991
PCCAO + 8% O ₂	2 hours	Neonatal Rat	Corticosterone Dexamethasone	40 to 80mg/kg 0.1mg/kg	SC IP	24 & 5 hours pre 24 hour pre	Reduced infarct area. Reduced infarct area. Dexamethasone effect inhibited by RU38486 suggesting a glucocorticoid receptor mediated effect	48 hours	Tuor 1996
PCCAO + 8% O ₂	1.5 hours	Neonatal Rat	Dexamethasone	0.5mg/kg	SC	5 hours pre hypoxia	Reduced lesion area Reduced mortality	48 hours During hypoxic period	Fleszeghy 2004
CC micro sphere injection + PCCAO		Rat	Dexamethasone	0.25mg/kg	IM	Immediately then 8 hourly	No reduction in cerebral oedema or mortality	Up to 72 hours	Siegel 1972
PCCAO		Gerbil	Dexamethasone	2.5mg/kg 5mg/kg	IP	Immediately & 24 hours post Immediately post	Reduced mortality. Reduced hemispheric swelling.	Up to 7 days 8 hours	Harrison 1973

Table 2.2 Continued. The Effect of Corticosteroid Administration in Experimental Acute Brain Injury.

Common carotid (CC), intramuscular (IM) intraperitoneal (IP), permanent common carotid artery occlusion (PCCAO), subcutaneous (SC).

Injury	Duration	Species	Drug	Dose	Route	Timing	Result	Assessed	Reference
PMCAO		Cat	Dexamethasone	4mg/kg	IM	20 minutes post then 12 hourly	Decreased oedema in infarcted tissue	48 hours	Hoppe 1974
PMCAO		Rat	Dexamethasone	3mg/kg	IP	10 minutes post	Reduced cortical TNF- α Reduced lesion volume	12 hours 24 hours	Bertorelli 1998
PCAO+ PMCAO				30mg/kg 15mg/kg 30mg/kg		Onset then at 2 hours then at 6 and 12 hours	No effect on infarct volume No effect on hemispheric volume or water content		
PCAO+ PMCAO		Rat	Methylprednisolone	105mg/kg	IA	Onset	No effect on infarct volume No effect on hemispheric volume or water content.	24 hours	Slivka 2001
TCCAO + TMCAO	3 hours			30mg/kg 15mg/kg 30mg/kg		Onset then at 3 hours then at 6 and 12 hours	Reduced infarct volume	24 hours	
PMCAO				75 or 100mg/kg	IP	Onset and 12 and 18 hours	Reduced MCP-1 mRNA	24 hours	
TMCAO	2 hours	Rat	Methylprednisolone	100mg/kg		Onset and 24 and 48 hours	Reduced macrophage accumulation No reduction in infarct area or neuronal damage	72 hours	Kim 1995
TMCAO	2 hours	Mouse	Dexamethasone	20mg/kg	IP	1 hour pre & 2 hours post onset 2 hours post onset	Reduced infarct volume + increased CBF. These effects were lost in eNOS(-/-) mice Dexamethasone effect on infarct volume blocked by RU486 Reduced infarct volume	24 and 72 hours (CBF at 1 hour)	Limbourg 2002
TMCAO	4 hours	Monkey	Dexamethasone	0.14mg/kg then 0.075 mg/kg	IM	Onset then 8 hourly until 48 hours	No effect on outcome or histopathology	Up to 7 days	Donley 1973

Table 2.2 Continued. The Effect of Corticosteroid Administration in Experimental Acute Brain Injury.

Endothelial nitric oxide synthase (eNOS), intra-arterial (IA), intramuscular (IM) intraperitoneal (IP), monocyte chemoattractant protein-1 (MCP-1), messenger ribonucleic acid (mRNA), permanent carotid artery occlusion (PCAO), permanent middle cerebral artery occlusion (PMCAO), transient common carotid artery occlusion (TCCAO), transient middle cerebral artery occlusion (TMCAO), tumour necrosis factor- α (TNF- α).

2.2.2.2.2. Anti-inflammatory Effects of Corticosteroids

In cell culture preparations of monocytes or fibroblast/astrocytes corticosteroids inhibit many aspects of the inflammatory response including the expression of adhesion molecules (Tessier et al., 1993) and the pro-inflammatory cytokines IL-1 β (Knudsen et al., 1987, Kern et al., 1988), TNF (Han et al., 1990) and IL-6 (Zanker et al., 1990, Zitnik et al., 1994, Grimaldi et al., 1998). Inhibition of chemokine expression by corticosteroids has been observed outwith the CNS. In human lung fibroblasts and large airway epithelial cells dexamethasone inhibited IL-8/CXCL8 mRNA and protein expression induced by TNF- α and IL-1 β (Tobler et al., 1992, Kwon et al., 1994). Similarly pre-treatment with dexamethasone reduced NF- κ B binding and MIP-2/CXCL2, KC/CXCL1, MCP-1/CCL2, MIP-1 α /CCL3, TNF- α and IL-6 expression in a mouse perfusion model of acute lung injury (Held et al., 2001). The expression of MIP-2/CXCL2 and KC/CXCL1 induced by the injection of staphylococcal enterotoxin B (SEB) into murine air pouches was also inhibited by dexamethasone treatment (Schramm & Thorlacius, 2003). Similarly for CNS derived cells, the IL-1 α induced IL-8/CXCL8 mRNA and protein expression by a human glioblastoma (astrocyte) cell line was inhibited by dexamethasone (Mukaida et al., 1994) and IL-1 β induced IL-8/CXCL8 production by another human astrocytoma line was also inhibited by dexamethasone (Bourke & Moynagh, 1999). MCP-1/CCL2 expression in cultured CNS endothelial cells, induced by TNF- α or IL-1 β , was inhibited by dexamethasone (Harkness et al., 2003), whilst *in-vivo*, systemic methylprednisolone commenced immediately after the onset of cerebral ischaemia reduced MCP-1/CCL2 mRNA expression and macrophage accumulation (Kim et al., 1995).

2.2.2.2.3. Corticosteroids in Clinical Brain Injury

Despite the experimental data, in clinical practice, the efficacy of steroid treatments in traumatic or ischaemic brain injuries has not been demonstrated by either clinical trial or meta-analysis (Alderson & Roberts, 2000, Qizilbash et al., 2002). The recently published corticosteroid randomisation after significant head injury (CRASH) trial, the largest clinical study of steroid treatment in TBI, reported an excess mortality in the treatment group, leading to termination of the trial (Roberts et al., 2004). In part, this failure to observe a benefit in clinical studies may be because the administration of steroid

has been delayed and prolonged relative to the time of injury and the expression of inflammatory mediators. For example in the CRASH study treatment was started within 1 hour of injury in only 27% of cases and then continued for 48 hours (Roberts et al., 2004). Further study of the effect of corticosteroids on the expression of inflammatory mediators is therefore warranted in order to avoid overlooking early potentially beneficial actions. The effect of glucocorticoid administration on MIP-2/CXCL2 and MCP-1/CCL2 production by rat glia has not been described. I have therefore investigated the effect of glucocorticoid treatment on chemokine expression by rat glial cells, concentrating on early administration to antagonise the action of pro-inflammatory cytokines known to be expressed within hours of CNS injury. The effect of steroid administration on chemokine expression *in-vivo* will also be examined in chapter 3.

2.2.2.3. FK506

FK506 is a macrolide lactone, isolated from cultures of the bacterium *Streptomyces tsukubaensis*, with immunosuppressive and anti-inflammatory properties (See Sharkey et al., 1997 and Kaminska et al., 2004). Clinically it is known as Tacrolimus and is used to prevent allograft rejection. It binds to the cytosolic immunophilin, FK506 binding protein (FKBP-12), to inhibit the action of the calcium/calmodulin-dependant protein phosphatase 2B, calcineurin. In T helper cells this results in the inhibition of the activation of nuclear factor for activated T cells (NFAT), its translocation into the nucleus and subsequent interleukin-2 (IL-2) transcription. This in turn inhibits the transition of resting T cells into the cell cycle and proliferation (Figure 2.2). FK506 also inhibits the transcription of several other cytokines including TNF- α (Sharkey et al., 1997).

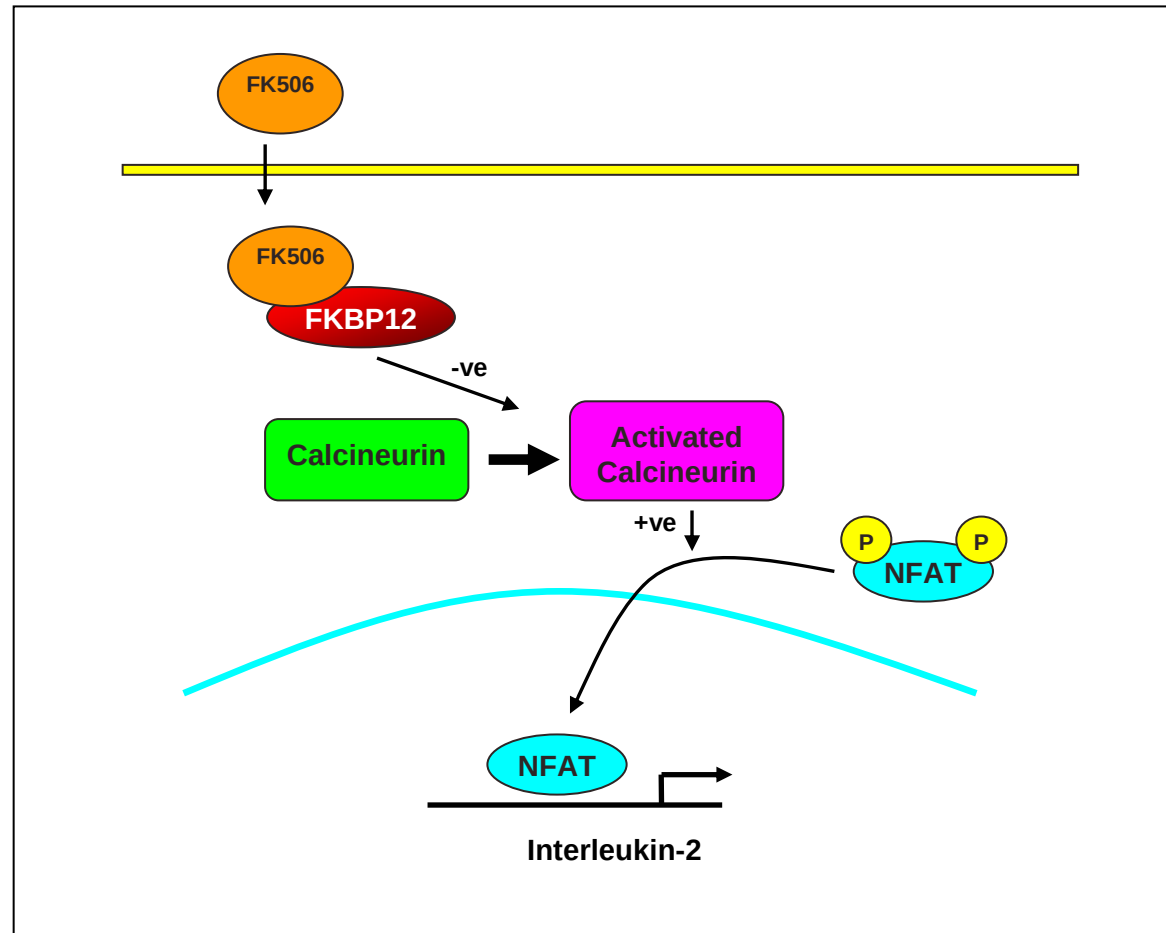


Figure 2.2. Immunophilin Mediated Inhibition of Gene Expression

FK506 binds to the immunophilin FK506 binding protein and inhibits the activation of the calcium/calmodulin dependant phosphatase, calcineurin. Dephosphorylation of nuclear factor of activated T cells (NFAT) is prevented which inhibits its movement into the nucleus and subsequent binding to the promoter region of the interleukin-2 gene. Adapted from Sharkey et al., 1997.

2.2.2.3.1. FK506 in Experimental Brain Injury

In-vivo FK506 has shown promise in pre-clinical studies reducing lesion volume and neuronal loss following ischaemic injury (Sharkey & Butcher, 1994, Tokime et al., 1996, Yagita et al., 1996, Butcher et al., 1997, Takamatsu et al., 2001, McCarter et al., 2001, Arai et al., 2001, Furuichi et al., 2003, Zawadzka & Kaminska, 2005) (Table 2.3). Inhibition of leucocyte accumulation has also been reported after transient ischaemia (Tsujikawa et al., 1998). Following experimental trauma, CSF levels of TNF- α and IL-6 (Stover et al., 2001) and evidence of axonal injury were reduced (Singleton et al., 2001)(Table 2.3). In addition FK506 can also promote the recovery of peripheral nerves after trauma and recovery of spinal cord function after injury (See Kaminska et al., 2004). However the mechanisms underlying these observations are incompletely defined.

In focal ischaemia, pre-treatment of animals with rapamycin, which also binds to FKBP12 with a similar affinity to FK506 but doesn't inhibit calcineurin, blocked the neuroprotection of FK506. This confirmed the role of immunophilin mediated inhibition of calcineurin in the neuroprotective action of FK506 to ischaemia (Sharkey & Butcher, 1994). Following experimental TBI calcineurin activity is increased in cortical and hippocampal homogenates (Kurz et al., 2005). Some of the other suggested mechanisms of neuroprotection by FK506 include inhibition of calcineurin mediated nitric oxide synthase activity and generation of free radicals, mitochondrial stabilisation and inhibition of apoptosis (Sharkey et al., 2000).

Injury	Duration	Species	Dose	Route	Timing	Result	Assessed	Reference
CCI		Rat	1 or 3mg/kg	IP	5, 30 minutes or 4 hr post 5 minutes	IL-6 and TNF- α undetectable in CSF compared to vehicle group. No reduction in CSF glutamate or hypoxanthine (ATP degradation product). Reduced hemispheric water content in the contralateral hemisphere only.	24 hours	Stover 2001
DAI		Rat	2mg/kg	IV	30 minutes prior	Significantly reduces β amyloid precursor protein immunostaining axonal swellings (marker of axonal injury) in the corticospinal tract and medial lemniscus.	24 hours	Singleton 2001
TMCAO	IC Endothelin 3 to 5 hours oligaemia	Rat	0.1-1.0mg/kg 1.0mg/kg	IV	1 minute post onset 1 hr post onset	Significant reduction in cortical lesion volume. No reduction on striatal lesion.	72 hours	Sharkey 1994
Bilateral TCCAO	5 minutes	Gerbil	0.5-1.0 mg/kg 1mg/kg	IP	1 minute post onset 1 minute, 24 and 48 hours post onset	Significant reduction in the loss of hippocampal CA1 neurones. Significant reduction in the loss of hippocampal CA1 neurones. No additional beneficial effect compared to a single dose.	7 days	Tokime 1996
Bilateral TCCAO	5 minutes	Gerbil	1mg/kg	IP	1, 24, 48 and 72 hours post reperfusion	Significant reduction in the loss of hippocampal CA1 neurones. Single treatment on first day or repeated treatment starting on the second day was not protective.	4 and 10 days	Yagita 1996
TMCAO	IC Endothelin 3 to 5 hours oligaemia	Rat	1.0-10mg/kg 1.0mg/kg	IP IV	30 minutes prior 1 minute post onset 1 to 120 minutes post onset	Significant reduction in cortical lesion volume. Significant reduction in cortical lesion volume. No reduction in striatal lesion. Significant reduction in cortical lesion volume.	72 hours	Butcher 1997
TRI	1 hour	Rat	3.2mg/kg	IM	10 minutes post onset	Significant reduction in leucocyte rolling. Significant reduction in leucocyte accumulation. Significant reduction in retinal damage.	12 and 24 hours 12 and 24 hours 7 days	Tsujikawa 1998
TMCAO	2 hours	Rat	0.3mg/kg	IV	30, 60 or 120 minutes post onset	Significant reduction in cortical infarct volume with treatment at 30 and 60 minutes. No reduction in striatal lesion volume.	24 hours	Arii 2001

Table 2.3. The Effect of FK506 Administration in Experimental Acute Brain Injury.

Adenosine triphosphate (ATP), cerebrospinal fluid (CSF), controlled cortical impact (CCI), diffuse axonal injury (DAI), intracerebral (IC), intramuscular (IM) intraperitoneal (IP), intravenous (IV), interleukin-6 (IL-6), transient common carotid artery occlusion (TCCAO), transient middle cerebral artery occlusion (TMCAO), transient retinal ischaemia (TRI), tumour necrosis factor- α (TNF- α).

Injury	Duration	Species	Dose	Route	Timing	Result	Assessed	Reference
PMCAO								
TMCAO	2 hours	Rat	1mg/kg	IV	5 minutes post onset	Significant reduction in cortical lesion volume.	72 hours	McCarter 2001
TMCAO	1 hour	Mouse		IP	30 minutes prior	Significant reduction in cortical lesion volume.	24 hours	
					5 minutes post onset			
TMCAO	3 hours	Monkey	0.1mg/kg	IV	175 minutes post onset	Significant reduction in cortical lesion volume. No effect on lesion volume in the basal ganglia.	8 hours	Takamatsu 2001
					0 to 180 minutes post onset	Significant reduction in cortical injury volume up to 120 minutes post onset.	24 hours	
PMCAO					Immediately post onset	Small decrease in lesion volume.	7 days	
		Rat				No reduction in lesion volume	14 days	
					0 to 180 minutes post onset	Significant reduction in cortical lesion volume up to 60 minutes post onset.	24 hours	Furuichi 2003
TMCAO	1 hour		1.0mg/kg	IV	Immediately post onset	Significant reduction in cortical lesion volume.	Up to 14 days	
					0 to 240 minutes post reperfusion	Significant reduction in the loss of hippocampal CA1 neurones up to 60 minutes post onset.	4 days	
Bilateral TCCAO	5 minutes	Gerbil			Immediately post reperfusion	Significant reduction in hippocampal CA1 neuronal loss.	28 days	
						Significant reduction in neurological deficit.	Up to 72 hours	
						Significant reduction in cortical infarct volume.	Up to 72 hours	
					60 minutes post reperfusion	Significant reduction in astrocyte staining in the cortex.	48 hours	Zawadzka 2004
TMCAO	1.5 hours	Rat	1.0mg/kg	IV		Significant reduction in mononuclear staining in the cortex.	24 and 48 hours	
						Significant reduction in cortical IL-1 β , TNF- α and IL-6 mRNA.	12 hours	
						Reduced in vivo astrocyte expression of IL-1 β .	24 hours	

Table 2.3 Continued. The Effect of FK506 Administration in Experimental Acute Brain Injury.

Intraperitoneal (IP), intravenous (IV), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), messenger ribonucleic acid (mRNA), permanent middle cerebral artery occlusion (PMCAO), transient common carotid artery occlusion (TCCAO), transient middle cerebral artery occlusion (TMCAO), tumour necrosis factor- α (TNF- α).

2.2.2.3.2. Anti-inflammatory Effects of FK506

In peripheral tissues such as heart, liver and skin FK506 has been shown to inhibit features of the inflammatory response (Sharkey et al., 2000)). In primary microglial or astrocyte cultures FK506 inhibits pro-inflammatory cytokine mRNA expression (Zawadzka & Kaminska, 2005) whilst *in-vivo* microglial/macrophage and astrocyte activation following both bilateral common carotid ligation or TMCAO are reduced (Wakita et al., 1998, Zawadzka & Kaminska, 2005) along with cortical expression of IL-1 β , TNF- α and IL-6 mRNAs (Zawadzka & Kaminska, 2005).

In-vitro FK506 inhibits the generation of superoxide radical by phorbol myristate acetate (PMA) activated neutrophils (Nishinaka et al., 1993). The inhibition of neutrophil/leucocyte infiltration by FK506 has also been reported following ischaemia in the liver (Suzuki et al., 1993, Wakabayashi et al., 1994, Garcia-Criado et al., 1997), gut (Kubes et al., 1991) and retina (Tsujikawa et al., 1998). This might be due to an inhibition of adhesion molecule or chemotactic factor expression. However the effect of FK506 on chemokine expression *in-vitro* has been variable. Inhibition (Okamoto et al., 1994, Staruch et al., 1998, Kohyama et al., 1999, Sasakawa et al., 2000), no effect (Kaplan et al., 1995, Saitoh et al., 1997) and induction (Borger et al., 2000) have been reported in various blood and epithelial cell types (Table 2.4). In patients with psoriasis, treatment with FK506 reduced the severity of the disease and this was associated with reduced IL-8/CXCL8 mRNA in skin biopsies and protein in plasma (Lemster et al., 1995) (Table 2.4). However to date the effect of FK506 on chemokine expression by glia has not been reported. Therefore I have investigated the effect of FK506 on chemokine expression by cultured astrocytes.

Concentration	Cell Type/Tissue	Species	Stimulus	Outcome	Reference
1-100nM	Jurkat T cell leukaemia derived cells	Human	PMA & Ionomycin	Reduced IL-8 protein.	Okamoto 1994
1-10nM	T Cells	Human	CD3/PMA or CD3/CD28	Reduced MIP-1 α and β and to a lesser degree RANTES protein Reduced MIP-1 α and β mRNA	Staruch 1998
0.1-10nM	Eosinophils	Human	Calcium inophore	Reduced IL-8 mRNA and protein and MCP-1 protein	Kohyama 1999
1.2-122nM	Peripheral Blood Mononuclear Cells	Human	Anti CD3, Anti CD2 antibodies	Suppressed IL-8 protein	Sasakawa 2000
Clinical study pre and during oral treatment	Skin Biopsies	Human	Psoriasis	Decreased IL-8 mRNA	Lemster 1995
	Plasma			Decreased IL-8 protein	
1nM - 5 μ M	Epidermal Keratinocytes	Human	Spontaneous or TNF- α & IFN- γ	No inhibition	Kaplan 1995
0.1-1000nM	Colonic Epithelial Cell Line HT-29	Human	IL-1 β or TNF- α	No effect on IL-8 mRNA or protein	Saitoh 1997
122nM-12.2 μ M	Respiratory Epithelial Cell Line A549	Human	-	Induced IL-8 Protein	Borger 2000

Table 2.4. The Effect of FK506 on Chemokine Expression *In-vitro*.

Cluster differentiation (CD), interferon- γ (IFN- γ), interleukin-1 β (IL-1 β), interleukin-8 (IL-8), macrophage inflammatory protein-1 (MIP-1 α , MIP-1 β), messenger ribonucleic acid (mRNA), monocyte chemoattractant protein-1 (MCP-1), phorbol myristate acetate (PMA), regulated on activation normal T cells expressed and secreted (RANTES), tumour necrosis factor- α (TNF- α).

2.3. Materials and Methods

2.3.1. Media & Reagents

Unless otherwise stated all cell culture media were purchased from Sigma-Aldrich Company Ltd (Poole, UK). Rat recombinant TNF- α (PRC3014) and IL-1 β (PRC0814) and human recombinant TNF- α (PHC3015) and IL-1 β (PHC0814) were obtained from Biosource International (Nivelles, Belgium). Recombinant human IL-6 (206-IL) and sIL-6R (227-SR) were purchased from R&D Systems (Abingdon, UK). Dexamethasone sodium phosphate was supplied by Organon Laboratories Ltd (Cambridge, UK). FK506 was a kind gift from Astellas Pharma Ltd (Staines, UK).

2.3.2. Primary Rat Microglia and Astrocyte Culture

Rat cortical microglia and astrocytes were isolated and cultured according to the method of Levison and McCarthy (Levison & McCarthy, 1991). Cerebral cortices were removed from day 0-2 Sprague Dawley rat pups, placed in cold Hank's balanced salt solution without calcium or magnesium (HBSS, H6648), the meninges removed and the tissue finely chopped. This tissue was then incubated at 37°C in the presence of 0.25% trypsin (T4665) and 0.05% deoxyribonuclease (DNase) (D4527) for 20 minutes and then triturated in a mixture of 0.05% DNase and culture medium, consisting of basal medium Eagle (BME, B1522) supplemented with 10% heat inactivated foetal calf serum (FCS, Invitrogen, Paisley, UK. No 10106-169), 0.5% dextrose (G5146), 2mM L-glutamine (G7513) and 10ml/L of penicillin (10,000units/ml) /streptomycin (10mg/ml) solution (P0781). The resulting cell suspension was then centrifuged at 500g for 10 minutes and re-suspended in culture media, filtered through a sterile nylon gauze (125 μ m), and divided into 75cm² flasks (equivalent to 2 pup cortices/flask). The cells were cultured at 37°C in air supplemented with 5% carbon dioxide and the media changed twice weekly.

Cultures were grown until confluent at 14-21 days. The flasks were sealed with Parafilm (Pechiney, Chicago, USA) and placed in a heated (37°C) orbital shaker rotating at 200rpm for 90 minutes. The supernatant containing microglia and some type I astrocytes was centrifuged at 500g for 10 minutes,

re-suspended in conditioned media and counted. The microglia were seeded into 24 well plates at 1×10^5 cells per well in 500 μ l and then returned to the incubator for 45 min at 37°C. The media was then aspirated to remove the astrocytes, the wells rinsed with serum free BME (B1522) and then 500 μ l of fresh culture media added to each well. The plates were then returned to the incubator.

In the remaining flasks fresh media was added and they were then incubated for 1 hour to allow re-equilibration of the carbon dioxide. The flasks were then resealed and placed in the orbital shaker overnight to separate type II astrocytes (French-Constant & Raff, 1986) and oligodendrocytes from the adherent astrocyte cell layer. After discarding the media, containing these cells, the adherent layer was rinsed in HBSS, trypsinised (0.25% trypsin EDTA, T4049) and centrifuged at 125g for 5 min. Once re-suspended in culture media the astrocytes were counted and seeded into 24 well plates at 2×10^5 cells per well in 500 μ l and returned to the incubator.

The cell densities were chosen based on previous laboratory experience in which increased cytokine expression by experimental cultures could be detected over that of control cells. Furthermore the density of astrocyte cultures were comparable to that of previous studies of chemokine expression *in-vitro* (Peterson et al., 1997, Oh et al., 1999, Wang et al., 2000, Otto et al., 2002). The lower density of the microglia was due to the lower yield of these cells from the available litter sizes.

2.3.2.1. Rat Primary Glial Cell Immunohistochemistry

To confirm the purity of the primary cultures, microglia and astrocytes were prepared as described but seeded onto a multi-welled slide (Lab-tek II Chamber Slide System-154534, Nalge Nunc International, Rochester, New York) at 1×10^4 cells per well for microglia and 2×10^4 cells per well for astrocytes, both in 500 μ l. The lower cell density was chosen to increase the separation between the cells and aid identification. After incubation for 24 hours the media was aspirated and the cells rinsed in phosphate buffered saline (PBS, Sigma-Aldrich Company Ltd, Poole, UK/BDH Laboratory Supplies, Gillingham, UK) then fixed in cold 4% paraformaldehyde (Fisher Scientific UK Ltd, Loughborough, UK) in PBS for 15 minutes, rinsed in PBS (3 x 5 minutes), permeabilised with 0.1% Triton X-100 (Sigma 93427) for 10 minutes, rinsed again in PBS (3 x 5 minutes) and then endogenous

peroxidase activity quenched with 3% hydrogen peroxide for 5 minutes. The cells were then rinsed in PBS (3 x 5 minutes) and blocked with normal horse serum (NHS) in PBS (200µl in 10ml) of 1 hour at room temperature. Primary antibodies were diluted in NHS/PBS. These were mouse anti-rat CD11b 1:100 (MCA 275G Serotec, Oxford, UK) for microglia and mouse anti-pig glial fibrillary acid protein (GFAP) 1:200 (Ab8136 Abcam, Cambridge, UK), which cross reacts with the rat protein, for astrocytes. The primary antibodies were omitted from the appropriate wells as a negative control. After incubation at 4°C overnight the cells were rinsed with PBS (3 x 5 minutes) and antigen visualisation performed using an anti-mouse Vectastain ABC kit (PK-4002) (Vector Laboratories Ltd, Peterborough, UK) according to the manufactures instructions. The biotinylated anti-mouse antibody (50µl) was diluted in NHS/PBS (150µl in 10ml) and applied for 1 hour. After rinsing with PBS (3 x 5 minutes), the avidin/biotinylated peroxidase enzyme complex (ABC) was added for 30 minutes and following another rinse with PBS (3 x 5 minutes) the antigen staining visualised with Vector stain VIP substrate (SK-4600) (Vector Laboratories Ltd). This reaction was terminated with water and then the cells counter stained in Harris' haematoxylin (BDH Laboratory Supplies) for 1 minute, dipped in 1% acid alcohol for 20 seconds and placed in Scott's tap water substitute (BDH Laboratory Supplies) for 5 minutes, dehydrated thorough 70, 90 and 100% ethanols for 2 minutes each, cleared in xylene for 10 minutes and mounted in DPX.

Light microscopy was performed with an Olympus BX51 microscope (Olympus UK Ltd, London, UK) and the images captured with a Qimaging MicroPublisher 3.3 RTV camera and Qcapture Pro 5.0 software (Media Cybernetics UK, Marlow, UK). Microglia and astrocyte cultures were found to be > 95% pure by this methodology (Figures 2.3 & 2.4).

2.3.3. Immortalised Cell Line Cultures

The rat astrocyte cell line CTX-TNA2 (Radany et al., 1992) was obtained from the European collection of cell cultures (ECACC, Porton Down, UK) and was maintained in Dulbecco's modified Eagle's medium (DMEM, D6546) supplemented with 10% FCS and 2mM L-glutamine. The human microglial cell line CHME-5 was a generous gift from Professor Marc Tardieu, Université Paris-Sud,

France (Janabi et al., 1995), and was maintained in Dulbecco's modified Eagle's medium (DMEM, D5671) with 10% heat inactivated FCS, 2mM L-glutamine and 0.05 mg/ml gentamycin (G1397). U373 human astrogloma cells (Ponten & Macintyre, 1968) were obtained from ECACC and maintained in minimal essential medium Eagle (MEME, M2279) supplemented with 10% FCS, 2mM L-glutamine, 1x non-essential amino acid solution (NEAA, M7145) and 1mM sodium pyruvate (S8636). The rat microglial HAPI cell line (Cheepsunthorn et al., 2001) was a generous gift from Professor James Connor, Pennsylvania State University, USA. These were maintained in DMEM (D5546) with 10% FCS, 2mM L-glutamine and 10ml/L of penicillin (10,000units/ml)/ streptomycin (10mg/ml) /amphotericin B (25µg/ml) solution (A5955). CTX-TNA2, CHME-5 and U373 cells were passaged up to +22, 16 and +11¹ respectively. All cell lines were maintained in an atmosphere of 5% carbon dioxide at 37°C.

For experiments the cells were rinsed in HBSS, trypsinised, centrifuged at 125g for 5 minutes and re-suspended in fresh culture media. Following counting they were seeded into 24 well plates at 2×10^5 cells per well in 500µl and returned to the incubator.

¹ For CTX-TNA2 and U373 cells the + represents the passages since resuspension. For the CHME-5 cell line the total number of passages was known.

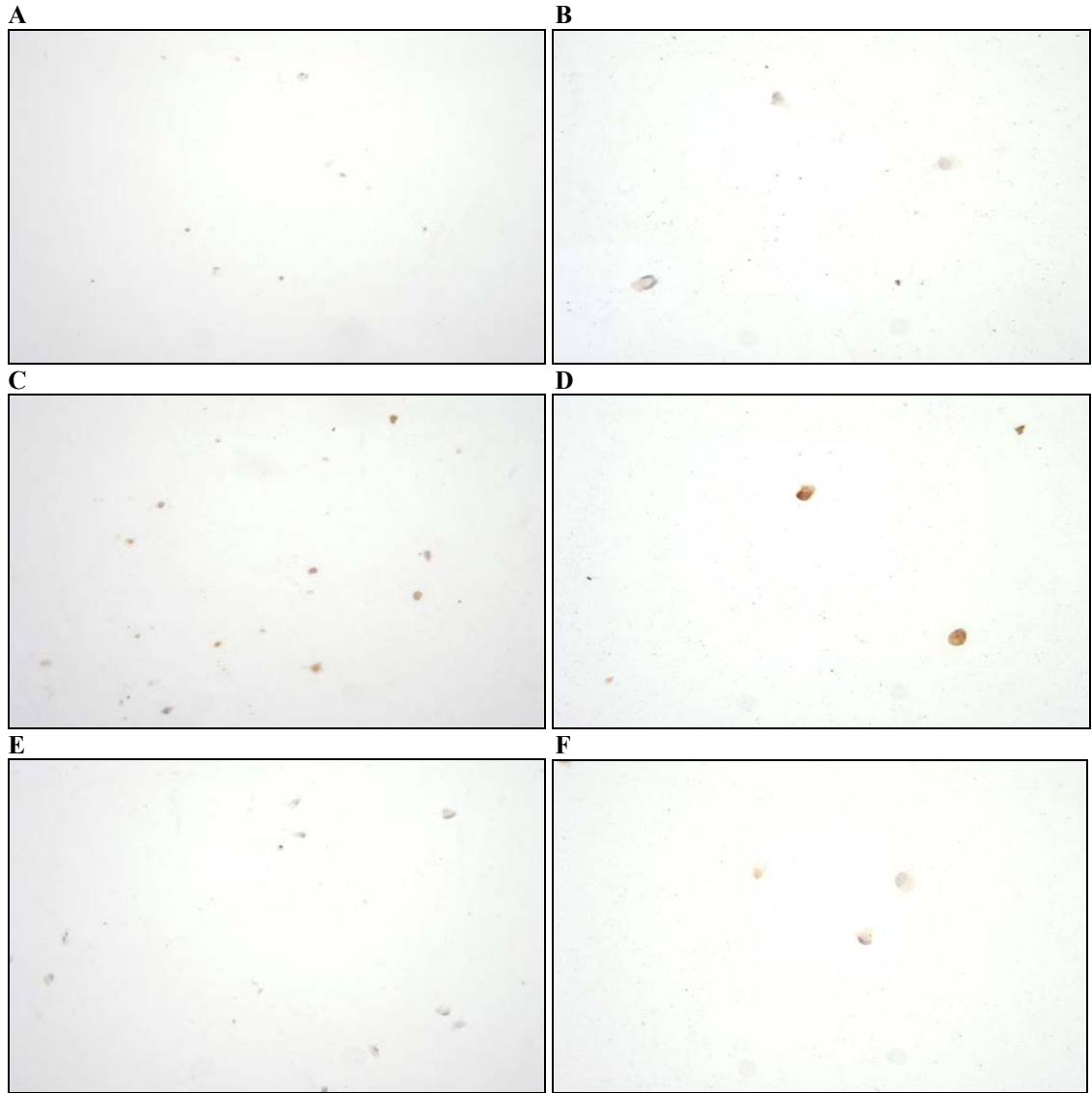


Figure 2.3. Purity of Rat Primary Microglial Culture.

Primary antibody omitted (A & B), CD11b (C & D) and GFAP (E & F). Immunohistochemistry indicated that cultures were more than 95% pure. Counter-stained with haematoxylin. A, C & E x 100, B, D & F x 200.

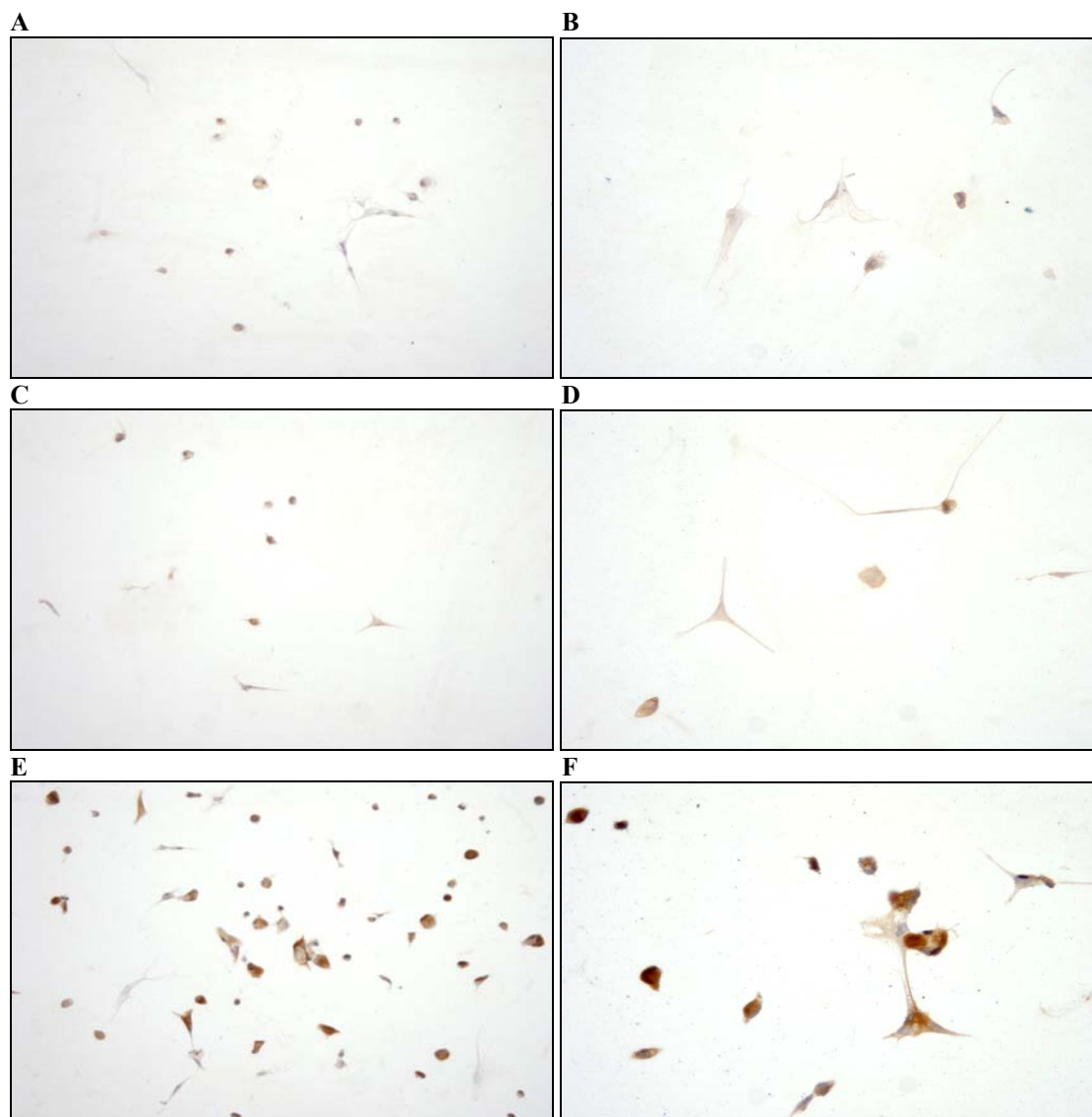


Figure 2.4. Purity of Rat Primary Astrocyte Cultures.

Primary antibody omitted (A & B), CD11b (C & D) & GFAP (E & F). Immunohistochemistry indicated that cultures were more than 95% pure. Counter-stained with haematoxylin. A, C & E x100, B, D & F x 200.

2.3.4. Induction of Chemokine Production by Pro-inflammatory Cytokines

2.3.4.1. Preliminary Investigation of Chemokine Expression by Glial Cells in Response to Pro-inflammatory Cytokines

Following overnight incubation of rat primary microglia, rat primary astrocytes, CTX-TNA2, CHME-5 and U373 cells the culture media was aspirated and the cells rinsed with serum free media. Fresh culture media containing species specific TNF- α (20ng/ml) or IL- β (10ng/ml) were then added. In addition, as a positive control, lipopolysaccharide (LPS 1 μ g/ml) was added to each cell line except the U373 astrocytes. These concentrations of pro-inflammatory cytokines were chosen based on published work on chemokine expression in human (the majority) and mouse glial cells (Aloisi et al., 1992, Barna et al., 1994, Peterson et al., 1997, Ehrlich et al., 1998, Oh et al., 1999, Otto et al., 2000, Hua & Lee, 2000, Otto et al., 2002). The expression of chemokines by U373 astrocytes in response to TNF- α and IL-1 β has been published and so LPS was omitted (Oh et al., 1999). Controls consisted of fresh culture media alone. Cells were incubated for 24 hours and subsequently the supernatants were aspirated and stored at -20°C until analysed. Each agonist treatment was performed in triplicate, as a single experiment.

2.3.4.2. Temporal Production by Glial Cells of Chemokines in Response to Pro-inflammatory Cytokines

Following overnight incubation of rat primary microglia, rat primary astrocytes, CTX-TNA2 and U373 cells the culture media was aspirated, the cells rinsed with serum free media and then fresh culture media containing species specific TNF- α (20ng/ml) or IL- β (10ng/ml) was added. Controls consisted of fresh culture media alone. Cells were then returned to the incubator for up to 24 hours. At 1, 3, 6, 12 and 24 hours the appropriate supernatants were aspirated and stored at -20°C until analysed. In each experiment the individual cytokine treatments were repeated in triplicate and each experiment repeated 3 times.

2.3.4.3. MIP-2/CXCL2 Production by Rat Primary Astrocytes verses TNF- α Concentration

Following overnight incubation of rat primary astrocytes the culture media was aspirated, the cells rinsed with serum free media and then fresh culture media containing species specific TNF- α (0-100ng/ml) added. Cells were then returned to the incubator for 12 hours. The supernatants were then aspirated and stored at -20°C until analysed. Each concentration of TNF- α was repeated in triplicate.

2.3.5. Inhibition of Chemokine Production

2.3.5.1. Inhibition of TNF- α induced MIP-2/CXCL2 Production by IL-6 and sIL-6R

Following overnight incubation of the rat primary microglia, rat primary astrocytes and CTX-TNA2 cells the culture media was aspirated, the cells rinsed with serum free media and then fresh culture media containing rat TNF- α (20ng/ml), human IL-6 (10ng/ml) and sIL-6R (10-1000ng/ml) were added as indicated. The concentrations of IL-6 and sIL-6R selected were based on those used in previously published reports of IL-6/sIL-6R modulating chemokine expression (Hurst et al., 2001), neuronal survival (Thier et al., 1999) or astrocyte differentiation (Marz et al., 1999). Controls consisted of fresh media or rat TNF- α (20ng/ml) alone. Rat primary microglia were cultured for a further 6 hours whilst the primary astrocytes and CTX-TNA2 cells were cultured for a further 12 hours. These times were based on the investigation of the temporal production of MIP-2/CXCL2 by these cells over 24 hours (2.3.4.2). At the appropriate time the supernatants were aspirated and stored at -20°C until analysed. For each experiment individual cytokine treatments were repeated in triplicate. Experiments involving microglia were repeated 6 times, primary astrocytes 3 times, and CTX-TNA2 cells 7 times.

Additionally, in order to indicate if IL-6 or sIL-6R, either individually or in combination, affected chemokine production by glia a single experiment was conducted in which all possible combinations of TNF- α , IL-6 and sIL-6R were added to glia. Each combination was repeated in triplicate and the cells cultured as described above.

2.3.5.2. Inhibition of TNF- α Induced Chemokine Production by Dexamethasone

2.3.5.2.1. Preliminary Dexamethasone Concentration Response Curve

To determine the concentration of dexamethasone which might inhibit TNF- α induced chemokine production in CTX-TNA2 astrocytes a range of concentrations were tested in a single experiment. Following overnight incubation culture media was aspirated from CTX-TNA2 astrocytes which were then rinsed with serum free media. Fresh culture media containing dexamethasone (0-100 μ M) was added and the cells incubated for 3 hours. Rat TNF- α to a final concentration of 1ng/ml was then added to the supernatant. Controls consisted of cells which were cultured for 3 hours in fresh culture media and then TNF- α or an equivalent volume of fresh media added. The cells were then incubated for a further 10 hours. Supernatants were subsequently aspirated and stored at -20°C until analysed. Each treatment condition was repeated in triplicate.

2.3.5.2.2. Inhibition of Chemokine Production by Dexamethasone

Following overnight incubation culture media was aspirated from CTX-TNA2 astrocytes which were then rinsed with serum free media. Fresh culture media containing dexamethasone (1 or 10 μ M) was added and the cells incubated for 3 hours. Rat TNF- α to a final concentration of 1ng/ml or an equivalent volume of fresh culture media was added to the supernatant to produce combinations of cells treated with TNF- α and dexamethasone or dexamethasone alone. Controls consisted of cells which were cultured for 3 hours in fresh culture media and then TNF- α or an equivalent volume of fresh media added. The cells were then incubated for a further 10 hours. In a separate experiment the period of incubation in the presence of dexamethasone, prior to the addition of TNF- α , was reduced to 1.5 and zero hours. Supernatants were subsequently aspirated and stored at -20°C until analysed. For each experiment individual treatments were repeated in triplicate and each experiment repeated 3 times.

2.3.5.3. Inhibition of Chemokine Production by FK506

Following overnight incubation culture media was aspirated from CTX-TNA2 astrocytes which were then rinsed with serum free media. Fresh culture media containing FK506 (100nM) was added and the cells incubated for 3 hours. FK506 (100nM) represents a supra-maximal concentration of drug based on previous studies of chemokine inhibition (Okamoto et al., 1994, Staruch et al., 1998, Kohyama et al., 1999). Rat TNF- α to a final concentration of 1ng/ml or an equivalent volume of fresh culture media was added to the supernatant to produce combinations of cells treated with TNF- α and FK506 or FK506 alone. Controls consisted of cells which were cultured for 3 hours in fresh culture media and then TNF- α or an equivalent volume of fresh media added. The cells were then incubated for a further 10 hours. Supernatants were subsequently aspirated and stored at -20°C until analysed. For each experiment individual treatments were repeated in triplicate and each experiment repeated 3 times.

2.3.6. Measurement of Chemokines

Chemokine concentrations were determined using commercially available solid phase enzyme linked immunosorbent assay (ELISA) kits (Biosource International, Nivelles, Belgium) to measure rat MIP-2/CXCL2 (KRC1022) and MCP-1/CCL2 (KRC1012) or human IL-8/CXCL8 (KHC0082) and MCP-1/CCL2 (KHC1012). For example, in the MIP-2/CXCL2 ELISA samples were diluted in standard dilutant buffer, to fit the concentration range of the assay standard. 50 μ l of this diluted sample was then added to a microtiter well coated with capture antibody to MIP-2/CXCL2 (Figure 2.5). A series of standards of known MIP-2/CXCL2 concentration were also prepared and 50 μ l of each added to microtiter wells containing capture antibody. 50 μ l of biotinylated anti-MIP-2/CXCL2 antibody was also added to each well. The samples and standards were then incubated for 2 hours at 37°C. This allowed the target protein in the sample/standard to be captured and sandwiched between the antibody coating the microtiter plate and the biotinylated antibody. The microtiter wells were then aspirated and washed 4 times with wash buffer, prior to the addition of 100 μ l of streptavidin-horse radish peroxidase (HRP) and then incubated for 1 hour at room temperature. During this time the streptavidin binds to the biotin of the biotinylated antibody. After further aspiration and washing 4

times with wash buffer, 100µl of stabilised chromogen was added to each well. The HRP bound to the streptavidin reacts with the colourless chromogen resulting in the liquid in the wells turning blue. The resultant colour change in a fixed period of time is proportional to the concentration of the HRP-streptavidin/biotinylated anti-MIP-2 antibody/MIP-2 complex. After 30 minutes this reaction was terminated with 100µl of stop solution. The colour change was read on a MRX microplate reader at 450nm and the output displayed using Revelation v3.04 software (Dynex Technologies, Worthing, UK).

2.3.7. Statistics

The values for optical density obtained from the cytokine ELISA standards were entered into a commercially available graphing package (Sigma Plot 8.0, Systat Software Inc, London, UK) and a 4 parameter logistic regression curve plotted. These parameters were used to derive the cytokine concentrations from the optical densities of the samples tested.

Data are presented as mean $\pm$ standard error of the mean (SEM) and were compared by either paired or un-paired Student's t test or, where appropriate, by one way analysis of variance (ANOVA) with multiple comparisons tested using the Holm-Sidak method. Differences were considered statistically significant at $p < 0.05$.

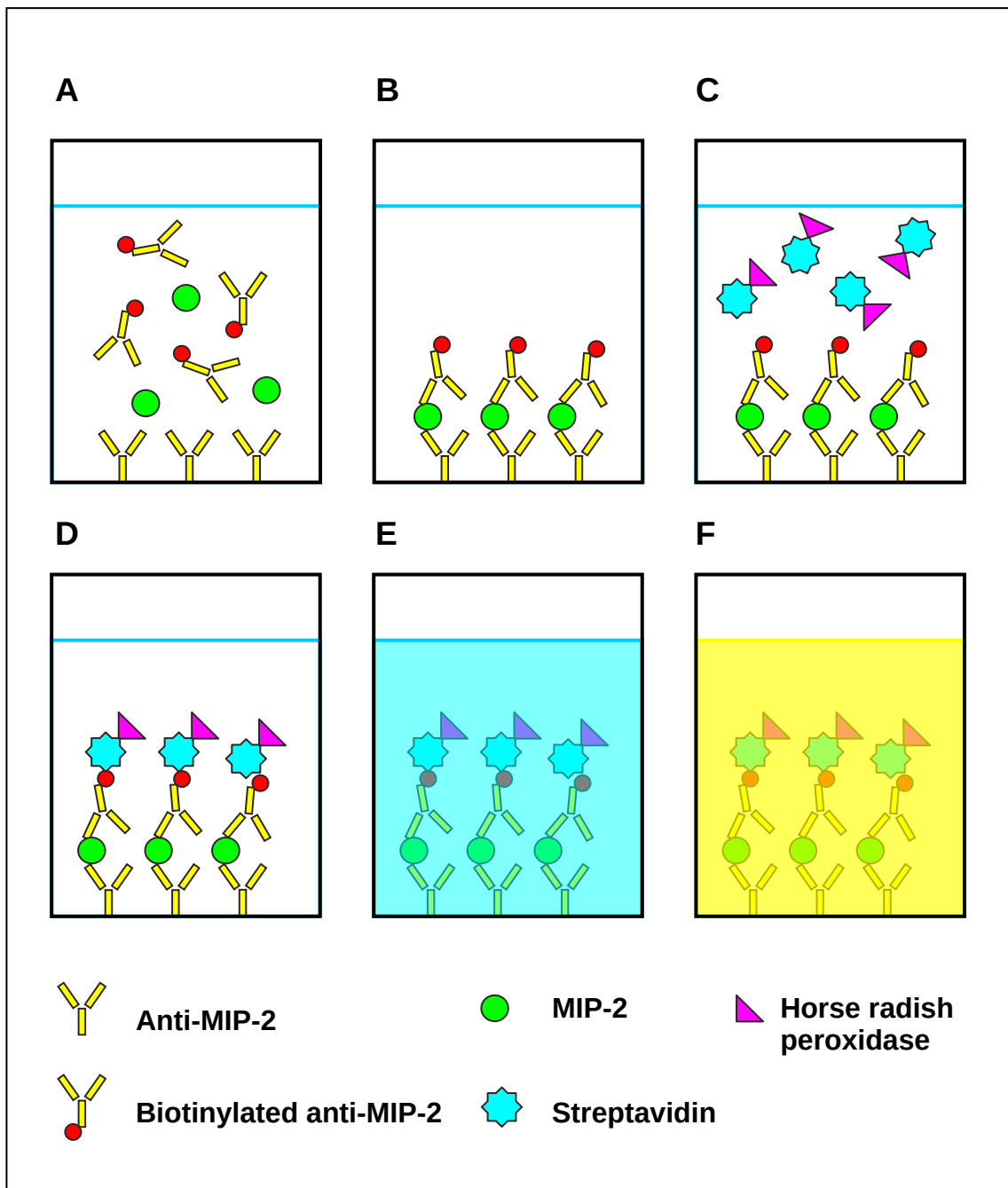


Figure 2.5. Principle of the Enzyme Linked Immunosorbent Assay

(A) Diluted sample or standard are added with biotinylated anti-MIP-2 antibody to a microtiter well coated with anti-MIP-2 capture antibody. (B) After incubation MIP-2 is sandwiched between the capture and biotinylated antibodies. (C) A streptavidin horse radish peroxidase complex is then added to the wells. (D) During a further incubation this binds to the biotinylated antibody. (E) Colourless chromogen solution reacts with the horse radish peroxidase and the solution turns blue. (F) The addition of a stopping solution terminates the reaction leaving a yellow coloured liquid. The density of the yellow colour is proportional to the concentration of MIP-2 in the sample or standard.

2.4. Results

2.4.1. Chemokine Production by Glia in Response to Pro-inflammatory Cytokines

2.4.1.1. Preliminary Investigation of Chemokine Expression by Glial Cells in Response to Pro-inflammatory Cytokines

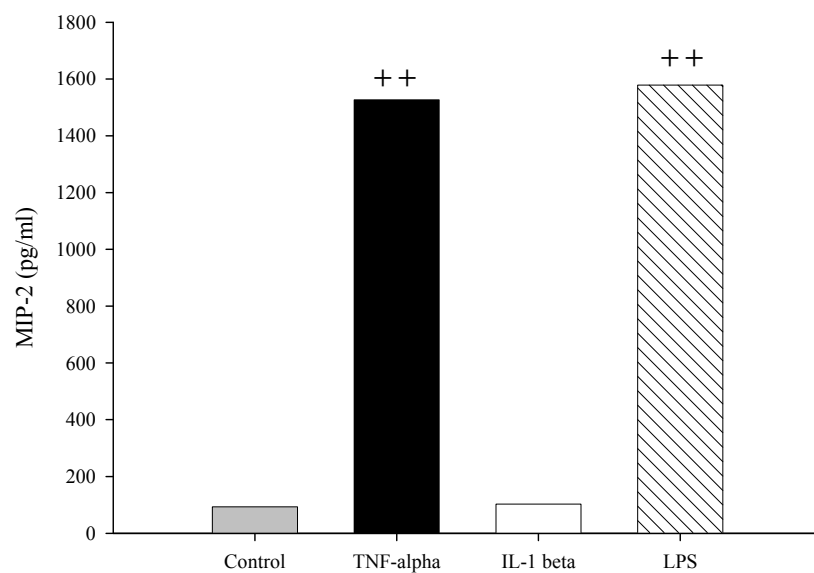
Both TNF- α and IL-1 β induced production of MIP-2/CXCL2 and MCP-1/CCL2 by rat primary astrocytes, CTX-TNA2 astrocytes and human U373 astrocytes but only TNF- α increased the MIP-2/CXCL2 and MCP-1/CCL2 concentrations in rat primary microglia (Figures 2.6-2.9).

In 2 independent experiments rat CHME-5 microglia were cultured with TNF- α , IL-1 β or LPS for 24 hours. MIP-2/CXCL2 and MCP-1/CCL2 were not detectable by ELISA in any supernatants and this cell line was not used further.

Two independent attempts were made to re-suspend the rat microglial HAPI cell line from storage in liquid nitrogen. However the cultures did not commence active cell division on either occasion. No further attempts to study this line were made.

During the course of this work temporal lobe resections to treat epilepsy were not performed at the Department of Clinical Neuroscience, Western General Hospital, Edinburgh. This department is the only neurosurgical centre in South East Scotland. Therefore despite ethical approval to do so, no material was available to attempt primary culture of adult human glial cells.

A



B

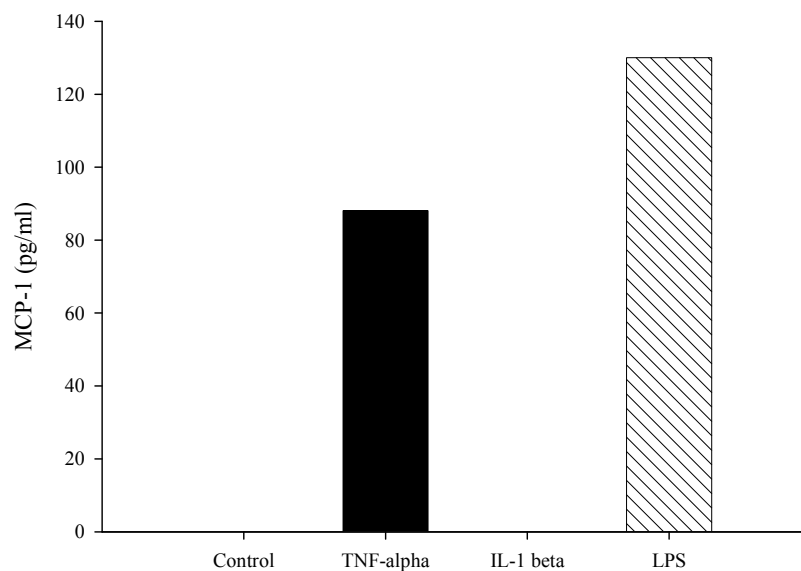
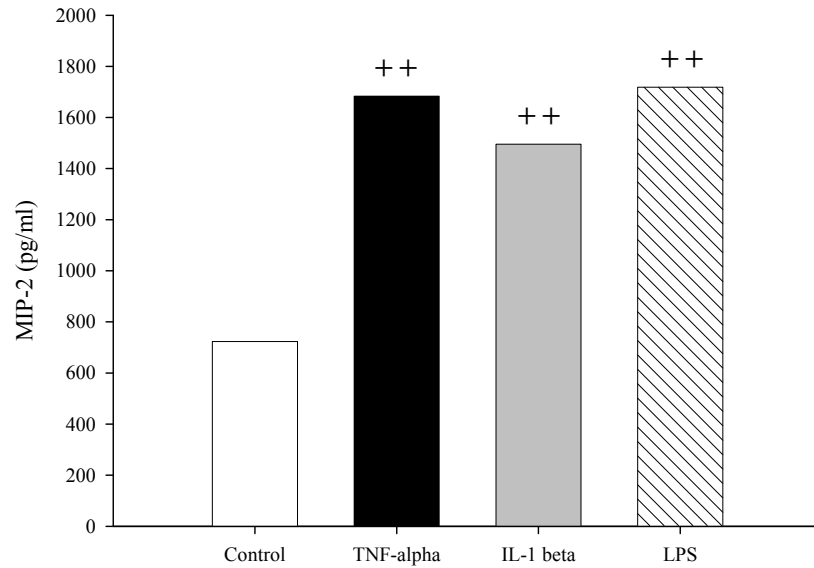


Figure 2.6. Chemokine Production by Rat Primary Microglia; Preliminary Data.

(A) MIP-2/CXCL2 and (B) MCP-1/CCL2 production after 24 hours in culture media alone (control) or with TNF- α (20ng/ml), IL-1 β (10ng/ml) or LPS (1 μ g/ml). ++ Optical densities in excess of standards.

A



B

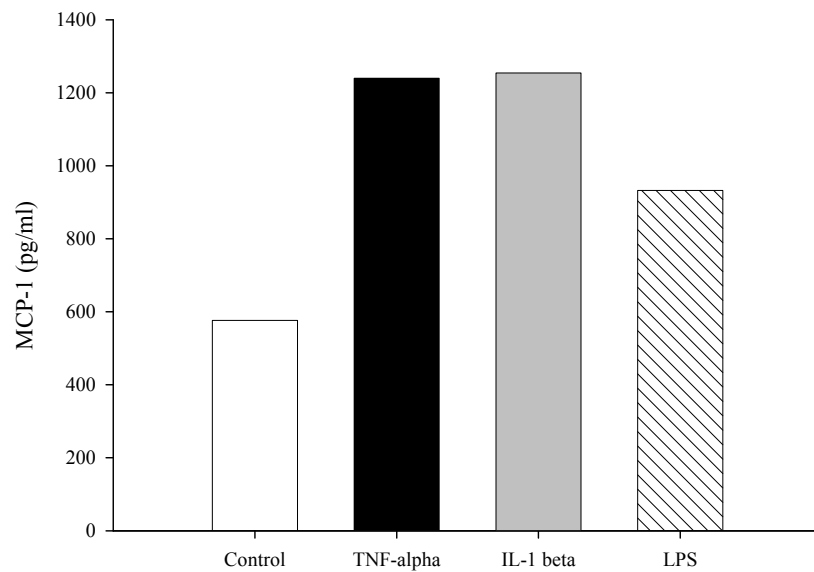
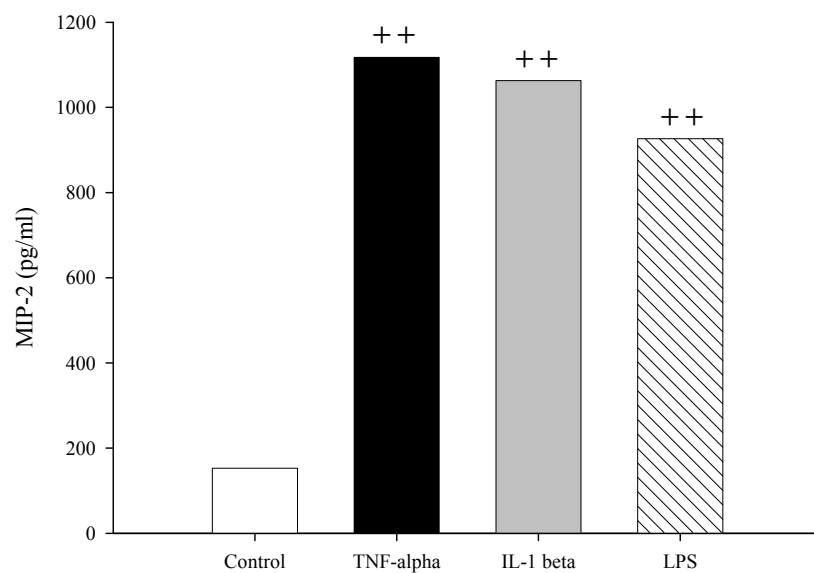


Figure 2.7. Chemokine Production by Rat Primary Astrocytes; Preliminary Data.

(A) MIP-2/CXCL2 and (B) MCP-1/CCL2 production after 24 hours in culture media alone (control) or with TNF- α (20ng/ml), IL-1 β (10ng/ml) or LPS (1 μ g/ml). ++ Optical densities in excess of standards.

A



B

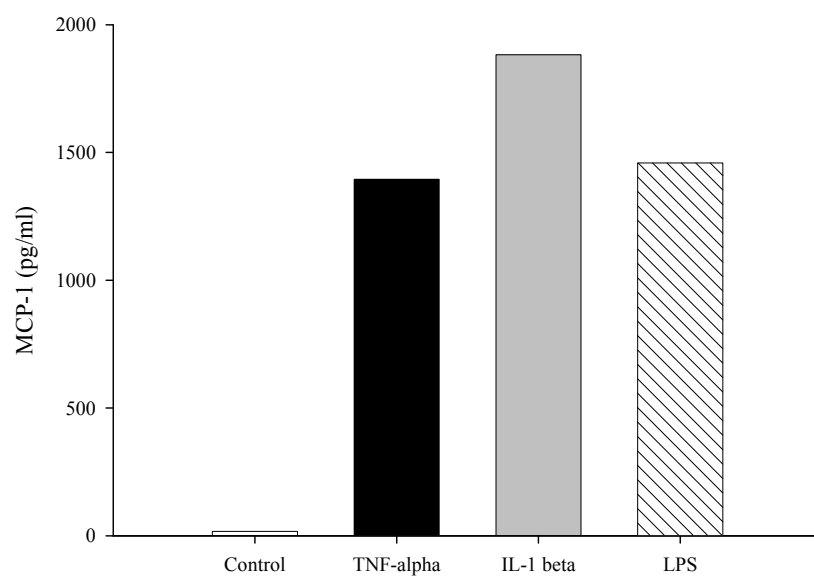
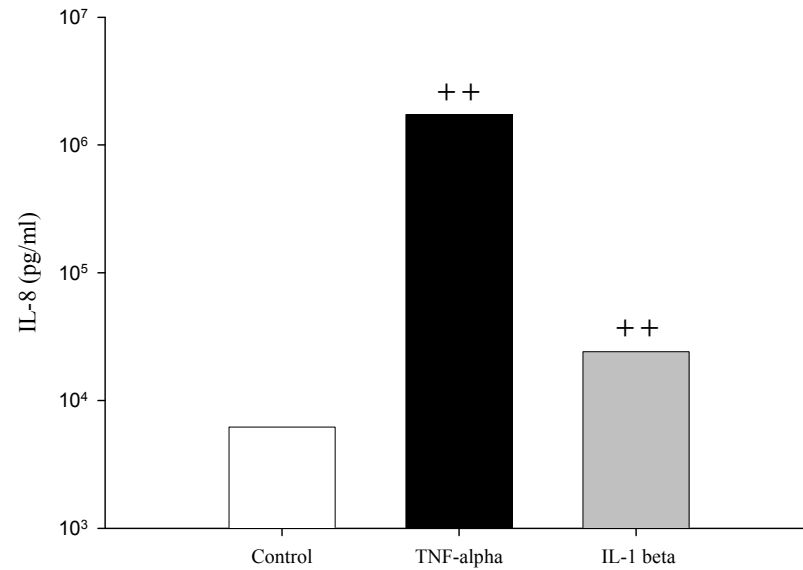


Figure 2.8. Chemokine Production by Rat CTX-TNA2 Astrocytes; Preliminary Data.

(A) MIP-2/CXCL2 and (B) MCP-1/CCL2 production after 24 hours in culture media alone (control) or with TNF- α (20ng/ml), IL-1 β (10ng/ml) or LPS (1 μ g/ml). ++ Optical densities in excess of standards.

A



B

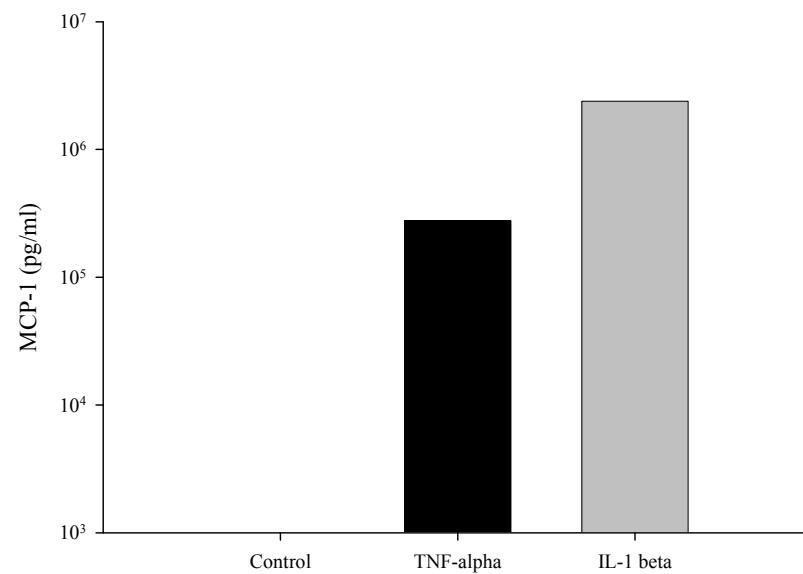


Figure 2.9. Chemokine Production by Human U373 Astrocytes; Preliminary Data.

(A) MIP-2/CXCL2 and (B) MCP-1/CCL2 production after 24 hours in culture media alone (control) or with TNF- α (20ng/ml), IL-1 β (10ng/ml) or LPS (1 μ g/ml). ++ Optical densities in excess of standards.

2.4.1.2. The Temporal Production by Glial Cells of Chemokines in Response to Pro-inflammatory Cytokines

Primary cultures and immortalised cell lines were treated with either TNF- α (20ng/ml) or IL-1 β (10ng/ml) for 24 hours and MIP-2/CXCL2 and MCP-1/CCL2 concentrations in the supernatant determined by ELISA.

TNF- α induced significant increases in MIP-2/CXCL2 by rat primary microglia (1560 ± 171 pg/ml vs. 120 ± 48 pg/ml in control media), primary astrocytes (17097 ± 2932 pg/ml vs. 563 ± 208 pg/ml in control media), rat CTX-TNA2 astrocytes (9865 ± 554 pg/ml vs. 370 ± 97 pg/ml in control media) and of IL-8/CXCL8 by human U373 astrocytes (284 ± 20 ng/ml vs. 5 ± 1 ng/ml in control media) (Figure 2.10A).

IL-1 β induced a significant production of MIP-2/CXCL2 by rat primary astrocytes (4219 ± 985 pg/ml vs. 473 ± 166 pg/ml in control media) and CTX-TNA2 astrocytes (4764 ± 158 pg/ml vs. 343 ± 86 pg/ml in control media) and of IL-8/CXCL8 by human U373 astrocytes (1085 ± 17 ng/ml vs. 5 ± 1 ng/ml in control media) but not rat primary microglia (276 ± 34 pg/ml vs. 170 ± 18 pg/ml in control media) (Figure 2.10B).

In the rat derived astrocytes TNF- α produced a significantly greater response over control than IL-1 β in the primary astrocyte cultures for MIP-2/CXCL2 (16534 ± 2796 pg/ml vs. 3746 ± 853 pg/ml for TNF- α vs. IL-1 β respectively)². For the rat CTX-TNA2 cells the response to TNF- α over control was also greater than for IL-1 β (9495 ± 457 pg/ml vs. 4421 ± 239 pg/ml for TNF- α vs. IL-1 β respectively). Conversely in the U373 human astrocyte line IL-8/CXCL8 production was significantly greater with IL-1 β (1080 ± 17 ng/ml vs. 278 ± 20 ng/ml for IL-1 β vs. TNF- α respectively).

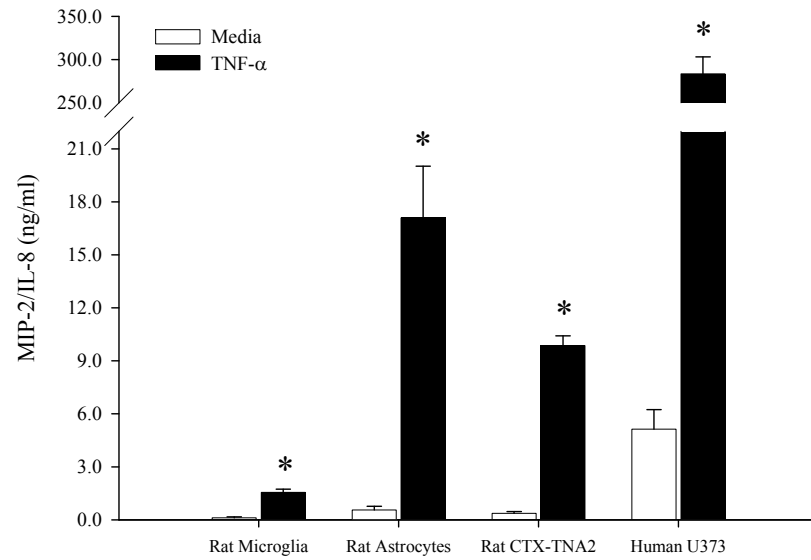
² Molecular masses of rat TNF- α = 17.0kDa, rat IL-1 β = 17.3kDa, human TNF- α = 17.5 kDa and human IL-1 β = 17.5kDa.

For rat reagents 20ng/ml TNF- α = 1.2nM and 10ng/ml IL-1 β = 0.6nM.

For human reagents 20ng/ml TNF- α = 1.1 nM and 10ng/ml = 0.6nM.

TNF- α is active as a dimer, therefore 1.2nM TNF- α has an equivalent functional molarity to 0.6nM IL-1 β .

A



B

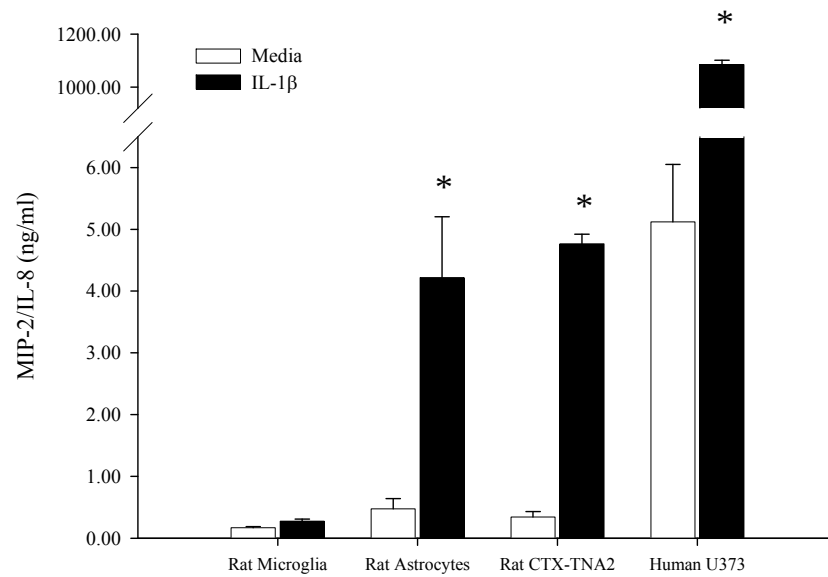
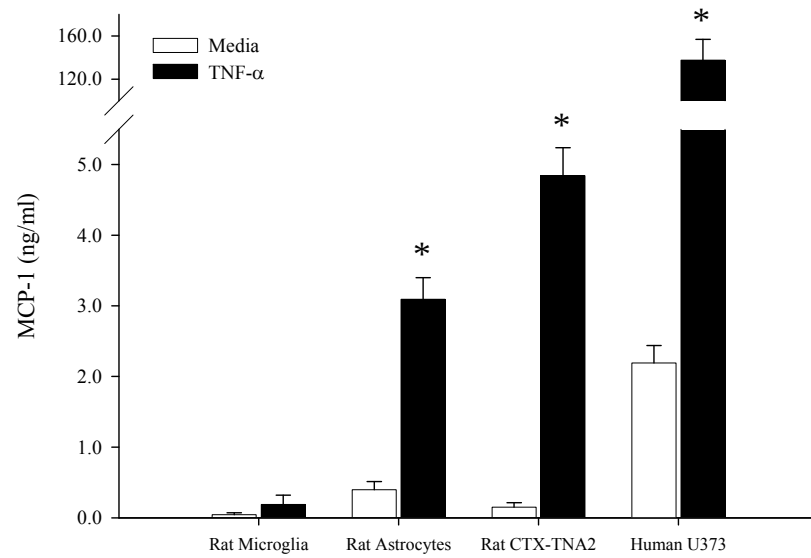


Figure 2.10. MIP-2/CXCL2 or IL-8/CXCL8 Production in Rat Primary Microglia, Rat Primary Astrocytes, Rat CTX-TNA2 astrocytes and Human U373 Astrocytes.

Cells were incubated for 24 hours in culture media alone (white bars) or with 20ng/ml of TNF- α (A) or 10ng/ml IL-1 β (B) (black bars). Data represents the mean + SEM of 3 independent experiments. * p < 0.05, compared to media.

A



B

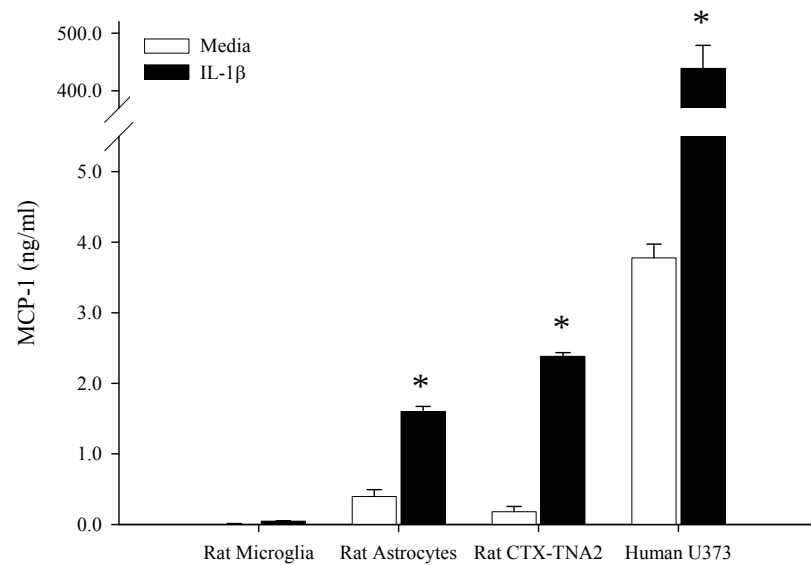


Figure 2.11. MCP-1/CCL2 Production in Rat Primary Microglia, Rat Primary Astrocytes, Rat CTX-TNA2 Astrocytes and Human U373 Astrocytes.

Cells were incubated for 24 hours in culture media alone (white bars) or with 20ng/ml of TNF- α (A) or 10ng/ml IL-1 β (B) (black bars). Data represents the mean + SEM of 3 independent experiments. * $p < 0.05$, compared to media.

TNF- α and IL-1 β both induced significant production of MCP-1/CCL2 by rat primary astrocytes (3092 $\pm$ 306 pg/ml with TNF- α vs. 397 $\pm$ 118 pg/ml in control media and 1599 $\pm$ 73 pg/ml with IL-1 β vs. 395 $\pm$ 99 pg/ml for control media), rat CTX-TNA2 astrocytes (4843 $\pm$ 397 pg/ml with TNF- α vs. 150 $\pm$ 64 pg/ml for control media and 2383 $\pm$ 53 pg/ml with IL-1 β vs. 180 $\pm$ 74 pg/ml for control media) and human U373 astrocytes (138 $\pm$ 19 ng/ml with TNF- α vs. 2 $\pm$ 2 ng/ml for control media and 439 $\pm$ 40 ng/ml with IL-1 β vs. 4 $\pm$ 0.2 ng/ml for control media). Neither cytokine induced significant production of MCP-1/CCL2 by rat primary microglia (190 $\pm$ 130 pg/ml with TNF- α vs. 43 $\pm$ 28 pg/ml for control media and 47 $\pm$ 6 pg/ml with IL-1 β vs. 8 $\pm$ 5 pg/ml for control media) (Figure 2.11A&B).

The increases in TNF- α induced MCP-1/CCL2 production over control were significantly greater than that for IL-1 β in both rat primary astrocytes (2695 $\pm$ 314 pg/ml vs. 1204 $\pm$ 182 pg/ml for TNF- α vs. IL-1 β) and rat CTX-TNA2 astrocytes (4693 $\pm$ 334 pg/ml vs. 2204 $\pm$ 28 pg/ml for TNF- α vs. IL-1 β). In contrast, IL-1 β induced a significantly greater increase in MCP-1/CCL2 over control than TNF- α in the human U373 astrocyte line (435 $\pm$ 40 ng/ml vs. 135 $\pm$ 19 ng/ml for IL-1 β vs. TNF- α).

Time course studies were performed for those cytokine cell combinations in which chemokine concentration in the supernatant was significantly increased at 24 hours (Figures 2.12-2.15). For the majority of this work all the samples, from three independent experiments, were analysed at each time point. With experience it was felt that this approach was unnecessarily expensive. For MIP-2/CXCL2 production by rat primary astrocytes incubated with IL-1 β (Figure 2.13A) and IL-8/CXCL8 and MCP-1/CCL2 production by human U373 astrocytes incubated with TNF- α or IL-1 β (Figure 2.15 A&B), only one full time course was completed in each case.

Over 24 hours TNF- α induced a significant increase in MIP-2/CXCL2 production by rat primary microglia (Comparison between treatment conditions at individual times by post hoc analysis confirming that this increase occurred between 6 and 24 hours in the TNF- α treated cells) (Figure 2.12) and primary astrocytes (Comparison between treatment conditions at individual times by post hoc analysis confirming that this increase occurred between 12 and 24 hours) (Figure 2.13A). For rat

CTX-TNA2 astrocytes TNF- α and IL-1 β induced significant increases in MIP-2/CXCL2 over 24 hours (Comparison between treatment conditions at individual times by post hoc analysis confirming that this increase occurred between 3 and 24 hours for TNF- α and IL-1 β) (Figure 2.14A).

Both TNF- α and IL-1 β induced significant increases in MCP-1/CCL2 production by rat primary astrocytes (Comparison between treatment conditions at individual times by post hoc analysis confirming that this increase occurred between 6 and 24 hours for TNF- α and between 3 and 24 hours for IL-1 β) (Figure 2.13B) and rat CTX-TNA2 astrocytes (Comparison between treatment conditions at individual times by post hoc analysis confirming that this increase occurred between 6 and 24 hours for TNF- α and between 3 and 24 hours for IL-1 β) (Figure 2.14B).

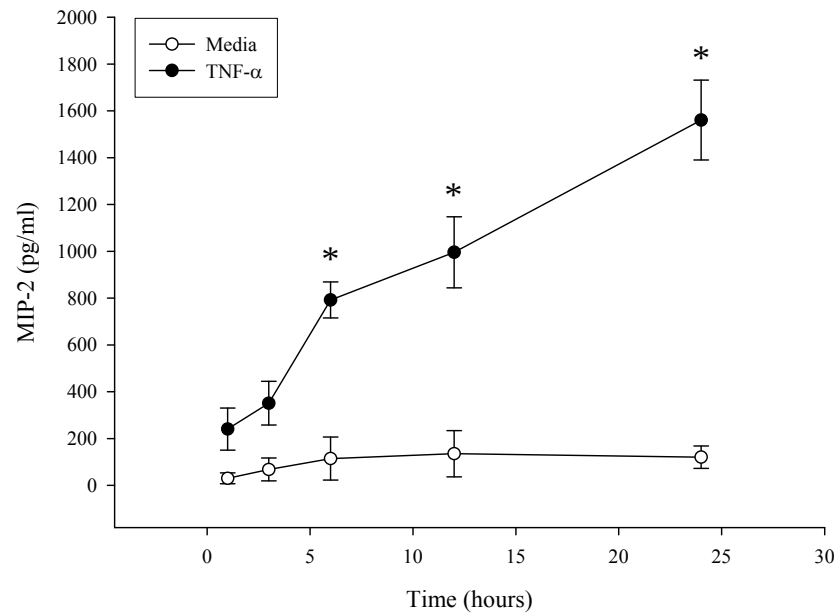
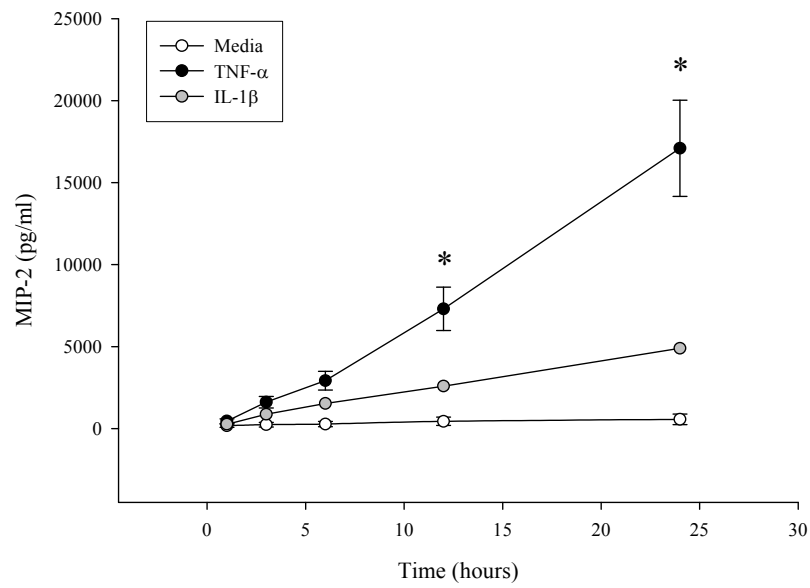


Figure 2.12. MIP-2/CXCL2 Production by Rat Primary Microglia Over 24 Hours.

Production with TNF- α (20ng/ml) vs. culture media. MIP-2/CXCL2 was not significantly increased by IL-1 β in rat primary microglia cultures at 24 hours. MCP-1/CCL2 was not significantly increased by TNF- α or IL-1 β in rat primary microglial cultures at 24 hours. Therefore the remaining supernatants from these experiments (1, 3, 6 & 12 hours were not analysed). Data represents the mean $\pm$ SEM of 3 independent experiments, * $p < 0.05$, compared to control at the time points indicated.

A



B

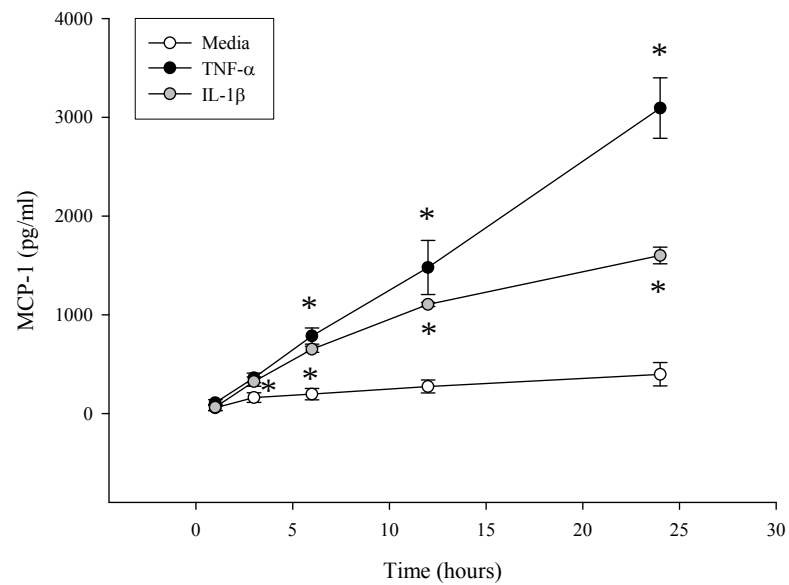
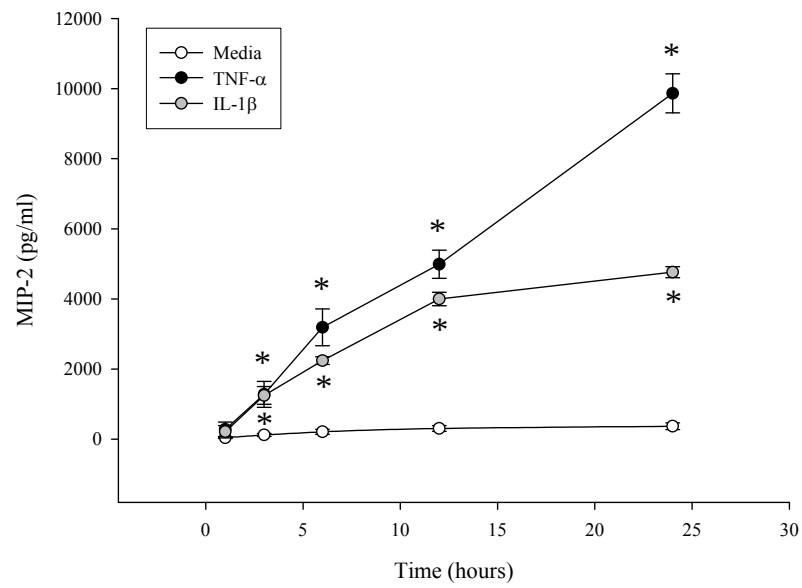


Figure 2.13. Chemokine Production by Rat Primary Astrocytes Over 24 Hours.

(A) MIP-2/CXCL2 and (B) MCP-1/CCL2 production with TNF-α (20ng/ml) or IL-1β (10ng/ml) vs. culture media. Data represents the mean ± SEM of 3 independent experiments, * $p < 0.05$, compared to control at the time points indicated, except for MIP-2/CXCL2 production by astrocytes incubated with IL-1β, where $n=1$.

A



B

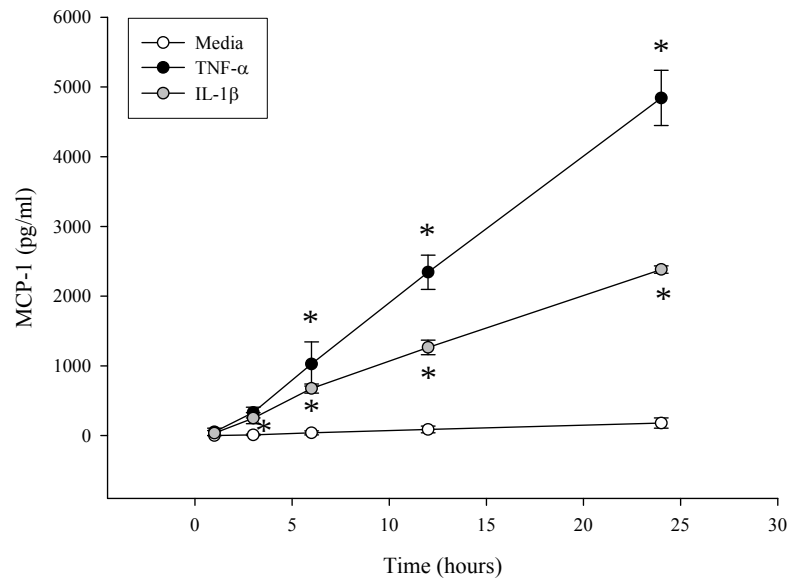
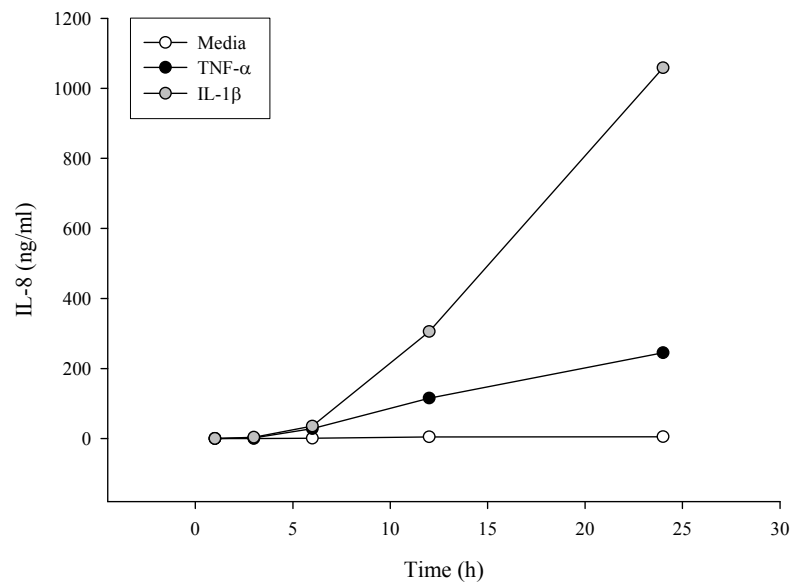


Figure 2.14. Chemokine Production by Rat CTX-TNA2 Astrocytes Over 24 Hours.

(A) MIP-2/CXCL2 and (B) MCP-1/CCL2 production with TNF- α (20ng/ml) or IL-1 β (10ng/ml) vs. culture media.). Data represents the mean $\pm$ SEM of 3 independent experiments, * $p < 0.05$, compared to control at the time points indicated.

A



B

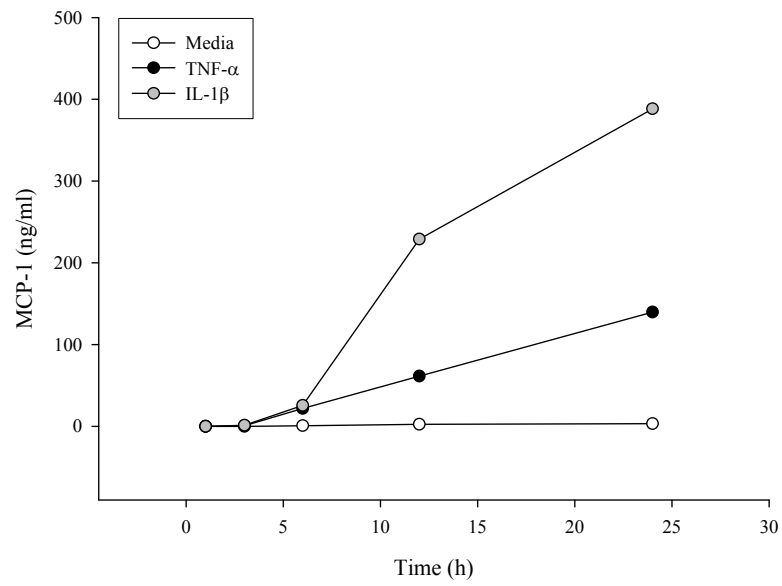


Figure 2.15. Chemokine Production by Human U373 Astrocytes Over 24 Hours.

(A) IL-8/CXCL8 and (B) MCP-1/CCL2 production with TNF-α (20ng/ml) or IL-1β (10ng/ml) vs. culture medial. n=1 independent experiment.

2.4.2. IL-6 and its Soluble Receptor Inhibit Production of MIP-2/CXCL2 by Rat Primary Microglia and CTX-TNA2 Astrocytes

The possible inhibitory effect of sIL-6R mediated signalling on the production of MIP-2/CXCL2 by rat primary microglia, rat primary astrocytes and rat CTX-TNA2 astrocytes, was investigated.

In order to determine if IL-6 or sIL-6R, either individually or in combination, affected chemokine production by glia, a single preliminary experiment was performed in which all possible combinations TNF- α (20ng/ml), IL-6 (10ng/ml) and sIL-6R (100ng/ml) were added to the culture media. Compared to controls IL-6 and/or sIL-6R did not appear to induce or inhibit MIP-2/CXCL2 production in the glial cells tested (Figures 2.16A, 2.17A & 2.18A).

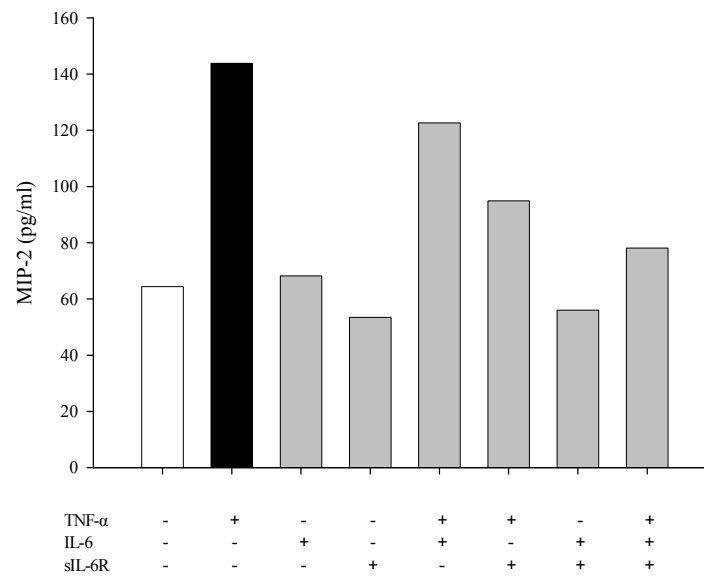
To investigate the possible inhibitory effect of sIL-6R mediated signalling on the production of MIP-2/CXCL2 by rat primary microglia, rat primary astrocytes or rat CTX-TNA2 astrocytes, cells were cultured with TNF- α (20ng/ml) alone and in the presence of IL-6 (10ng/ml) and its soluble receptor (10, 100 or 1000ng/ml). In rat primary microglia, the addition of IL-6 with various concentrations of sIL-6R resulted in a reduction in the MIP-2/CXCL2 concentration by $17.3 \pm 5.2\%$, $11.7 \pm 8.1\%$ and $28.0 \pm 6.7\%$ for sIL-6R 10, 100 and 1000ng/ml respectively. For TNF- α alone, and TNF- α with IL-6 and sIL-6R (10 or 100ng/ml) the MIP-2/CXCL2 concentration was significantly increased above that of the control media, comparison between groups by post hoc analysis (266.1 ± 42.3 pg/ml with TNF- α alone, 210.0 ± 24.0 pg/ml with TNF- α , IL-6 and sIL-6R 10ng/ml, 233.9 ± 30.0 pg/ml with TNF- α , IL-6 and sIL-6R 100ng/ml vs. 118.4 ± 23.9 pg/ml for control media). For TNF- α with IL-6 and sIL-6R 1000ng/ml the concentration of MIP-2/CXCL2 was not significantly increased over that of control media (188.2 ± 31.1 pg/ml for TNF, IL-6 and sIL-6R vs. 118.4 ± 23.9 pg/ml for control media) (Figure 2.16B).

In the CTX-TNA2 astrocytes IL-6 and sIL-6R were also associated with reductions in the MIP-2/CXCL2 concentration of $10.4 \pm 1.6\%$, $17.7 \pm 3.7\%$ and $28.8 \pm 5.0\%$ with IL-6 and sIL-6R 10, 100 or 1000ng/ml respectively. The reduction in MIP-2/CXCL2 production with the addition of IL-6 and sIL-6R 1000ng/ml was significant compared to TNF- α alone, comparison between groups by post hoc

analysis (4421.2 ± 468.0 pg/ml for TNF- α , IL-6 and sIL-6R 1000ng/ml vs. 6514.6 ± 930.1 pg/ml for TNF- α (Figure 2.17B).

In the rat primary astrocytes the addition of IL-6 and sIL-6R was also associated with reduced MIP-2/CXCL2 concentration in the supernatants of $10.7 \pm 1.9\%$, $10.6 \pm 6.2\%$ and $16.8 \pm 2.7\%$ with IL-6 and sIL-6R 10, 100 or 1000ng/ml respectively. However these were not significant (Figure 2.18B). To determine if the response of the rat primary astrocytes to TNF- α was sigmoidal and saturated at 20ng/ml of TNF- α , an experiment to determine the relationship of MIP-2/CXCL2 production to TNF- α concentration was performed. Rat primary astrocytes were incubated with 0-100ng/ml of TNF- α for 12 hours (Figure 2.19). This plot suggested that TNF- α 20ng/ml was on the linear portion of the relationship of MIP-2/CXCL2 production and TNF- α concentration.

A



B

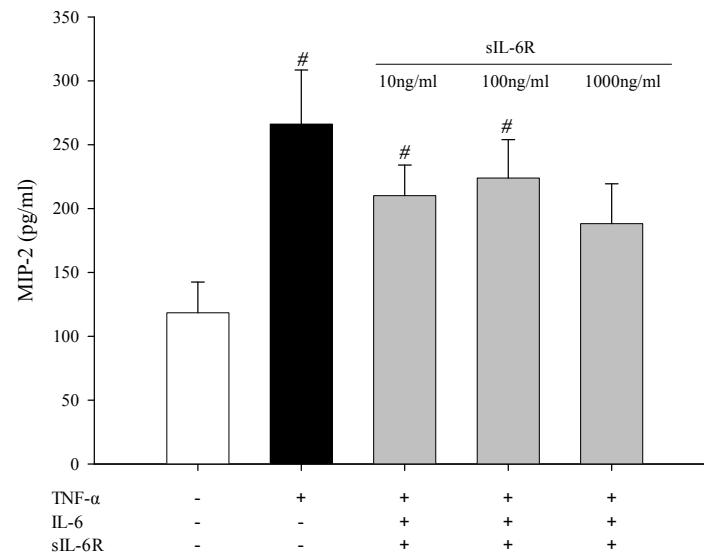
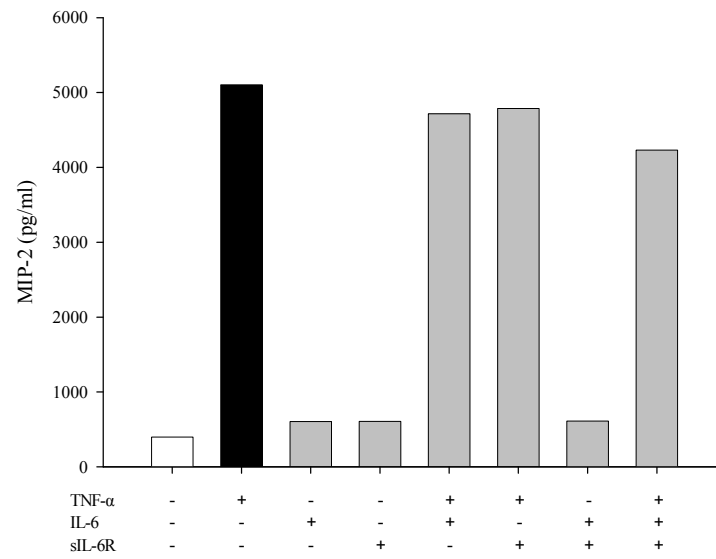


Figure 2.16. Inhibition of MIP-2/CXCL2 Production in Rat Primary Microglia by IL-6 and sIL-6R.

(A) Microglia were incubated for 6 hours in culture media alone (white bar), TNF- α 20ng/ml (black bar) or a complete battery of TNF- α 20ng/ml, IL-6 10ng/ml and sIL-6R 100ng/ml combinations (grey bars), n=1. (B) Under the same conditions cells were incubated in culture media alone (white bar), TNF- α 20ng/ml (black bar) or TNF- α 20ng/ml with IL-6 10ng/ml and sIL-6R 10, 100 or 1000 ng/ml (grey bars). Data represents the mean + SEM of 6 independent experiments. # p < 0.05, compared to media.

A



B

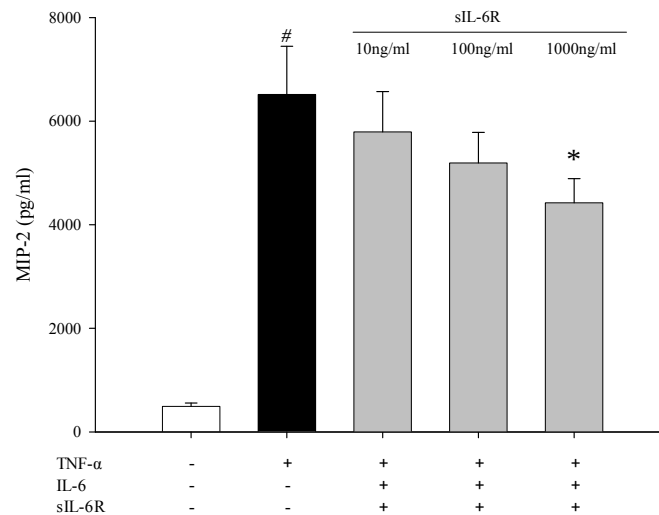
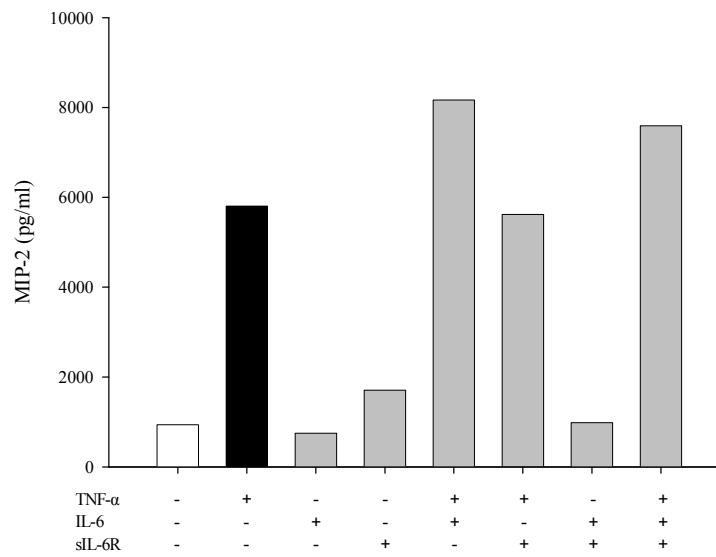


Figure 2.17. Inhibition of MIP-2/CXCL2 Production in Rat CTX-TNA2 Astrocytes by IL-6 and SIL-6R.

(A) Astrocytes were incubated for 12 hours in culture media alone (white bar), TNF- α 20ng/ml (black bar) or a complete battery of TNF- α 20ng/ml, IL-6 10ng/ml and sIL-6R 100ng/ml combinations (grey bars), n=1. (B) Under the same conditions cells were incubated in culture media alone (white bar), TNF- α 20ng/ml (black bar) or TNF- α 20ng/ml with IL-6 10ng/ml and sIL-6R 10, 100 or 1000ng/ml (grey bars). Data represents the mean + SEM of 7 independent experiments. * p < 0.05, compared to TNF- α alone. # p < 0.05, compared to media.

A



B

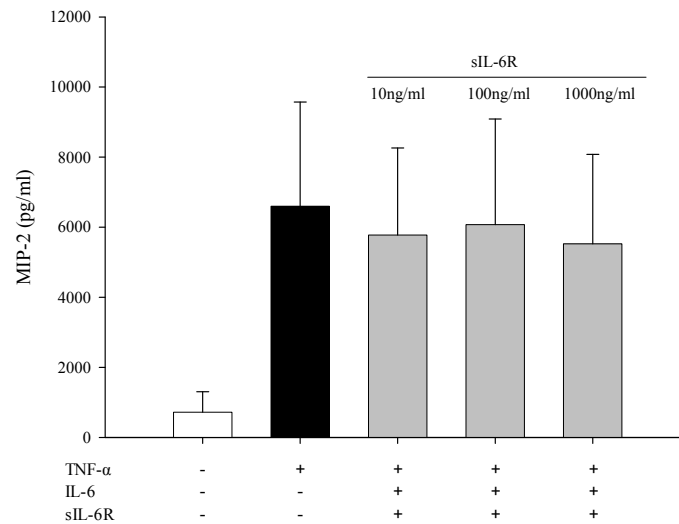


Figure 2.18. MIP-2/CXCL2 Production is not Inhibited in Rat Primary Astrocytes by IL-6 and sIL-6R.

(A) Astrocytes were incubated for 12 hours in culture media alone (white bar), TNF- α 20ng/ml (black bar) or a complete battery of TNF- α 20ng/ml, IL-6 10ng/ml and sIL-6R 100ng/ml combinations (grey bars), n=1. (B) Under the same conditions cells were incubated in culture media alone (white bar), TNF- α 20ng/ml (black bar) or TNF- α 20ng/ml with IL-6 10ng/ml and sIL-6R 10, 100 or 1000ng/ml (grey bars). Data represents the mean + SEM of 3 independent experiments.

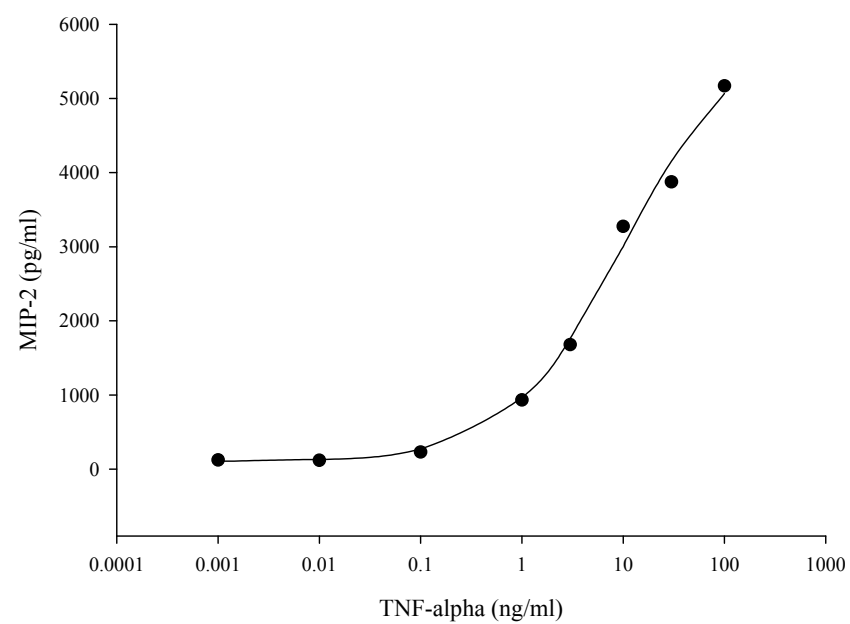


Figure 2.19. MIP-2/CXCL2 Production vs. TNF- α Concentration by Rat Primary Astrocytes.
Cells were cultured with 0-100ng/ml of TNF- α for 12 hours, n=1.

2.4.3. Pre-incubation of Rat CTX-TNA2 Astrocytes with Dexamethasone but not FK506 Prior to the Addition of TNF- α Inhibits the Production of MIP-2/CXCL2 and MCP-1/CCL2

In-vivo the tissue concentration of TNF- α reaches a peak at 3-4 hours after TBI (Taupin et al., 1993, Shohami et al., 1994) and 8-24 hours following both permanent (Liu et al., 1994, Buttini et al., 1996) and transient (Ohtaki et al., 2004) ischaemic insults. Therefore in order to maximize the potential inhibitory effect of dexamethasone or FK506 on chemokine expression, in a clinically meaningful manner, rat CTX-TNA2 astrocytes were pre-treated with these agents for 3 hours prior to the addition of the TNF- α . TNF- α (20ng/ml) had originally been used in these studies to induce chemokine expression by glia. However the increase in chemokine concentration with this concentration of TNF- α required multiple dilutions of the supernatant to avoid exceeding the range of the ELISA kit. Therefore for the investigation of the effect of dexamethasone and FK506 on chemokine production a lower concentration of TNF- α (1ng/ml) was chosen. This still lead to a significant increase in chemokine concentration vs. control media (Figures 2.21 & 2.23).

A pilot experiment, in which CTX-TNA2 astrocytes were pre-incubated with increasing concentrations of dexamethasone prior to the addition of TNF- α , was performed (Figure 2.20). This suggested that the greatest inhibition in MIP-2/CXCL2 production occurred with 1 and 10 μ M dexamethasone. When formally tested dexamethasone 10 μ M significantly reduced the concentration of MIP-2/CXCL2 induced by TNF- α by 48.8 ± 8.4 % (142 ± 30 pg/ml for TNF- α with dexamethasone 10 μ M vs. 288 ± 61 pg/ml for TNF- α alone, comparison between groups by post hoc analysis confirming that the reduction was significant with the addition of dexamethasone 10 μ M) (Figure 2.21A). The inhibition of MIP-2/CXCL2 production was more pronounced, at 74.9 ± 5.9 %, when the pre-incubation time was reduced to 1.5 hours (77 ± 7 pg/ml for TNF- α with dexamethasone 10 μ M vs. 329 ± 47 pg/ml for TNF- α alone, comparison between the groups by post hoc analysis confirming that the reduction was significant with the addition of dexamethasone 10 μ M) compared to that with a 3 hour pre-incubation but this was not significantly different. However inhibition was no longer significant when the dexamethasone and TNF- α were added simultaneously (51.5 ± 13.2 %, $167 \pm$

69pg/ml for TNF- α with dexamethasone 10 μ M vs. 316 ± 64 pg/ml for TNF- α alone) (Figure 2.22). Pre-incubation with dexamethasone 10 μ M for 3 hours also significantly inhibited the production of MCP-1/CCL2 in CTX-TNA2 astrocytes by $62.8 \pm 9.2\%$ (164 ± 23 pg/ml for TNF- α with dexamethasone 10 μ M vs. 497 ± 151 pg/ml for TNF- α alone, comparison between the groups by post hoc analysis confirming that the reduction was significant with the addition of dexamethasone 10 μ M) (Figure 2.21B).

In contrast pre-incubation with FK506 for 3 hours prior to TNF- α increased the MIP-2/CXCL2 concentration by $38.1 \pm 23.9\%$ (411 ± 89 pg/ml for TNF- α with FK506 100nM vs. 314 ± 84 pg/ml for TNF- α alone) and reduced the MCP-1/CCL2 concentration by $12.9 \pm 9.2\%$ (425 ± 72 pg/ml for TNF- α with FK506 100nM vs. 516 ± 142 pg/ml for TNF- α alone). These changes were not significant when compared to TNF- α alone (Figure 2.23).

The effects of dexamethasone or FK506 on chemokine production by rat primary microglia or astrocytes were not investigated.

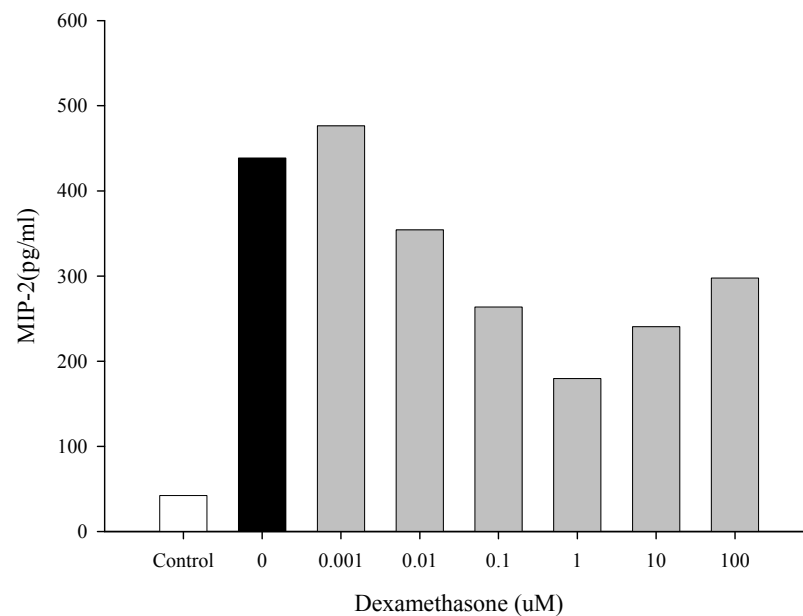


Figure 2.20. Inhibition of MIP-2/CXCL2 Production in Rat CTX-TNA2 Astrocytes by Dexamethasone.

Rat CTX-TNA-2 astrocytes were pre-incubated for 3 hours with 0-100 μ M dexamethasone. 1ng/ml of TNF- α (grey bars) was then added as indicated and the cells cultured for a further 10 hours. Controls consisted of cells treated for 3 hours in fresh culture media and then 1ng/ml TNF- α (black bar) or an equivalent volume of fresh media (Control).

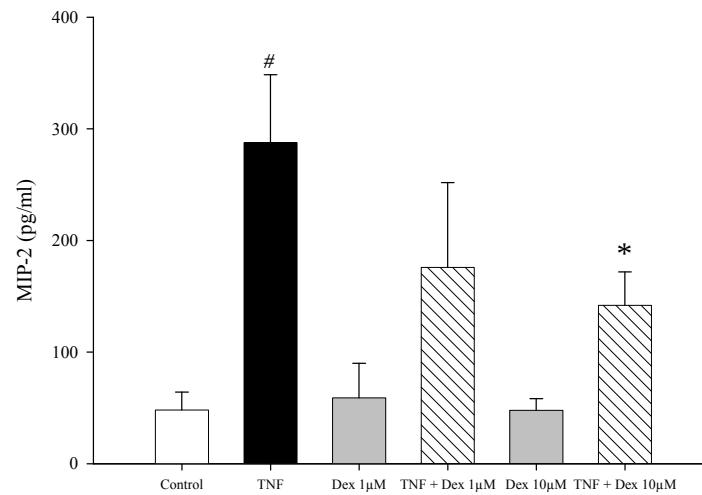
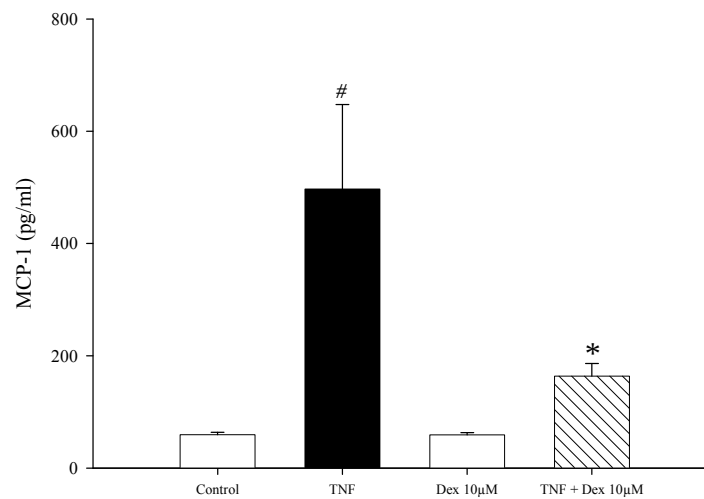
A**B**

Figure 2.21. Inhibition of MIP-2/CXCL2 (A) and MCP-1/CCL2 (B) Production in Rat CTX-TNA2 Astrocytes by Dexamethasone.

Cells were pre-incubated for 3 hours with 1 or 10 µM dexamethasone. 1ng/ml of TNF- α (TNF + Dex 1µM, TNF + Dex 10µM) or an equivalent volume of fresh culture media (Dex 1µM, Dex 10µM) were then added as indicated and the cells cultured for a further 10 hours. Controls consisted of cells treated for 3 hours in fresh culture media and then 1ng/ml TNF- α (TNF) or an equivalent volume of fresh media (Control). Data represents the mean + SEM of 3 independent experiments. * $p < 0.05$, compared to TNF- α alone. # $p < 0.05$, compared to control.

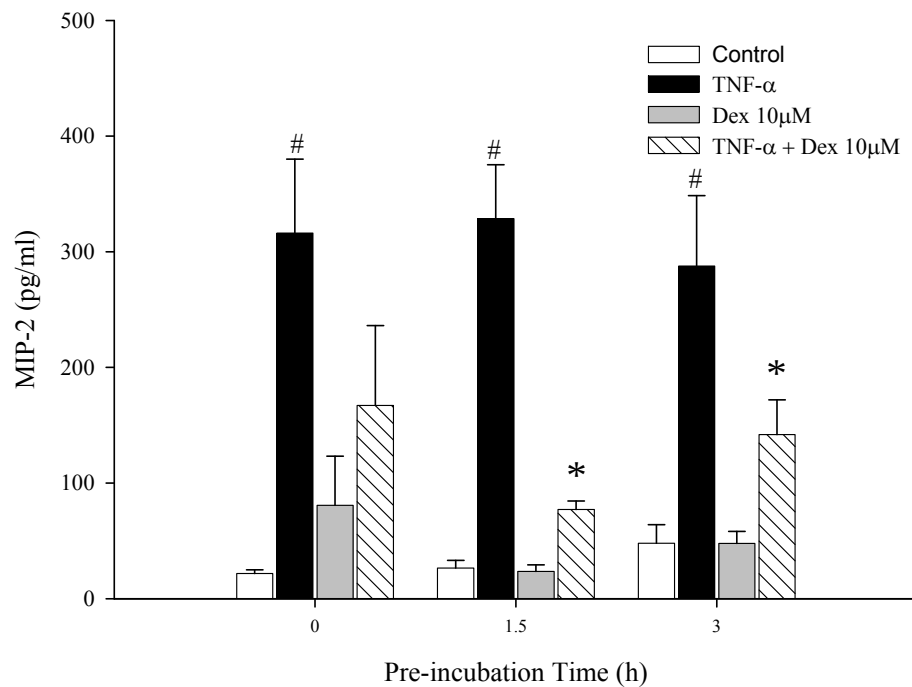


Figure 2.22. The Effect of Reducing Pre-incubation Time on the Inhibition of MIP-2/CXCL2 Production in Rat CTX-TNA2 Astrocytes by Dexamethasone.

Cells were pre-incubated for 0, 1.5 or 3 hours with 10 μ M dexamethasone. 1 ng/ml of TNF- α (TNF + Dex 10 μ M) or an equivalent volume of fresh culture media (Dex 10 μ M) were then added as indicated and the cells cultured for a further 10 hours. Controls consisted of cells treated for 0, 1.5 or 3 hours in fresh culture media and then 1 ng/ml TNF- α (TNF) or an equivalent volume of fresh media (Control) added. Data represents the mean + SEM of 3 independent experiments. * $p < 0.05$, compared to TNF- α alone in that pre-incubation period. # $p < 0.05$, compared to control in that pre-incubation period.

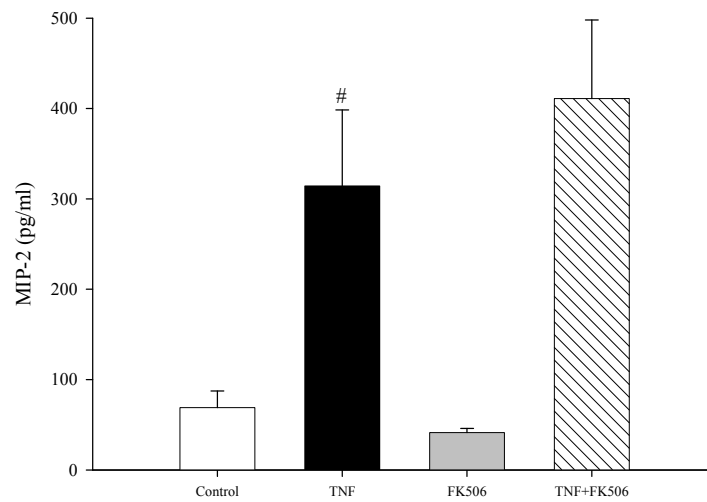
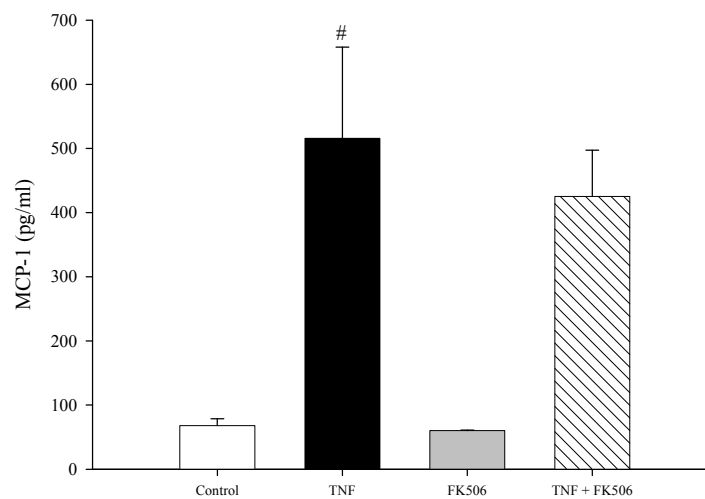
A**B**

Figure 2.23. MIP-2/CXCL2 (A) and MCP-1/CCL2 (B) Production in Rat CTX-TNA2 Astrocytes are not Inhibited by FK506.

Rat CTX-TNA-2 astrocytes were pre-incubated for 3 hours with 100nM FK506. 1ng/ml of TNF- α (TNF + FK506) or an equivalent volume of fresh culture media (FK506) were then added as indicated and the cells cultured for a further 10 hours. Controls consisted of cells treated for 3 hours in fresh culture media and then 1ng/ml TNF- α (TNF) or an equivalent volume of fresh media (Control) added. Data represents the mean + SEM of 3 independent experiments. # $p < 0.05$, compared to control.

2.5. Discussion

2.5.1. Summary of Findings

- Rat astrocytes produce MIP-2/CXCL2 and MCP-1/CCL2 in culture with pro-inflammatory cytokines and this is similar over time to the production of IL-8/CXCL8 and MCP-1/CCL2 by human derived astrocytes. For MIP-2/CXCL2 the rat derived cells demonstrated a greater response to TNF- α than IL-1 β whereas in the human derived cells the reverse was seen, for the same concentrations of cytokine. For MCP-1 the response of rat derived cells to TNF- α and IL-1 β were similar but for the human derived cells IL-1 β produced a greater increase in chemokine.
- Rat microglia produce MIP-2/CXCL2 in culture with TNF- α .
- Rat microglia do not produce MCP-1/CCL2 following incubation with TNF- α or IL-1 β
- Chemokine production by astrocytes is inhibited by dexamethasone but not FK506.
- Chemokine production by microglia and astrocytes can be inhibited by IL-6/sIL-R.

2.5.2. Chemokine Expression by Glia

I have compared the expression of chemokines by rat astrocytes and microglia with that of a human astrocyte cell line. With additional consideration of the available literature I sought to establish if rat and human cells respond in a similar manner to stimulation with pro-inflammatory mediators, thereby supporting the study of chemokine biology in a rat model of traumatic brain injury. I also investigated strategies to modulate chemokine expression in rat glial cell cultures in order to select candidate agents for further study *in-vivo*.

I have demonstrated that rat astrocytes produce MIP-2/CXCL2 and MCP-1/CCL2 in response to TNF- α or IL-1 β (Figures 2.10 & 2.11). This expression is similar over time to the induction of IL-8/CXCL8 and MCP-1/CCL2 by human U373 astrocytes (Figures 2.13 to 2.15). For the rat derived astrocytes TNF- α produced a greater increase in chemokine concentration than IL-1 β but in the human cells the reverse was seen, for the same concentrations of cytokine agonist. In contrast rat microglia produce MIP-2/CXCL2 in response to incubation with TNF- α but not IL-1 β and do not increase MCP-1/CCL-2 expression with either cytokine (Figures 2.10 & 2.11).

Human U373 astrocytes are derived from a human glioblastoma astrocytoma (Ponten & Macintyre, 1968) and have functional, histological and immunological features of native astrocytes. The expression of IL-8/CXCL8 and MCP-1/CCL2 mRNA and protein by these cells following treatment with TNF- α or IL-1 β has been described previously by Oh and colleagues (Oh et al., 1999). They also demonstrated the similar induction of IL-8/CXCL8 and MCP-1/CCL2 in another astrogloma cell line, U251, and adult human astrocytes cultured from biopsy material. Pro-inflammatory cytokines have also been shown to induce IL-8/CXCL8 (Aloisi et al., 1992, Ehrlich et al., 1998, Janabi et al., 1999, Croitoru-Lamoury et al., 2003) and MCP-1/CCL2 (Hurwitz et al., 1995, Peterson et al., 1997, Croitoru-Lamoury et al., 2003) in human embryonic/foetal primary astrocyte cultures. In keeping with these responses the present data indicates that in the rat, both neonatal primary astrocytes and the transfected cortical astrocyte cell line CTX-TNA2 (Radany et al., 1992) express MIP-2/CXCL2 and MCP-1/CCL-2 in response to the same pro-inflammatory mediators. This is consistent with the

finding that mouse neonatal primary astrocyte cultures also produce MIP-2/CXCL-2 (Otto et al., 2000) and MCP-1/CCL2 (Hayashi et al., 1995) (Table 2.1).

A consistent feature of the literature is that in human cells the reported IL-8/CXCL8 response is greater with IL-1 β than TNF- α (Aloisi et al., 1992, Oh et al., 1999, Croitoru-Lamoury et al., 2003) and this was also found in my study of the U373 human astrocyte cell line. The units used to quantify the IL-1 β and TNF- α concentrations in these different studies vary, with units/ml (Aloisi et al., 1992), international units/ml (Croitoru-Lamoury et al., 2003) and ng/ml (Aloisi et al., 1992, Oh et al., 1998) being employed. However a direct comparison between my study of human U373 astrocytes and those of Oh with both U373 cells and adult human astrocytes can be made as the same cell number (2×10^5 cells per well) and similar concentrations of TNF- α (20ng/ml vs. 10ng/ml) and IL-1 β (10ng/ml vs. 4ng/ml) were used in both studies. In each case the IL-8/CXCL8 increase over controls in the human U373 cells and adult human astrocytes was greater with IL-1 β than TNF- α . This is in contrast to MIP-2/CXCL2 produced by rat astrocytes, where TNF- α produces a greater response than IL-1 β for the same cell density and concentration of cytokines used between the species.

The significant IL-8/CXCL8 production by human foetal astrocyte cultures reported by Ehrlich and colleagues was induced by IL-1 β but not TNF- α (Ehrlich et al., 1998). In the Ehrlich paper the TNF- α (20ng/ml) and IL-1 β (10ng/ml) stimulus were the same as in my study. However in contrast to the studies discussed in which cell densities of 2 to 8×10^5 cells per well were used (Aloisi et al., 1992, Oh et al., 1999, Croitoru-Lamoury et al., 2003) the cell number used by Ehrlich and colleagues was considerably lower at 5×10^3 cells per well. This raises the possibility that the lesser response to TNF- α compared to IL-1 β , coupled with a lower cell count, was responsible for the failure to detect a significant increase in IL-8/CXCL8 production in human foetal astrocytes. Croitoru and colleagues found that both TNF- α and IL-1 β could induce IL-8/CXCL8 production in similarly aged human foetal astrocytes to Ehrlich (Croitoru-Lamoury et al., 2003). This would argue against a lack of TNF- α responsiveness in foetal cells.

For MCP-1/CCL2 production by rat astrocytes I found that TNF- α also induced a greater increase in chemokine concentration than IL-1 β in both the primary cultures and the CTX-TNA2 cell line. In the human U373 cells IL-1 β produced a greater response than TNF- α for the same cell density and concentrations of cytokines used. The comparison with the literature is less consistent than it was for IL-8/CXCL8 production by astrocytes. The data from Oh and colleagues indicated that the response of U373 cells to TNF- α was greater than that for IL-1 β but in the primary adult astrocytes the reverse was found (Oh et al., 1999). Peterson also found that in human primary astrocytes the increase in MCP-1/CCL2 over controls was greater with IL-1 β than TNF- α (Peterson et al., 1997).

I have also demonstrated that in rat microglia the production of MIP-2/CXCL2 is significantly increased in response to TNF- α but not IL-1 β (Figure 2.10). The culture of microglia and astrocytes from neonatal tissue generates cultures that are >90% pure (2.3.2.1, figures 2.3 & 2.4). Even allowing for the lower density of the microglial cultures compared with the astrocytes (1×10^5 vs. 2×10^5 cells per well) the increase in MIP-2/CXCL2 concentration seen in the microglia was small compared to that of the astrocyte cell lines. This raises the possibility that the observed increase in MIP-2/CXCL2 is due to the TNF- α acting on contaminating astrocytes and not microglia. This would be consistent with the greater chemokine response in the rat astrocytes to TNF- α than IL-1 β . However if the observed MIP-2/CXCL2 production in microglia was due to contaminating astrocytes the absolute increase in MIP-2/CXCL2 concentration over control, allowing for the different cell densities would be at best 5% of that seen in primary astrocytes (50% reduction due to half the cell density, 10% (contaminating astrocytes worst case) of 50%=5%). However in my study the increase in MIP-2/CXCL2 in the microglia culture was 15% of that seen in astrocytes. This would therefore suggest that the production of MIP-2/CXCL2 is indeed from rat microglia and not contaminating astrocytes.

A small non-significant increase in MIP-2/CXCL2 concentration was seen in rat primary microglia treated with IL-1 β . However, the low number of experiments ($n=3$) and the small increase in chemokine concentration resulted in the analysis failing to reach a power of 0.8. Therefore it is unsafe, without further study, to conclude that IL-1 β does not induce MIP-2/CXCL2 in rat primary microglia. Ehrlich and colleagues reported a significant increase in IL-8/CXCL8 production by

human foetal microglia cultured with IL-1 β and a smaller, non-significant increase, with TNF- α (Ehrlich et al., 1998). This difference would be in keeping with a species difference in the response of human cells to IL-1 β and TNF- α , as discussed for astrocytes.

Small increases in MCP-1/CCL2 concentration were seen in rat primary microglia treated with either TNF- α or IL-1 β but these were not significant (Figure 2.11). The same result was reported in human foetal microglia (Peterson et al., 1997), although pro-inflammatory cytokines did significantly increase MCP-1/CCL2 mRNA expression in rat embryonic microglial culture (Calvo et al., 1996). Again, for my work the sample size (n=3) combined with a small treatment effect resulted in the analysis being under powered. It is therefore unsafe to conclude that TNF- α or IL-1 β do not induce MCP-1/CCL2 production in rat primary microglia. However, like the possible effect of IL-1 β on MIP-2/CXCL2 production in rat primary microglia, this effect, if real, is small compared to that seen in rat primary astrocytes.

The comparison of chemokine production by microglia was relatively limited compared to that of astrocytes. I was unable to culture the rat HAPI microglial cell line (Cheepsunthorn et al., 2001) to compare with the primary neonatal cultures. Despite ethical approval no tissue was available from which to obtain human primary cultures of microglia to compare with the rat primary cultures. In the immortalised human CHME-5 microglial cell line (Janabi et al., 1995), chemokines were undetectable in the supernatants, in both the control and cytokine treated cells. This finding was consistent with the experience of colleagues in our laboratory with these cells. Unlike human foetal primary microglia (Lee et al., 1993), the incubation of CHME-5 cells with LPS failed to cause IL-1 β or TNF- α production (Findlayson K, unpublished observations). This would suggest that these cells lacked either the ability transduce cytokine signals or were missing cellular elements crucial for cytokine synthesis and released.

Despite these difficulties I have been able to demonstrate that the overall response of rat derived glia to pro-inflammatory cytokines was similar to that of human U373 astrocytes in this study and other human glial cell cultures described in the literature.

2.5.3. Inhibition of Chemokine Production by Rat Glia

The inhibition of chemokine expression represents a possible means of limiting leukocyte recruitment and microglial activation, reducing secondary brain injury. I have investigated 3 different potential antagonists of chemokine production by glia, namely IL-6/sIL-6R signalling, the glucocorticoid dexamethasone and the calcineurin antagonist FK506.

2.5.3.1. IL-6/sIL-6R

In rat primary microglia and the immortalised rat astrocyte cell line CTX-TNA2 the incubation of IL-6 and sIL-6R inhibited the production of MIP-2/CXCL2 induced by TNF- α (Figures 2.16 & 2.17). IL-6 acting via its soluble receptor to modulate chemokine expression and the inflammatory response has been demonstrated previously in human peritoneal mesothelial cells (Hurst et al., 2001). Whilst the inhibition of MIP-2/CXCL2 production in glial cells is a novel finding it raises some important questions. Firstly in my experiments the inhibition was not present in all the cell lines tested. I was unable to demonstrate a significant reduction in MIP-2/CXCL2 production by rat primary astrocytes (Figure 2.18). Although some reduction was seen it was considerably less than that observed in the CTX-TNA2 cells ($16.8 \pm 2.7\%$ for primary astrocytes vs. $28.8 \pm 5.0\%$ for CTX-TNA2 astrocytes). In part, this may be due to the large variability in the astrocyte response making the number of experiments required to reduce the risk of a type 2 errors prohibitive. It is also possible that the relatively immature neonatal astrocytes are lacking a critical element required in order to respond to IL-6/sIL-6R.

In rats the peak cortical TNF- α concentration following TBI was approximately 40ng/ml (Shohami et al., 1994) and IL-6 levels in excess of 100ng/ml been reported following both experimental trauma (Shohami et al., 1994) and ischaemia (Legos et al., 2000). In humans IL-6 CSF concentrations of 6-10ng/ml have been measured after TBI (Csuka et al., 1999, Hans et al., 1999b) and 0.3ng/ml after stroke (Tarkowski et al., 1995). Assuming that the cortical concentration of IL-6 is greater than the CSF level, then concentrations of TNF- α (20ng/ml) and IL-6 (10ng/ml) used in my study are likely to be clinically relevant. However for sIL-6R the CSF concentration after human TBI has been found to

be less than 10ng/ml (Hans et al., 1999b). Since inhibition of MIP-2/CXCL2 only occurred with 1000ng/ml of sIL-6R it is difficult to be confident that this concentration is appropriate without *in-vivo* measurement. I am not aware of any published data on cortical sIL-6R concentration after injury.

Finally, *in-vitro*, the reduction in MIP-2/CXCL2 production by IL-6/sIL-6R seen in primary microglia or CTX-TNA2 astrocytes occurred in optimised conditions. This reduction of approximately 28% at best was relatively small and may prove difficult to replicate *in-vivo*.

2.5.3.2. Dexamethasone

The glucocorticoids are archetypal anti-inflammatory agents widely used for this effect in clinical practice. The effect of a glucocorticoid on MIP-2/CXCL2 or MCP-1/CCL2 expression by rodent glia has not been described previously. I have shown that in the rat CTX-TNA2 astrocyte cell line the TNF- α induced production of both MIP-2/CXCL2 and MCP-1/CCL2 is inhibited by pre-incubation with dexamethasone (Figure 2.21). The pre-incubation period could be reduced to 1.5 hours with significant inhibition of MIP-2/CXCL2 production remaining but this was lost when the dexamethasone and TNF- α were added concurrently (Figure 2.22). However rigorous regard of the data requires the mention that this analysis was underpowered and so the possibility of significant inhibition at zero hours of pre-incubation cannot be safely discounted. Whilst the mechanism by which dexamethasone inhibited chemokine expression in glia is outwith the scope of this study, consideration of the known regulatory elements of inflammatory genes and the presence of these elements in chemokine genes suggests mechanisms which could be pertinent.

The expression of many cytokines are governed by key regulators of transcription such as nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1). Inflammatory signals trigger cytoplasmic protein kinases which activate these transcription factors allowing their translocation into the nucleus and binding to specific recognition sequences in the 5' regulatory or promoter region of a cytokine gene, resulting in the subsequent transcription of that gene (Saklatvala, 2002). NF- κ B exists in the cytoplasm of cells in a latent form due to its association with an inhibitory protein I κ B. Activation

leads to dissociation of the I κ B releasing active NF- κ B free to translocate in the nucleus (Bourke & Moynagh, 1999).

Glucocorticoids influence gene expression and protein production via many mechanisms both genomic and non-genomic (For review see Saklatvala 2002 & Stellato 2004). The genomic actions of steroids classically involve the regulation of gene transcription. Glucocorticoid binding to the glucocorticoid receptor (GR) in the cytoplasm allows translocation of this complex to the nucleus where the GR can bind its own recognition or consensus sequence, the glucocorticoid response element (GRE), on the 5' regulatory region of a gene. Induction of the transcription and translation of an anti-inflammatory or regulatory gene such as I κ B (Auphan et al., 1995, Scheinman et al., 1995) or glucocorticoid-induced leucine zipper (GILZ) (Mittelstadt & Ashwell, 2001) can then occur. Rarely GR can also bind to a negative GRE inhibiting gene transcription. These actions involving GREs are termed DNA dependant mechanisms. Alternatively DNA independent mechanisms occur in which ligand activated GR interacts with transcription factors, such NF- κ B (Ray & Prefontaine, 1994)(De Bosscher et al., 2000)(Reichardt et al., 2001) or AP-1 (Jonat et al., 1990) directly, interfering with their function at regulatory sites and thereby inhibiting transcription (Saklatvala, 2002, Stellato, 2004).

Transcription factor recognition sites have been identified in the regulatory regions of chemokine genes and there is some evidence that glucocorticoids can modulate transcription factor function at these sites. In a human glioblastoma cell line, deletion and mutation analysis demonstrated that in the case of IL-1 α induced IL-8/CXCL8 production the NF- κ B binding site was crucial and the inhibition of IL-8/CXCL8 mRNA and protein expression by dexamethasone was associated with reduced binding of NF- κ B to DNA (Mukaida et al., 1994) (Figure 2.24). An NF- κ B binding region in the murine MIP-2/CXCL2 promoter has also been described. This promoter and its regulatory elements are highly conserved in the related human homologues Gro- α /CXCL1, GRO- β /CXCL2 and GRO- γ /CXCL3. The NF- κ B element has been demonstrated, in 5' deletion mutants, to be essential for conferring promoter responsiveness to lipopolysaccharide (Widmer et al., 1993, Lee et al., 2005) (Figure 2.24). Consistently, the TNF- α induced MIP-2/CXCL2 mRNA expression in activated rat

hepatic stellate cells was associated with a reduction in I κ B α and an increase in the nuclear translocation and DNA binding of NF- κ B (Hellerbrand et al., 1998).

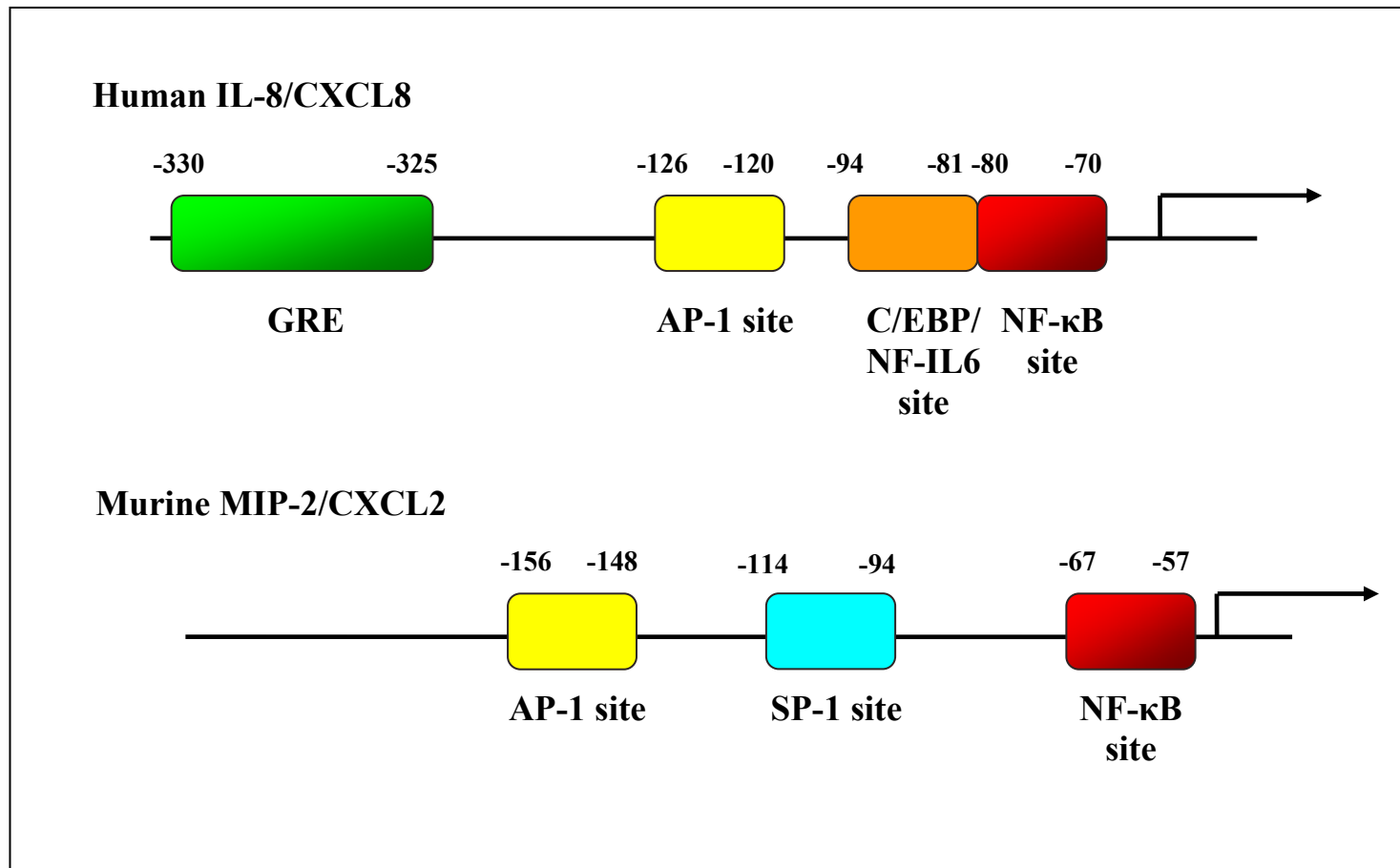


Figure 2.24. Transcription Factor Binding Sites in the Promoter Region of the IL-8/CXCL8 and MIP-2/CXCL2 Genes.

Human IL-8/CXCL8 from Mukaida *et al.*, 1994. Murine MIP-2/CXCL2 from Widmer *et al.*, 1993 and Lee *et al.*, 2005. Activator protein-1 (AP-1), CCAAT enhancer binding protein (C/EBP), glucocorticoid response element (GRE), interleukin-6 (IL-6), nuclear factor (NF).

Similar transcription factor binding sites have been identified in the 5' regulatory region of the MCP-1/CCL2 gene. In human glioblastoma, fibrosarcoma and leiomyosarcoma cells deletion/mutation analysis has localized basal transcriptional activity to a GC box, a binding site for the transcription factor SP-1 (Figure 2.25). Transcription in response to TNF- α and IL-1 β is localised to another region containing NF- κ B and AP-1 consensus regions, with the NF- κ B site being crucial (Ueda et al., 1994). Similarly in human umbilical vein endothelial cells IL-1 β induced activation of the MCP-1/CCL2 promoter required both NF- κ B and AP-1 binding sites to be present for maximal effect (Martin et al., 1997). MCP-1/CCL2 mRNA and protein expression are also increased in human glomerular endothelial cells by TNF- α treatment which was associated with increased DNA binding of both NF- κ B and AP-1 in nuclear extracts. MCP-1/CCL2 mRNA transcription was inhibited by decoy oligonucleotides to these transcription factors. In this example the antagonism of MCP-1/CCL2 expression by dexamethasone treatment was associated with the inhibition of AP-1 but not NF- κ B DNA binding (Park et al., 2004). Similar homology of response elements and transcription factor binding following TNF- α stimulation has also been described for the mouse MCP-1/CCL2 gene, in fibroblasts (Ping et al., 1996) (Figure 2.26). In perfused mouse lung, LPS treatment increases nuclear NF- κ B binding and the MCP-1/CCL2 concentration measured in the perfusate. Dexamethasone inhibited the formation of nuclear NF- κ B complexes and this was associated with inhibition of MCP-1/CCL2 production (Held et al., 2001).

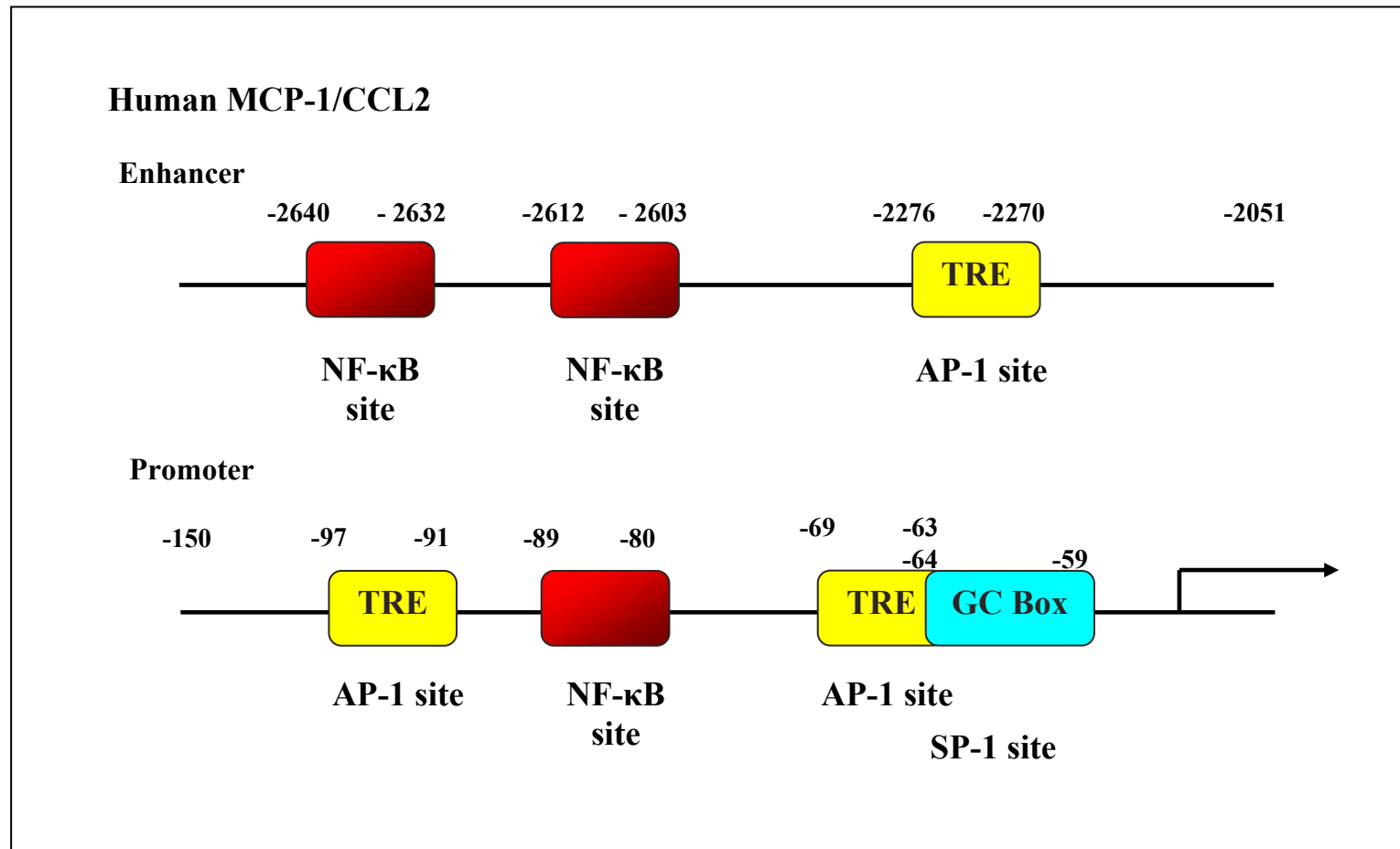


Figure 2.25. Transcription Factor Binding Sites in the Promoter and Enhancer Regions of the Human MCP-1/CCL2 Gene.

From Ueda *et al.*, 1994. Activator protein-1 (AP-1), nuclear factor- κ B (NF- κ B), tetradecanoylphorbol acetate (TPA), TPA response element (TRE).

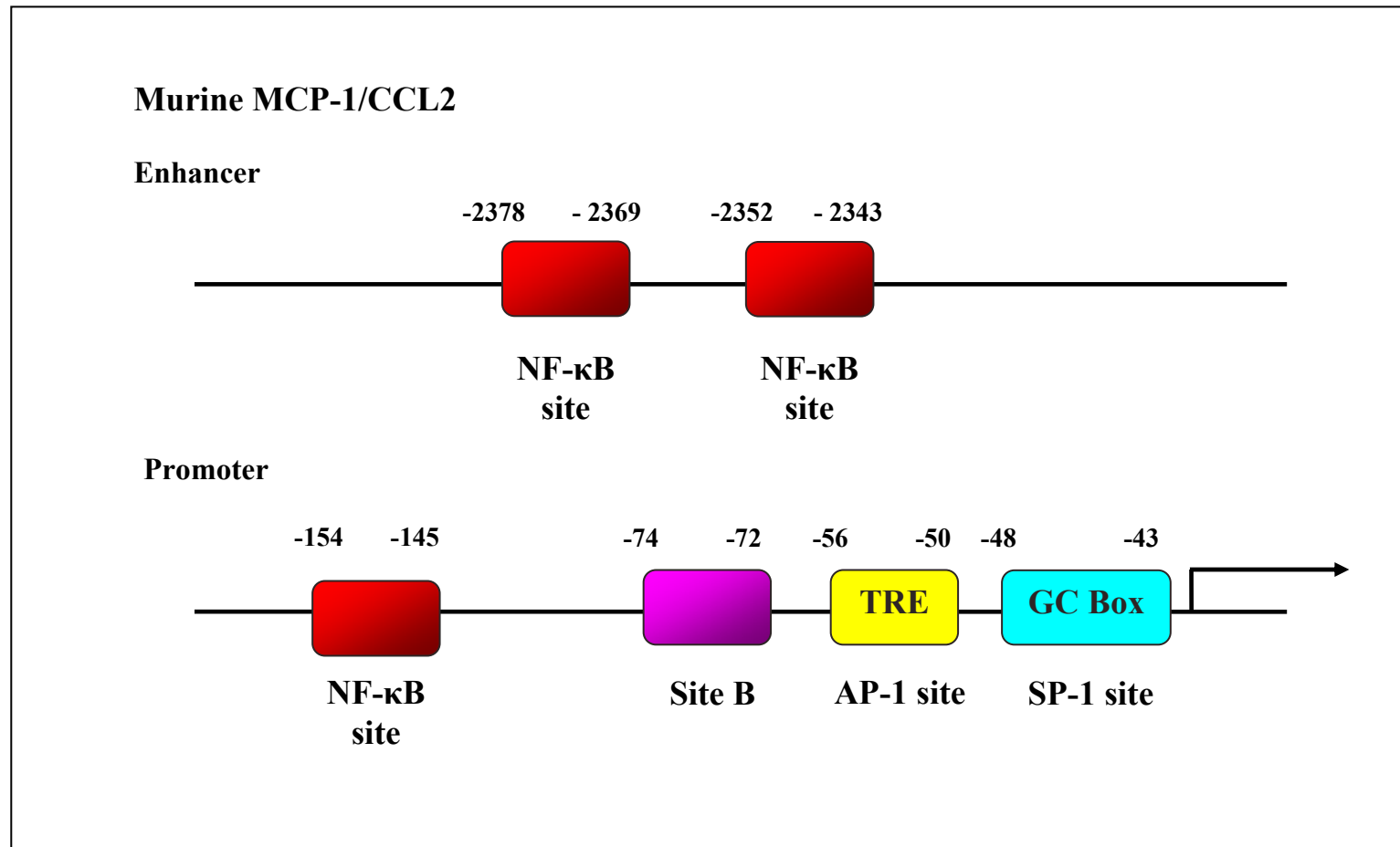


Figure 2.26. Transcription Factor Binding Sites in the Promoter and Enhancer Regions of the Murine MCP-1/CCL2 Gene.

From Ping *et al.*, 1996. Activator protein-1 (AP-1), nuclear factor- κ B (NF- κ B), tetradecanoylphorbol acetate (TPA), TPA response element (TRE).

The ability of glucocorticoids to regulate inflammatory gene expression at a post transcriptional level has also been recognized recently (Stellato, 2004) and this too could be relevant to the inhibition of chemokine production in glia. The stability and translation of mRNA can be influenced by elements within the 3' untranslated region (UTR) of mRNAs, particularly adenylate/uridylate-rich elements (AREs), regulated by ARE binding proteins (ABPs). Stimulation by pro-inflammatory stimuli leads to the activation of mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. The activation of these kinases, in addition to transcriptional activity, is associated with ARE dependant mRNA stabilisation and translation. It is suggested that the induction of kinase inhibitors such as MAPK phosphatase-1 (MKP-1) by glucocorticoids are responsible for reducing mRNA stability and translation (Stellato, 2004) (Figure 2.27). For both IL-8/CXCL8 and MCP-1/CCL2 glucocorticoid treatment decrease mRNA stability. In human embryonic lung fibroblasts, treatment with TNF- α induced IL-8/CXCL8 mRNA and protein expression. Both of these were inhibited by dexamethasone, which reduced the half-life of the IL-8/CXCL8 mRNA. Furthermore experiments with the transcriptional inhibitor actinomycin D and the translational inhibitor cyclohexamide indicated that the reduction in mRNA stability required intact mRNA and protein synthesis. This would be consistent with the requirement for the induction of an inhibitory protein such as MKP-1 to antagonise the action of MAPKs (Tobler et al., 1992). For MCP-1/CCL2 a region of the 5'UTR of the mRNA has been identified which contains sequences mediating glucocorticoid induced acceleration of mRNA decay. Deletion analysis localised a dexamethasone sensitive region of the MCP-1/CCL2 mRNA to the initial 224 nucleotides of the 5' UTR. For activated cells expressing MCP-1/CCL2 mRNA the destabilising effect of dexamethasone only occurred if cells were incubated in the presence of the steroid. If dexamethasone was added later to cytosolic extracts from activated cells cultured in the absence of steroid the half-life of the MCP-1 mRNA was unaffected. This suggests that the destabilising effect was not a direct one. Furthermore the destabilising effect of dexamethasone was not prevented by transcriptional or translational inhibitors but was lost after exposure to heat or proteinase K. Taken together this suggests that the effect of dexamethasone was mediated by activation of a constitutively expressed protein such as an RNase (Poon et al., 1999).

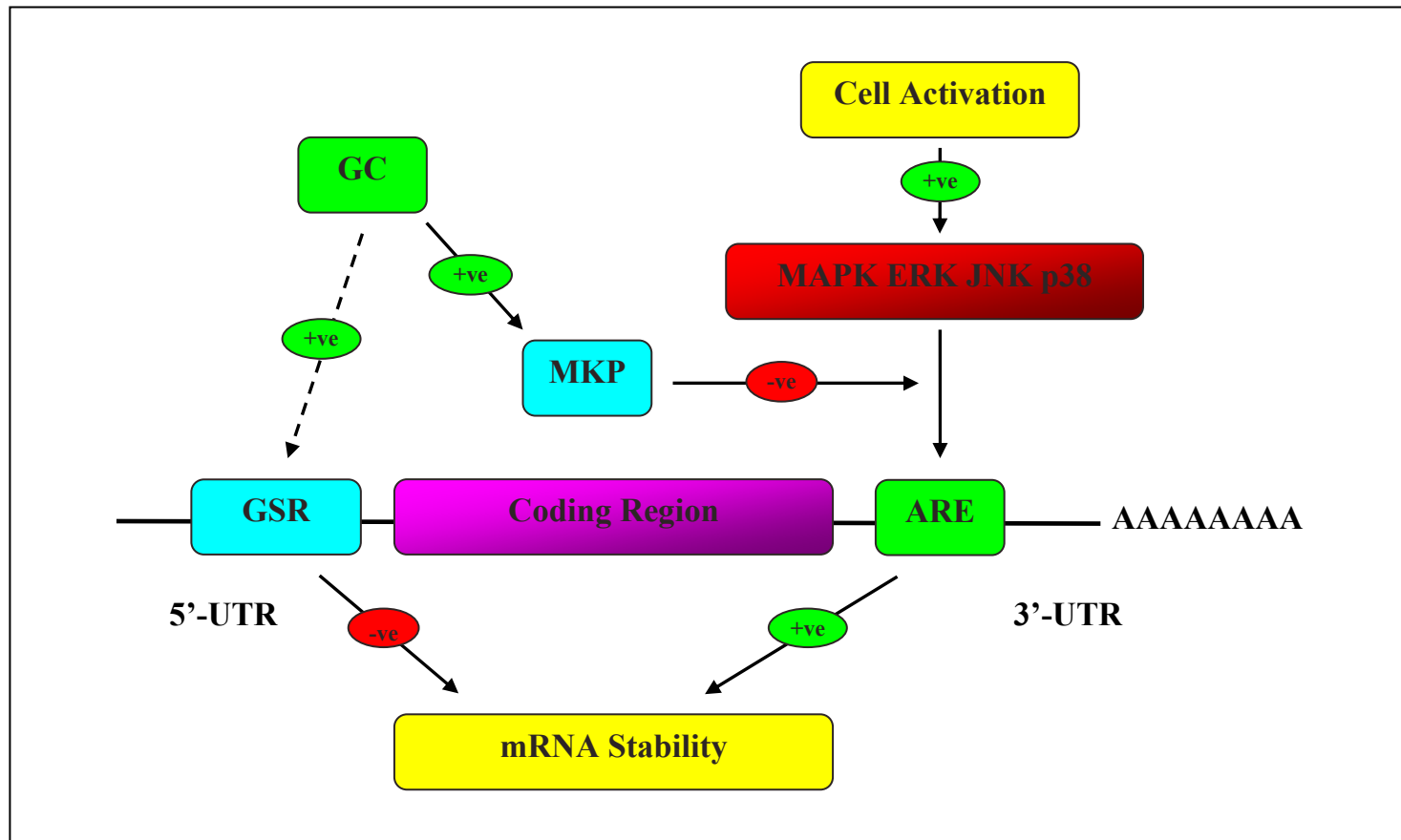


Figure 2.27. Post Transcriptional Effects of Glucocorticoids on mRNA Stability

Cell activation by pro-inflammatory stimuli is associated with the activation of protein kinases and adenylate/uridylylate-rich elements (AREs). Stabilisation of mRNA is increased promoting translation. Glucocorticoids (GC) by inducing kinase inhibitors such as a MAPK phosphatase (MKP) inhibit the action of kinases and reduce mRNA stability. A GC sensitive region (GSR) in the 5' untranslated region (5'UTR) of mRNA has also been identified which has also been implicated in GC mediated reduction in mRNA stability. Extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), mitogen activated protein kinase (MAPK). Adapted from Stellato 2004.

As mentioned earlier the mechanism of the inhibition by dexamethasone of chemokine production by astrocytes was not investigated in this present study. The presence of an NF- κ B consensus region in the 5' flanking region of the rodent MIP-2/CXCL2 (Widmer et al., 1993) and MCP-1/CCL2 (Ping et al., 1996) genes and the increased NF- κ B DNA binding associated with production (Hellerbrand et al., 1998, Held et al., 2001) would make the inhibition of NF- κ B mediated transcription by dexamethasone a plausible mechanism. Furthermore the loss of significant steroid inhibition when the pre-incubation time was less than 90 minutes would also be consistent with the time required for the synthesis of I κ B (Auphan et al., 1995). Alternatively the induction of an inhibitor of mRNA stabilisation is also possible. The human gene for GRO- β /CXCL2, one of the 3 human homologues of rodent MIP-2/CXCL2, contains an ARE, making post transcriptional modulation a possibility (Khabar, 2006).

2.5.3.3. FK506

FK506 is an immunosuppressive agent and its action as a calcineurin antagonist is well described (Sharkey et al., 1997, Kaminska et al., 2004). Significant beneficial effects associated with FK506 administration have been reported in both ischaemic and traumatic acute brain injury (Sharkey & Butcher, 1994, Tokime et al., 1996, Yagita et al., 1996, Butcher et al., 1997, Singleton et al., 2001, Stover et al., 2001, Takamatsu et al., 2001, McCarter et al., 2001, Arii et al., 2001, Furuichi et al., 2003, Zawadzka & Kaminska, 2005) (Table 2.3) including the suppression of glial activation and pro-inflammatory cytokine expression (Stover et al., 2001, Zawadzka & Kaminska, 2005).

In activated human T lymphocytes the production of IL-8/CXCL8 (Okamoto et al., 1994) or the CC chemokines MIP-1 α /CCL3 and MIP-1 β /CCL4 (Staruch et al., 1998) were inhibited by FK506 at concentrations of 1-100 nM. Similarly IL-8/CXCL8 and MCP-1/CCL2 production in activated human eosinophils were also inhibited by FK506 at 0.1-10nM (Kohyama et al., 1999). In activated peripheral blood mononuclear cells IL-8/CXCL8 production was inhibited by FK506 over a similar concentration range of 1.2-122nM (Sasakawa et al., 2000)(Table 2.4). However in my study a concentration of 100 nM FK506 did not inhibit TNF- α induced MIP-2/CXCL2 or MCP-1/CCL2

expression by CTX-TNA2 astrocytes. In fact there was an increase in MIP-2/CXCL2 production by CTX-TNA2 astrocytes treated with FK506. Although this was not significant, the small sample size (n=3) meant that the analysis was underpowered. Similarly, although the reduction in MCP-1/CCL2 production in CTX-TNA2 astrocytes was not significant the analysis was underpowered. A small inhibitory effect of FK506 on MCP-1/CCL2 may have been missed in this study. Alternatively the inhibitory effect of FK506 on chemokine expression previously reported might be specific to the cell type studied or the activating stimulus.

**3. The Temporal Expression,
Cellular Localisation and
Inhibition of the Chemokines
MIP-2 and MCP-1 Following
Traumatic Brain Injury in the
Rat**

3.1. **Chapter Aims**

- To establish and validate the fluid percussion model of focal TBI in Edinburgh.
- Describe the cortical expression of MIP-2/CXCL2 and MCP-1/CCL2 after fluid percussion injury.
- Confirm the cellular source of MIP-2/CXCL2 and MCP-1/CCL2 expression.
- Investigate the effect of immunomodulatory strategies on chemokine expression *in-vivo*.

3.2. Modelling Traumatic Brain Injury

Human traumatic brain injury (TBI) is a heterogeneous condition. The balance of contact and inertial forces applied to the head differs widely between injuries and individuals, resulting in different patterns of skull and brain deformation. Many models of TBI have been developed that aim to replicate pathological features of TBI pertinent to human disease. Because of the advantages of size, economy, wide availability and acceptance these models have frequently been applied or adapted to rodents (Reviewed in Morales et al., 2005). Some of these, mainly, replicate contact focal injury and include the weight drop closed head injury (CHI) model or the controlled cortical impact model (CCI). In the weight drop model a guided weight is dropped either onto a footplate placed on the dura via a craniotomy (Feeney et al., 1981) or, in later variants, the intact exposed skull (Shohami et al., 1994). The model is quick to perform, generates contusions and haematomas but risks skull fracture. In the controlled cortical impact model a contact injury is delivered to the intact dura via a craniotomy resulting in contusion formation extra and subdural haematomas (Dixon et al., 1991). This can be applied by a pneumatically driven impactor and the magnitude of the injury readily controlled by adjusting the pneumatic device. The impact acceleration model (Marmarou et al., 1994) is a model of inertial diffuse injury. The animal is supported on a deformable foam block as a guided weight falls onto a metal disc cemented to the vertex of the skull. The disc prevents skull fractures and allows the energy of the falling weight to briefly accelerate the head. The lateral fluid percussion injury (LFPI) model (McIntosh et al., 1989) reproduces features of both contact and inertial loading. Via a craniotomy a fluid pulse is applied to the intact dura deforming the underlying brain tissue¹ for a clinically relevant period (Figure 3.1) (Morales et al., 2005).

The experimental results obtained with the LFPI model and their validity to human TBI has been extensively reviewed by Thompson et al., 2005. Since its introduction the LFPI model has become the most extensively used and characterised model of TBI. It reproduces an injury with both focal and diffuse characteristics showing good association between the severity of the delivered injury and the extent of the lesion. Whilst the aim of this thesis is to investigate the inflammatory response to focal

¹ A more comprehensive description is given in Materials and Methods (3.4.2)

TBI, the introduction of a model with both focal and diffuse pathological characteristics has attractive practical and financial implications, providing more flexible research options for the future.

Both pathological and neurological features of LFPI are pertinent to those seen following human TBI (Graham et al., 2000). Cortical contusion is generated lateral to the site of force application and cell death is observed in the cortex, hippocampus and thalamus. Stretching and shearing of axons occurs both near the injury and remotely. Contusion is accompanied by petechial or intraparenchymal haemorrhage. In addition to the primary injury of cellular rupture and vascular disruption with consequent necrosis, secondary injury mechanisms are activated. EAAs are released with subsequent intracellular accumulation of calcium, cell swelling and destruction. Inflammation is an important part of the response to the contusional injury with the early generation of pro-inflammatory cytokines followed by the recruitment of neutrophils and monocytes. Neutrophils are seen lining the subarachnoid/subdural spaces and large blood vessels by 4 hours after injury. Thereafter they enter the parenchyma around the contused tissue and accumulation peaks within the first 2 days. Monocytes also line the vasculature by 12 hours and then accumulate in the contused tissue, predominating in the lesion by 3-6 days (Soares et al., 1995). Whilst macrophages accumulate in the area of focal injury activated microglia appear more widespread and have been found to be distributed throughout the injured hemisphere over days (Aihara et al., 1995). Reactive astrocytes are seen from one day after injury (Hill et al., 1996). Apoptotic and necrotic cell death continues over months after injury. As the lesion enlarges a cavity lined by glia is formed.

In addition to the pathological lesion LFPI imparts defects in neuromotor and cognitive function which tend to lessen with time but which have been found to persist up to a year after injury (Thompson et al., 2005).

Although many of features of the inflammatory response have been described in other models of TBI, the expression of inflammatory cytokines (Fan et al., 1995, Fan et al., 1996, Knoblach et al., 1999, Taupin et al., 1993, Zhu et al., 2004)(Table 1.2) and recruitment of leucocytes (Aihara et al., 1995, Hill et al., 1996, Soares et al., 1995) have been extensively described following LFPI. This thesis is

concerned with the study of chemokine expression and modulation following TBI. In-vitro data would suggest the expression of chemokines follows that of other pro-inflammatory cytokines. Leucocytes would be expected accumulate after the expression of chemokines. Therefore the existing descriptions of the timing of pro-inflammatory cytokine expression and leucocyte recruitment in this model serve to guide the design of experiments. This had the advantage of avoiding the uncertainties caused by translating observations made in different models, to LFPI.

Finally, experience gained from using the Marmarou weight drop model was useful in the decision to adopt LFPI as a model of focal injury. The Marmarou model had been difficult to reproduce in Edinburgh and no direct help was available. It occurred to me that in the future it would be wise, when introducing a significant piece of equipment, to enlist help from a centre already using the method. To this end my supervisor Peter Andrews already knew Professor Tracy McIntosh, whose group had published extensively on the use of LFPI in TBI research. I had already made contact with his team for advice on sources of equipment. Professor McIntosh was willing to allow a visit in order to experience the LFPI model in person and to insure the method could be accurately reproduced in Edinburgh.

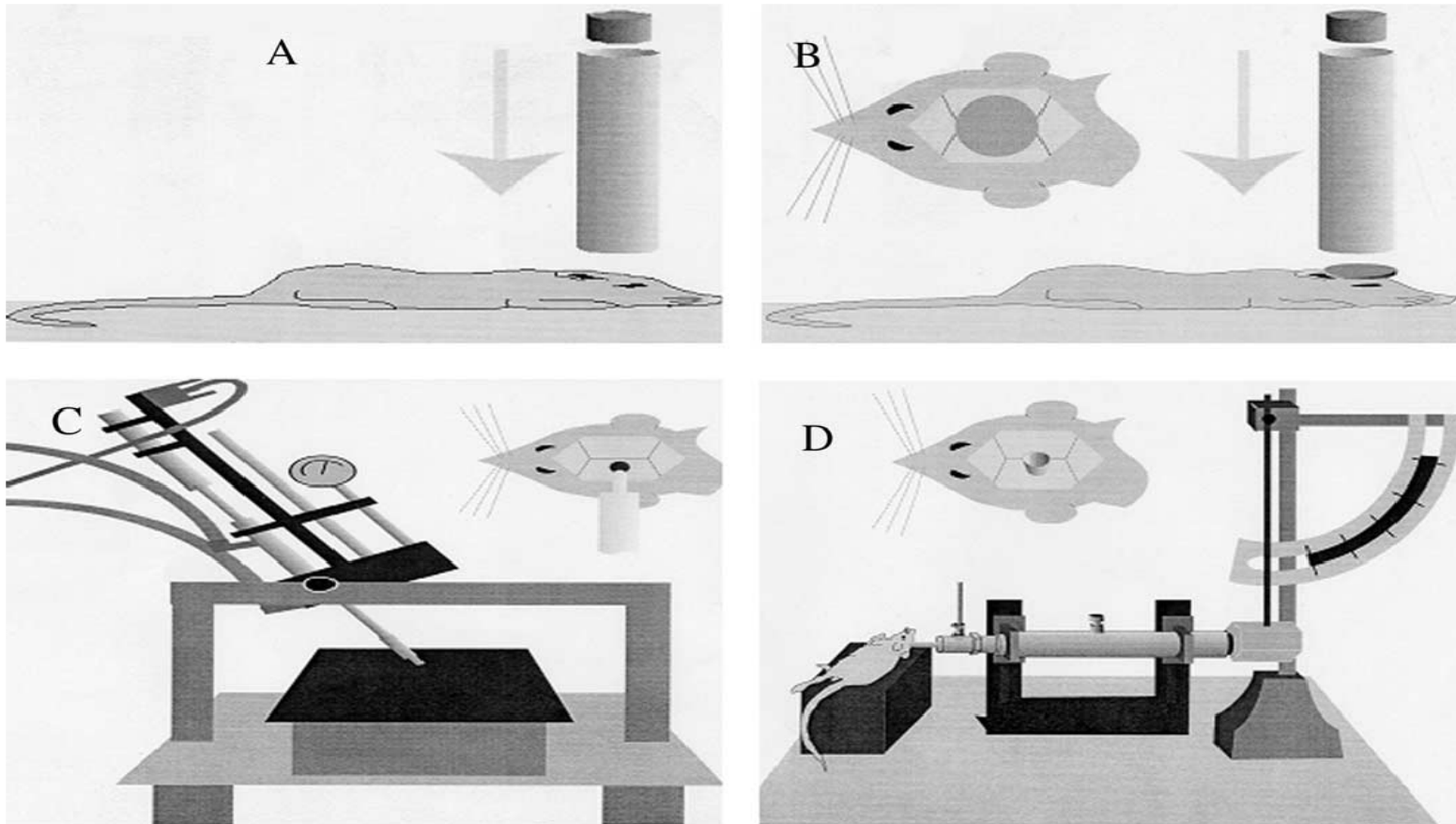


Figure 3.1. Common Rodent Models of Traumatic Brain Injury

(A) Closed head injury, (B) impact acceleration injury, (C) controlled cortical impact and (D) lateral fluid percussion injury. From Morales et al., 2005.

3.3. Chemokine Expression in Traumatic Brain Injury

Experimental studies have confirmed that chemokines contribute to the recruitment of leucocytes into the brain (See section 1.4.2.3.2.2). *In-vivo* chemokines have been identified following a variety of acute injuries to the CNS including traumatic, ischaemic, excitotoxic, neurodegenerative and inflammatory lesions (See section 1.4.2.3.2.3 and table 1.4). However, many of the experimental traumatic brain injuries in which chemokine expression has been demonstrated involve penetration of the cerebral cortex (Babcock et al., 2003, Berman et al., 1996, Glabinski et al., 1996) or resection of tissue (Hausmann et al., 1998, Muessel et al., 2000) and might therefore model inflammatory processes that would occur following surgical procedures on the brain rather than blunt trauma. Chemokine expression after cortical injury produced by freezing (Grzybicki et al., 1998) or following diffuse axonal injury (Rancan et al., 2001) have also been described. Less information on the induction of chemokine expression associated with cerebral contusion formation is available. This is limited to MIP-2/CXCL2 which has been shown to increase acutely in the ipsilateral hemisphere of the closed head injury model (Otto et al., 2001) and following controlled cortical impact (Valles et al., 2006). Peak expression of protein occurred between 4 and 8 hours after injury (Table 1.4). It is not clear if MCP-1/CCL2 is increased following focal contusional TBI.

3.3.1. Cellular Source of Chemokines Following Traumatic Brain Injury

The expression of chemokine mRNA or production of protein has been repeatedly reported in glial cells. *In-vitro* microglia and/or astrocytes have been shown to produce MIP-2/CXCL2 (Otto et al., 2000), IL-8/CXCL8 (Aloisi et al., 1992, Croitoru-Lamoury et al., 2003, Ehrlich et al., 1998, Hua & Lee, 2000, Janabi et al., 1999, Oh et al., 1999) or MCP-1/CCL2 (Barna et al., 1994, Calvo et al., 1996, Croitoru-Lamoury et al., 2003, Hayashi et al., 1995, Hurwitz et al., 1995, Oh et al., 1999, Peterson et al., 1997) when incubated with pro-inflammatory cytokines such as TNF- α or IL- β (Section 2.2.1, table 2.1). Brain microvascular endothelial cells (MVEC) also release MIP-2/CXCL2 (Otto et al., 2000) and MCP-1/CCL2 (Harkness et al., 2003) in culture with TNF- α and/or IL-1 β .

These findings are supported *in-vivo* where following non-contusional traumatic injury MCP-1/CCL2 expression has been localised to glial cells (Babcock et al., 2003, Glabinski et al., 1996, Muessel et al., 2000). Glia have also been found to be a consistent source of MCP-1/CCL2 after ischaemic (Che et al., 2001, Gourmala et al., 1997, Kim et al., 1995b, Minami & Satoh, 2003), excitotoxic (Calvo et al., 1996, Minami & Satoh, 2003) and neurodegenerative (Little et al., 2002) injuries, although early expression by neurones has also been described following ischaemia, by one group, in mice (Che et al., 2001) (Table 1.4).

However *in-vivo* there are no reports describing the cellular source of MIP-2/CXCL2 or MCP-1/CCL2 in cerebral contusions. Taking the available evidence from the *in-vitro* and *in-vivo* literature described above it would be reasonable to hypothesise that the likely source of this acute expression following contusional TBI would be microglia and/or astrocytes. Therefore, using the lateral fluid percussion model of TBI I have investigated the temporal expression of the chemokines MIP-2/CXCL2 and MCP-1/CCL2 in the rat. I then attempted to identify the cells expressing these chemokines, *in-vivo*, acutely after injury.

3.3.2. Corticosteroids and Acute Brain Injury

3.3.2.1. Beneficial Effects of Corticosteroids in Experimental Brain Injury

The studies of animal models of acute CNS injury have frequently demonstrated that corticosteroid treatment is associated with reduced lesion volume, cerebral oedema and mortality or improvement in function, if administered either prior to or very early after the injury (See section 2.2.2.2.1, table 2.2).

3.3.2.2. Inhibition of Cerebral Inflammation

In CNS models of inflammation corticosteroids inhibit elements of the inflammatory process. Dexamethasone administration pre and post intra-hippocampal injection of lipopolysaccharide (LPS) significantly reduced the expression of IL-1 β in the hippocampus (Jones P, Heron I, Findlayson K unpublished observations). In the 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) model of

Parkinson's disease, pre-treatment with dexamethasone, continuing during lesion development decreased dopaminergic neuronal loss, lymphocyte infiltration, microglial activation, expression of major histocompatibility complex (MHC) antigens and ICAM-1 (Kurkowska-Jastrzebska et al., 1999). Similarly LPS has been injected into the substantia nigra to induce an acute inflammatory response with monocyte activation and degeneration of dopaminergic neurons, also modelling Parkinson's disease. However when dexamethasone was administered systemically prior to the LPS injection microglial/macrophage activation and loss of dopaminergic markers was reduced (Castano et al., 2002). Modulation of the neurochemical response to injury has also been observed in spinal cord injury. In the rat methylprednisolone IV post weight drop injury reduces TNF- α expression and NF- κ B binding at 24 hr (Xu et al., 1998) and in the cat inhibits lipid peroxidation following ferrous chloride injection into the spinal grey matter (Anderson & Means, 1985).

3.3.2.2.1. *Inhibition of Chemokine Expression by Corticosteroids*

Generally corticosteroids inhibit many aspects of the biochemical response to injury including the expression of the pro-inflammatory cytokines IL-1 β (Kern et al., 1988, Knudsen et al., 1987), TNF (Han et al., 1990) and IL-6 (Grimaldi et al., 1998, Zanker et al., 1990, Zitnik et al., 1994), adhesion molecules (Tessier et al., 1993) and the progression of lipid peroxidation (Braugher, 1985). Outside the CNS the inhibition of chemokine expression by corticosteroids has also been described. Dexamethasone inhibited IL-8/CXCL8 mRNA and protein expression induced by TNF- α (Tobler et al., 1992) and IL-1 β (Kwon et al., 1994) in non-CNS cells. Pre-treatment with dexamethasone also reduced the expression MIP-2/CXCL2 and MCP-1/CCL2 in a mouse model of acute lung injury (Held et al., 2001) and MIP-2/CXCL2 in response to staphylococcal enterotoxin B injection (Schramm & Thorlacius, 2003) (See section 2.2.2.2.2).

When considering the CNS specifically, data on the effect of steroids on brain derived chemokine expression is more limited. The production of MCP-1/CCL2 by CNS endothelial cells, induced by IL-1 β or TNF- α , was reduced by dexamethasone (Harkness et al., 2003). In chapter 2, I demonstrated that dexamethasone could significantly inhibit the production of MIP-2/CXCL2 and MCP-1/CCL2 in

response to TNF- α in the rat astrocyte cell line CTX-TNA2. *In-vivo*, the systemic administration of methylprednisolone, commenced immediately after the onset of cerebral ischaemia, inhibited MCP-1/CCL2 mRNA expression and macrophage accumulation (Kim et al., 1995a).

Following TBI the effect of steroid administration on chemokine expression has not been described. I have therefore investigated the effect of dexamethasone on cerebral MIP-2/CXCL2 and MCP-1/CCL2 concentrations using the LFPI model in the rat.

3.3.3. FK506 and Acute Brain Injury

In-vivo FK506 has shown promise in pre-clinical studies, reducing both ischaemic lesion volume and ameliorating some features of TBI. It can also promote recovery of peripheral nerves after trauma and spinal cord function after injury (See section 2.2.2.3.1, table 2.3). However the mechanisms underlying these observations are incompletely defined. In focal ischaemia pre-treatment of animals with rapamycin, which binds to FKBP12 with a similar affinity to FK506 but doesn't inhibit calcineurin, blocked the neuroprotective effect of FK506. This confirmed the role of immunophilin mediated inhibition of calcineurin in the neuroprotective action of FK506 to ischaemia (Sharkey & Butcher, 1994). Following experimental TBI calcineurin activity is increased in cortical and hippocampal homogenates (Kurz et al., 2005). Some of the other suggested mechanisms of neuroprotection by FK506 include inhibition of calcineurin mediated nitric oxide synthase activity and generation of free radicals, mitochondrial stabilisation and inhibition of apoptosis (Sharkey et al., 2000).

3.3.3.1. Anti-inflammatory Effect of FK506 in the Brain

In primary microglia or astrocyte cultures FK506 inhibits pro-inflammatory cytokine mRNA expression (Zawadzka & Kaminska, 2005). *In-vivo* microglial/macrophage and astrocyte activation following both permanent and transient ischaemia (Wakita et al., 1998, Zawadzka & Kaminska, 2005) along with cortical expression of IL-1 β , TNF- α and IL-6 mRNAs following transient ischaemia (Zawadzka & Kaminska, 2005) are reduced.

3.3.3.1.1. *Inhibition of Neutrophil Recruitment and Chemokine Expression*

In-vitro FK506 inhibits the generation of superoxide radical by phorbol myristate acetate (PMA) activated neutrophils (Nishinaka et al., 1993). The inhibition of neutrophil/leucocyte infiltration by FK506 has also been reported following ischaemia in tissues such as the liver (Garcia-Criado et al., 1997, Suzuki et al., 1993, Wakabayashi et al., 1994), gut (Kubes et al., 1991) and retina (Tsujikawa et al., 1998). However the mechanism of this action is not yet clear as the effect of FK506 on chemokine expression has been variable, with inhibition, no effect or induction reported in various blood and epithelial cell types (See section 2.2.2.3.2, table 2.4).

To date the effect of FK506 on chemokine expression following TBI has not been reported. In chapter 2 I demonstrated that pre-treatment of the rat astrocyte cell line CTX-TNA2 with FK506 did not inhibit the production of MIP-2/CXCL2 or MCP-1/CCL2 induced by TNF- α . The finding of significant neuroprotective effects of FK506 administration in acute brain injury raises the possibility that *in-vivo* a reduction in lesion volume or cell death might be associated with an indirect reduction in glial expression of chemokines. It is also possible that *in-vivo* chemokines are not expressed by glia after injury and so the response to FK506 might be quite different to that seen *in-vitro*. I have therefore investigated the effect of FK506 on MIP-2/CXCL2 and MCP-1/CCL2 concentrations in the cortex following LFPI in the rat.

3.4. Materials and Methods

3.4.1. Animals

Adult male Sprague-Dawley rats (Charles River UK Ltd, Margate, UK) weighing 350-400g were housed in a registered animal unit on a 12 hour day/night cycle with free access to standard food and water. All procedures in this study were approved after review by the university ethics committee and the United Kingdom Home Office. Procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986.

3.4.2. Lateral Fluid Percussion Injury Device

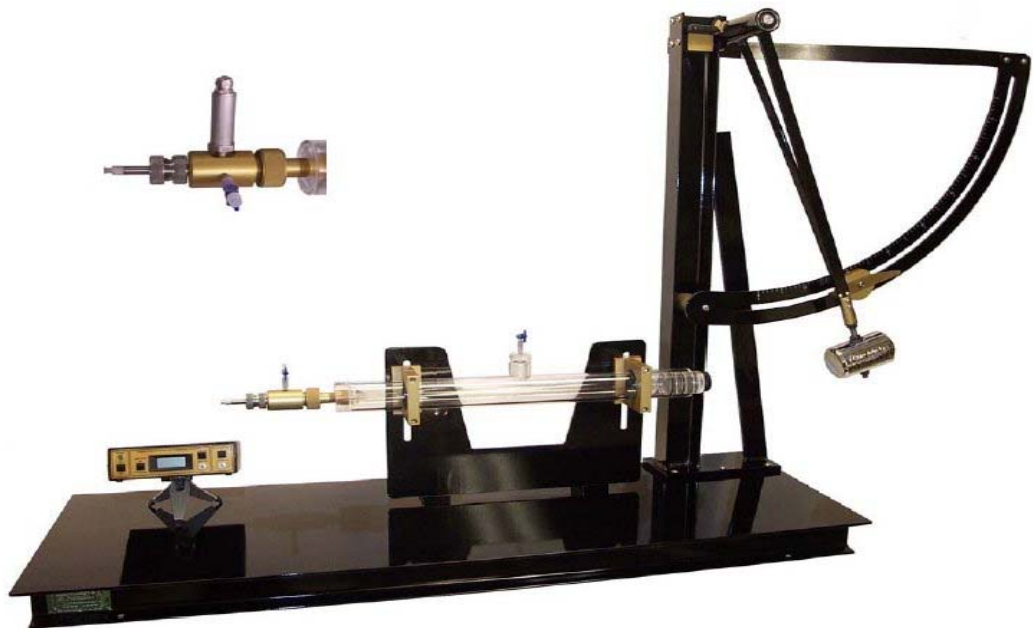
3.4.2.1. Visit to the University of Pennsylvania

The fluid percussion model of traumatic brain injury is new to the University of Edinburgh. Therefore prior to ordering the fluid percussion device I spent 3 days visiting the Department of Neurosurgery, Haden Hall, University of Pennsylvania as the guest of Professor Tracy McIntosh. This allowed me to experience the technique first hand and understand the technical aspects of the procedure with a level of detail which is not possible from published descriptions. I was also able to witness the level of injury sustained by the animals and then answer specific questions from both the University of Edinburgh ethical review committee and the Home Office inspector.

3.4.2.2. Installation, Filling and Waveform of the Fluid Percussion Device

The lateral fluid percussion device was built to order by Custom Design and Fabrication (Virginia Commonwealth University, Richmond, USA) and delivered to be assembled on site. The base of the unit was attached to a fixed bench and the device constructed following the manufactures instructions. The fluid percussion machine consists of a saline filled acrylic cylinder and transfer tube, securely attached to a metal base by clamps (Figure 3.2). At one end of the cylinder is a plunger which is free to move within the cylinder. Adjacent to the plunger is a large metal hammer. This can be displaced from the vertical by up to 80° and when released impacts the plunger imparting a pressure increase in the cylinder fluid. The amplitude of this pressure wave is proportional to the deflection of the hammer from the vertical. The pressure wave is detected by a pressure transducer, shown to the left of the cylinder, the output from which is amplified and then displayed on an oscilloscope (Gould 400, LDS Test and Measurement Ltd, Royston, UK) as millivolts (mV) vs. time. This allows the pressure wave to be measured and its character assessed. The duration of the wave is typically 20ms. When the machine is being tested a three way tap, fitted to the transfer tube, is closed preventing fluid escaping from the system and allowing the pressure wave to be measured.

A



B

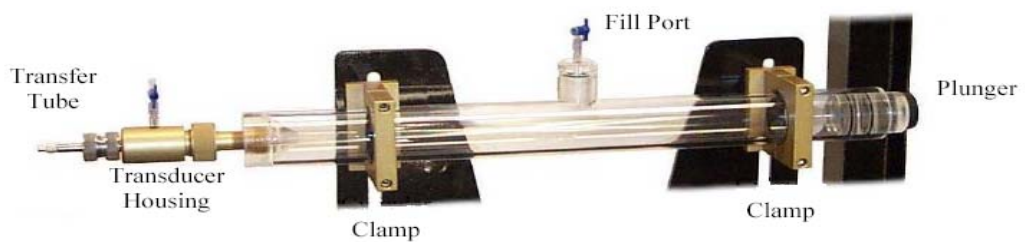
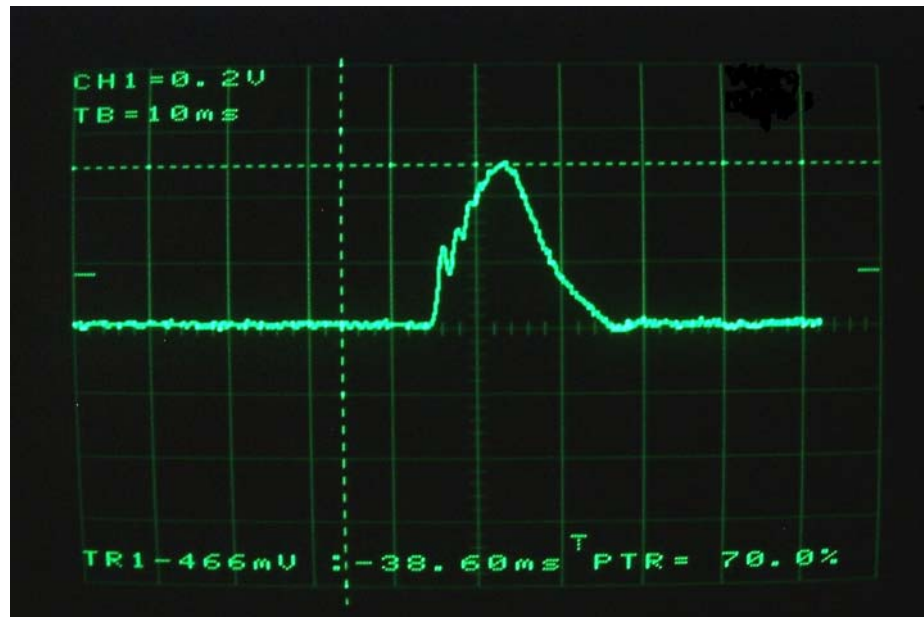


Figure 3.2. The Fluid Percussion Injury Device.

(A) The complete machine is shown with the hammer deflected from the vertical as it would be prior to release and striking the plunger. The hammer is held in position by a trigger which can be moved around the large curved protractor 0 to 80°. Also shown to the left of the machine is the amplifier unit. This receives the signal from the pressure transducer (Insert) which is fed to an oscilloscope. The fulcrum of the hammer incorporates a switch, the output from which is also fed to the external trigger input on the oscilloscope. The movement of the hammer on release therefore marks the start of the pressure wave recording. (Insert) Enlarged view of the pressure transducer, the housing has been rotated through 90° about its long axis. (B) Detail of the cylinder, plunger, filling port and transducer housing. Photographs with permission from G.M.Blankinship III, Custom Design & Fabrication.

The fluid percussion injury (FPI) machine is filled with 0.9% sterile saline (MacoPharma, London, UK) which was degassed by subjecting it to a sustained negative pressure. It is essential that no air bubbles remain in the cylinder, pressure transducer housing or transfer tube, as these degrade the pressure wave with oscillations and this prevents the applied stimulus from being reproducible. The effect of air in the system is shown in figure 3.3 where 1ml of air was introduced into the device through the port in the transducer housing (Figure 3.2). Degassing the saline allowed small bubbles, which form on the inner surface of the cylinder during filling, to dissolve.

A



B

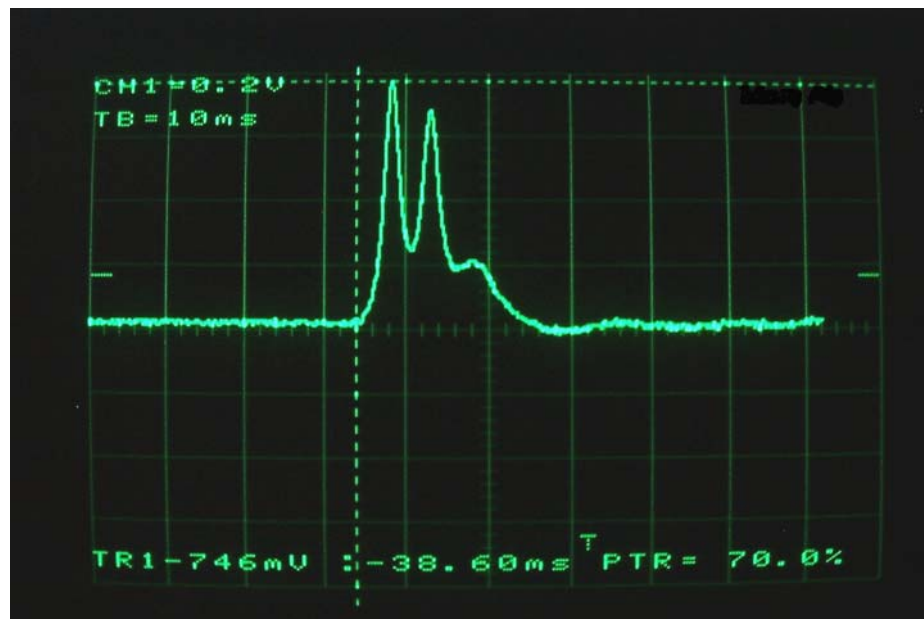


Figure 3.3. The Effect of Air on the Character of the Fluid Percussion Waveform.

(A) Using degassed saline an acceptable waveform with a single peak and 20ms duration is obtained.

(B) Note that the wave character is dramatically altered by 1ml of air introduced at the transducer housing.

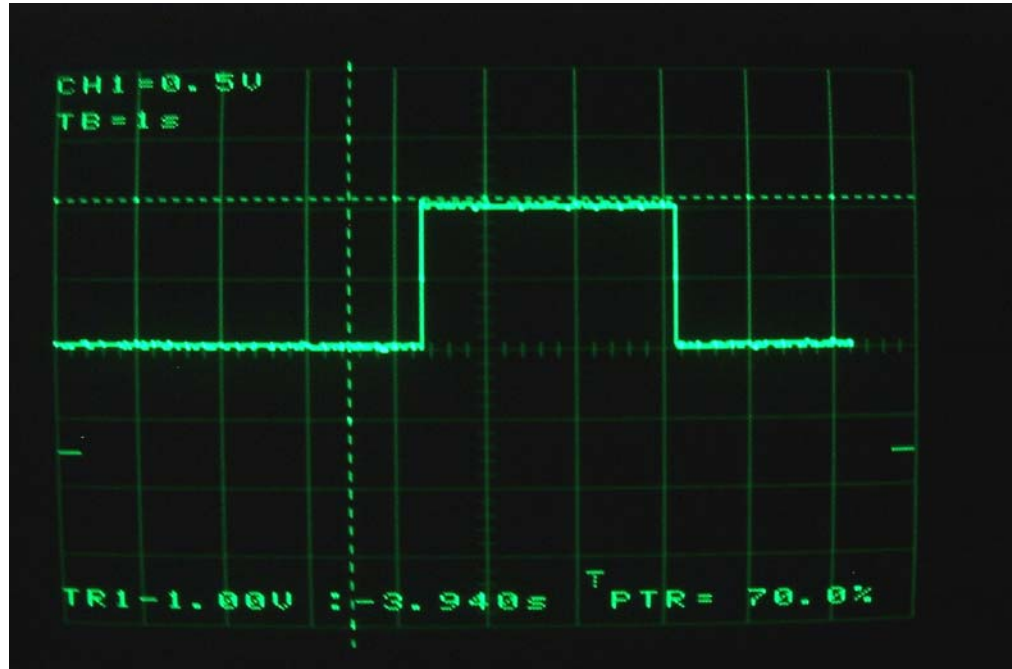
3.4.2.2.1. Amplifier Output and the Oscilloscope

The amplifier outputs the pressure transducer signal at 10mV/pounds per square inch (psi) of pressure. The pressure wave was therefore displayed in the form of millivolts (mV) against time on the oscilloscope (Figure 3.4). To determine the injury severity delivered by the fluid percussion device the peak amplitude of the wave was read from the oscilloscope and converted to psi and then to atmospheres (atm) of pressure (14.7psi= 1atm).

3.4.2.2.2. Calibration Check

Prior to each use of the FPI apparatus a 2 point calibration check of the amplifier and oscilloscope was performed. For the first point the three-way tap on the transfer tube was opened to atmospheric pressure. The pressure transducer output in this condition was zero V. The position of this signal was then adjusted to the zero V reference line on the oscilloscope. The second calibration point was a 100 psi signal delivered from the amplifier by operation of a calibration switch. In correct operation this was read as 1V on the oscilloscope (Figure 3.4).

A



B



Figure 3.4. Calibration Check of the Amplifier Output.

(A) The 1000mV signal from the amplifier, equivalent to a pressure of 100psi, is displayed on the oscilloscope. The baseline was set to zero V. The reference line (dashed horizontal) correctly indicates 1.0V. (B) Close up view of the pressure transducer amplifier. The 100psi calibration switch is shown to the right of the picture.

3.4.2.2.3. Determining the Angle of Hammer Deflection

The FPI device was used to deliver an injury of moderate severity. This corresponds to a peak pressure amplitude of 1.5-2.0 atm. This level of injury produces histological and functional pathology with features pertinent to human TBI but with an acceptable mortality (McIntosh et al., 1989). Moderate lateral FPI is widely practiced in literature. Mild injury results in minimal pathology and with severe injury mortalities > 50% can be expected (McIntosh et al., 1989).

To determine the angle of hammer deflection required to deliver a pressure wave of suitable amplitude a graph of deflection vs. peak pressure was constructed. For each angle of deflection the FPI device was operated in the following way. The hammer was deflected from the vertical by a predetermined angle. It was then held in this position by a trigger which could be moved around the large protractor to this angle (Figure 3.2). After ensuring that the three-way tap attached to the transfer tube was closed the hammer was then released to strike the plunger once. It was then caught on the back swing and clicked back into the trigger. The pressure wave on the oscilloscope was inspected. The hammer was then released a second time, caught on the back swing and placed back into the trigger. If the pressure wave remained consistent the hammer was then released for a third time, caught and replaced into the trigger. This pressure wave was then measured and recorded². This was repeated for deflections of 10-40° (Figure 3.5). A deflection of 11-16° was required for a pressure wave of 1.6-1.9 atm peak amplitude.

Prior to the commencing experiments photographs of the pressure waves were discussed with both the machines manufacturer and the staff at Haden Hall. The quality of the fluid percussion wave was judged to be satisfactory and *in-vivo* work commenced.

² When the device was used to deliver FPI to the animals the amplitude of the pressure wave was checked twice, prior to connecting the animal to the machine, and the injury delivered on the third swing.

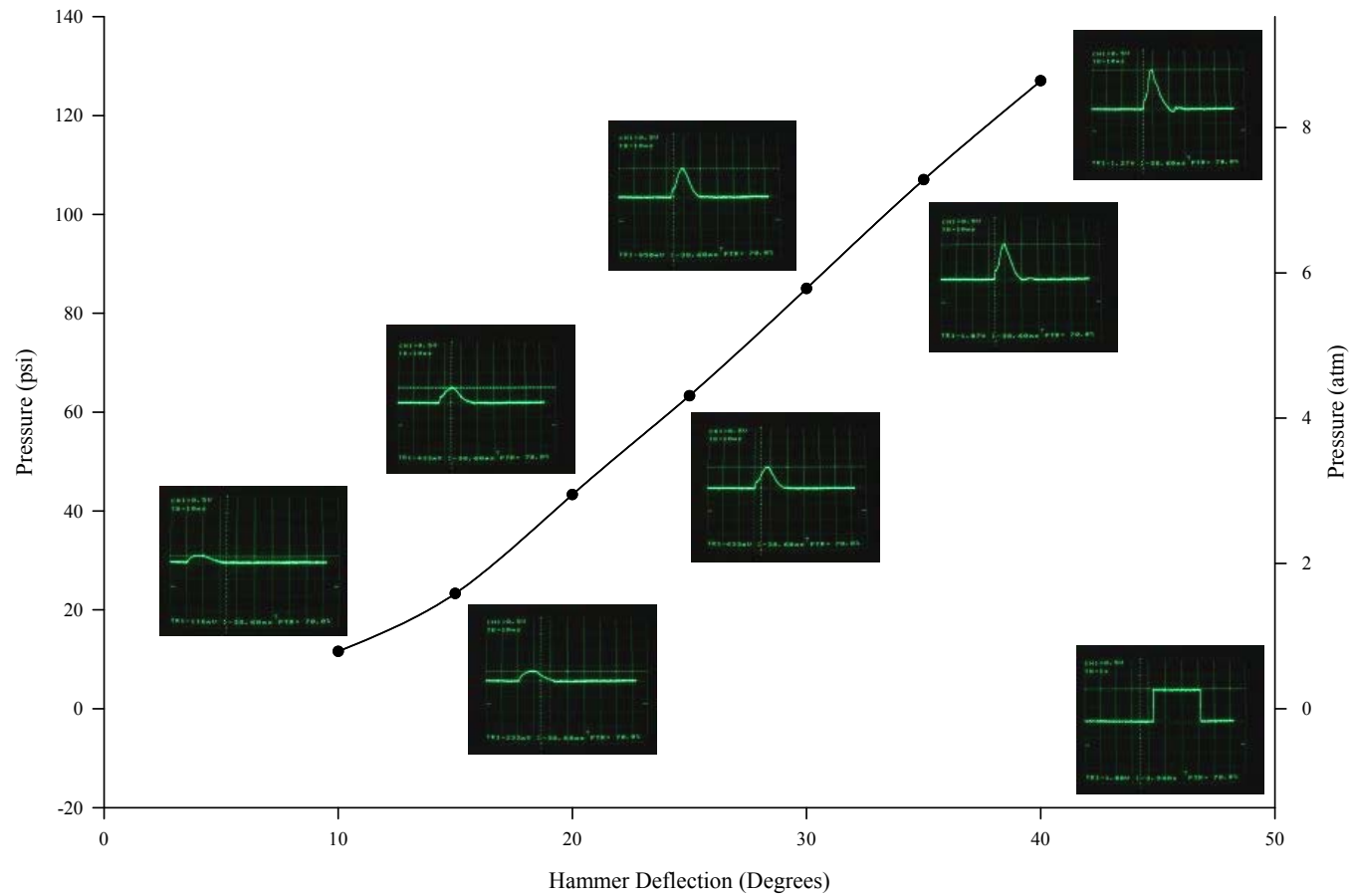


Figure 3.5. The Relationship Between Hammer Deflection and the Magnitude of the Peak Pressure Delivered by the FPI device.

For a moderate injury (1.6-1.9 atm) a deflection of 11-16° was required.

3.4.3. Anaesthesia Protocols

In order to commence using the FPI model a preliminary program of work was agreed with the Home Office. This allowed the animals to receive a moderate injury and then be sacrificed up to 4 hours after the injury. Unlike the main body of work, anaesthesia was maintained throughout this period (non-recovery). 16 animals were used in the preliminary experiments. The histology of the lesion and expression of chemokine mRNA were investigated. The fluid percussion injury, harvesting of brain tissue and tissue preparation were performed as for the main body of work (see below).

The results of the preliminary study were discussed with the Home Office and subsequently the protocol amended to allow the animals to recover from anaesthesia. They could then be observed and sacrificed up to 24 hours after the injury.

3.4.3.1. Anaesthesia: Protocol 1

For the preliminary (non-recovery) study anaesthesia was induced with halothane (Merial Animal Health, Harlow, UK) in oxygen (BOC Ltd, Manchester, UK) and then maintained with sodium pentobarbital (60mg/kg, intra-peritoneal (IP), Merial Animal Health Care Ltd, Harlow, UK). The halothane and oxygen were discontinued. To continue anaesthesia over the entire course of the experiment (up to 6 hours) additional pentobarbital (15-30mg/kg, IP) was given as required.

3.4.3.2. Anaesthesia: Protocol 2

For the main body of work (recovery) anaesthesia was also induced with halothane in oxygen and maintained with a single bolus of sodium pentobarbital (60mg/kg, IP). The halothane and oxygen were discontinued. If further anaesthesia was required during the surgery pentobarbital (15mg/kg, IP) was given. Prior to the administration of the FPI the animals were given 100% oxygen to breath via a nose cone. This was continued after the injury until a stable respiratory pattern was established.

3.4.3.3. Anaesthesia: Protocol 3

The integrity of the blood brain barrier (BBB) was investigated in a small group of animals. These animals were anaesthetised as per protocol 2. Five hours after the injury they were then induced with halothane in oxygen and maintained with this mixture via a nose cone. At 6 hours after injury additional pentobarbitone (150mg/kg, IP) was administered and the animals sacrificed by transcardiac perfusion.

3.4.4. Lateral Fluid Percussion Injury

The animals were positioned in a stereotaxic frame (Model 900, David Kopf Instruments, Tujunga, California, USA). Body temperature was maintained at $37\pm0.5^{\circ}\text{C}$ by means of a heating pad and measured by a rectal temperature probe connected to the heating pad controller (Homeothermic blanket control unit, Harvard Apparatus Ltd, Edenbridge, UK). The animals were prepared for fluid percussion according to the method described by McIntosh *et al.*, in 1989. Via a midline incision the scalp, temporal muscle and periosteum were exposed and dissected from the skull (Figure 3.6A). A 5mm craniotomy was trephined (26-140 Michele Trephine, Miltex, 953-1390 Henry Schein, Melville, New York, USA) freehand, without disrupting the dura, over the left parietal cortex, midway between the bregma and lambda and 4mm lateral to the sagittal suture (Figure 3.6B & C). The skull was dripped with saline during the trephining to prevent heating. A hole was then drilled into the bregma just lateral to the mid-line, without penetrating the cranial cavity, and a 2 mm self tapping screw (560-530, RS Components Ltd, Corby, UK) was then fixed into the bregma (Figure 3.6D). A hollow female Luer-Lock fitting, cut from a 19 gauge hypodermic needle (301750, Becton, Dickson & Company, Drogheda, Ireland), was then secured to the margins of the craniotomy with cyanoacrylate adhesive (B&Q PLC, Eastleigh, UK) and filled with saline (Figure 3.6E). The 2 mm screw served to stabilise an acrylic cap which was formed by dental cement (Simplex Rapid, Associated Dental Products, Swindon, UK) poured around the Luer-Lock fitting and encompassing the screw (Figure 3.6F). This assembly was then allowed to harden. Ninety minutes after the administration of the pentobarbital the animals were pre-oxygenated with 100% oxygen (recovery animals only) and additional saline added to the Luer-Lock fitting as necessary. Prior to performing the injury the

hammer of the FPI device was deflected from the vertical to between 11 and 16°. It was then held in this position by the trigger which moved around the large protractor to this angle. After ensuring that the three-way tap attached to the transfer tube was closed the hammer was then released to strike the plunger once. It was then caught on the back swing and clicked back into the trigger. The pressure wave on the oscilloscope was inspected and if it was of sufficient amplitude the hammer was released a second time, caught on the back swing and placed back into the trigger. If the pressure wave remained constant the three way tap was opened and the animal attached to the fluid percussion device by the Luer-Lock, taking care not to trap air bubbles. The FPI device thus consisted of a fluid filled cylinder in continuity with the dura. The hammer was then released for a third time to impact with the plunger. The pressure wave generates a pulse of elevated intracranial pressure with transient displacement and deformation of the cerebral tissue. The hammer was again caught on the back swing and placed back into the trigger. The pressure wave was then measured and recorded. Sham animals underwent the same anaesthetic regime and operative procedure, including connection to the FPI device, except that the hammer was not released. After the injury the dental acrylic cap and Luer-Lock fitting were removed. The scalp wound was sutured and infiltrated with bupivacaine (2mg/kg, AstraZeneca UK Ltd, Luton, UK). The animals were then placed in a heated incubator (Animal intensive unit, Lyon Electric Company, Los Angeles, USA). Rectal temperature was checked whilst the animals were in the incubator. The non-recovery animals were observed in the incubator until they were sacrificed. Recovery animals were returned to their cages once they were awake and mobilising.

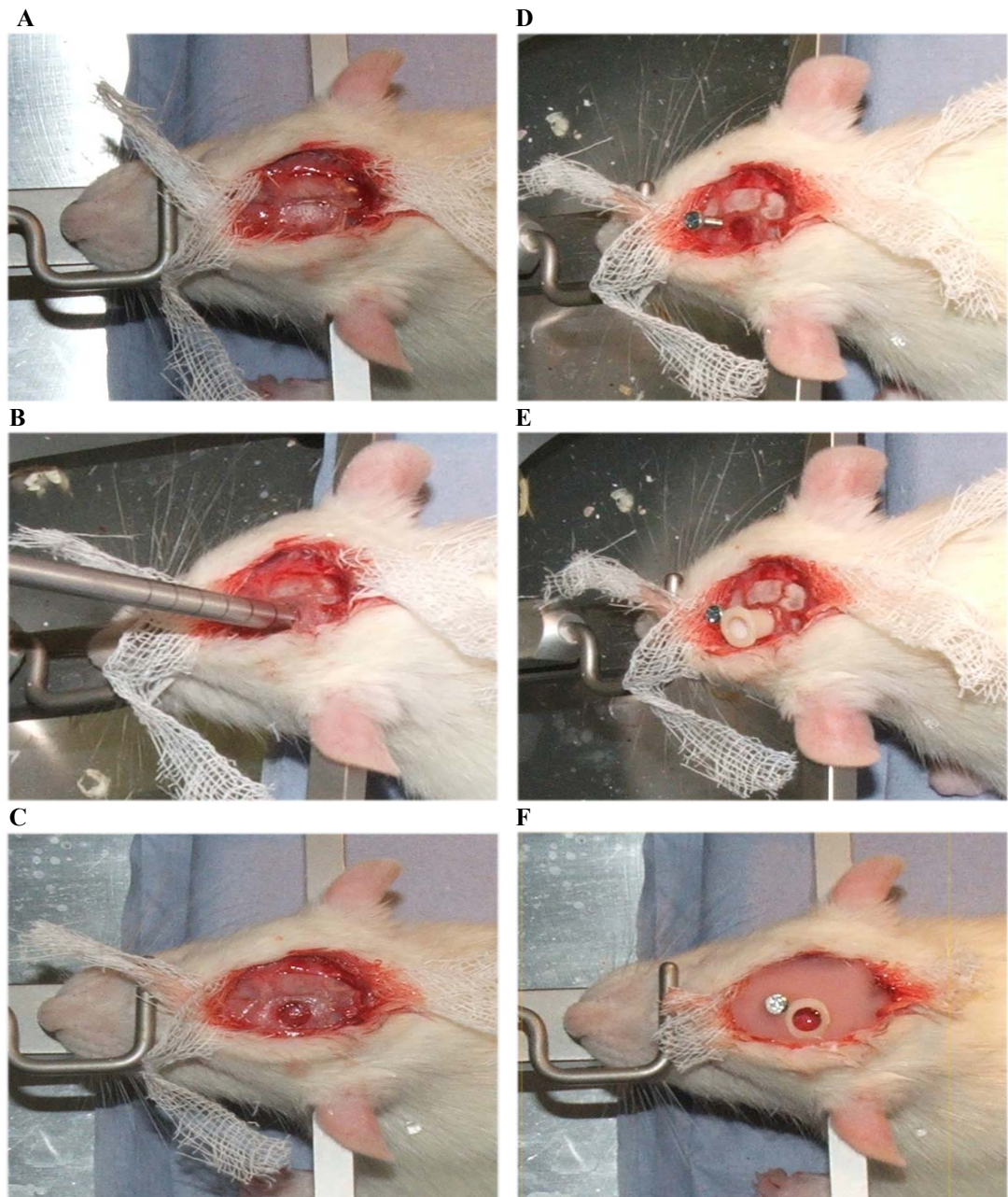


Figure 3.6. Craniotomy and Luer-Lock Fitting for Lateral Fluid Percussion Injury.

(A) Periosteum and muscle dissected from the skull. (B) Position of the trephine. (C) Craniotomy with intact dura. (D) A 2mm screw fixed into the bregma. (E) The hub of a 19G needle cemented to the margins of the craniotomy and filled with saline. (F) Dental acrylic poured around the Luer-Lock hub and screw, then left to harden.

3.4.5. Gross Pathology and Blood Brain Barrier Integrity

For the study of gross pathology and integrity of the BBB, 5 hours after FPI animals were re-anaesthetised as per protocol 3, placed on a heating pad and body temperature monitored with a rectal probe. The right femoral vessels were exposed using blunt dissection and the femoral vein separated from the surrounding connective tissue. A small incision was made in the vessel whilst it was held taut with silk thread (4/0 Mersilk, Ethicon, Edinburgh, UK) to briefly reduce blood flow. A polythene catheter (external diameter 0.96 mm, internal diameter 0.58mm diameter, 15 cm long; SIMS Portex Ltd, Hythe, UK) containing heparinised saline was inserted 1cm into the vein and then secured with 2/0 silk suture. The cannula was then flushed with 0.5ml of heparinised saline (10 iu/ml). 3.5-4.0 ml of 1% Evan's blue (Sigma-Aldrich Company Ltd, Poole) in saline, 100mg/kg, was administered slowly and flushed with a further 0.5ml of heparinised saline (Cortez et al., 1989). One hour later, under deep halothane and pentobarbitone (150mg/kg, IP) anaesthesia a transverse sub-diaphragmatic incision was made. The diaphragm was divided anteriorly where it meets the costal margin, to expose the thoracic cavity. The ribs were divided bilaterally at the mid-thoracic level allowing the anterior chest wall to be folded cephalad. The left ventricle was then cannulated with a blunt 19G (Becton, Dickson & Company) needle, which was held in place by arterial forceps and then connected to an air free perfusing system. The right atrium was then incised. Phosphate buffered saline (PBS, Sigma-Aldrich Company Ltd, Poole, UK/BDH Laboratory Supplies, Gillingham, UK) was perfused at 32ml/min until the fluid from the right atrium ran clear. This was followed by followed by 4% paraformaldehyde (Fisher Scientific UK Ltd, Loughborough, UK) in PBS which was continued until muscle spasm ceased. The brain was then dissected from the skull and placed in 4% paraformaldehyde at 4°C over night. The next day each brain was inspected and photographed. They were then placed on a rat brain matrix (Zivic Miller Inc, Pittsburgh, USA) and cut into 2mm coronal sections, which were again photographed (Figure 3.7).

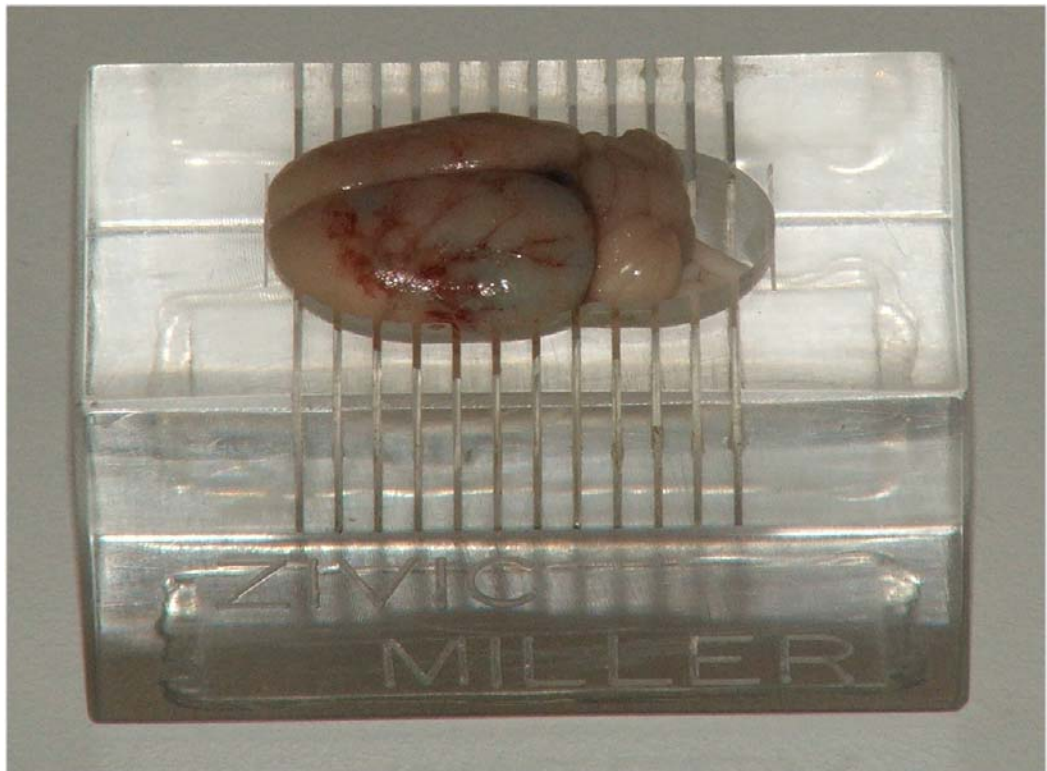


Figure 3.7. Whole Paraformaldehyde Fixed Cerebral Cortex and Cerebellum

6 hours post moderate lateral fluid percussion injury. Evan's blue was administered IV 1 hour prior to sacrifice. Tissue was placed on a rat brain matrix to guide coronal sectioning.

3.4.6. Chemokine mRNA & Protein Expression

Following FPI, animals were deeply anaesthetised with halothane and pentobarbitone (150mg/kg, I.P.), decapitated and the cerebral tissue dissected free. The brain was then sectioned coronally, isolating a 4mm slice of tissue at the level of the craniotomy, containing the anterior and mid hippocampus. From this coronal section the medial extremes of the cortex were trimmed. The major remaining segments of the ipsi and contralateral cortex and the ipsi and contralateral hippocampus were dissected free, separated, snap frozen in liquid nitrogen and stored at -80°C (Figure 3.8).

For cortical chemokine mRNA analysis animals were sacrificed 4 hours after injury. For the study of chemokine proteins animals were sacrificed at 0, 4, 8, 12 or 24 hours after injury.

Blood was also aspirated from the left ventricle prior to decapitation and dissection of the brain. These samples were allowed to clot for 15 minutes and then centrifuged at 4500 rpm for 15 minutes at 4°C. The serum was aspirated and frozen at -80°C until analysed.

The time to peak cortical chemokine concentration was assessed and this information used to determine the post-trauma recovery period required for the subsequent studies of chemokine inhibition and immunohistochemical staining for chemokine expression.

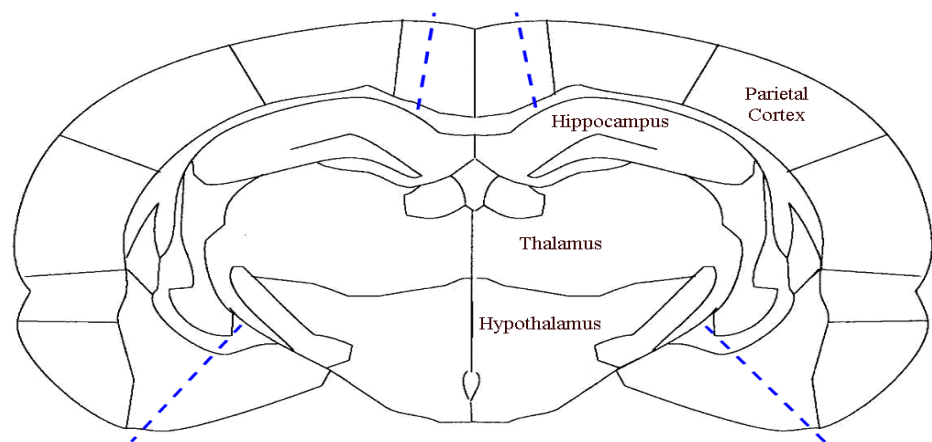


Figure 3.8. Illustrative Coronal Section of the Brain at the Level of the Anterior Hippocampus.
The FPI was centred over the parietal cortex. The dashed lines indicate where the cortex was trimmed.

3.4.6.1. RNA Extraction and Chemokine mRNA Reverse Transcriptase Polymerase Chain Reaction

3.4.6.1.1. RNA Extraction from Cortical Tissue

RNA was recovered from frozen cortical tissue using a commercially available kit (RNeasy, Qiagen, Crawley, UK) according to the manufacturer's instructions. β (2) mercaptoethanol was mixed with lysis buffer (RNeasy lysis buffer- a denaturing guanidine isothiocyanate (GITC) containing buffer) 1:100. This buffer inactivates RNase to ensure isolation of RNA. 600 μ l of this buffer was added to each tissue sample which was then homogenised first with a Polytron PT1200 motorised homogenizer (Kinematica, Lucerne, Switzerland) and then by passing through a QIAshredder spin column centrifuged at 8000g for 2 minutes. The tissue lysate was then centrifuged for 3 minutes and the supernatant collected and mixed with 600 μ l of 70% ethanol by pipetting. This solution was then added to an RNeasy mini-column containing a silica gel membrane designed to bind total RNA. The addition of ethanol aids this binding. The column was centrifuged for 15 seconds. The bound RNA was then washed 3 times by the addition of 500 μ l of a washing buffer to the mini-column and centrifuging for 15 seconds, 15 seconds and 2 minutes respectively. The RNA sample was eluted from the column by adding 50 μ l of RNase free water and centrifuging for a further minute (Figure 3.9). For each sample the RNA concentration was then measured spectrophotometrically. For a single sample from an ipsilateral injured cortex the concentration of RNA was particularly low compared to the other samples (70 μ g/ml vs. 162-291 μ g/ml).

3.4.6.1.2. cDNA Synthesis

cDNA was prepared from the RNA samples using a commercially available kit (First strand cDNA synthesis kit, Amersham Biosciences, Little Chalfont, UK) as per the manufactures instructions. 2 μ g of RNA from each sample was diluted up to 20 μ l in RNase free water. For one sample from an ipsilateral injured cortex, mentioned in the proceeding section, only 1.4 μ g of RNA was available. The RNA was heated to 65°C for 10 minutes and then placed on ice. Bulk first-strain reaction mix (containing murine reverse transcriptase, dATP, dCTP, dGTP and dTTP in buffer) 10 μ l, cDNA

random primer pd(N)₆ 1μl and 200mM dithiothreitol (DTT) solution 1μl were added together with the RNA and then incubated at 37°C for 1 hour.

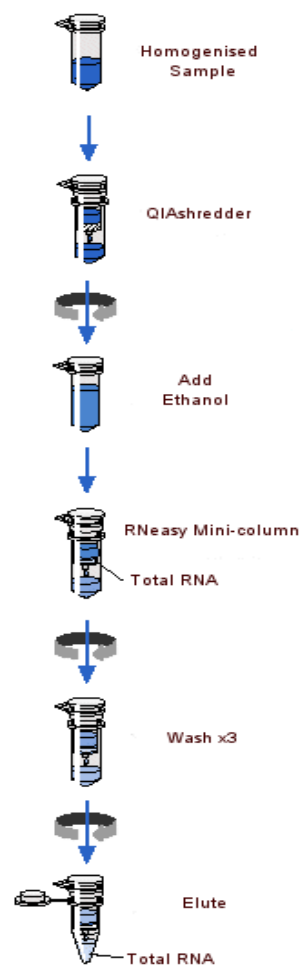


Figure 3.9. Summary of the RNA Extraction Method.

The tissue sample was suspended in a denaturing buffer and homogenised manually. Further homogenisation occurred through the QIAshredder. The RNA is then captured on the RNeasy mini-column, washed and eluted. Adapted from the RNeasy Mini Handbook, Qiagen.

3.4.6.1.3. Polymerase Chain Reaction (PCR)

Primers were selected after reviewing the recent literature for suitable sequences and performing a search of the National Centre for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) database to confirm that these sequences were complimentary to the target mRNAs. The final selection of primers was made with the help of Dr Stewart Milne. The melting temperatures of the primer pairs were compared using the Gene Jockey Program (Biosoft, Ferguson, Missouri, USA) and the suitable candidates obtained from Invitrogen Ltd (Paisley, UK). The primer pairs selected were: MIP-2/CXCL2, sense 5'-ACC CTG CCA AGG GTT GAC TTC-3' and antisense 5'-GGC ACA TCA GGT ACG ATC CAG-3' (Accession Number NM 053647)(Gilmour et al., 2004), MCP-1/CCL2, sense 5' -TCC ACC ACT ATG CAG GTC TC-3' and antisense 5'-TGG ACC CAT TCC TTA TTG GG-3' (Accession Number NM 031530)(Kono et al., 2004) and β -actin as a control, sense 5' -TCA TGA AGT GTG ACG TTG ACA TCC GT -3' and antisense 5' -CCT AGA AGC ATT TGC GGT GCA CGA TG- 3'(Accession Number NM 031144) (Nudel et al., 1983). The primers were diluted to 100pM with RNase free water. For each PCR reaction 5 μ l of cDNA was added to 45 μ l of PCR reaction mixture containing, 1 μ l of each paired primer, 25 μ l of REDTaq ReadyMix (R2523 (Taq DNA Polymerase, dNTP mix, MgCL₂), Sigma-Aldrich Company Ltd) and 18 μ l of RNase free water. The PCR conditions were 94°C for 5 minutes, then 30 cycles of 94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute followed by a final extension step of 72°C for 7 minutes. For MIP-2/CXCL2, MCP-1/CCL2 and β -actin this resulted in 283, 260 and 285 base pair products respectively. The amplified DNA fragments were separated by electrophoresis on a 1% agarose gel (Sigma-Aldrich Company Ltd) in tris-acetate-EDTA (TAE) buffer (Sigma-Aldrich Company Ltd) with 3 μ l/100ml ethidium bromide (Sigma-Aldrich Company Ltd) and the resulting bands visualized under ultra violet illumination on a Fluro-Chem gel imaging system (Alpha Innotech Corportion, San Leandro, California, USA).

3.4.6.2. Protein Extraction and Chemokine ELISAs

3.4.6.2.1. Protein Recovery

Frozen samples of cortex or hippocampus were weighed and homogenised on ice in 4 volumes of extraction buffer consisting of 150mM sodium chloride, 50mM tris(hydroxymethyl)aminomethane hydrochloride and 1% Triton X-100 (pH to 7.2) with proteinase inhibitor cocktail (10µl/10ml) (P8340)(All Sigma-Aldrich Company Ltd). These were then agitated for 90 minutes at 4°C and centrifuged for 15 minutes at 3000g. The resulting supernatants were aspirated and then stored at -80°C prior to analysis.

3.4.6.2.2. MIP-2/CXCL-2, MCP-1/CCL-2 and IL-1 β ELISAs

Cytokine concentrations of the supernatants were determined using commercially available solid phase enzyme linked immunosorbent assay (ELISA) kits to measure rat MIP-2/CXCL2 (KRC1022, Biosource International, Nivelles, Belgium) and MCP-1/CCL2 (KRC1012, Biosource) and IL-1 β (RLB00, R&D Systems Europe Ltd, Abingdon, UK) according to the manufacturers instructions. The colour change was read on a MRX microplate reader at 450nm and the output displayed using Revelation v3.04 software (Dynex Technologies, Worthing, UK). Each sample was measured in duplicate and the average value used.

3.4.6.2.3. Protein Assay

Cytokine concentrations were standardised against total protein concentration in the homogenates using a bicinchoninic acid (BCA) protein assay (Pierce Biotechnology Inc, Rockford, Illinois, USA). Samples were diluted 1:50. A series of standards of known concentration of bovine serum albumin were also prepared. 10µl of each sample or standard was added to a microtiter well. The BCA reagents A (bicinchoninic acid in alkaline buffer) and B (cupric sulphate solution) were mixed in a 50:1 by volume ratio and 200µl of this mix added to each sample/standard. The whole microtiter plate was then incubated at 37°C for 30 minutes. Cupric (Cu⁺⁺) ions are reduced to cuprous (Cu⁺) ions by protein in an alkaline medium. The resulting cuprous ions are chelated by BCA to form a purple

coloured reaction product, the absorbance of which is read on a MRX microplate reader at 560nm and the output displayed using Revelation v3.04 software (Dynex Technologies). Each experimental sample was analysed in triplicate and the average value used.

Cytokine concentrations expressed as pg/ml were divided by the protein concentration in mg/ml to yield a standardised cytokine concentration in pg/mg total protein.

3.4.7. Inhibition of Chemokine Expression

Dexamethasone 21-phosphate disodium salt (D1159, Sigma-Aldrich Company Ltd) was reconstituted in saline and administered IP in a final volume of 1ml, 0-15mg/kg, immediately after moderate LFPI. This range included doses that had previously been shown to inhibit chemokine expression *in-vivo* (Schramm & Thorlacius, 2003) (See section 2.2.2.2.2). FK506 0mg/kg (vehicle) or 1mg/kg were made up to a final volume of 1ml in saline and administered IP, 30 minutes prior to moderate LFPI. This dose route and timing of FK506 administration has previously been shown to reduce lesion volume following transient ischaemia (Butcher et al., 1997, McCarter et al., 2001) (See section 2.2.2.3.1, table 2.3). Animals were sacrificed at 4 hours and 9 hours after injury for the determination of cortical MIP-2/CXCL-2 and MCP-1/CCL2 concentrations respectively. These times were based on the time to peak chemokine expression identified in the study of the temporal expression of these chemokines (3.4.6). Cortical tissue was isolated and MIP-2/CXCL2 and MCP-1/CCL2 concentrations subsequently determined as described in section 3.4.6.

3.4.8. Histology & Immunohistochemistry

For histopathological study animals were sacrificed at 4 and 24 hours after injury for the study of the FPI lesion histology and 4 and 9 hours after injury for chemokine immunohistochemistry. Animals were deeply anaesthetised with halothane and pentobarbitone (150mg/kg, IP), and for fixed tissue, transcardially perfused with PBS followed by 4% paraformaldehyde in PBS as described (3.4.5). The brain was then dissected free and stored in paraformaldehyde in PBS at 4°C. This fixed tissue was

embedded in wax, cut into 5µm coronal sections on a microtome and then floated onto charged slides (VWR International, Lutterworth, UK). Non-fixed tissue was also obtained. Under halothane and pentobarbitone anaesthesia the animals were decapitated, the brain quickly dissected free and then frozen in cold (-38°C) isopentane (Fischer Scientific UK Ltd) prior to storage at -80°C. 10µm coronal cryostat sections were prepared from this tissue, mounted on gelatine (BDH Laboratory Supplies) coated slides (Raymond A Lamb Ltd, Eastbourne, UK) and stored at -80°C

3.4.8.1. Cresyl Violet and Acid Fuchsin

To identify contused tissue coronal sections were stained with 0.1% cresyl violet (BDH Laboratory Supplies) with 2.5µl/ml of glacial acetic acid (Fisher Scientific UK Ltd) and 1% acid fuchsin (Sigma-Aldrich Company Ltd) with 3 drops/100ml of glacial acetic acid. Wax embedded sections were soaked in xylene (Genta Medical, York, UK) for 10 minutes and rehydrated through 100, 90, 70 and 50% ethanol (Fisher Scientific UK Ltd) for 2 minutes each. Cryostat sections were dried in an oven overnight at 40°C and fixed in cold 4% paraformaldehyde in PBS for 1 hour. All slides were then rinsed in deionised water for 1 minute, stained with cresyl violet for 2 minutes, briefly rinsed with deionised water and then stained with acid fuchsin for a further 2 minutes prior to being differentiated in 70% alcohol. Finally the slides were dehydrated in 100% ethanol, cleared in xylene for 10 minutes and then mounted in DPX (44581 Fluka BioChemika, Sigma-Aldrich Company Ltd).

All light microscopy was performed with an Olympus BX51 microscope (Olympus UK Ltd, London, UK) and captured with a Qimaging MicroPublisher 3.3 RTV camera and Qcapture Pro 5.0 software (Media Cybernetics UK, Marlow, UK).

3.4.8.2. Immunohistochemistry for Myeloperoxidase and CD11b

In order to confirm that the pattern of leucocyte infiltration after LFPI was the same as that described in the literature coronal brain sections were stained for myeloperoxidase (to identify neutrophils) and CD11b (to identify mononuclear cells including tissue macrophages and microglia).

3.4.8.2.1. *Myeloperoxidase Immunohistochemistry*

To identify neutrophil accumulation post LFPI myeloperoxidase (MPO) immunohistochemistry was performed on frozen and paraformaldehyde fixed tissue.

Frozen 10µm coronal cryostat sections from rats sacrificed 24 hours after moderate LFPI were dried at 40°C overnight. These were then fixed in cold 4% paraformaldehyde in PBS for 30 seconds and then rinsed in PBS (3 x 5 minutes). The slides were dipped in acetone at -20°C for 5 minutes and rinsed again in PBS (3 x 5 minutes). Paraformaldehyde fixed wax embedded 5µm coronal sections from an animal sacrificed at 24 hours after moderate LFPI were soaked in xylene for 10 minutes and rehydrated through 100, 90, 70 and 50% ethanol for 2 minutes each, then placed in PBS. The sections were then permeabilised with 0.1% Triton X-100 (Sigma-Aldrich Company Ltd) for 10 minutes and washed in PBS (3 x 5 minutes). For both unfixed and fixed tissue endogenous peroxidase activity was then quenched with 3% hydrogen peroxide (Sigma-Aldrich Company Ltd) for 5 minutes and the slides rinsed in PBS (3 x 5 minutes). Blocking was performed with normal goat serum (NGS) in PBS (200µl in 10ml) for 1 hour at room temperature. A polyclonal rabbit anti-human myeloperoxidase antibody (A0398-cross-reacts with rat protein, Dako UK Ltd, Ely, UK) was diluted in NGS/PBS, 1:200 and added to each slide. The primary antibody was omitted from consecutive sections as a negative control. The slides were then incubated at room temperature for 2 hours and subsequently rinsed in PBS (3 x 5 minutes). Antigen visualisation was performed using the goat anti-rabbit IgG Vectastain ABC kit (PK-6101, Vector Laboratories Ltd, Peterborough, UK) according to the manufacturer's instructions. The biotinylated antibody (50µl) was diluted in NGS/PBS (150µl in 10ml) and applied for 1 hour at room temperature. After rinsing with PBS (3 x 5 minutes), avidin/biotinylated peroxidase enzyme complex (ABC) was added for 30 minutes and following another rinse with PBS the antigen staining visualised with 3,3'-diaminobenzidine (DAB, SK-4100, Vector Laboratories Ltd) or Vector stain VIP substrate (SK-4600, Vector Laboratories Ltd). This reaction was terminated with water and then the slides counter stained in Harris' haematoxylin (BDH Laboratory Supplies) for 1 minute, dipped in 1% acid alcohol for 20 seconds and placed in Scott's tap water substitute (BDH Laboratory Supplies) for 5 minutes, dehydrated thorough 70, 90 and 100% ethanols for 2 minutes each, cleared in xylene for 10 minutes and mounted in DPX.

3.4.8.2.2. *CD11b Immunohistochemistry*

Frozen 10µm coronal cryostat sections from rats sacrificed 4 and 24 hours after moderate lateral fluid percussion (recovery) were dried at 40°C overnight. These were then fixed in cold 4% paraformaldehyde in PBS for 30 seconds and then rinsed in PBS (3 x 5 minutes). The slides were dipped in acetone at -20°C for 5 minutes and rinsed in PBS (3 x 5 minutes). They were then placed back into cold 4% paraformaldehyde for 15 minutes. After rinsing in PBS (3 x 5 minutes), the tissue was permeabilised with 0.1% Triton X-100 for 10 minutes followed by a further rinse with PBS (3 x 5 minutes). Endogenous peroxidase activity was then quenched with 3% hydrogen peroxide for 5 minutes. After rinsing in PBS (3 x 5 minutes), blocking was performed with normal horse serum (NHS) in PBS (200µl in 10ml) for 1 hour at room temperature. The monoclonal mouse anti-rat cluster differentiation-11b (CD11b) antibody (OX-42, MCA275G, Serotec, Oxford, UK) was diluted in NHS/PBS, 1:100 and added to each slide. The primary antibody was omitted from consecutive sections as a negative control. The slides were then incubated at room temperature for 1 hour and subsequently rinsed in PBS (3 x 5 minutes). Antigen visualisation was performed using a horse anti-mouse IgG Vectastain ABC kit (PK-6102, Vector Laboratories Ltd) according to the manufacturer's instructions. The biotinylated antibody (50µl) was diluted in NHS/PBS (150µl in 10ml) and applied for 1 hour at room temperature. After rinsing with PBS (3 x 5 minutes), avidin/biotinylated peroxidase enzyme complex (ABC) was added for 30 minutes and following another rinse with PBS the antigen staining visualised with Vector stain VIP substrate (SK-4600, Vector Laboratories Ltd). This reaction was terminated with water and then the slides counter stained in Harris' haematoxylin (BDH Laboratory Supplies) for 1 minute, dipped in 1% acid alcohol for 20 seconds and placed in Scott's tap water substitute (BDH Laboratory Supplies) for 5 minutes, dehydrated through 70, 90 and 100% ethanols for 2 minutes each, cleared in xylene for 10 minutes and mounted in DPX.

3.4.8.3. *Development of a MIP-2/CXCL2 Immunohistochemistry Protocol*

In order to identify the cells producing MIP-2/CXCL2 after LFPI I planned to perform immunohistochemistry for this protein on coronal sections containing contused tissue. This region could be identified grossly and histologically at the level of the anterior hippocampus. This was the

same region of the brain in which chemokine mRNA and protein expression were investigated. At the time of commencing this project there was no anti-rat MIP-2/CXCL2 antibody marketed as suitable for immunohistochemistry. However the Biosource MIP-2/CXCL2 ELISA kit (KRC1022) used a polyclonal rabbit anti-rat MIP-2/CXCL2 antibody (ARC1074) as a capture antibody. This was available for sale separately and its use to identify MIP-2/CXCL2 positive cells *in-vitro* had been described (Wang et al., 2000).

3.4.8.3.1. Identification of Cellular MIP-2/CXCL2 in CTX-TNA2 Astrocytes Using the Biosource anti-MIP-2/CXCL2 Antibody and a Fluorescent Secondary Antibody

To determine if cellular MIP-2/CXCL2 could be identified with the ARC1074 antibody rat CTX-TNA2 astrocytes were used as a positive control. In chapter 2, I demonstrated that these cells produce MIP-2/CXCL2 when cultured with TNF- α (Figures 2.10 & 2.14).

3.4.8.3.1.1. Cell Culture

The rat astrocyte cell line CTX-TNA2 was cultured as described (See section 2.3.3), seeded into 6 well plates at 1×10^5 cells per well in 3ml and returned to the incubator. This lower cell density was chosen to aid clear visualisation of individual cells.

Following overnight incubation the culture media was aspirated, the cells rinsed with serum free media and then fresh culture media containing rat TNF- α (20ng/ml) added. The controls consisted of cells to which fresh culture media alone was added. Cells were then returned to the incubator for 24 hours.

3.4.8.3.1.2. MIP-2/CXCL2 Immunohistochemistry in CTX-TNA2 Astrocytes

After 24 hours the CTX-TNA2 cells were rinsed with PBS and fixed in 4 % paraformaldehyde in PBS for 20 minutes. Following a further wash in PBS (3 x 5minutes), the cells were permeabilised with 0.1% Triton X-100 (Sigma-Aldrich Company Ltd) for 10 minutes, washed in PBS (3 x 10 minutes)

and blocked with NGS in PBS (200µl in 10ml) for 1 hour at room temperature. The polyclonal rabbit anti-rat MIP-2/CXCL2 antibody was diluted in NGS/PBS, 1:200 (Wang et al., 2000) and 2 ml of this is mix added to the wells. For a negative control the primary antibody was omitted from one well of TNF- α stimulated cells. The cells were then incubated at 4°C overnight and then washed in PBS (3 x 10 minutes). A fluorescent goat anti-rabbit Alexa Fluro® 488 secondary antibody (Invitrogen) was diluted in NGS/PBS (150µl in 10ml), 1:1000 and 2 mls added to each well, the whole plate wrapped in foil and then incubated for 1 hour at room temperature. The cells were rinsed in PBS for a final time (2 x 10 minutes). Counter staining was performed with 1µM TO-PRO®-3 (Invitrogen) in PBS for 5 minutes followed by a further rinse in PBS. Staining was visualised on a Nikon Eclipse E600FN microscope (Nikon Instruments Europe B.V, Badhoevedorp, Netherlands) fitted with a Bio Rad Radiance 2000 confocal laser scanning system (Argon 488nm, Red diode 633nm lasers) and the images captured through Lasersharp 2000 software (Biorad, Hemel Hempstead, UK) .

3.4.8.3.2. *Immunohistochemistry for MIP-2/CXCL2 After Lateral Fluid Percussion Injury;*

unfixed tissue and visualisation with a fluorescent secondary antibody

Having identified MIP-2/CXCL2 staining *in-vitro* with the Biosource anti rat MIP-2/CXCL2 antibody, I then used it to attempt to identify MIP-2/CXCL2 positive cells in the rat brain after LFPI. As some antibodies can be affected by prolonged paraformaldehyde fixation or the wax embedding process I elected to use unfixed frozen tissue sections initially.

Frozen 10µm coronal cryostat sections from rats sacrificed 4 hours after moderate LFPI (non-recovery) were dried at 40°C overnight. These were then fixed in 4% paraformaldehyde in PBS at room temperature for 20 minutes and washed in PBS (3 x 5 minutes). The tissue was then permeabilised with 0.1% Triton X-100 for 10 minutes and following a further wash in PBS (3 x 5 minutes), blocked with NGS in PBS (200µl in 10ml) for 1 hour at room temperature. The polyclonal rabbit anti-rat MIP-2/CXCL2 antibody was diluted in NGS/PBS, 1:200 and added to each slide. The primary antibody was omitted from consecutive sections as a negative control. The slides were then incubated at 4°C overnight. After washing in PBS (3 x 5 minutes) the goat anti-rabbit Alexa Fluro®

488 secondary antibody, diluted in NGS/PBS (150µl in 10ml), 1:1000, was added to each slide. The slides were then wrapped in foil and then incubated for 1 hour at room temperature. After washing in PBS (2 x 5 minutes), 1µM TO-PRO®-3 in PBS was applied for 5 minutes followed by a further rinse in PBS. The slides were then covered with Mowoil mounting medium (Consisting of Mowoil 4-88 (Calbiochem, Merk Chemicals Ltd, Nottingham ,UK), glycerol (Fisher Scientific UK, Ltd, Loughborough, UK), tris(hydroxymethyl) aminomethane hydrochloride (Tris)(Sigma-Aldrich Company Ltd) and diazabicyclo octane (DABCO) (Sigma-Aldrich Company Ltd)) and allowed to dry for 30 minutes. Staining was visualised on a Nikon Eclipse E600FN microscope as described in section 3.4.8.3.1.

3.4.8.3.3. *Immunohistochemistry for MIP-2/CXCL2 After Lateral Fluid Percussion Injury; unfixed tissue and visualisation with a biotinylated secondary antibody*

MIP-2/CXCL2 positive cells could not be identified by confocal microscopy. Another search of the literature revealed a paper by Sharafeldin and colleagues in which the Biosource MIP-2/CXCL2 antibody had been used on unfixed rat brain tissue sections and staining visualised with a biotinylated secondary antibody (Sharafeldin et al., 2000). This was directly applicable to the identification of cells expressing MIP-2/CXCL2 after FPI. It also offered the advantage of not using a fluorescent secondary antibody which had suffered from bleaching in the previous attempt. After discussion with Dr Mikhail Pashenkov, a co-author of this paper, 2 fixation protocols were compared.

3.4.8.3.3.1. *Fixation Protocol 1*

Frozen 10µm coronal cryostat sections from rats sacrificed 4 hours after moderate LFPI (recovery) were dried at 40°C overnight. These were fixed in cold 4% paraformaldehyde in PBS for 30 seconds and rinsed in PBS (3 x 5 minutes). The slides were dipped in acetone (BDH Laboratory Supplies) at -20°C, for 5 minutes and then rinsed in PBS (3 x 5 minutes).

3.4.8.3.3.2. *Fixation Protocol 2*

Frozen 10µm coronal cryostat sections from rats sacrificed 4 hours after moderate LFPI (recovery) were dried at 40°C overnight. These were then fixed in cold 4% paraformaldehyde in PBS for 30seconds and then rinsed in PBS (3 x 5 minutes). The slides were dipped in acetone at -20°C for 5 minutes and rinsed in PBS (3 x 5 minutes). To increase cross-linking and reduce chemokine loss the slides were then placed back into cold 4% paraformaldehyde for 15 minutes. After rinsing in PBS (3 x 5 minutes), the tissue was permeabilised with 0.1%Triton X-100 for 10 minutes followed by a further rinse with PBS (3 x 5 minutes).

3.4.8.3.3.3. *Immunohistochemistry; common steps*

For each fixation protocol the endogenous peroxidase activity was then quenched with 3% hydrogen peroxide for 5 minutes and then the sections rinsed in PBS (3 x 5 minutes). Blocking was performed with NGS in PBS (200µl in 10ml) for 1 hour at room temperature. The polyclonal rabbit anti-rat MIP-2/CXCL2 antibody was diluted in NGS/PBS, 1:50 and added to each slide. The primary antibody was omitted from consecutive sections as a negative control. The slides were then incubated at 4°C overnight and subsequently rinsed in PBS (3 x 5 minutes). Antigen visualisation was performed using the goat anti-rabbit IgG Vectastain ABC kit (PK-6101) and DAB (SK-4100), the slides counter stained and mounted as described in section 3.4.8.2.1.

The intensity of MIP-2/CXCL2 staining was rated on a simple subjective scale, as absent (-), light (+), moderate (++) or dense (+++). The contralateral tissue was used as a reference between animals.

3.4.8.3.4. Immunohistochemistry for MIP-2/CXCL2 After Lateral Fluid Percussion Injury; fixed tissue and visualisation with biotinylated secondary antibody

In an attempt to improve the quality of the tissue morphology I went on to repeat the immunohistochemistry for MIP-2/CXCL2 on paraformaldehyde fixed tissue. Animals were sacrificed 4 hours after LFPI.

5µm coronal wax embedded sections were soaked in xylene for 10 minutes and rehydrated through 100, 90, 70 and 50% ethanol for 2 minutes each, then placed in PBS. Antigen retrieval was then performed by immersing the slides in 10mM citric acid (Sigma-Aldrich Company Ltd) (pH to 6.0) and heating in a microwave on full power for 10 minutes. The slides were then allowed to cool and were rinsed in PBS (3 x 5 minutes). Sections were then permeabilised with 0.1% Triton X-100 for 10 minutes, washed in PBS (3 x 5 minutes) and endogenous peroxidase activity quenched with 3% hydrogen peroxide for 5 minutes. After further washing in PBS the slides were blocked with NGS in PBS (200µl in 10ml) for 1 hour prior to the addition of the primary antibodies. The rabbit anti-rat MIP-2/CXCL2 antibody was diluted in NGS/PBS, 1:50 and applied to the sections for 1 hour at room temperature. For negative controls the primary antibodies were omitted from consecutive sections. The slides were subsequently rinsed in PBS (3 x 5 minutes) then antigen visualisation performed using the goat anti-rabbit IgG Vectastain ABC kit (PK-6101) and DAB (SK-4100), the slides counter stained and mounted as described in section 3.4.8.2.1.

The intensity of the staining was rated on a simple subjective scale, as absent (-), light (+), moderate (++) or dense (+++). The contralateral tissue was used as a reference between animals.

3.4.8.4. Immunohistochemistry for MCP-1/CCL2 After Lateral Fluid Percussion Injury; fixed tissue and visualisation with biotinylated secondary antibody

To identify the cells expressing MCP-1/CCL2 animals were sacrificed 9 hours after LFPI. The immunohistochemistry protocol was the same as that for MIP-2/CXCL2 on wax embedded sections described in the previous section (3.4.8.3.4), and similar to that described by Berman and colleagues,

who identified MCP-1/CCL2 positive cells in the rat CNS (Berman et al., 1996). The primary antibody was a polyclonal rabbit anti-rat MCP-1/CCL2 (AB7202, Abcam Ltd, Cambridge, UK) which was diluted in NGS/PBS, 1:500 and applied to the sections for 1 hour at room temperature.

A semi-quantitative and qualitative assessment of MCP-1/CCL2 staining was made. Within the contused area the outer cortex (molecular (I) and external granular cell (II) layers) and deep cortex (external pyramidal (III), internal granular (IV) and internal pyramidal (V) cell layers) were assessed separately. The centre of the contused outer and deep cortex, the corresponding regions of the contralateral cortex and the ipsi and contralateral cortex of sham animals were photographed at x200. Three slides from the same level of the brain per animal were photographed. The number of MCP-1/CCL2 positive cells were counted in each frame. The difference in the intensity of MCP-1/CCL2 staining between animals was also assessed. This was scored subjectively as absent (-), light (+) and moderate (++). The contralateral tissue was used as a reference between animals.

3.4.8.5. Identification of Cell Staining for Chemokine Protein by Double Staining With Cell Specific Markers

3.4.8.5.1. Double Staining for Chemokines and NeuN, GFAP or CD11b: visualisation with biotinylated secondary antibodies

Once the sections stained with primary antibodies to MIP-2/CXCL2 and MCP-1/CCL2 had been photographed the slides were soaked in xylene overnight to remove the coverslips and rehydrated through 100, 90, 70 and 50% ethanols for 2 minutes each then placed in PBS. The slides were then blocked with NHS in PBS (200µl in 10ml) for 1 hour at room temperature. The second primary antibodies were diluted in NHS/PBS and applied for a further hour, also at room temperature. These were mouse anti-mouse neuronal nuclei (NeuN) (MAB377, Chemicon Europe Ltd, Chancellors Ford, UK) (diluted 1:100) which cross-reacts with rat neurones, mouse anti-pig glial fibrillary acid protein (GFAP) (G3893, Sigma-Aldrich Company Ltd) (diluted 1:400) which cross-reacts with rat astrocytes and mouse anti-rat cluster differentiation-11b (CD11b) (MCA275G, Serotec, Oxford, UK) (diluted 1:100) for microglia, infiltrating monocytes and neutrophils. For a negative control primary

antibodies were omitted from consecutive sections. After rinsing in PBS (3 x 5 minutes) antigen visualisation was performed using a horse anti-mouse IgG Vectastain ABC kit (PK-6102) and Vector stain VIP substrate (SK-4600) according to the manufactures instructions as described (3.4.8.2.2). This reaction was terminated with water then the slides where dehydrated thorough 70, 90 and 100% ethanols for 2 minutes each then cleared in xylene for 10 minutes and mounted in DPX. Cryostat sections of rat spleen were used as a positive control for the CD11b antibody.

3.4.8.5.2. *Double Staining for CXCL2/MIP-2 or CCL2/MCP-1 and the Neuronal Marker NeuN; fixed tissue and fluorescent secondary antibodies*

To complete the immunohistochemistry, paraformaldehyde fixed brain sections from animals, sacrificed at 4 and 9 hours after LFPI, were double stained for MIP-2/CXCL2 or MCP-1/CCL2 respectively and with the neuronal marker NeuN (MAB377).

5µm coronal wax embedded sections were rehydrated, antigen retrieval performed and permeablised as described (3.4.8.3.4). The slides were then blocked with NGS in PBS (200µl in 10ml) for 1 hour at room temperature, prior to the addition of the primary antibodies. The rabbit anti-rat MIP-2/CXCL2 antibody (diluted in NGS/PBS (1:50)) or the rabbit anti-rat MCP-1/CCL2 antibody (diluted in NGS/PBS (1:250)) were applied to the sections for 1 hour at room temperature. For negative controls the primary antibodies were omitted from consecutive sections. After washing in PBS (3 x 5 minutes) a goat anti-rabbit Alexa Fluro® 546 (Invitrogen) secondary antibody, diluted in NGS/PBS (150µl in 10ml), 1:500 was added to each slide. The slides where kept wrapped in foil for the remaining steps. Following incubation for 1 hour at room temperature and after washing in PBS (2 x 5 minutes), the sections were blocked with NHS in PBS (200µl in 10ml) for 1 hour at room temperature. The second primary antibody, mouse anti-rat NeuN (diluted 1:100) in NHS/PBS was added and incubated at 4°C overnight. Again for negative controls the primary antibodies were omitted from consecutive sections. The sections were washed again in PBS (3 x 5minutes) and a horse anti-mouse Alexa Fluro® 488 (Invitrogen) secondary antibody, diluted in NHS/PBS (150µl in 10ml), 1:500 was added. The sections were incubated for a further hour at room temperature followed by another wash in PBS

(3 x5 minutes). 1 μ M TO-PRO®-3 (Invitrogen) in PBS was then applied for 5 minutes followed by a further rinse in PBS. The slides were then covered with Vectorsheild® mounting medium (Vector Laboratories Ltd) and sealed with clear nail polish. Visualisation was performed on a LSM 510 confocal microscope (Argon 488nm, Helium/Neon 543nm and Helium/Neon 633nm lasers) running LSM software (Carl Zeiss Ltd, Welwyn Garden City, UK).

3.4.9. Randomisation, Allocation Concealment and Blinding

All the animals in this study were identified by an individual number. At the start of each operating day the animals for that day were weighed and assigned the next available number in the sequence. If an animal died during the procedure it was replaced by the next animal in the sequence. Animals were randomised to trauma, sham and treatment groups by ballot at the end of the surgical procedure prior to the delivery of LFPI. If an animal died immediately after LFPI it was replaced by the next animal in the sequence which received the same injury and treatment, if indicated. Allocation concealment after injury and treatment was not possible on the day of surgery. However no animals were withdrawn from the study after injury was delivered reducing this potential for bias. For blinding, specimens were identified by the animal number with no reference to if they came from an animal receiving trauma, sham, drug or vehicle treatment. For the analysis of chemokine protein concentrations the treatment of the animal was not identified until the analysis was complete. For the interpretation of histological sections blinding was not possible as it was clear from the histology if an animal had received LFPI or sham injury.

3.4.10. Data and Statistics

The values for optical density obtained from the cytokine ELISA and protein assay standards were entered into a commercially available graphing package (Sigma Plot 8.0, Systat Software Inc, London, UK) and a 4 parameter logistic regression curve plotted. These parameters were used to derive the cytokine and protein concentrations from the optical densities of the samples tested.

Parametric data are presented as mean $\pm$ SEM and were compared by 1 or 2 way analysis of variance (ANOVA) with multiple comparisons tested using the Holm-Sidak method. Non parametric data are presented as median and range. These were analysed by Kruskal-Wallis analysis of variance on ranks. Differences are considered statistically significant at $p < 0.05$.

The limit of detection of the MIP-2/CXCL2 ELISA was 1pg/ml. For the study of serum chemokine concentration after lateral FPI this value was substituted for those samples in which MIP-2/CXCL2 was not detected.

3.5. Results

3.5.1. Preliminary Study

In order to satisfy the Home Office that the FPI could be performed with acceptable mortality the initially studies were performed with non-recovery from anaesthesia. The aims were to confirm that a lesion with appropriate histology could be produced and to provide data supporting the feasibility of investigating chemokine production after FPI. For the latter the expression of MIP-2/CXCL2 and MCP-1/CCL2 mRNAs was determined.

All animals were sacrificed at 4 hours after injury. This was the maximum time permitted by the project licence, initially. It was hoped that this would be sufficient to allow histological and molecular changes to be detected.

3.5.1.1. Mortality

16 animals were used in this study. 2 were withdrawn due to surgical errors. 4 animals died after anaesthesia with IP pentobarbitone. In 3 of these this was associated with the repeat administration of barbiturate to maintain anaesthesia. 3 animals received a FPI which was followed immediately by prolonged apnoea, pulmonary oedema, cyanosis and cardiac arrest. Mortality from the anaesthetic deaths and apnoeas after injury was 44%.

3.5.1.2. Histology

Animals were sacrificed at 4 hours after LFPI. In order to allow prompt assessment of the lesion histology frozen sections were chosen over paraformaldehyde wax embedded tissue. 3 brains were examined with cresyl violet and acid fuchsin staining. Cresyl violet stains normal nuclei blue whilst acid fuchsin has been used to identify acidophilic irreversibly damaged neurones, which appear red (Auer et al., 1985, Cortez et al., 1989). The lateral FPI was centred over the parietal cortex (Figure 3.10). In the ipsilateral hemisphere traumatic haemorrhage was apparent in the corpus callosum, external capsule, within the lateral ventricle, hippocampal fissure and across the surface of the cortex. In each animal the cortical injury was defined by the loss of cresyl violet staining and hypocellularity. Within this lesion acidophilic pink/red neurones were prominent. These cells had contracted nuclei and shrunken cytoplasm (Figure 3.11). The architecture of the ipsilateral hippocampus was also disturbed and acidophilic neurones were present in the CA2/3 pyramidal cell layer (Figure 3.12). In contrast the morphology and staining of the neurones in the contralateral cortex and hippocampus remained regular with no evidence of neuronal necrosis. These observations were consistent with previous descriptions of this model (Cortez et al., 1989, Hicks et al., 1996).

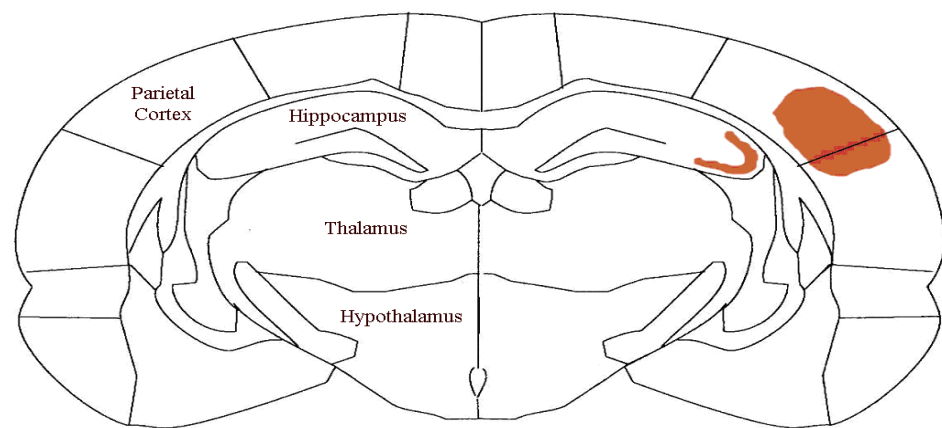
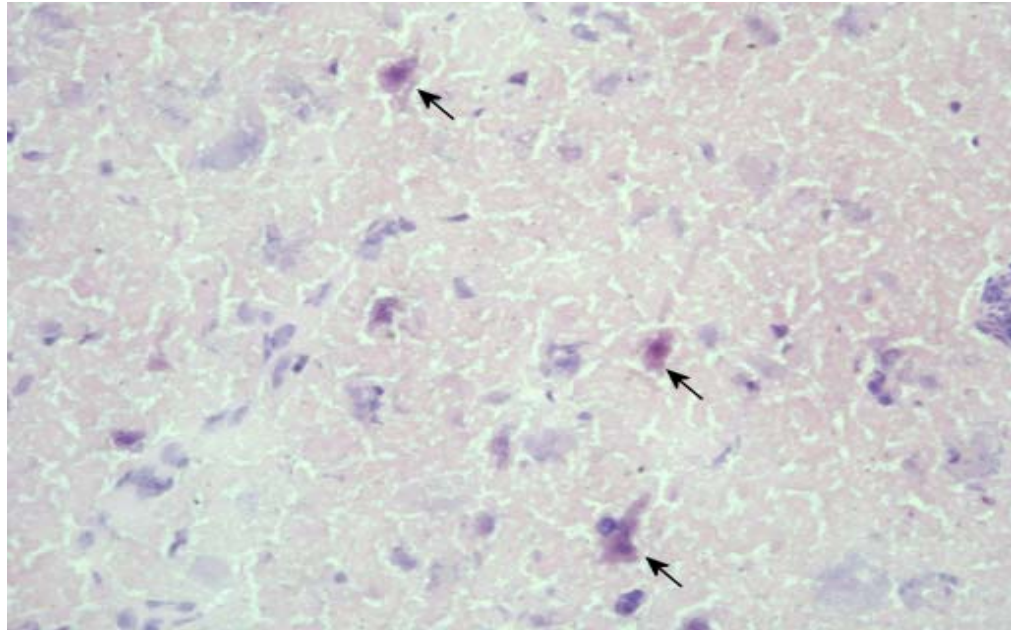


Figure 3.10. Illustrative Coronal Section of the Brain at the Level of the Anterior Hippocampus.

The LFPI was centred over the parietal cortex. The red shading in the cortex and hippocampus indicates where necrotic neurones could be identified at 4 hours after injury.

A



B

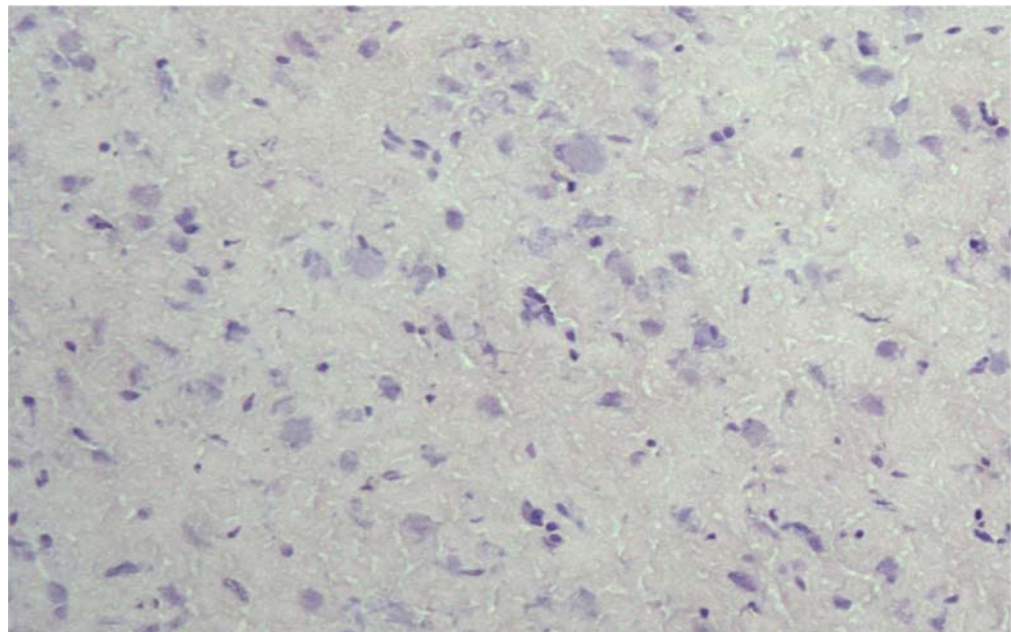
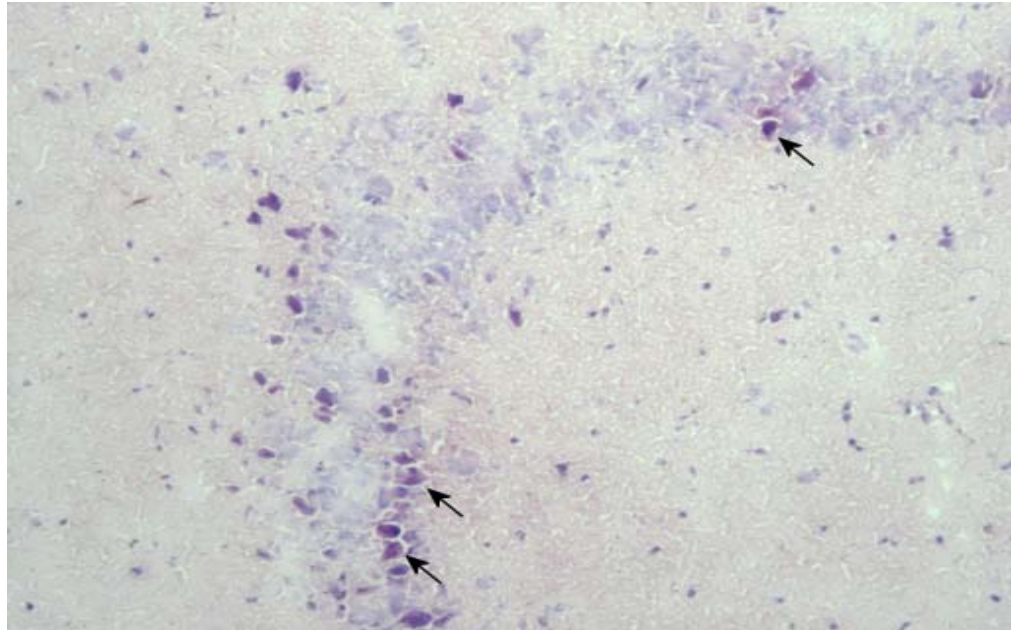


Figure 3.11. Damaged Neurones are Apparent in the Parietal Cortex at 4 hours After Lateral Fluid Percussion Injury.

(A) In the ipsilateral parietal cortex acidophilic neurones with condensed nuclei and contracted cytoplasm stain a pink/red colour (Arrows). (B) In the contralateral parietal cortex the cell bodies of neurones maintain a rounded morphology. Both cresyl violet and acid fuchsin x200.

A



B

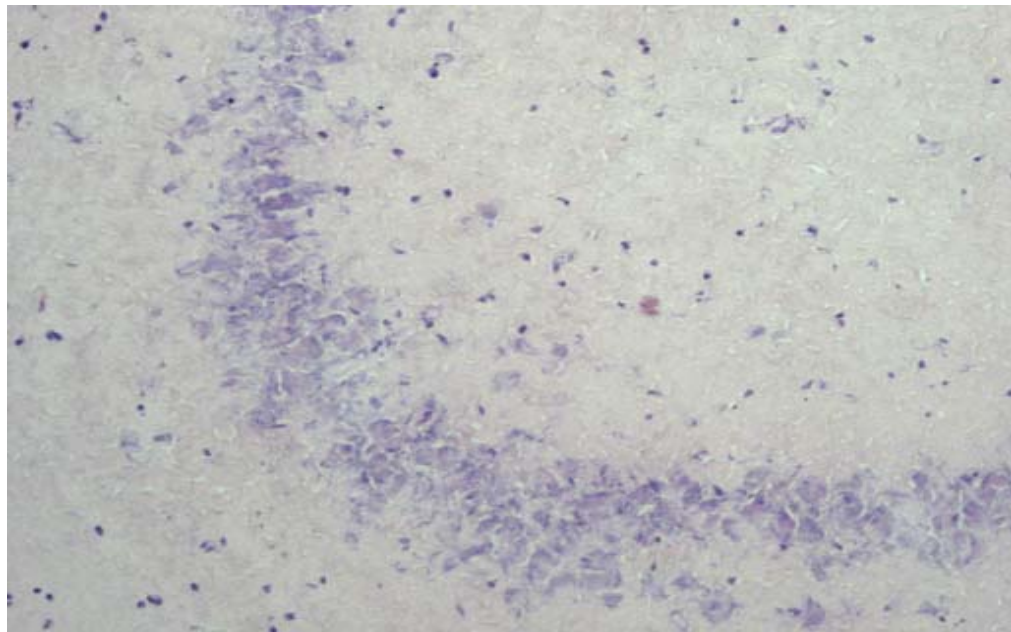


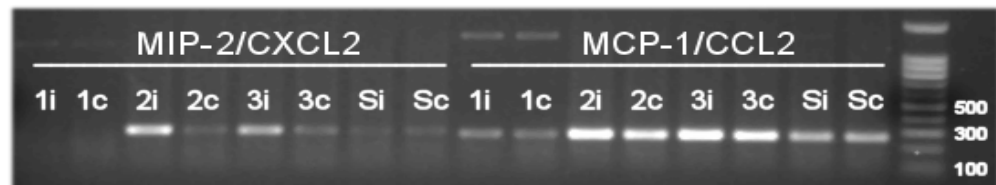
Figure 3.12. Damaged Neurones are Apparent in the Ipsilateral Hippocampus at 4 Hours after Lateral Fluid Percussion Injury.

(A) Acidophilic CA2/3 pyramidal layer cells (Arrows) in the disrupted lateral hippocampus. (B) Contralateral hippocampus. Both cresyl violet and acid fuchsin x100.

3.5.1.3. Induction of Chemokine mRNA by Lateral Fluid Percussion Injury

Chemokine mRNA expression in cortical tissue after LFPI was investigated by reverse transcriptase-polymerase chain reaction (RT-PCR) to obtain an early indication of whether MIP-2/CXCL2 and MCP-1/CCL2 expression were increased by this injury. 4 animals, 3 injury and 1 sham received a FPI of moderate severity and were sacrificed at 4 hours after injury. Additionally 2 animals, 1 sham and 1 trauma died under anaesthesia after randomisation and were not included in the analysis. Both MIP-2/CXCL2 and MCP-1/CCL2 mRNA were detectable in the sham animal. Following injury, increased MIP-2/CXCL2 mRNA was present in the ipsilateral cortex of 2 of the 3 injured animals. Similarly MCP-1/CCL2 mRNA induction was seen in the ipsilateral cortex of all 3 of the injured animals (Figure 3.13). As noted in the materials and methods section (3.4.6.1.1, 3.4.6.1.2) the RNA available to convert to cDNA was less in sample 1i and this can be seen as a less intense β actin product. This could explain the lack of a visible band for MIP-2/CXCL2 for this sample.

A



B



Figure 3.13. Induction of MIP-2/CXCL2 and MCP-1/CCL2 mRNA by Lateral Fluid Percussion Injury.

4 animals (3 injury and 1 sham) were sacrificed 4 hours after moderate LFPI and cortical mRNA amplified by reverse transcriptase polymerase chain reaction with specific primers for rat MIP-2/CXCL2 and MCP-1/CCL2. (A) In 2 of the 3 injured animals MIP-2/CXCL2 mRNA (283 base pair product) was increased in the ipsilateral (i) cortex compared to the contralateral (c) and sham (S) cortices. For MCP-1/CCL2 (260 base pair product) increased mRNA was detected in all 3 injured ipsilateral cortices compared to contralaterals and shams. (B) β -Actin amplification product (285 base pairs) from the same cortical samples shown in A.

3.5.1.4. Preliminary Study Review

The histology from the cryostat sections was consistent with the literature for lateral FPI of moderate severity (Cortez et al., 1989, Hicks et al., 1996). The injury was also associated with increased expression of chemokine mRNA detectable at 4 hours. This would suggest that MIP-2/CXCL2 and MCP-1/CCL2 protein could be increased after FPI.

However the mortality rate was unacceptably high at 44%. Four of the animals died after the administration of barbiturate, without receiving an injury and in 3 of these, death was associated with the administration of additional barbiturate to maintain anaesthesia. In 3 animals apnoea and hypoxia following injury were a significant problem leading to death.

Barbiturate anaesthesia has the advantage of not requiring an anaesthetic machine or breathing circuit. It was the technique used in the Philadelphia laboratory I visited and because I was aiming to replicate the model I used it for this study. Also because of its long duration of action it was a suitable agent for non-recovery experiments. The disadvantages of barbiturates are that the anaesthetic depth cannot be titrated and respiratory depression or airway obstruction due to secretions are common.

A search of the recent literature for units using LFPI was performed and I then contacted staff by email from 3 of the units identified. Linda Philips (Associate Professor of Anatomy, Virginia Commonwealth University), Alan Hazell (Assistant Professor, Department of Medicine, University of Montreal) and David Diebold (Senior Research Technician, Haden Hall, University of Pennsylvania) were kind enough to reply. Mortality rates for moderate injury were quoted between 10-30%. It was suggested that inhalational anaesthesia would be better and that recovery could be improved by the administration of oxygen immediately prior to and following the injury.

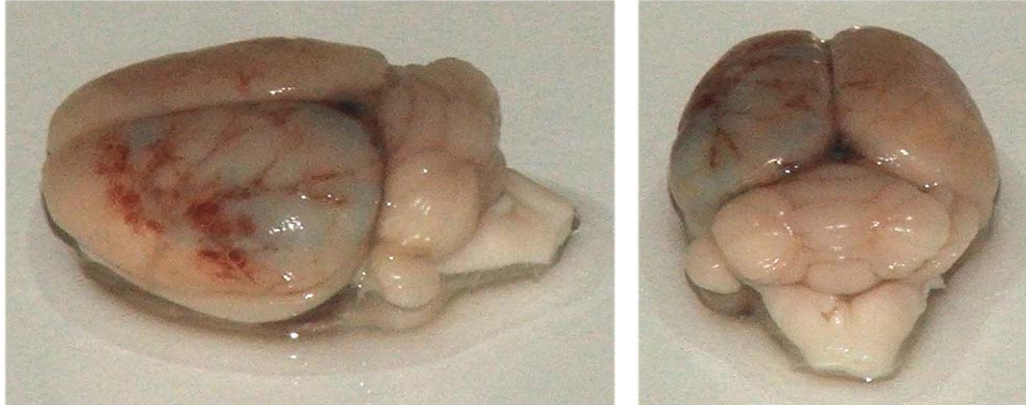
I then discussed the experimental results, mortality and my enquiries with the Home Office inspector. As the repeated administration of barbiturate was associated with excess deaths the requirement for non-recovery was lifted. The experimental protocol also was changed to include the administration of 100% oxygen prior to injury and immediately after, until a regular respiratory pattern was established.

Having demonstrated appropriate histology and induction of chemokine mRNA the maximum permitted period of recovery after injury was increased to 24 hours.

3.5.2. Gross pathology and Blood Brain Barrier Disruption

To evaluate gross pathological changes and BBB disruption after LFPI 7 animals (4 injury, 3 shams) underwent surgery and were recovered for 6 hours. One hour prior to sacrifice they were perfused with Evan's blue solution. FPI was associated with extensive traumatic haemorrhage across the surface of the cortex. Evan's blue extravasation could also be seen in the ipsilateral hemisphere indicating compromise of the BBB (Figure 3.14). On coronal sections, haemorrhage was identified in the ipsilateral corpus callosum, external capsule, and hippocampal fissure. Lacerations were also seen in the ipsilateral parietal cortex. Evan's blue extravasation could be seen in the ipsilateral external capsule and parietal cortex. This was associated with haemorrhage (Figure 3.15). These observations were consistent with the previous description of the pathology of this model (Cortez et al., 1989, McIntosh et al., 1989) at 6 hours.

A



B

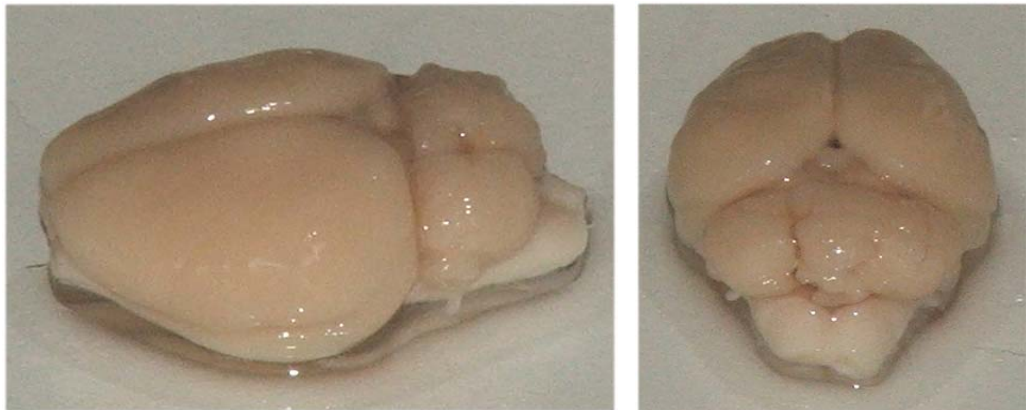


Figure 3.14. Gross Pathology of Lateral Fluid Percussion Injury.

Five hours after moderate LFPI animals were administered Evan's blue solution IV and sacrificed 1 hour later by transcardiac perfusion. (A) Representative specimen after LFPI. Extensive haemorrhage extends across the ipsilateral cortex with evidence of Evan's blue extravasation also visible. (B) Sham injury.

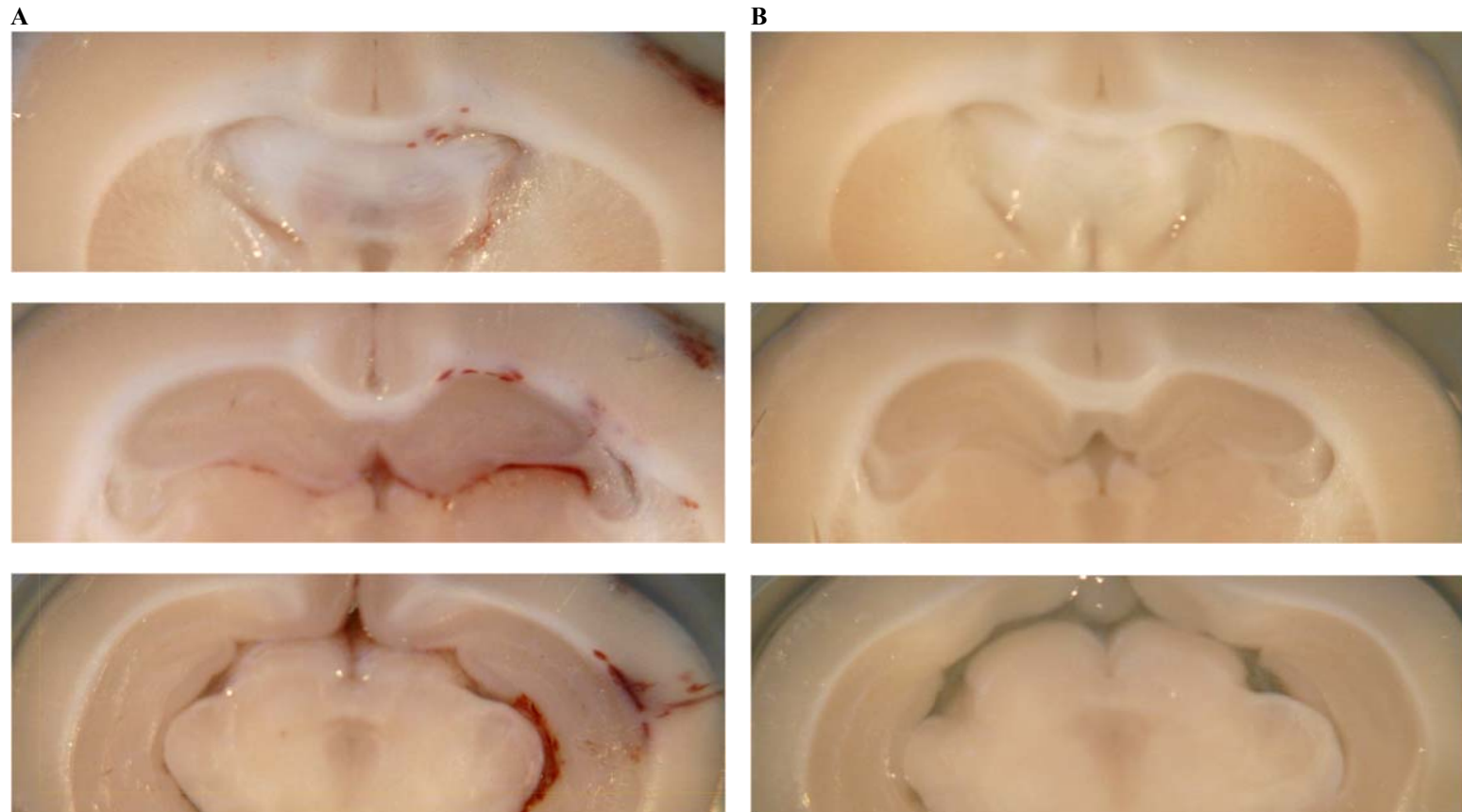


Figure 3.15. Coronal Sections Illustrating the Gross Pathology of Lateral Fluid Percussion Injury.

Five hours after moderate LFPI, animals were administered Evan's blue solution IV and sacrificed 1 hour later by transcardiac perfusion. (A) Representative specimen after LFPI. Extensive haemorrhage is visible in the ipsilateral corpus callosum, external capsule and hippocampal fissure. Lacerations are also present in the ipsilateral parietal cortex. Evan's blue extravasation can be seen in the ipsilateral external capsule and cortex. (B) Sham injury.

3.5.3. Induction of MIP-2/CXCL2 and MCP-1/CCL2 by Lateral Fluid

Percussion Injury

3.5.3.1. Cerebral Chemokine Concentrations Over 24 Hours

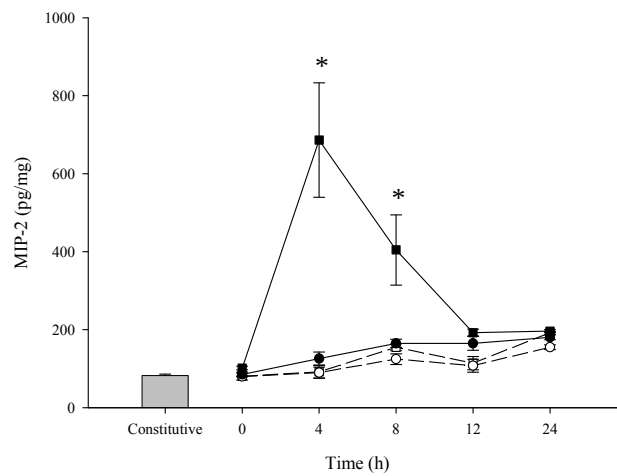
Chemokine protein expression in cortical tissue was examined by ELISA over 24 hours following moderate LFPI. 7 animals were used per time point, 5 injury and 2 shams. Additionally 2 animals died after randomisation, both immediately after LFPI and were not included in the analysis. 2 animals were also sacrificed without surgery to determine constitutive chemokine expression.

LFPI led to an early and transient increase in both MIP-2/CXCL2 and MCP-1/CCL2 concentrations in the ipsilateral cortex (Figure 3.16). The ipsilateral MIP-2/CXCL2 concentration peaked at 4 hours and was significantly increased over the contralateral cortex in injured animals and the ipsilateral and contralateral cortex of shams at 4 (686.3 ± 146.6 pg/mg vs. 126.0 ± 16.6 pg/mg, ipsilateral vs. contralateral injury and 91.9 ± 16.4 pg/mg vs. 90.0 ± 14.0 pg/mg, ipsilateral vs. contralateral sham respectively) and 8 hours (404.5 ± 90.2 pg/mg vs. 165.0 ± 10.6 pg/mg, ipsilateral vs. contralateral injury and 154.9 ± 8.8 pg/mg vs. 124.5 ± 13.7 pg/mg, ipsilateral vs. contralateral sham respectively), comparison between sites at specified times by post hoc analysis confirming that the increase occurred in the ipsilateral cortex of injured animals at 4 and 8 hours. The expression of MCP-1/CCL2 was lower and delayed in comparison to MIP-2/CXCL2, peaking between 8 and 12 hours after injury in the injured cortex. Ipsilateral cortical MCP-1/CCL2 concentration was significantly increased over the contralateral cortex in injured animals and the ipsilateral and contralateral cortex of shams at 8 (58.3 ± 7.6 pg/mg vs. 23.7 ± 2.6 pg/mg, ipsilateral vs. contralateral injury and 22.5 ± 1.7 pg/mg vs. 11.9 ± 4.4 pg/mg ipsilateral vs. contralateral sham respectively) and 12 hours (58.2 ± 11.7 pg/mg vs. 33.8 ± 10.3 pg/mg, ipsilateral vs. contralateral injury and 13.9 ± 0.6 pg/mg vs. 19.8 ± 5.7 pg/mg ipsilateral vs. contralateral sham respectively), comparison between sites at specified times by post hoc analysis confirming that the increase occurred in the ipsilateral cortex of injured animals at 8 and 12 hours. In addition to the increase in MCP-1/CCL2 concentration in the ipsilateral cortex, the MCP-1/CCL2 concentration was also increased significantly in the contralateral cortex of injured animals at 12 and 24 hours compared to 0 and 4 hours after injury in the same cortex (10.6 ± 0.8 pg/mg, $13.1 \pm$

0.9 pg/mg, 33.8 ± 10.3 pg/mg and 32.2 ± 6.1 pg/mg, at 0, 4, 12 and 24 hours post injury respectively, comparison between times within site by post hoc analysis confirming that the increase occurred at 12 and 24 hours in the contralateral cortex of injured animals) (Figure 3.16).

The hippocampal MIP-2/CXCL2 concentration at 4 hours after injury was also examined. As in the cortex the ipsilateral MIP-2/CXCL2 concentration was significantly increased over the contralateral hippocampus in injured animals and the ipsilateral and contralateral hippocampus of shams (580.8 ± 62.2 pg/mg vs. 207.7 ± 30.8 pg/mg, ipsilateral vs. contralateral injury and 90.1 ± 13.5 pg/mg vs. 77.7 ± 3.2 pg/mg, ipsilateral vs. contralateral sham respectively, with comparison between the sites by post hoc analysis confirming that the increase occurred in the ipsilateral hippocampus of injured animals) (Figure 3.17).

A



B

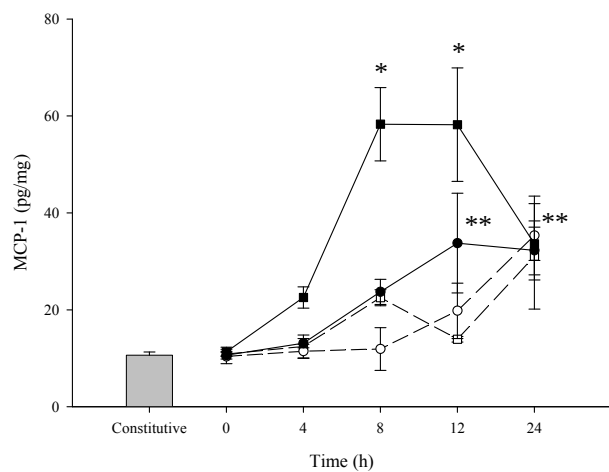


Figure 3.16. Cortical MIP-2/CXCL2 and MCP-1/CCL2 Protein Concentration after Lateral Fluid Percussion Injury.

(A) MIP-2/CXCL2 protein was significantly increased in the ipsilateral cortex at 4 and 8 hours after injury. (B) MCP-1/CCL2 protein concentration was significantly increased in the ipsilateral cortex at 8 and 12 hours after injury. The MCP-1/CCL2 concentration was also significantly increased at 12 and 24 hours in the contralateral cortex of injured animals. Cortical chemokine concentrations from 2 non-operated animals are shown for comparison (grey filled columns). Ipsilateral injury (■), contralateral injury (●), ipsilateral sham (□) and contralateral sham (○). * $p < 0.05$ vs. contralateral injury and ipsilateral sham. ** $p < 0.05$ in contralateral injury at 12 and 24 hours compared to 0 and 4 hours.

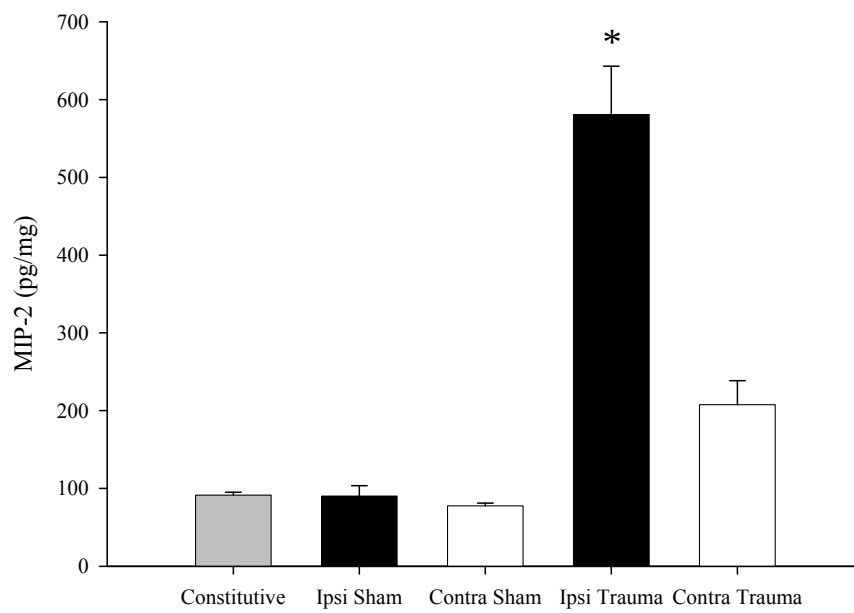


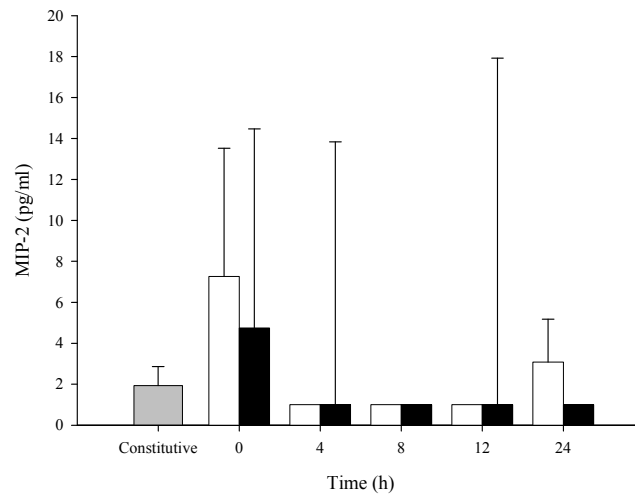
Figure 3.17. Hippocampal MIP-2/CXCL2 Protein Concentration after Lateral Fluid Percussion Injury.

MIP-2/CXCL2 protein was significantly increased in the ipsilateral hippocampus of injured animals by 4 hours after injury. Hippocampal MIP-2/CXCL2 concentration in 2 non-operated animals is shown for comparison (grey filled columns). * $p < 0.05$ vs. contralateral injury and ipsilateral and contralateral sham.

3.5.3.2. Serum Chemokine Concentration

The chemokine protein concentration in serum was also examined, over 24 hours following moderate LFPI, in the animals used to define the cortical expression. Despite significant increases in cortical chemokine concentrations, serum MIP-2/CXCL2 was undetectable in 77% (23 of 30) of the samples tested and the concentrations of both MIP-2/CXCL2 and MCP-1/CCL2 in injured animals were indistinguishable from those of shams (Figure 3.18). For MIP-2/CXCL2 the range of values were lower (0 to 17.9 pg/ml vs. 0 to 13.5 pg/ml trauma vs. sham respectively) than those of MCP-1/CCL2 (126.6 to 479.5 pg/ml vs. 149.3 to 455.4 pg/ml trauma vs. sham respectively). The concentration of MCP-1/CCL2 in serum increased significantly in both trauma and sham samples (203.3 ± 21.1 pg/ml, 195.8 ± 37.1 pg/ml and 227.0 ± 21.3 pg/ml at 0, 4 and 8 hours vs. 334.0 ± 39.9 pg/ml at 24 hours post injury respectively and 186.1 ± 18.6 pg/ml and 149.3 pg/ml at 0 and 4 hours vs. 387.4 ± 3.0 pg/ml at 24 hour post sham respectively, comparison between times within groups by post hoc analysis confirming the increase occurred at 24 hours in both injured and sham groups) over the 24 hour period.

A



B

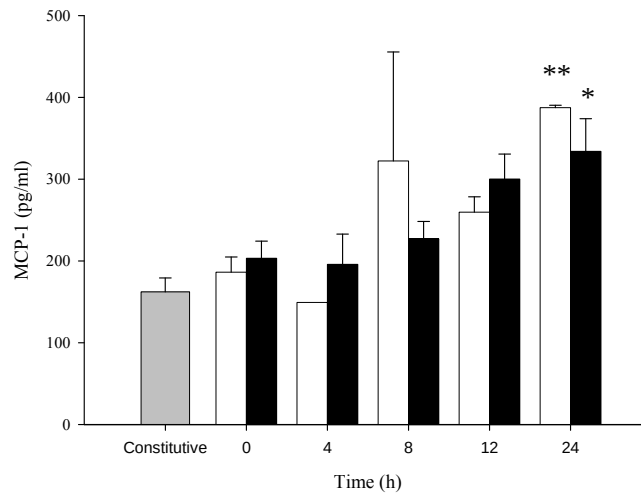


Figure 3.18. Serum Concentration of MIP-2/CXCL2 and MCP-1/CCL2 after Lateral Fluid Percussion Injury.

For both MIP-2/CXCL2 (A) and MCP-1/CCL2 (B) protein concentrations in the serum of injured animals were indistinguishable from those of shams over 24 hours. However there was a significant increase in MCP-1/CCL2 at 24 hours in both injured and sham animals. Serum chemokine concentrations from 2 non-operated animals are shown for comparison (grey filled columns). Fluid percussion injury (■) and sham (□). For MIP-2/CXCL2 data is shown as median and maximum values. The lower limit of detection of the MIP-2/CXCL2 ELISA was 1pg/ml and for clarity this value is substituted for those samples in which MIP-2/CXCL2 was not detected. For MCP-1/CCL2 data is shown as mean and SEM. * $p < 0.05$ for 24 hours post injury vs. 0, 4 and 8 hours post injury. ** $p < 0.05$ for 24 hours post sham vs. 0 and 4 hours post sham.

3.5.4. Inhibition of Chemokine Expression by Dexamethasone and FK506

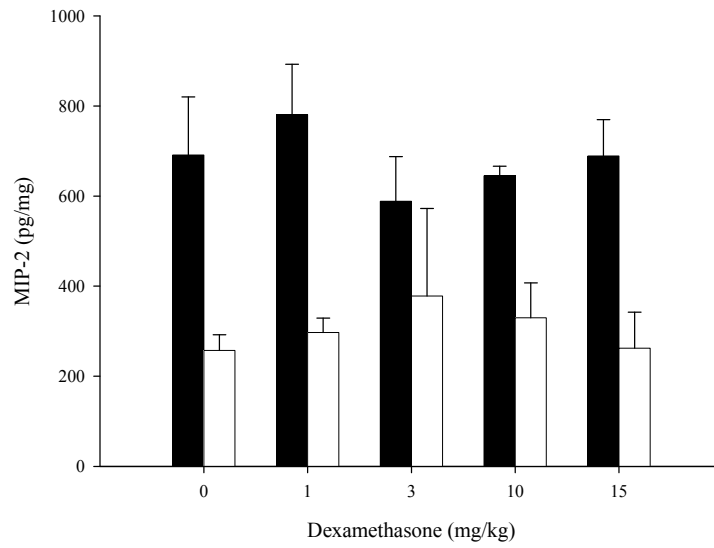
The effect of the anti-inflammatory glucocorticoid dexamethasone or the neuroprotectant FK506 on chemokine expression after moderate LFPI was investigated. Dexamethasone was administered (0, 1, 3, 10 or 15 mg/kg IP) immediately after injury, to 3 animals per dose. Additionally 2 animals randomised to receive saline (Dexamethasone 0 mg/kg) died immediately after injury and were not included in the analysis. FK506 (0 or 1mg/kg IP) was administered 30 minutes prior to injury, 3 animals per dose. For FK506, 1mg/kg IP 30 minutes prior to injury had previously been found to be neuroprotective for ischaemic injury in both rats and mice (Butcher et al., 1997, McCarter et al., 2001). Based on the timing of the peak cortical MIP-2/CXCL2 and MCP-1/CCL2 protein concentrations, animals were sacrificed at 4 or 9 hours after injury and cortical MIP-2/CXCL2 or MCP-1/CCL2 determined by ELISA.

The ipsilateral cortical MCP-1/CCL2 concentration at 9 hours was inhibited in a dose dependant manner by dexamethasone (83.9 ± 18.0 pg/mg, 43.9 ± 2.5 pg/mg, 31.0 ± 4.7 pg/mg, 30.1 ± 2.8 pg/mg and 30.8 ± 8.3 pg/mg for 0, 1, 3, 10 and 15 mg/kg respectively, comparison between ipsilateral and contralateral cortex at individual dexamethasone doses by post hoc analysis confirming that the difference occurred in the ipsilateral cortex between 1 and 15mg/kg of dexamethasone) (Figure 3.19). Conversely at 4 hours post injury no inhibition of ipsilateral cortical MIP-2/CXCL2 was found (691.0 ± 129.1 pg/mg, 780.6 ± 111.9 pg/mg, 588.3 ± 99.5 pg/mg, 644.7 ± 21.4 pg/mg and 689.2 ± 80.7 pg/mg for 0, 1, 3, 10 and 15 mg/kg respectively) (Figure 3.19). Increased cortical IL-1 β has been reported following experimental TBI (Fan et al., 1995, Taupin et al., 1993). In order to support the penetration of significant steroid into the CNS at the time points chosen, the concentration of IL-1 β protein was also measured in the cortical samples. IL-1 β protein concentration was significantly reduced in the ipsilateral cortex at both 4 (216.7 ± 51.5 pg/mg, 100.0 ± 21.5 pg/mg, 58.5 ± 18.9 pg/mg, 43.6 ± 6.6 pg/mg and 48.9 ± 8.4 pg/ml for 0, 1, 3, 10 and 15 mg/kg of dexamethasone respectively, comparison between the ipsilateral and contralateral cortex at individual dexamethasone doses by post hoc analysis confirming that the difference occurred in the ipsilateral cortex between 1 and 15mg/kg of dexamethasone) and 9 hours (146.6 ± 52.0 pg/mg, 41.3 ± 12.7 pg/mg, 28.9 ± 13.5 pg/mg, 18.2 ± 4.0 pg/mg and 25.9 ± 6.3 pg/mg of dexamethasone 0, 1, 3, 10 and 15 mg/kg respectively, comparison

between the ipsilateral and contralateral cortex at individual dexamethasone doses by post hoc analysis confirming that the difference occurred in the ipsilateral cortex between 1 and 15mg/kg of dexamethasone) after LFPI for all the doses of dexamethasone tested (Figure 3.20) suggesting that an effective anti-inflammatory concentration of the drug was present in the cortex at these time points.

FK506 (1mg/kg) did not reduced the concentration of either MIP-2/CXCL2 in the ipsilateral cortex at 4 hours (814.0 ± 132.2 pg/ml vs. 1059.2 ± 187.9 pg/ml, FK506 vs. vehicle) or MCP-1/CCL2 at 9 hours (40.8 ± 2.0 pg/ml vs. 37.9 ± 0.8 pg/ml, FK506 vs. vehicle) post injury (Figure 3.21). Although the IL-1 β concentration was also not reduced significantly in the ipsilateral cortex by FK506 at 4 hours post injury (252.5 ± 49.3 pg/mg vs. 308.3 ± 23.0 pg/ml, FK506 vs. vehicle) it was significantly increased at 9 hours post injury (145.2 ± 11.0 pg/mg vs. 96.2 ± 12.5 pg/ml, FK506 vs. vehicle, comparison between ipsilateral and contralateral cortex by post hoc analysis confirming that the difference occurred in the ipsilateral cortex with FK506) (Figure 3.22).

A



B

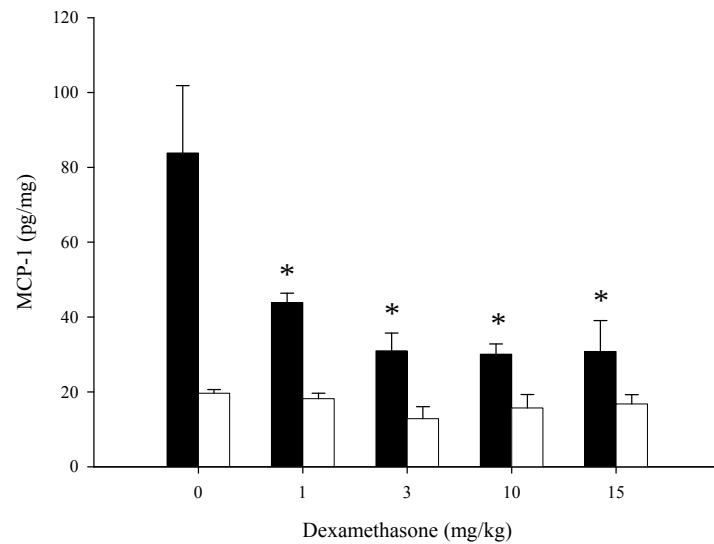
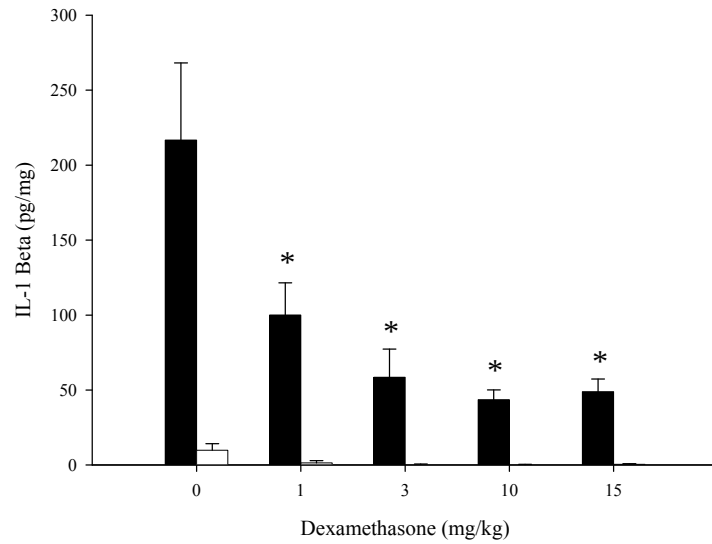


Figure 3.19. Modulation of Cortical MIP-2/CXCL2 and MCP-1/CCL2 by Dexamethasone after Lateral Fluid Percussion Injury.

Animals were then sacrificed 4 or 9 hours after injury for the analysis of cortical MIP-2/CXCL2 or MCP-1/CCL2 respectively. (A) MIP-2/CXCL2 protein concentration was not reduced in the ipsilateral cortex at any of the doses tested. (B) MCP-1/CCL2 protein concentration was significantly reduced in the ipsilateral cortex by all the doses tested. Ipsilateral cortex (Black bars), contralateral cortex (White bars). * $p < 0.05$ vs. vehicle (0mg/kg).

A



B

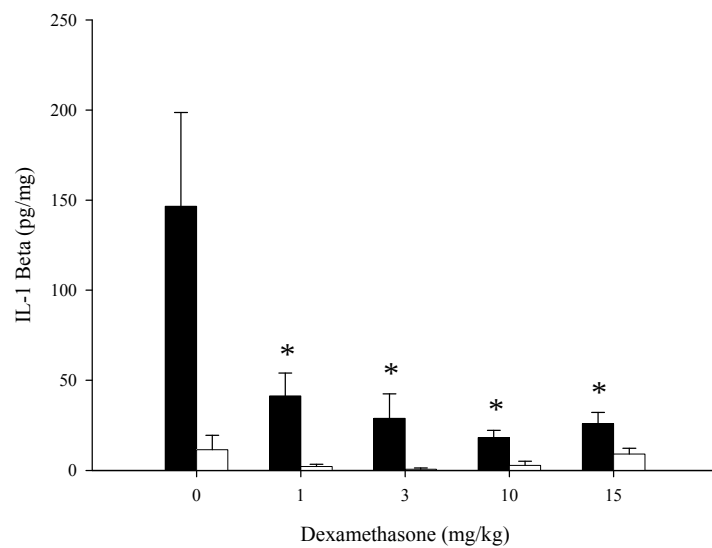
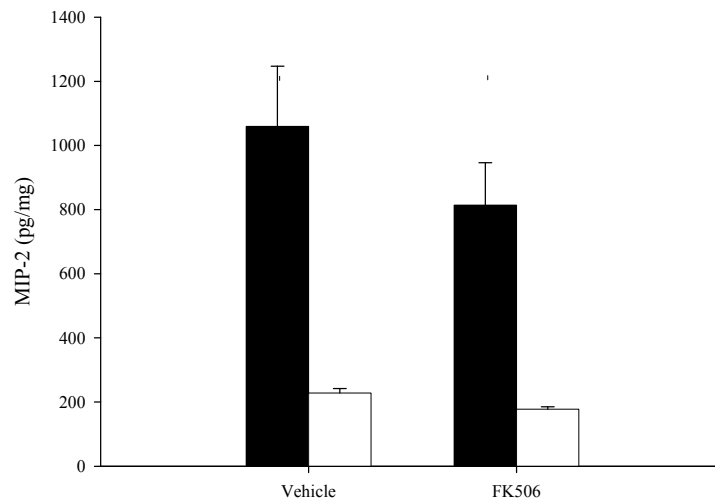


Figure 3.20. Inhibition of IL-1 β by Dexamethasone after Lateral Fluid Percussion Injury.

Cortical IL-1 β concentration in the ipsilateral cortex was significantly inhibited at 4 (A) and 9 hours (B) by all the doses tested. Ipsilateral cortex (Black bars), contralateral cortex (White bars). * $p < 0.05$ vs. vehicle (0mg/kg).

A



B

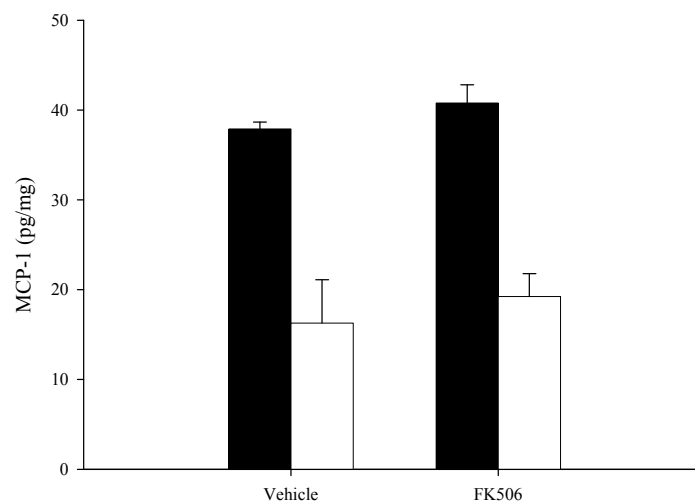
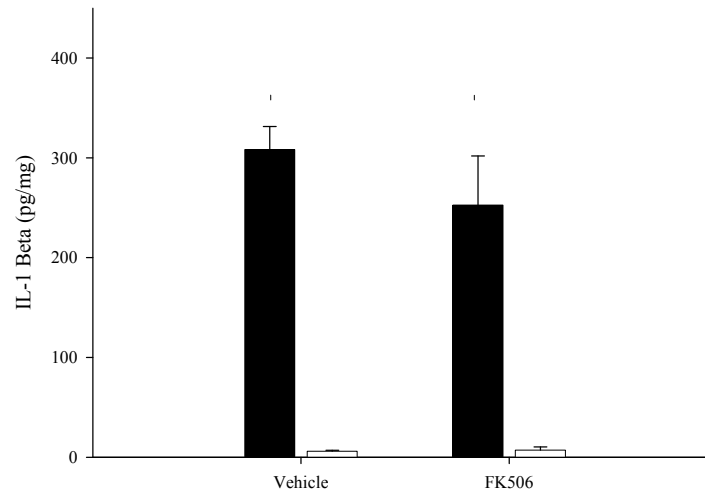


Figure 3.21. The Effect of FK506 on Cortical MIP-2/CXCL2 and MCP-1/CCL2 after Lateral Fluid Percussion Injury.

Animals were sacrificed 4 or 9 hours after injury for the analysis of cortical MIP-2/CXCL2 or MCP-1/CCL2 respectively. Neither MIP-2/CXCL2 (A) nor MCP-1/CCL2 (B) protein concentrations were inhibited by FK506. Ipsilateral cortex (Black bars), contralateral cortex (White bars).

A



B

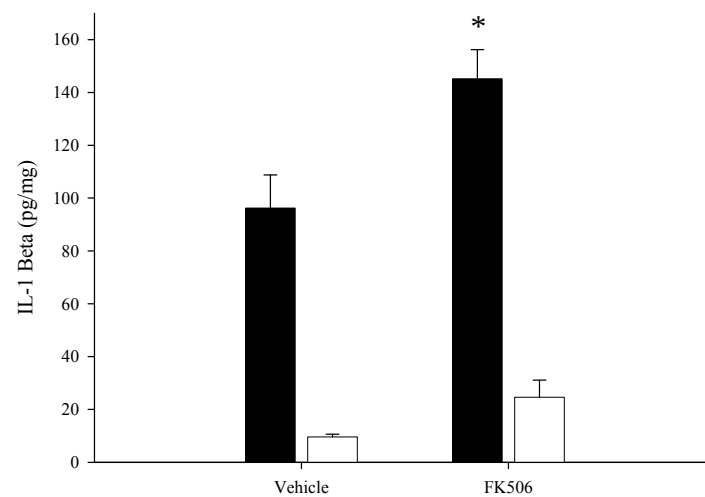


Figure 3.22. The Effect of FK506 on IL-1 β Protein Concentration after Lateral Fluid Percussion Injury.

Ipsilateral cortical IL-1 β concentration was not changed compared to vehicle at 4 hours (A) but was significantly increased over vehicle at 9 hours (B) post injury. Ipsilateral cortex (Black bars), contralateral cortex (White bars). * $p < 0.05$ vs. vehicle (0mg/kg).

3.5.5. Histopathology and Immunohistochemistry

3.5.5.1. Cresyl Violet & Acid Fuchsin

Cresyl violet and acid fuchsin staining facilitates the identification of the contused tissue and was performed on fixed or frozen tissue sections from each animal used in the immunohistochemistry studies, to map the extent of the lesion. As described for frozen tissue (3.5.1.2), in fixed tissue traumatic subarachnoid blood, cerebral laceration and haemorrhage into the corpus callosum and external capsule, lateral ventricle and hippocampal fissure was present. In each animal the cortical injury was defined by the loss of cresyl violet staining and hypocellularity. Acid fuchsin identified intensely pink staining damaged neurones, within the contusion, with shrunken nuclei contracted cytoplasm and surrounding vacuolation (Figures 3.33, 3.34, 3.40 & 3.41). These damaged cells were also seen in the CA2/3 region of the ipsilateral hippocampus (Figures 3.35 & 3.42). These findings are consistent with previous reports of the histology of LFPI (Cortez et al., 1989, Hicks et al., 1996).

3.5.5.2. Immunohistochemistry for Myeloperoxidase and CD11b

In order to confirm that the pattern of leucocyte infiltration after LFPI was the same as that described in the literature, coronal brain sections were stained for MPO and CD11b.

3.5.5.2.1. *Myeloperoxidase*

MPO staining was performed in coronal brain sections from 3 animals (2 frozen tissue and 1 paraformaldehyde fixed) all sacrificed at 24 hours post LFPI, corresponding to the time when numerous neutrophils have been identified entering the cerebral parenchyma (Biagas et al., 1992, Clark et al., 1994, Holmin et al., 1995, Soares et al., 1995). With both fixation methods numerous MPO positive cells were present in the ipsilateral cortex and absent from the contralateral side (Figure 3.23). The MPO staining was clearest in the paraformaldehyde fixed tissue.

3.5.5.2.2. *CD11b*

Immunohistochemistry was performed on frozen coronal brain sections from 2 animals, sacrificed at 4 and 24 hours after LFPI. Four hours after injury only occasional small densely staining CD11b positive cells, probably representing tissue macrophages were visible in the cortex. These were seen bilaterally. At this early time point many positive cells were also seen in the menigeal layer below the ipsilateral hippocampus (Figure 3.24). By 24 hours after injury numerous positive cells could be seen in the ipsilateral cortex. These had 2 distinct morphologies. Small densely staining cells probably representing infiltrating circulating monocytes were mixed with larger more diffusely staining activated microglia. Some less intensely staining microglia were also seen in the contralateral hemisphere (Figure 3.25). The accumulation of macrophages in the injured cortex, visible from 24 hours, has been described previously after LFPI (Soares et al., 1995).

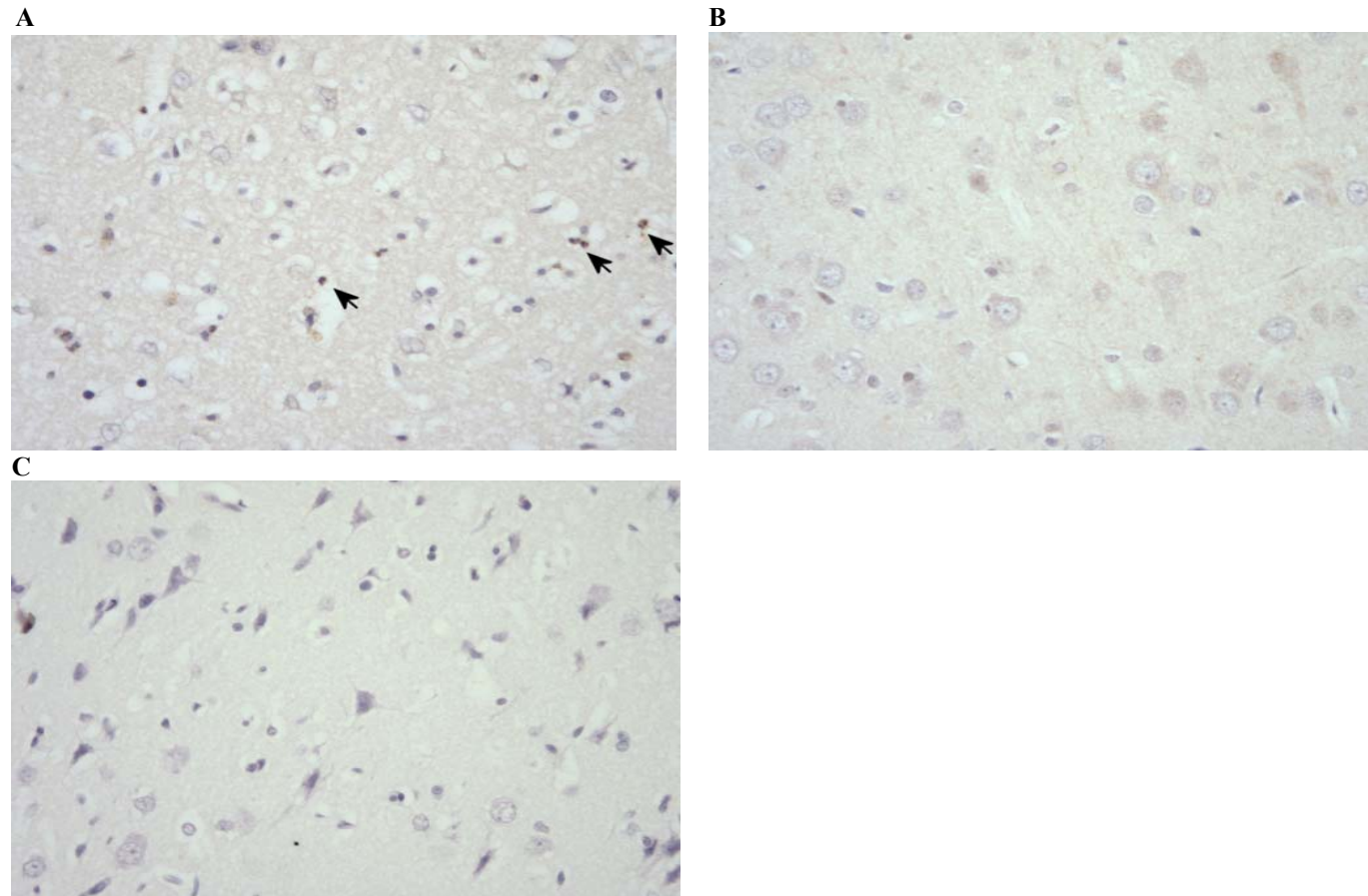


Figure 3.23. Cortical Myeloperoxidase (MPO) Staining 24 Hours after Lateral Fluid Percussion Injury

(A) Numerous positive cells are present in the ipsilateral cortex (Arrows). (B) Contralateral cortex. (C) Ipsilateral cortex with omission of the MPO primary. Visualised with DAB and counter-stained with haematoxylin. Paraformaldehyde fixed tissue. All x 200.

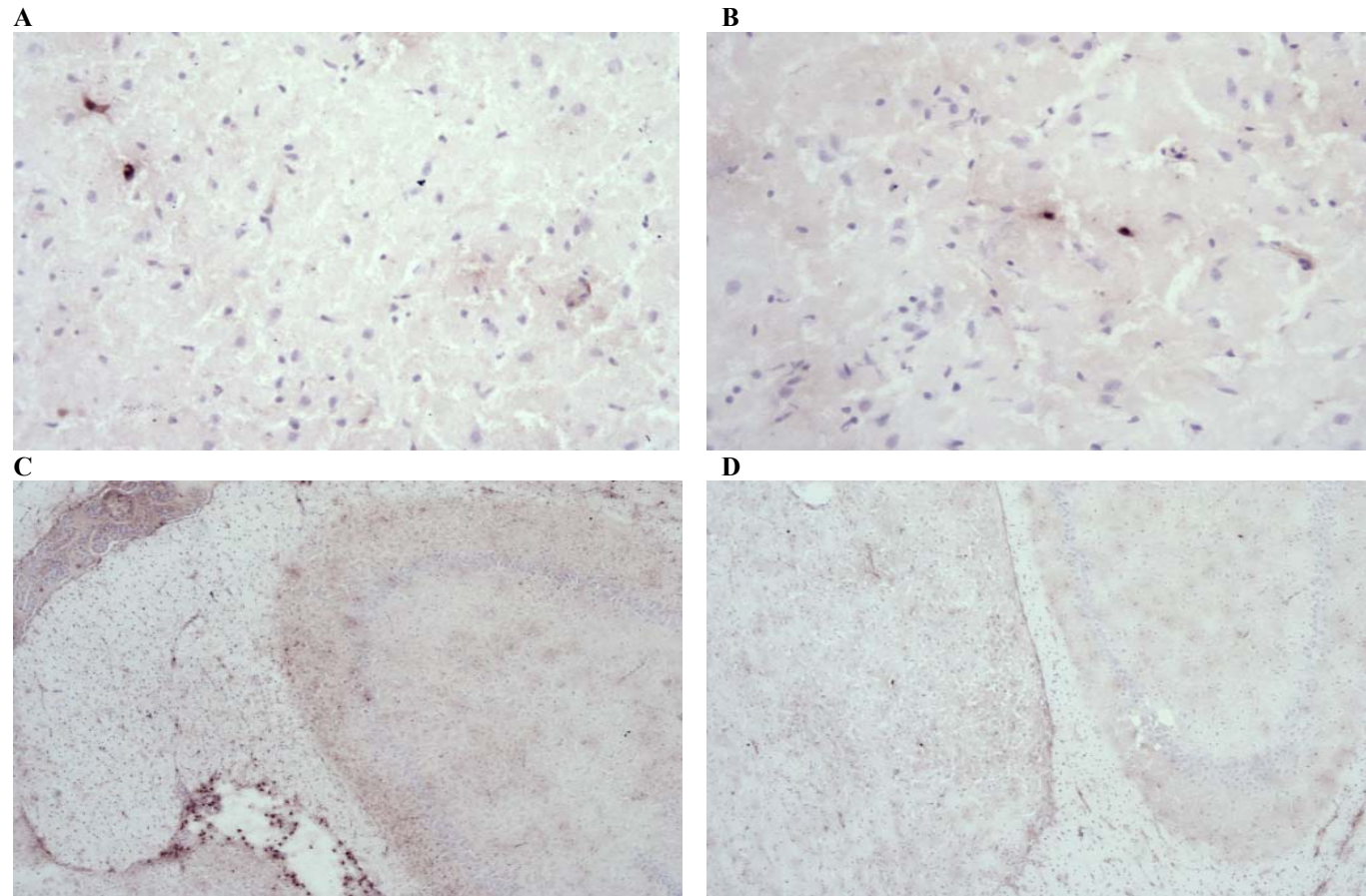


Figure 3.24. CD11b Staining 4 hours Post Lateral Fluid Percussion Injury

(A) Occasional positive cells in the ipsilateral and (B) contralateral cortex. (C) Around the ipsilateral hippocampus positive cells are located mainly in the sub hippocampal fissure. (D) Contralateral hippocampus. Visualised with VIP and counter stained with haematoxylin. Unfixed frozen tissue. A&B x200, C&D x40.

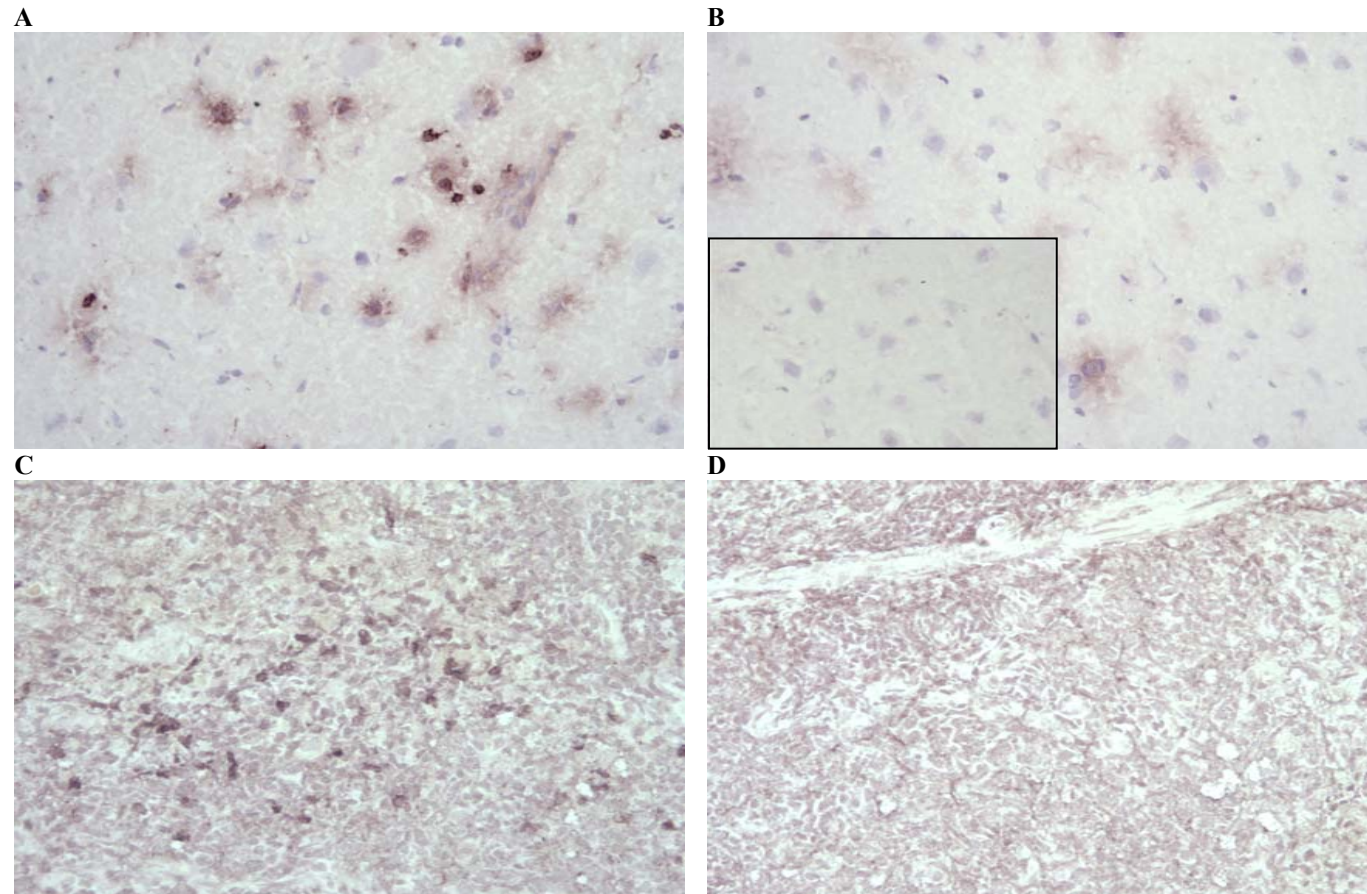


Figure 3.25. CD11b Staining 24 Hours Post Lateral Fluid Percussion Injury.

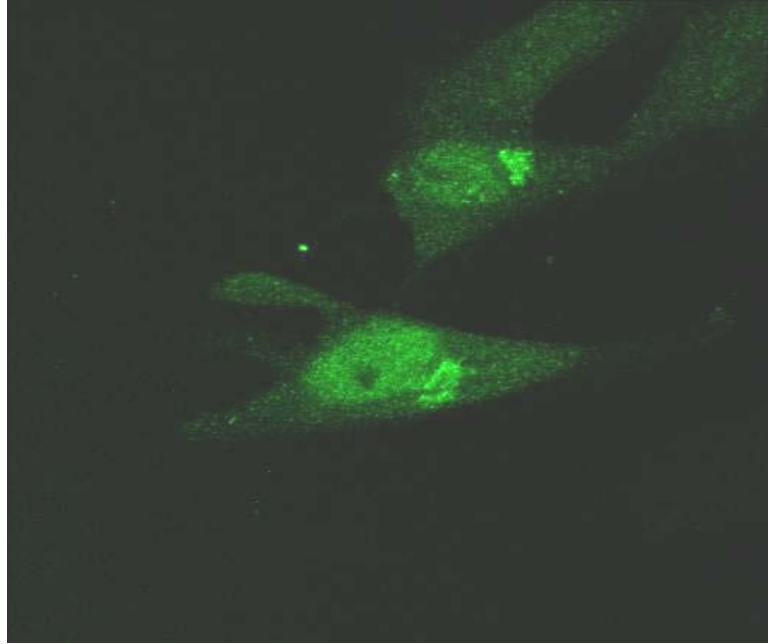
(A) Numerous positive cells in the ipsilateral cortex consist of 2 types, with small densely staining macrophages discernable from larger more diffusely staining microglia. (B) Contralateral cortex with some activated microglia (Insert, omission of primary). (C) Section of rat spleen as a CD11b positive control. (D) Spleen with omission of CD11b primary. Visualised with VIP and counter stained with haematoxylin. Frozen unfixed tissue. All x 200.

3.5.5.3. Development of a Protocol for Chemokine Immunohistochemistry

3.5.5.3.1. *Identification of Cellular MIP-2/CXCL-2 in CTX-TNA2 Astrocytes Using the Biosource anti-MIP-2/CXCL2 Antibody and a Fluorescent Secondary Antibody*

Rat CTX-TNA2 astrocytes produce MIP-2/CXCL2 when cultured in the presence of TNF- α (2.4.1.2). In stimulated cells immunocytochemistry with the MIP-2/CXCL2 antibody revealed discrete positive bodies adjacent to the nuclei which were much less prominent in untreated control cells (Figures 3.26 & 3.27). The images from the confocal microscope were stored and transported on recordable compact discs. Unfortunately some the data on one disc were corrupted. The damaged data were recovered, with partial success, using Badcopy Pro (Jufsoft Brandenton, USA). This confirmed the lack of MIP-2/CXCL2 staining with omission of the primary antibody. Overall this experiment suggested that the MIP-2/CXCL2 antibody might be used to identify MIP-2/CXCL2 staining in tissue.

A



B

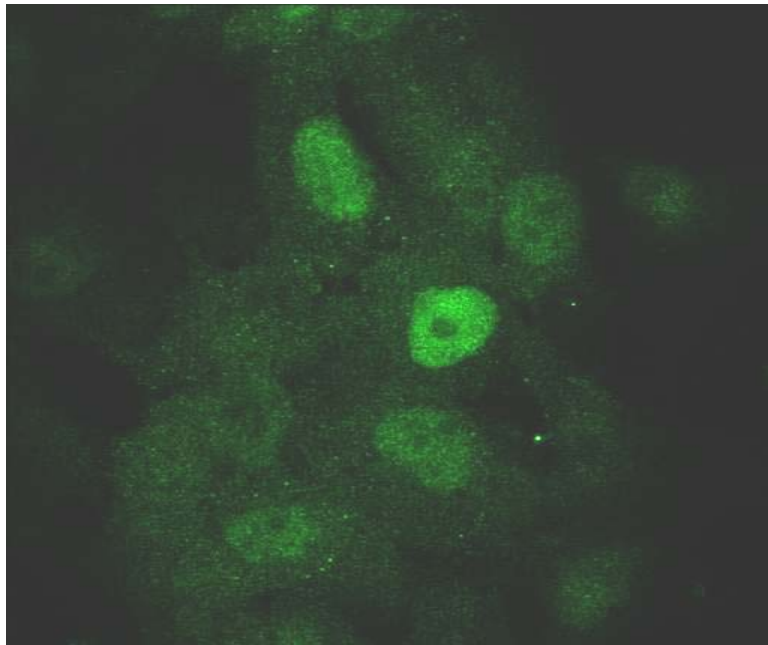


Figure 3.26. Immunocytochemistry for MIP-2/CXCL-2 in TNF- α Stimulated Rat CTX-TNA2 Astrocytes.

(A) Discrete MIP-2/CXCL2 staining adjacent to the nuclei is apparent in the CTX-TNA2 cells cultured with TNF- α (20ng/ml) for 24 hours. (B) CTX-TNA2 cells cultured in the absence of TNF- α . Visualised with Alexa Fluro® 488. A x1200, B x960.

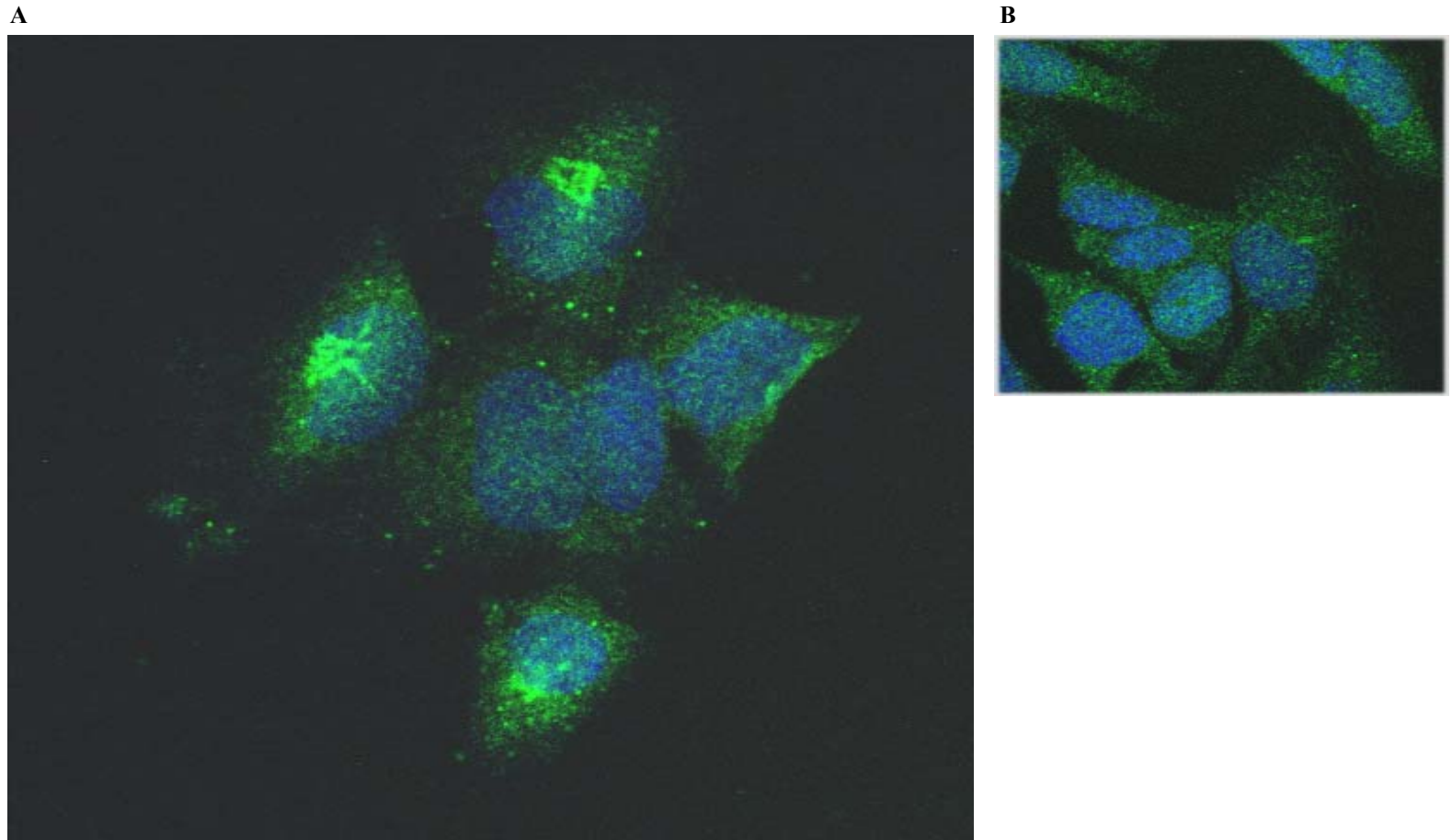


Figure 3.27. Immunocytochemistry for MIP-2/CXCL-2 in TNF- α Stimulated Rat CTX-TNA2 Astrocytes.

(A) Discrete MIP-2/CXCL-2 staining adjacent to the nuclei is apparent in CTX-TNA2 cells cultured with TNF- α (20ng/ml) for 24 hours. (B) CTX-TNA2 cells cultured in the absence of TNF- α . Visualised with Alexa Fluro® 488 and counterstained with TO-PRO®-3. A x1200, B x900 & C x 500.

3.5.5.3.2. *Immunohistochemistry for MIP-2/CXCL2 After Lateral Fluid Percussion Injury; unfixed tissue and visualisation with a fluorescent secondary antibody*

Immunohistochemistry for MIP-2/CXCL2 was attempted in cryostat coronal brain sections from rats sacrificed 4 hours after LFPI. The 4 hour time point was chosen as this was the time of maximal MIP-2/CXCL2 protein concentration in the cortical homogenates (3.5.3.1). Overall the results were disappointing. Only a very weak background MIP-2/CXCL2 signal could be seen and in the absence of a positive control it was unclear if any cells were positively staining for MIP-2/CXCL2. Furthermore there was no difference in staining between the ipsilateral and contralateral cortex. Analysis of the slides was further impeded by rapid fading of the Alexa Fluoro® 488 (Figure 3.28).

This prompted a further review of the literature where I discovered a reference to chemokine expression in experimental African Trypanosomiasis (Sleeping sickness). The Biosource MIP-2/CXCL2 antibody had been used to stain for MIP-2/CXCL2 expressing cells in cryosections of cerebral tissue (Sharafeldin et al., 2000). To visualise the signal a biotinylated secondary antibody was used with an avidin/biotinylated peroxidase enzyme complex. I therefore contacted one of the authors of this paper (Mikhail Pashenkov) to discuss a protocol for MIP-2/CXCL2 staining. Two tissue fixation methods were suggested. MIP-2/CXCL2 staining with the Biosource antibody and a biotinylated secondary was then attempted and the effect of the different fixation methods on the quality of the staining compared.

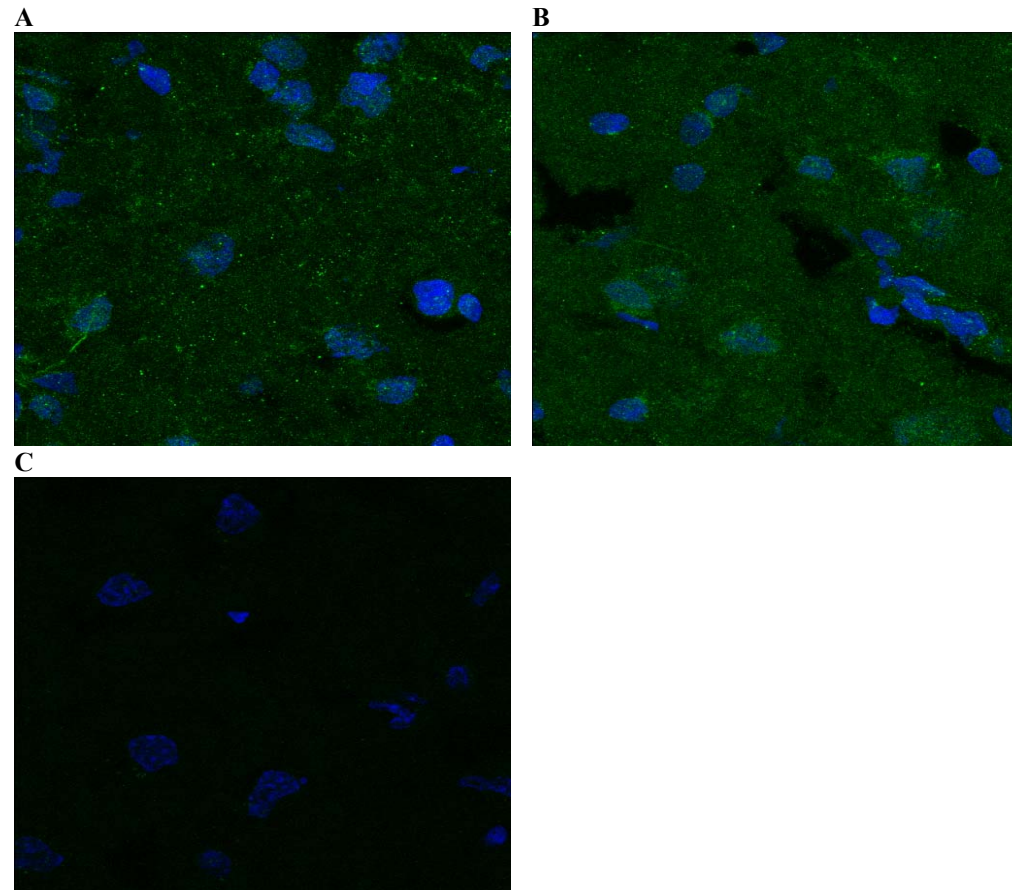


Figure 3.28. Immunohistochemistry for MIP-2/CXCL2 4 hours after Lateral Fluid Percussion Injury.

(A) Ipsilateral and (B) contralateral cortex. (C) Ipsilateral cortex with omission of MIP-2/CXCL2 primary antibody. Visualised with Alexa Fluro® 488 and counterstained with TO-PRO®-3. Unfixed frozen tissue. All x1200.

3.5.5.3.3. *Immunohistochemistry for MIP-2/CXCL2 After Lateral Fluid Percussion Injury; unfixed tissue and visualisation with a biotinylated secondary antibody*

In cryostat sections of a rat brain sacrificed 4 hours after lateral FPI densely staining MIP-2/CXCL2 positive cells could be seen in the ipsilateral cortex and the CA2/3 pyramidal cell layer of the hippocampus with both fixation protocols (Figures 3.29-3.32). The distribution of these cells was the same as that of the acidophilic neurones seen on cresyl violet and acid fuchsin histology (Figures 3.11 & 3.12). These cells also had condensed nuclei and contracted cytoplasm suggesting further that they were neurons. Compared with protocol 1 the sections fixed with protocol 2 had less background DAB staining. Furthermore less intensely staining cells in the contralateral cortex and contralateral pyramidal cell layer of the hippocampus could be discriminated (Figure 3.31 & 3.32).

MIP-2/CXCL2 expression was subsequently investigated in brain tissue from 5 animals (3 trauma and 2 shams) fixed with protocol 2. The intensity of the staining was rated on a simple subjective scale, as absent (-), light (+), moderate (++) or dense (+++). The contralateral tissue was used as a reference between animals. The distribution and intensity of MIP-2/CXCL2 staining in these animals was the same as described and is summarised in table 3.1.

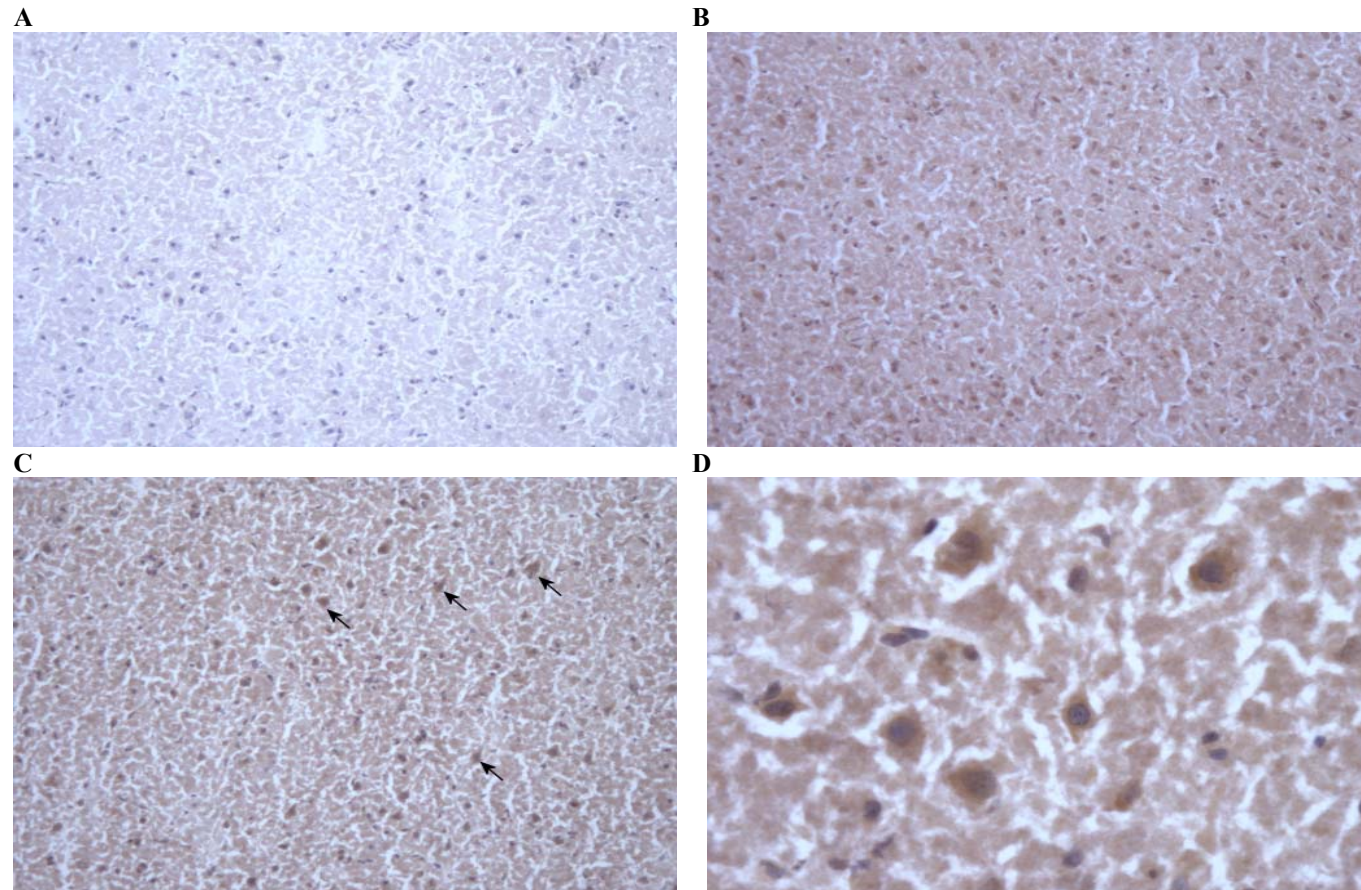


Figure 3.29. Immunohistochemistry for MIP-2/CXCL2 in the Cortex 4 Hours after Lateral Fluid Percussion Injury, Fixation Protocol 1.

(A) Ipsilateral cortex, no primary antibody. (B) Contralateral cortex. (C) Ipsilateral cortex, densely staining MIP-2/CXCL2 positive cells can be seen (Arrows). (D) Higher power view of C. Visualised with DAB and counter stained with haematoxylin. Unfixed frozen tissue. A, B & C x 100. D x 400.

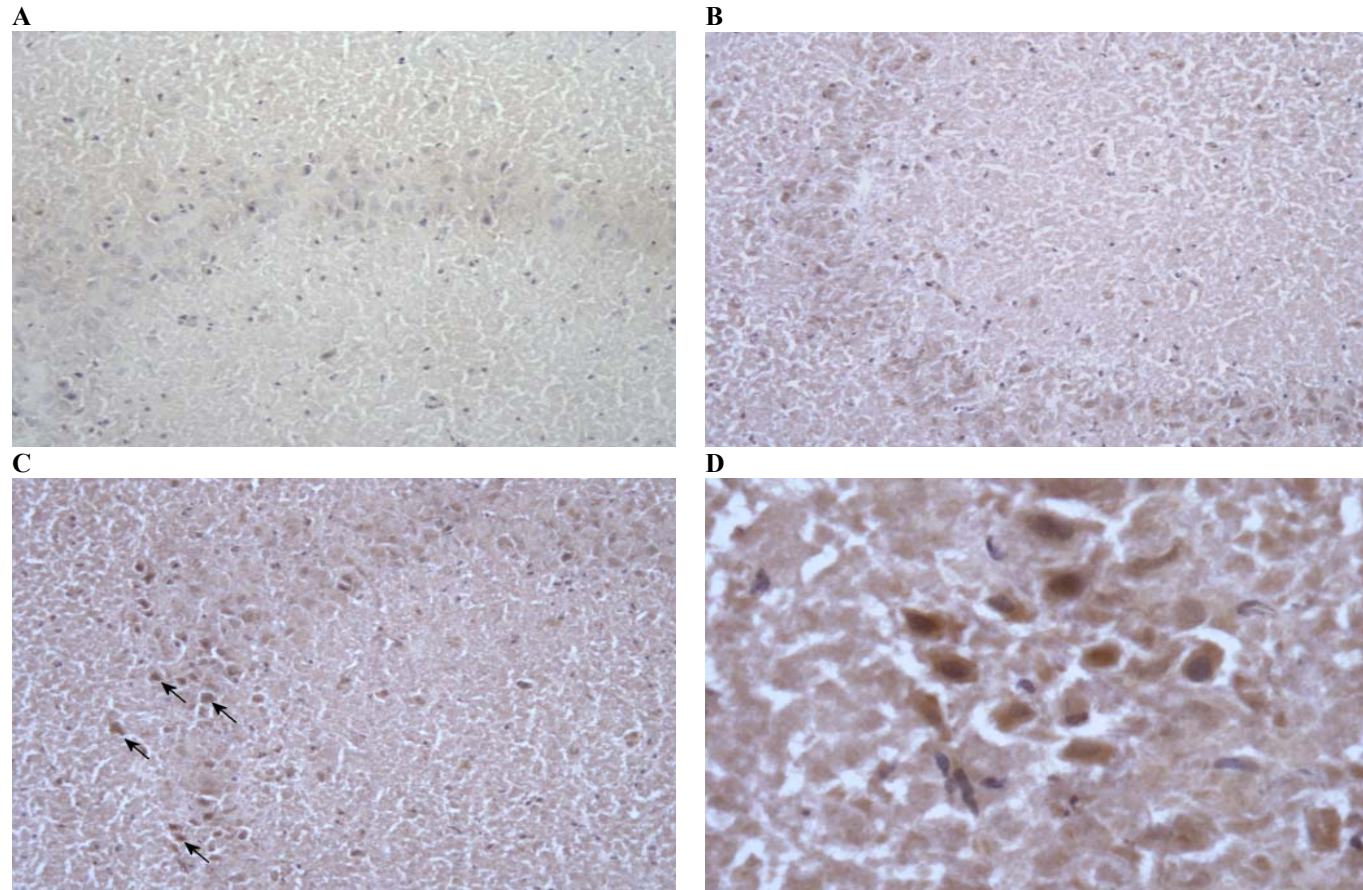


Figure 3.30. Immunohistochemistry for MIP-2/CXCL2 in the Hippocampus 4 hours after Lateral Fluid Percussion Injury, Fixation Protocol 1.

A) Ipsilateral hippocampus, no primary antibody. (B) Contralateral hippocampus. (C) Ipsilateral hippocampus, densely staining MIP-2/CXCL2 positive cells can be seen (Arrows) in the CA2/3 pyramidal cell layer. (D) Higher power view of C. Visualised with DAB and counterstained with haematoxylin. Unfixed frozen tissue. A, B & C x 100. D x 400.

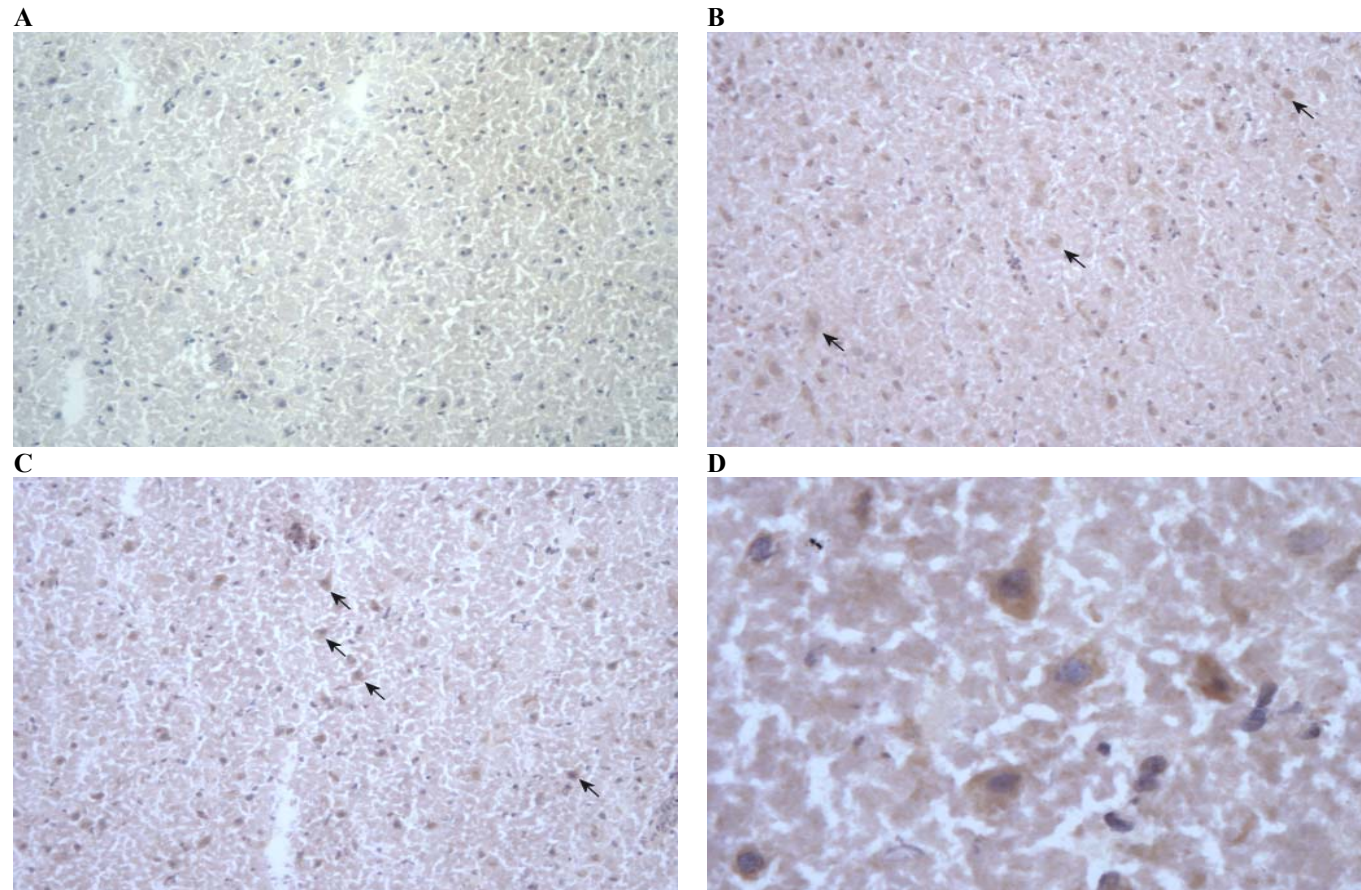


Figure 3.31. Immunohistochemistry for MIP-2/CXCL2 in the Cortex 4 Hours after Lateral Fluid Percussion Injury, Fixation Protocol 2.

(A) Ipsilateral cortex, no primary antibody. (B) Contralateral cortex, low intensity MIP-2/CXCL2 positive cells (Arrows) are visible. (C) Ipsilateral cortex, densely staining MIP-2/CXCL2 positive cells can be seen (Arrows). (D) Higher power view of C. Visualised with DAB and counter stained with haematoxylin. Unfixed frozen tissue. A, B & C x 100. D x 400.

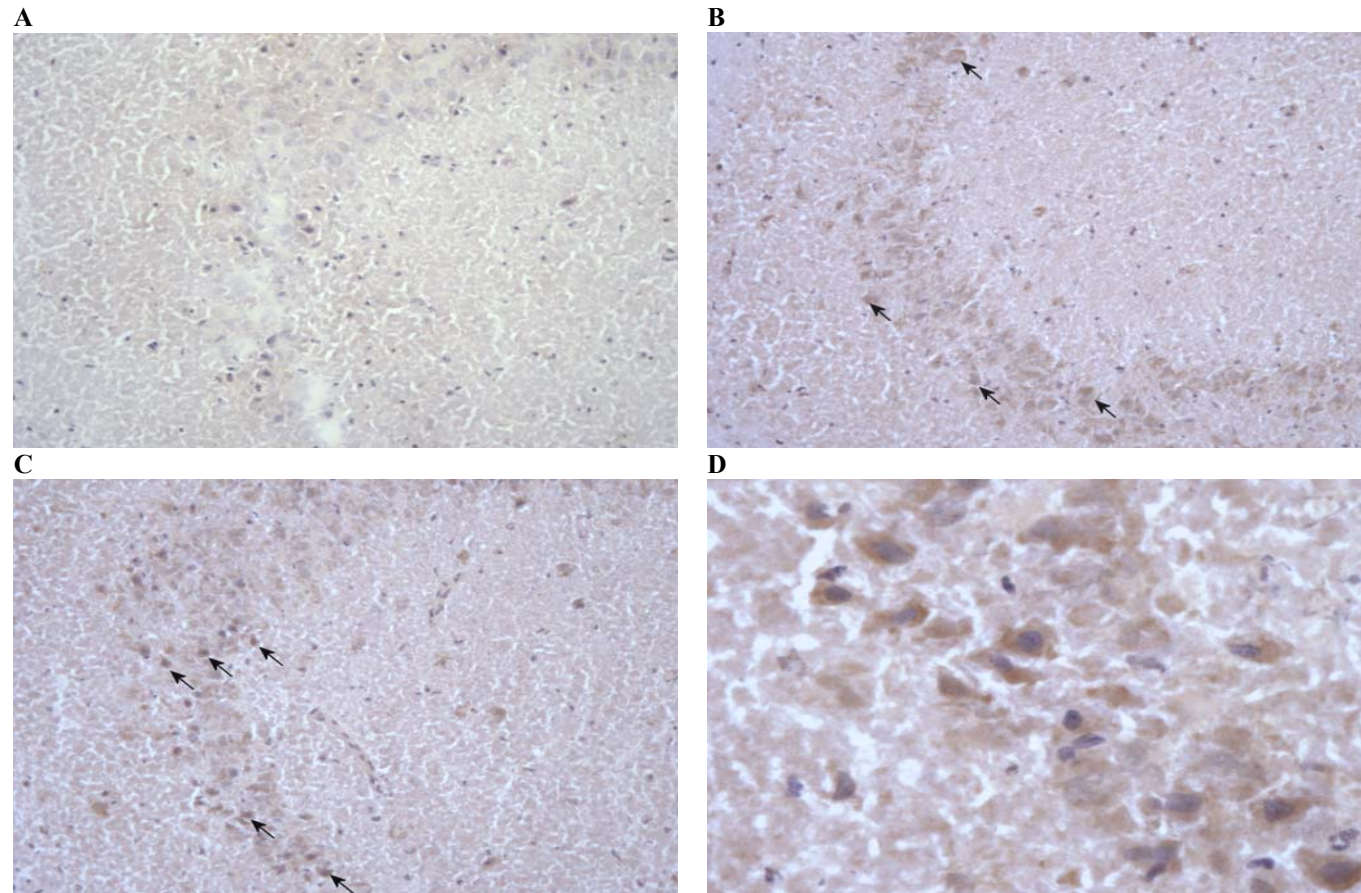


Figure 3.32. Immunohistochemistry for MIP-2/CXCL2 in the Hippocampus 4 hours after Lateral Fluid Percussion Injury, Fixation Protocol 2.

(A) Ipsilateral hippocampus, no primary antibody. (B) Contralateral hippocampus, low intensity MIP-2/CXCL2 positive cells (Arrows) are visible. (C) Ipsilateral hippocampus, densely staining MIP-2/CXCL2 positive cells can be seen (Arrows). (D) Higher power view of C. Visualised with DAB and counter stained with haematoxylin. Unfixed frozen tissue. A, B & C x 100. D x 400.

Number		Ipsilateral		Contralateral	
		Cortex	Hippocampus	Cortex	Hippocampus
1	Truama	+++	+++	++	+
2	Truama	+++	+++	+	+
3	Truama	+++	+++	+	+
4	Sham	-/+	+	+	+
5	Sham	+	+	+	+

Table 3.1. The Intensity of MIP-2/CXCL2 Staining in Unfixed Frozen Tissue 4 Hours after Lateral Fluid Percussion Injury

Staining intensity scored as absent (-), light (+), moderate (++) and dense (+++). MIP-2/CXCL2 staining was increased in the cortical contusion and CA2/3 pyramidal cell layer of the ipsilateral hippocampus after LFPI.

3.5.5.3.4. *Immunohistochemistry for MIP-2/CXCL2 After Lateral Fluid Percussion Injury; fixed tissue and visualisation with a biotinylated secondary antibody*

Immunohistochemistry using paraformaldehyde perfused tissue resulted in sections with more specific staining, superior morphology and improved definition when compared with the unfixed cryostat sections. With this method of fixation it was possible to discriminate some of the anatomy of the cerebral cortical layers. In the figures presented outer cortex refers to the molecular (I) and external granular cell (II) layers. Deep cortex refers to the external pyramidal (III), internal granular (IV) and internal pyramidal (V) cell layers.

6 animals (4 injury and 2 shams) underwent LFPI and were sacrificed at 4 hours after injury. MIP-2/CXCL2 staining was clearly visible in both the contralateral hemisphere and hippocampus of injured animals and was the same as that seen in the cortex and hippocampus of shams (Figure 3.33-3.38). This expression was distributed throughout the cortex, particularly in the pyramidal cell layers and external granular layer (Figure 3.33, 3.34, 3.36 & 3.37), and also in the pyramidal cell layer of the hippocampus (Figure 3.35 & 3.38).

Following injury increased MIP-2/CXCL2 expression was visible within the contusion localised to shrunken cells with the morphology of necrotic neurones (Figure 3.33 & 3.34) and these were distributed in the same regions as the acidophilic necrotic neurones seen with cresyl violet and acid fuchsin staining. A similar increase in MIP-2/CXCL2 expression and co-localisation with acidophilic cells was also apparent in the CA2/3 region of the ipsilateral hippocampus (Figure 3.35). The distribution of increased MIP-2/CXCL2 staining is shown in figure 3.39.

The intensity of the staining in each area was rated on a simple scale, as absent (-), light (+), moderate (++) or dense (+++). The contralateral tissue was used as a reference between animals. The distribution and intensity of MIP-2/CXCL2 staining in animals receiving LFPI and shams is summarised in table 3.2.

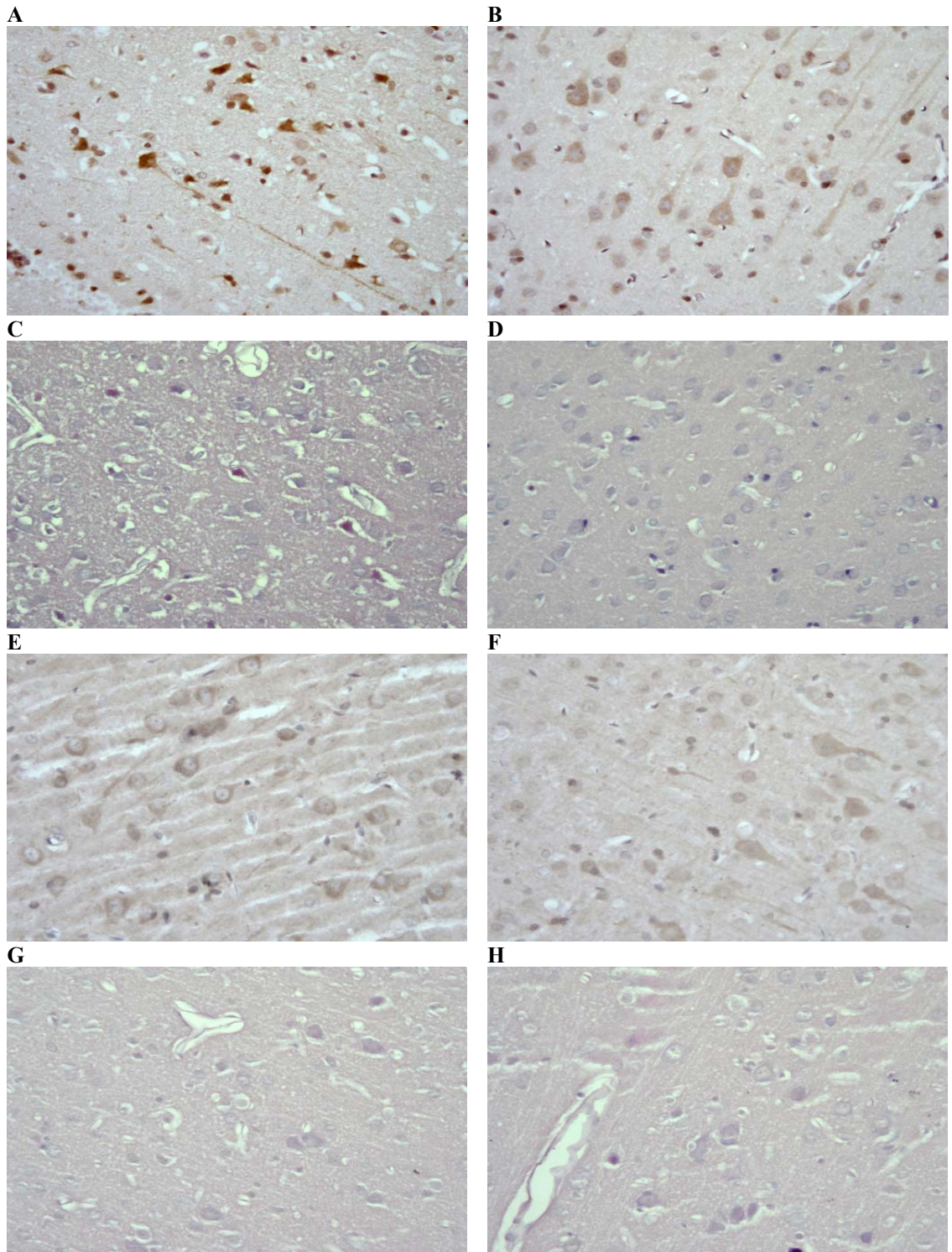


Figure 3.33. Immunohistochemistry for MIP-2/CXCL2 in the Deep Cortex 4 Hours after Fluid Percussion Injury vs. Sham.

Ipsilateral (A, C, E, G) and contralateral deep cortex (B, D, F, H). MIP-2/CXCL2 staining visualized by DAB following FPI (A&B) and sham (E&F). Immunohistochemical sections counterstained with haematoxylin. Cresyl violet and acid fuchsin staining of corresponding sections following FPI (C&D) and sham (G&H). Paraformaldehyde fixed tissue. All x200.

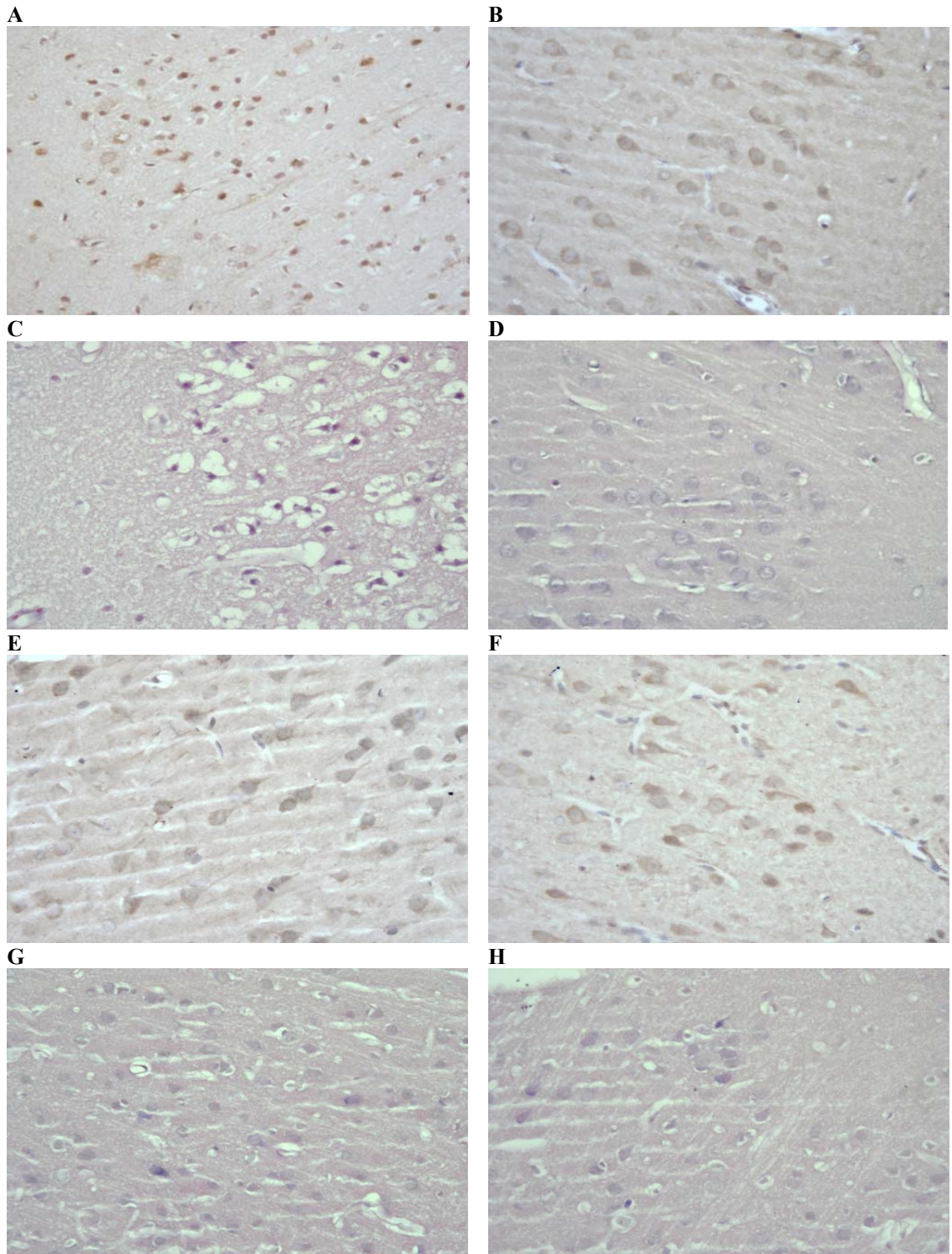


Figure 3.34. Immunohistochemistry for MIP-2/CXCL2 in the Outer Cortex 4 hours after Fluid Percussion Injury vs Sham.

Ipsilateral (A, C, E, G) and contralateral outer cortex (B, D, F, H). MIP-2/CXCL2 staining visualized by DAB following FPI (A&B) and sham (E&F). Immunohistochemical sections counterstained with haematoxylin. Cresyl violet and acid fuchsin staining of corresponding sections following FPI (C&D) and sham (G&H). Paraformaldehyde fixed tissue. All x200.

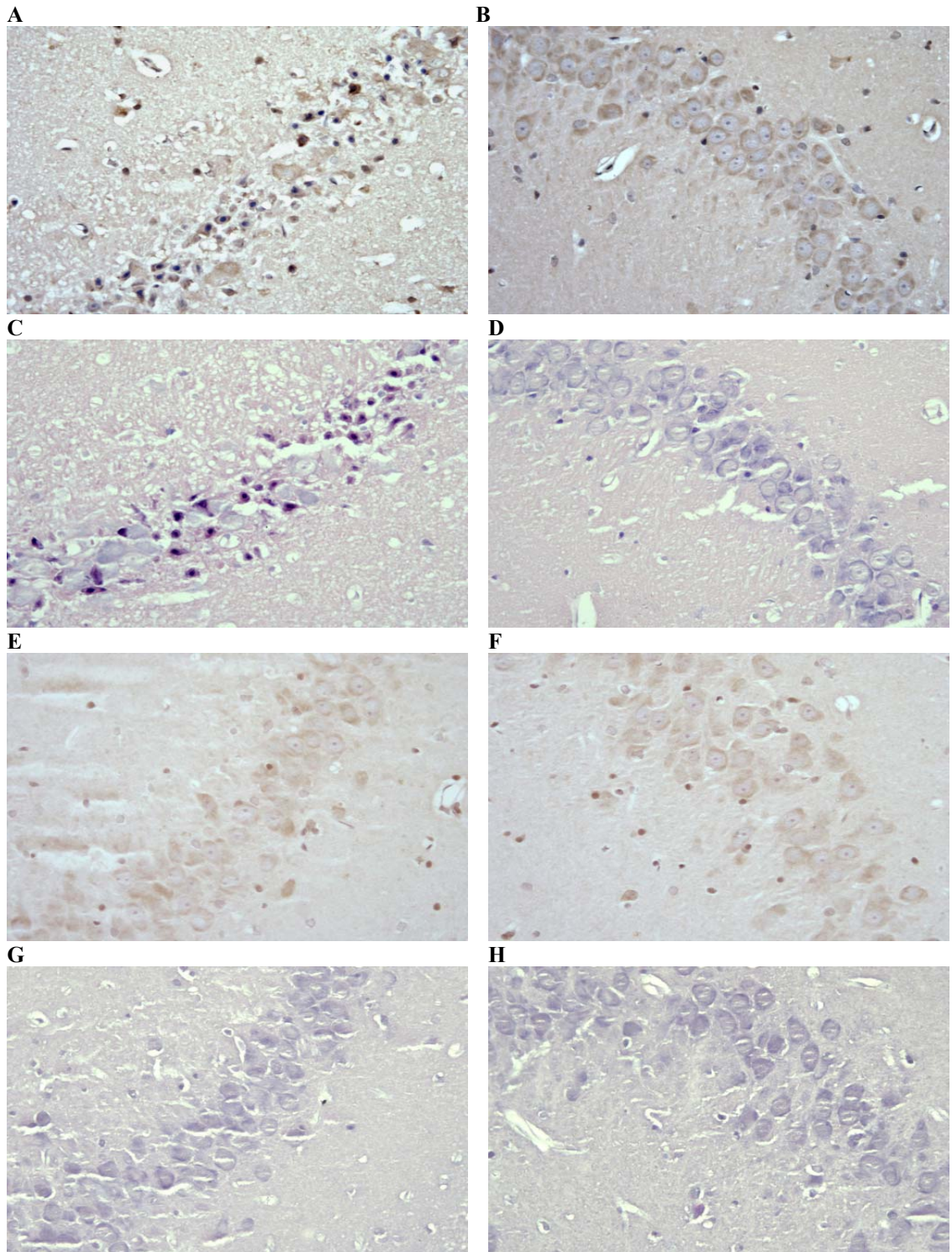


Figure 3.35. Immunohistochemistry for MIP-2/CXCL2 in the Hippocampus 4 Hours after Fluid Percussion Injury vs. Sham.

Ipsilateral (A, C, E, G) and contralateral hippocampus (B, D, F, H). MIP-2/CXCL2 staining visualized by DAB following FPI (A&B) and sham (E&F). Immunohistochemical sections counterstained with haematoxylin. Cresyl violet and acid fuchsin staining of corresponding sections following FPI (C&D) and sham (G&H). Paraformaldehyde fixed tissue. All x200.

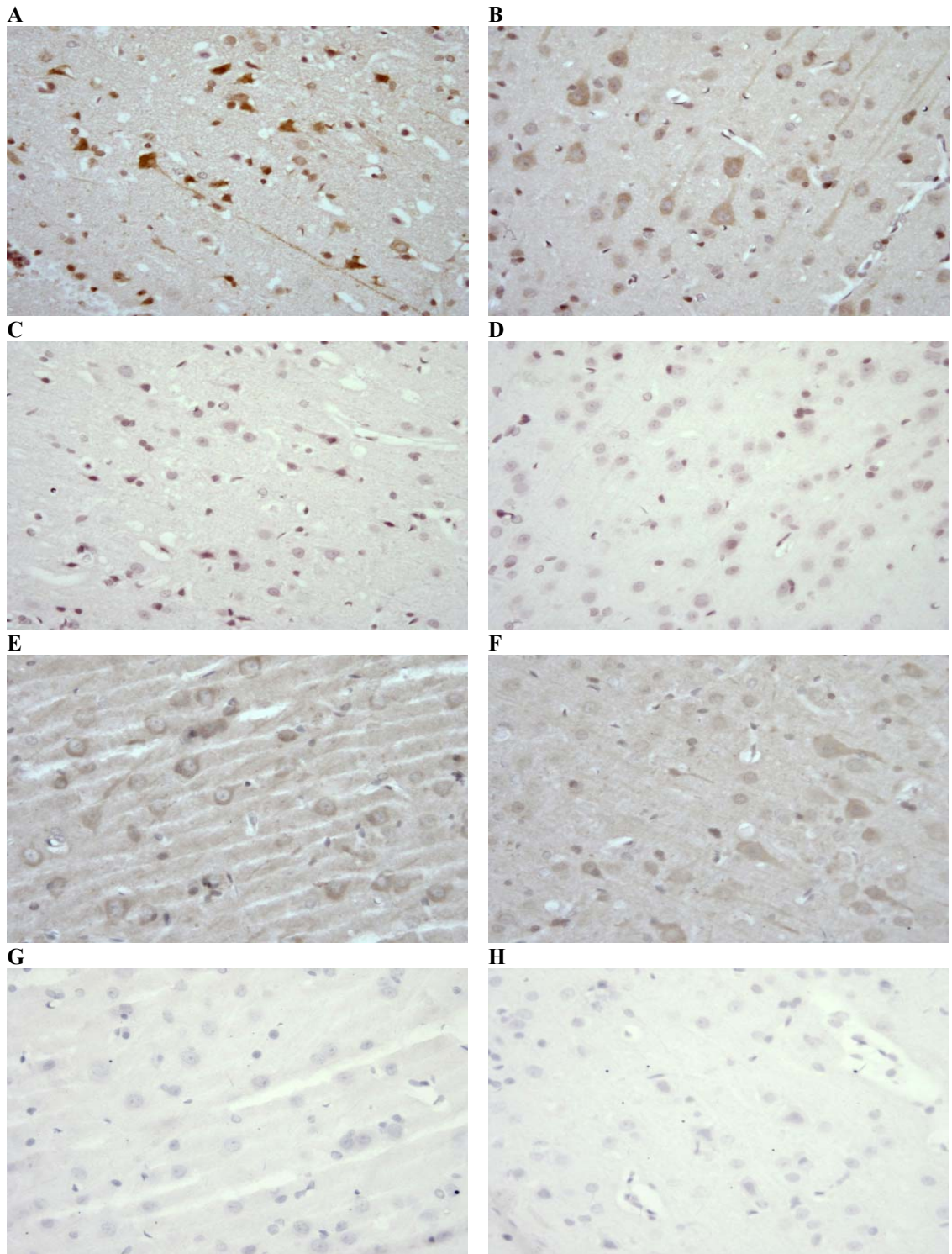


Figure 3.36. Immunohistochemistry for MIP-2/CXCL2 vs. Control Sections. Deep Cortex 4 Hours after Fluid Percussion Injury and Sham.

Ipsilateral (A, C, E, G) and contralateral deep cortex (B, D, F, H). Following FPI, MIP-2/CXCL2 staining visualized by DAB (A&B) and with omission of primary (C&D). Following sham, MIP-2/CXCL2 staining visualized by DAB (E&F) and with omission of primary (G&H). Immunohistochemical sections counterstained with haematoxylin. Paraformaldehyde fixed tissue. All x200.

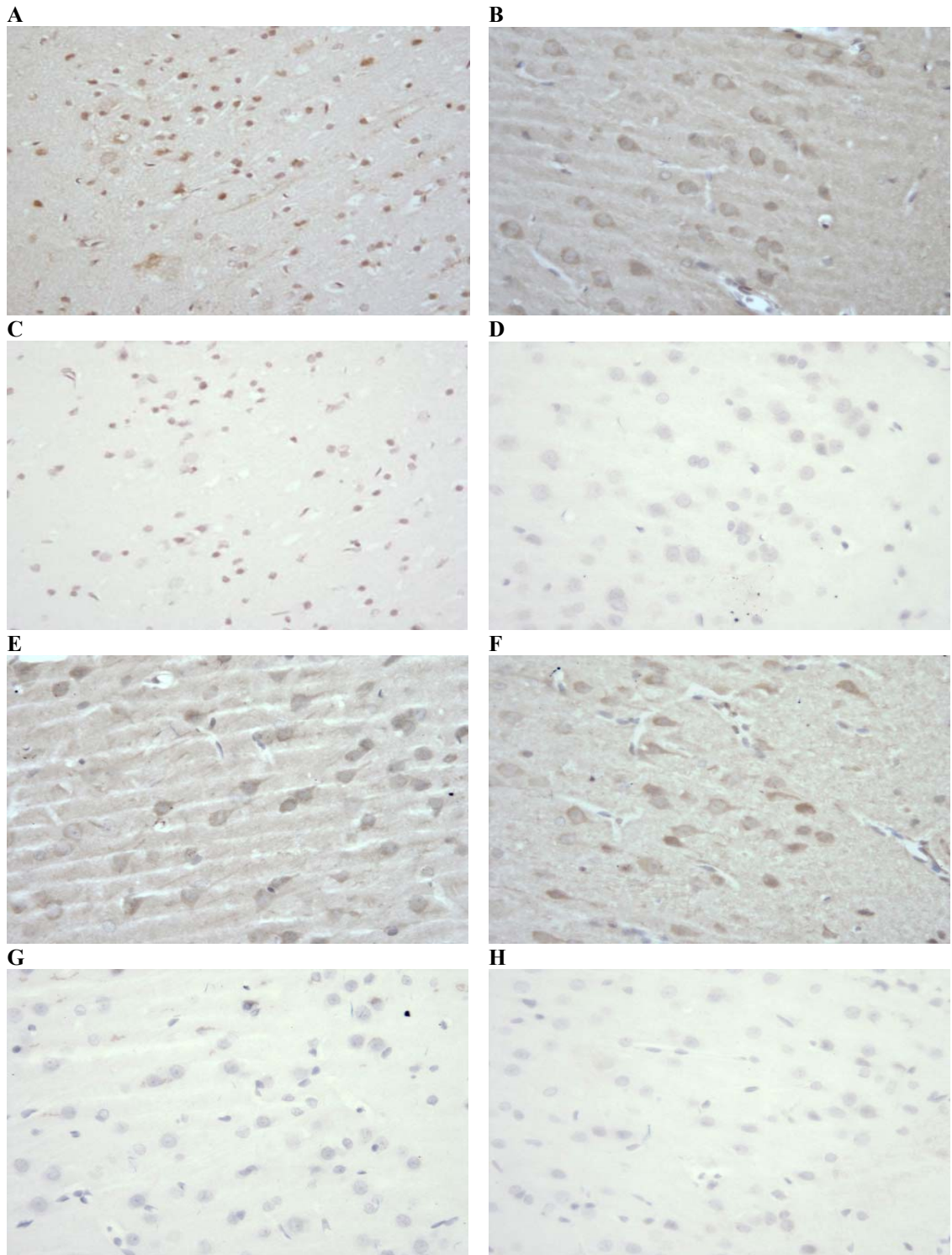


Figure 3.37. Immunohistochemistry for MIP-2/CXCL2 vs. Control Sections. Outer Cortex 4 hours after Fluid Percussion Injury and Sham.

Ipsilateral (A, C, E, G) and contralateral outer cortex (B, D, F, H). Following FPI, MIP-2/CXCL2 staining visualized by DAB (A&B) and with omission of primary (C&D). Following sham, MIP-2/CXCL2 staining visualized by DAB (E&F) and with omission of primary (G&H). Immunohistochemical sections counterstained with haematoxylin. Paraformaldehyde fixed tissue. All x200.

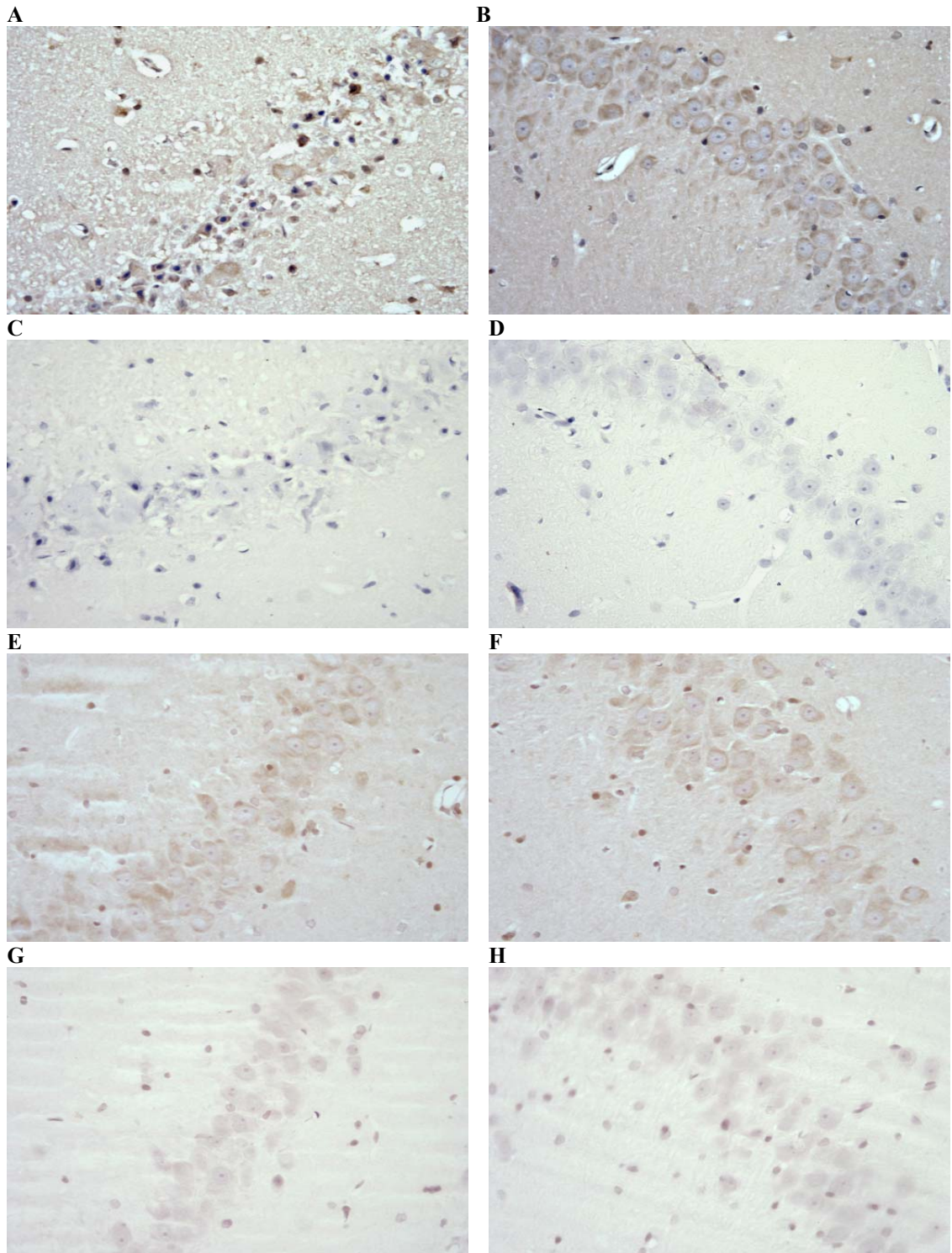


Figure 3.38. Immunohistochemistry for MIP-2/CXCL2 vs. Control Sections. Hippocampus 4 Hours after Fluid Percussion Injury and Sham.

Ipsilateral (A, C, E, G) and contralateral hippocampus (B, D, F, H). Following FPI, MIP-2/CXCL2 staining visualized by DAB (A&B) and with omission of primary (C&D). Following sham, MIP-2/CXCL2 staining visualized by DAB (E&F) and with omission of primary (G&H). Immunohistochemical sections counterstained with haematoxylin. Paraformaldehyde fixed tissue. All x200.

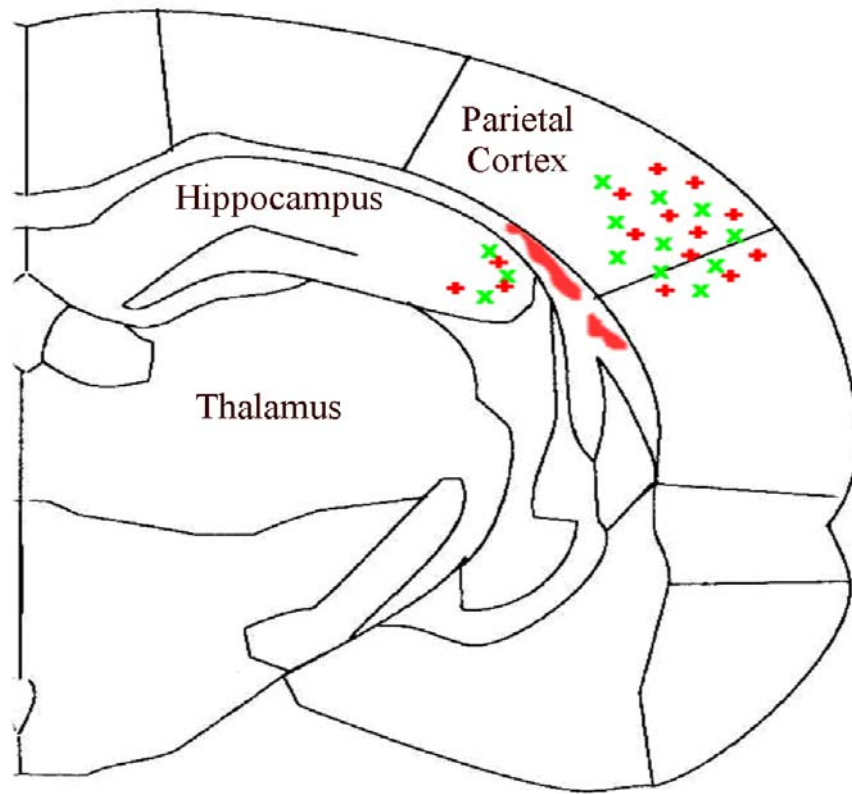


Figure 3.39. The Distribution of Increased MIP-2/CXCL2 Staining 4 Hours after Lateral Fluid Percussion Injury.

Intensely stained MIP-2/CXCL2 positive cells (x) 4 hours after injury co-distribute with acidophilic neurons (+).

		Ipsilateral		Contralateral	
Number		Cortex	Hippocampus	Cortex	Hippocampus
1	Trauma	+++	+++	++	+
2	Trauma	+++	+++	++	+
3	Trauma	+++	+++	++	+
4	Trauma	+++	+++	++	+
5	Sham	++	++	++	++
6	Sham	++	+	++	+

Table 3.2. The Distribution and Intensity of MIP-2/CXCL2 Staining in Paraformaldehyde Fixed Tissue 4 Hours after Lateral Fluid Percussion Injury.

Staining intensity was scored as absent (-), light (+), moderate (++) and dense (+++). MIP-2/CXCL2 staining was clearly increased in the cortical contusion and ipsilateral hippocampus after LFPI.

3.5.5.3.5. *Immunohistochemistry for MCP-1/CCL2 Staining After Lateral Fluid Percussion Injury*

To identify the cells expressing MCP-1/CCL2, animals were sacrificed at 9 hours after injury, the point of maximal cortical MCP-1/CCL2 concentration.

Staining for MCP-1/CCL2 was originally attempted in non-fixed cryostat sections. However the staining intensity was low and difficult to confidently distinguish from that of non-specific staining seen when the primary antibody was omitted. MCP-1/CCL2 immunohistochemistry was then performed in paraformaldehyde fixed tissue (3 injury and 2 sham). Compared with MIP-2/CXCL2, MCP-1/CCL2 staining was less intense. MCP-1/CCL2 staining was the same in both the contralateral cortex of injured animals and the ipsilateral and contralateral cortices of shams. Small numbers of MCP-1/CCL2 positive cells were seen mainly in the outer granular layer (Figure 3.40, 3.41, 3.43 & 3.44). Occasional MCP-1/CCL2 positive cells were seen in the pyramidal layer of the undamaged hippocampus (Figure 3.42 & 3.45).

Following injury and consistent with the ELISA data (3.5.3.1), the increase in MCP-1/CCL2 staining was much less than that seen for MIP-2/CXCL2. In order to define the change in expression after LFPI more clearly the number of positive cells were counted and the intensity of the staining scored. Following LFPI there was an increase in the number of positively staining cells in the deep cortex and an increase in the number of cells staining positive and the intensity of MCP-1/CCL2 staining in the outer cortex (Table 3.3). Many of these MCP-1/CCL2 positive cells had the morphology of shrunken neurones (Figure 3.41) and resembled the acidophilic neurones seen with cresyl violet and acid fuchsin staining. This distribution is summarised in figure 3.46. No increase in hippocampal staining was seen with injury (Figure 3.42).

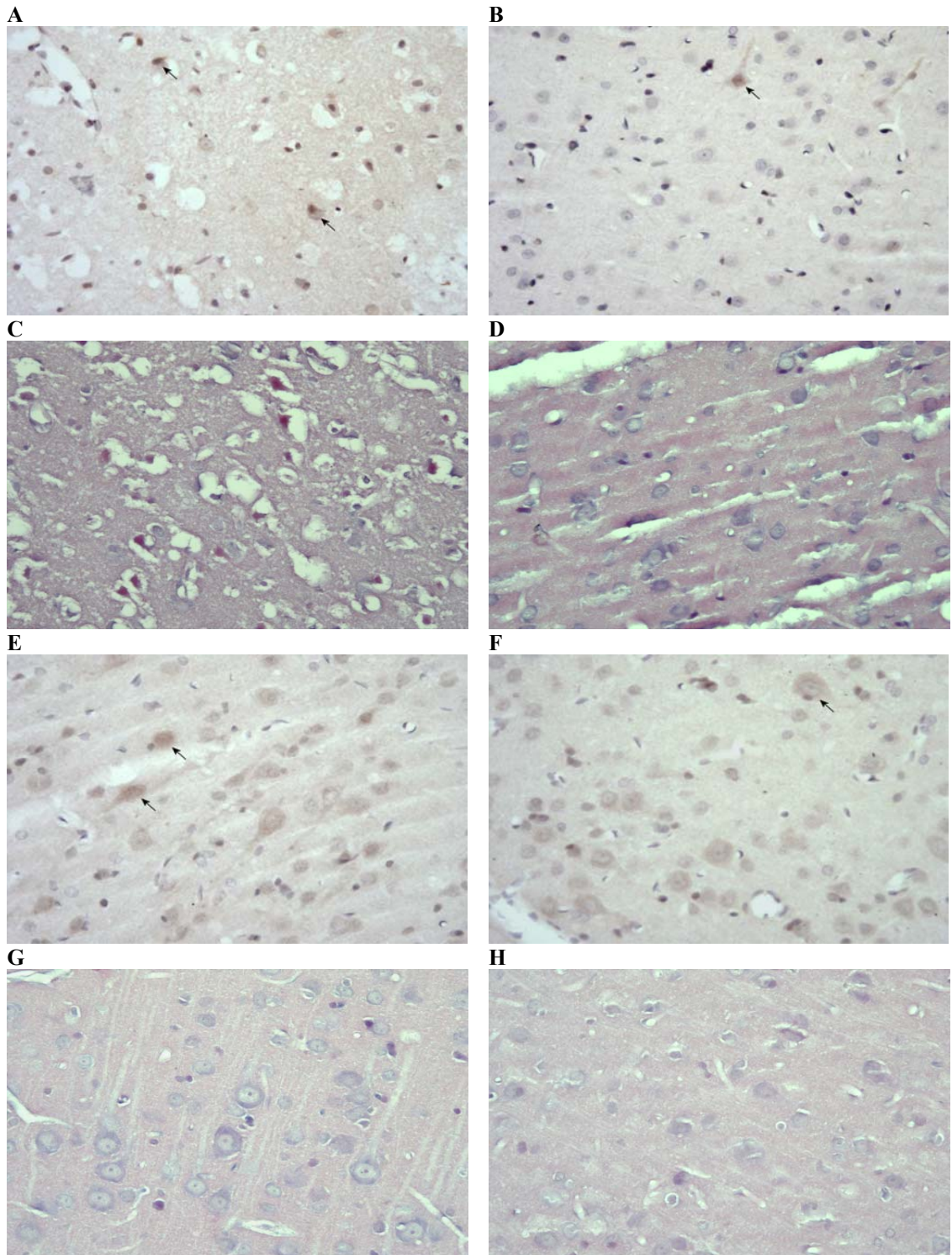


Figure 3.40. Immunohistochemistry for MCP-1/CCL2 in the Deep Cortex 9 Hours after Fluid Percussion Injury vs. Sham.

Ipsilateral (A, C, E, G) and contralateral deep cortex (B, D, F, H). MCP-1/CCL2 staining visualized by DAB following FPI (A&B) and sham (E&F). Faint MCP-1/CCL2 is visible in a small number of cells (arrows). Immunohistochemical sections counterstained with haematoxylin. Cresyl violet and acid fuchsin staining of corresponding sections following FPI (C&D) and sham (G&H). Paraformaldehyde fixed tissue. All x200.

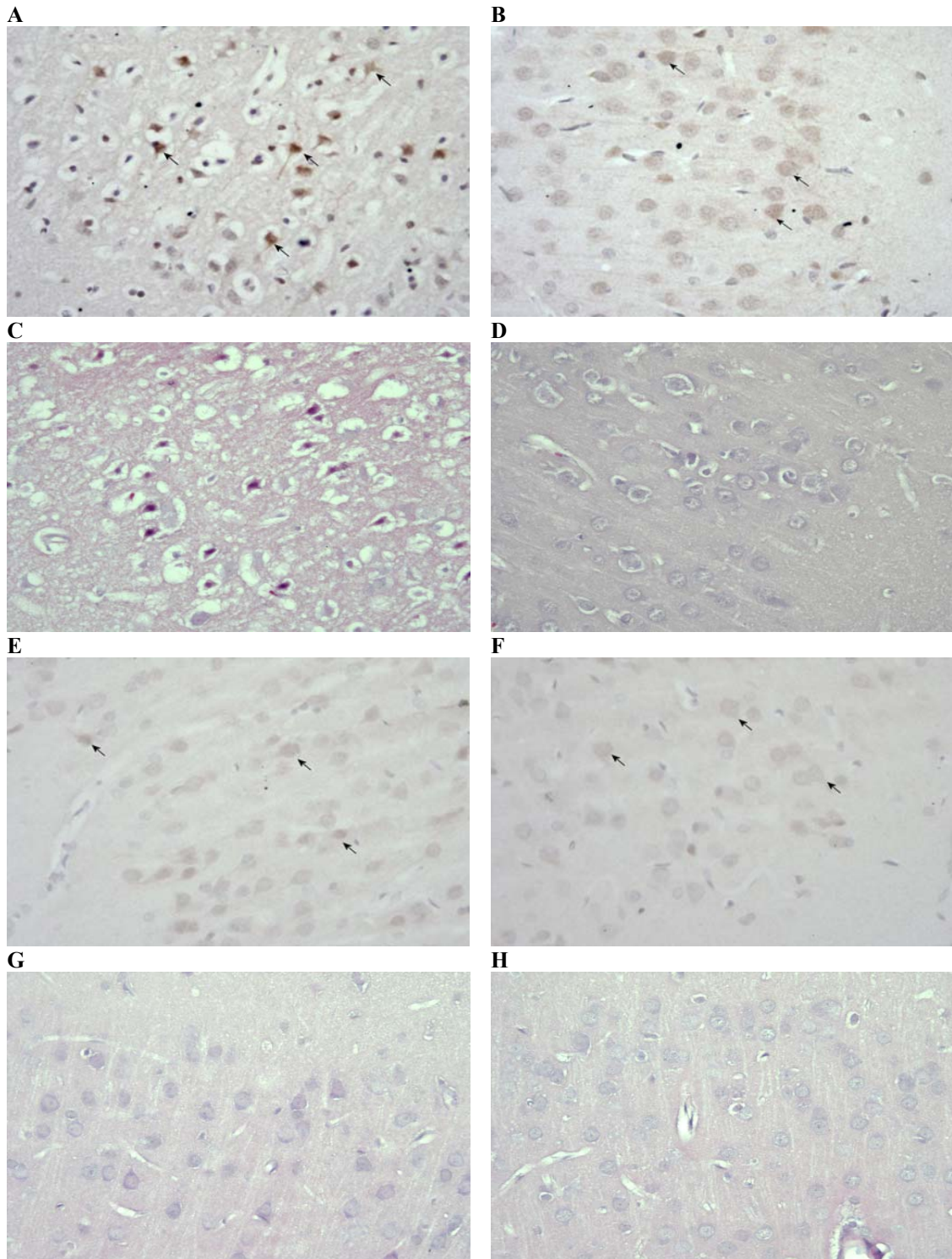


Figure 3.41. Immunohistochemistry for MCP-1/CCL2 in the Outer Cortex 9 Hours after Fluid Percussion Injury vs Sham.

Ipsilateral (A, C, E, G) and contralateral outer cortex (B, D, F, H). MCP-1/CCL2 staining visualized by DAB following FPI (A&B) and sham (E&F). MCP-1/CCL2 staining (arrows) is more intense in the ipsilateral damaged tissue compared to the contralateral. Immunohistochemical sections counterstained with haematoxylin. Cresyl violet and acid fuchsin staining of corresponding sections following FPI (C&D) and sham (G&H). Paraformaldehyde fixed tissue. All x200.

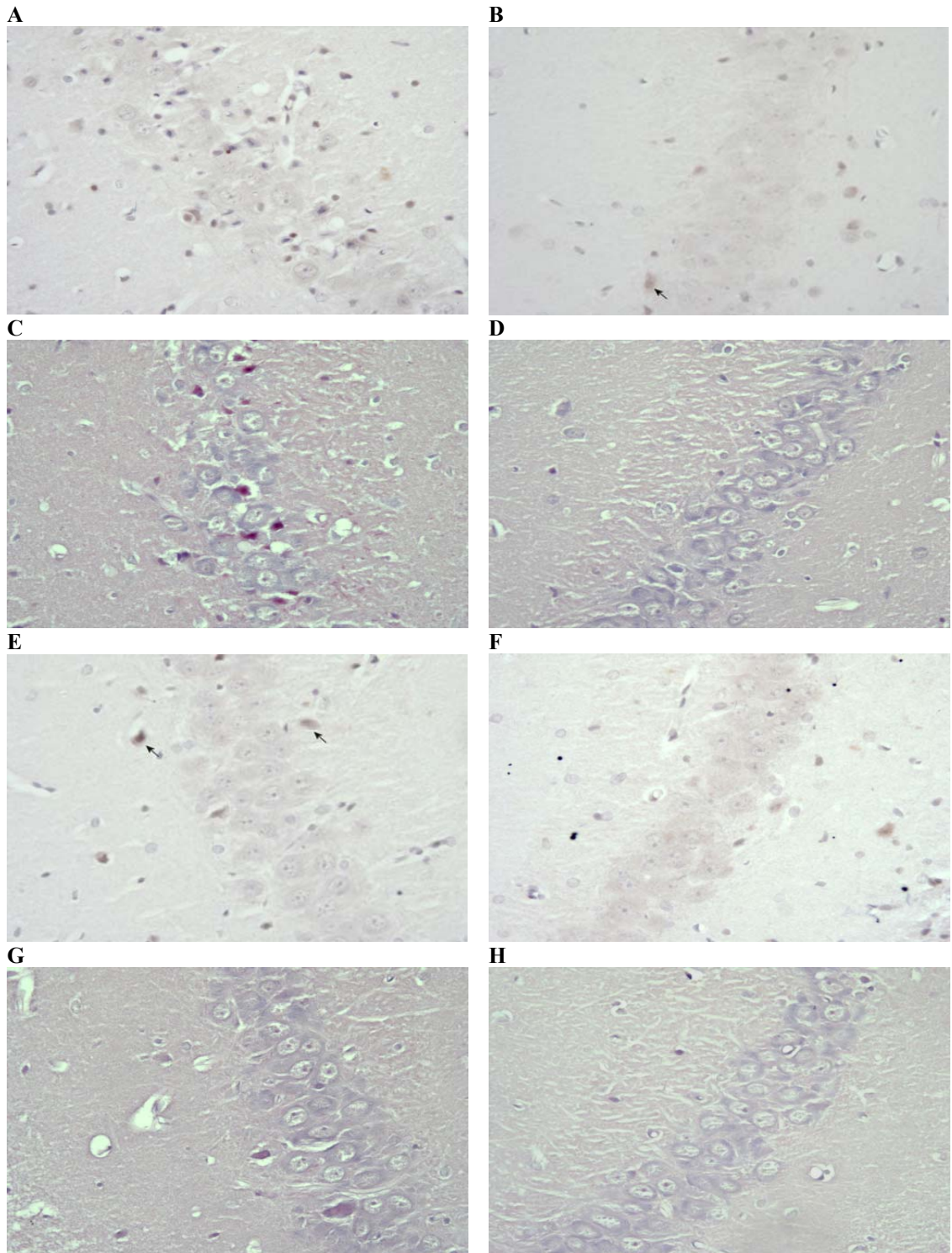


Figure 3.42. Immunohistochemistry for MCP-1/CCL2 in the Hippocampus 9 hours after Lateral Fluid Percussion Injury vs. Sham.

Ipsilateral (A, C, E, G) and contralateral hippocampus (B, D, F, H). MCP-1/CCL2 staining visualized by DAB following FPI (A&B) and sham (E&F). An occasional MCP-1/CCL2 positive cell can be seen in the undamaged hippocampus (arrows). Immunohistochemical sections counterstained with haematoxylin. Cresyl violet and acid fuchsin staining of corresponding sections following FPI (C&D) and sham (G&H). Paraformaldehyde fixed tissue. All x200.

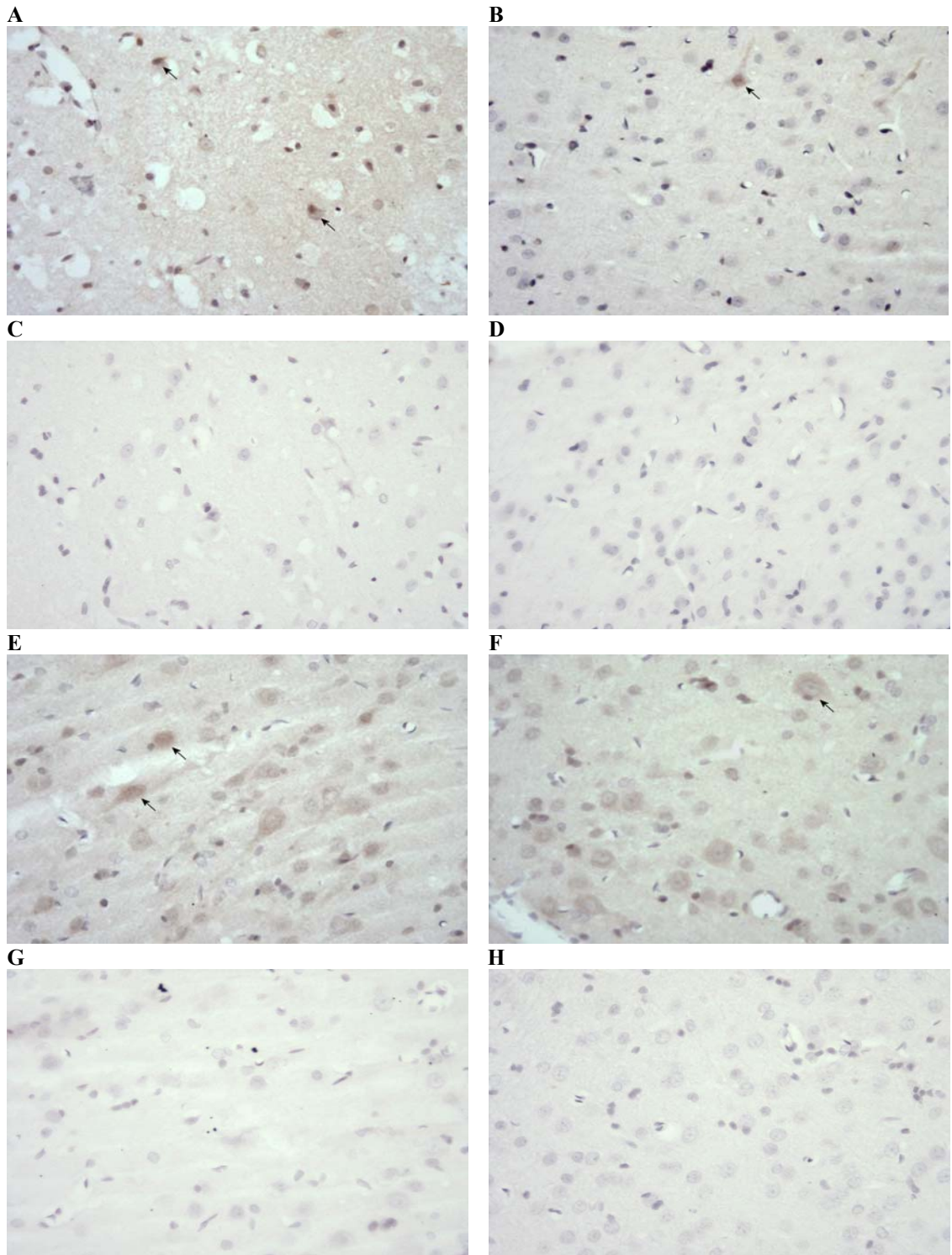


Figure 3.43. Immunohistochemistry for MCP-1/CCL2 vs. Control Sections. Deep Cortex 9 Hours after Fluid Percussion Injury and Sham.

Ipsilateral (A, C, E, G) and contralateral deep cortex (B, D, F, H). Following FPI, MCP-1/CCL2 staining visualized by DAB (A&B) and with omission of primary (C&D). Following sham, MCP-1/CCL2 staining visualized by DAB (E&F) and with omission of primary (G&H). Immunohistochemical sections counterstained with haematoxylin. Paraformaldehyde fixed tissue. All x200.

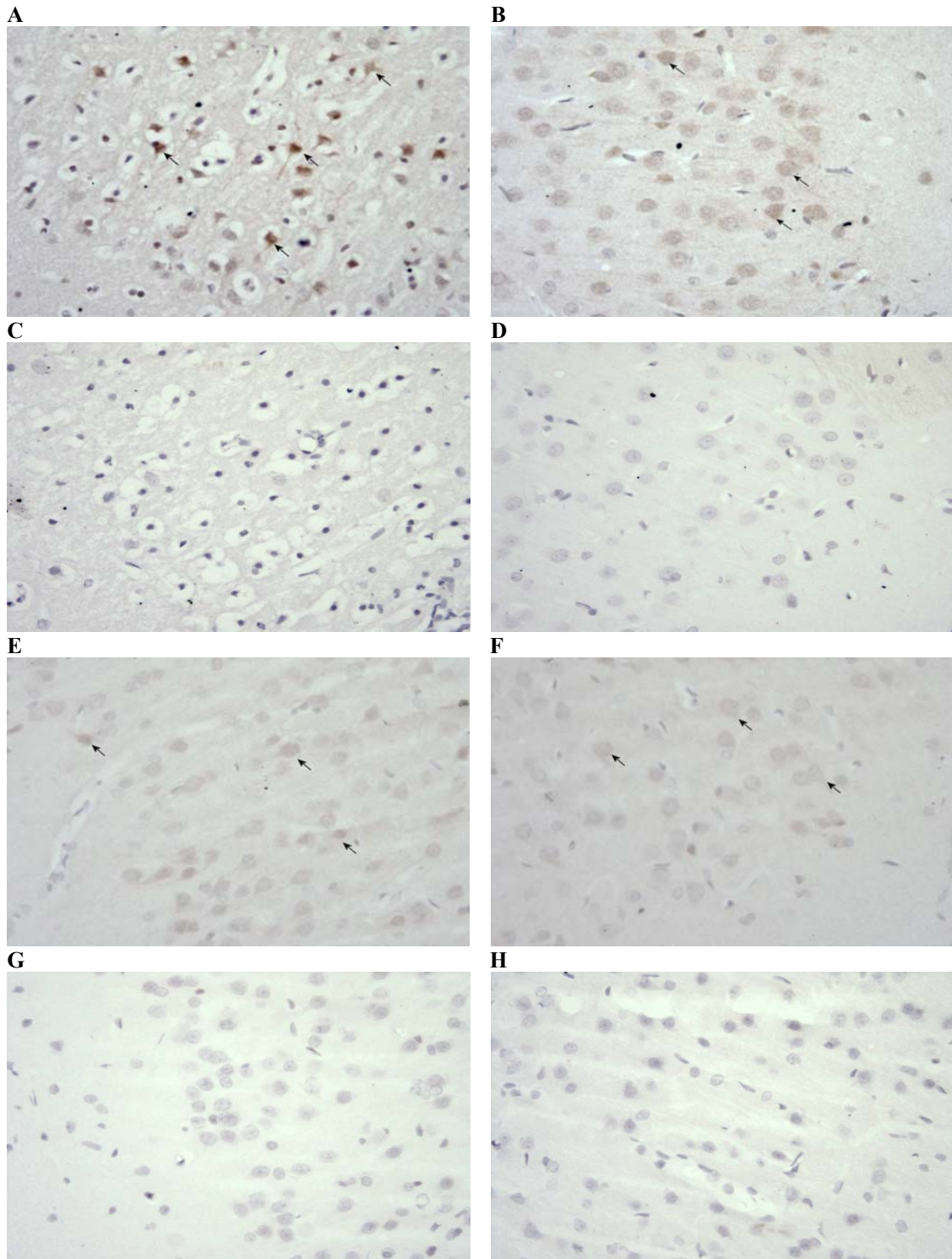


Figure 3.44. Immunohistochemistry for MCP-1/CCL2 vs. Control Sections. Outer Cortex 9 Hours after Fluid Percussion Injury and Sham.

Ipsilateral. (A, C, E, G) and contralateral outer cortex (B, D, F, H). Following FPI, MCP-1/CCL2 staining visualized by DAB (A&B) and with omission of primary (C&D). Following sham, MCP-1/CCL2 staining visualized by DAB (E&F) and with omission of primary (G&H). Immunohistochemical sections counterstained with haematoxylin. Paraformaldehyde fixed tissue. All x200.

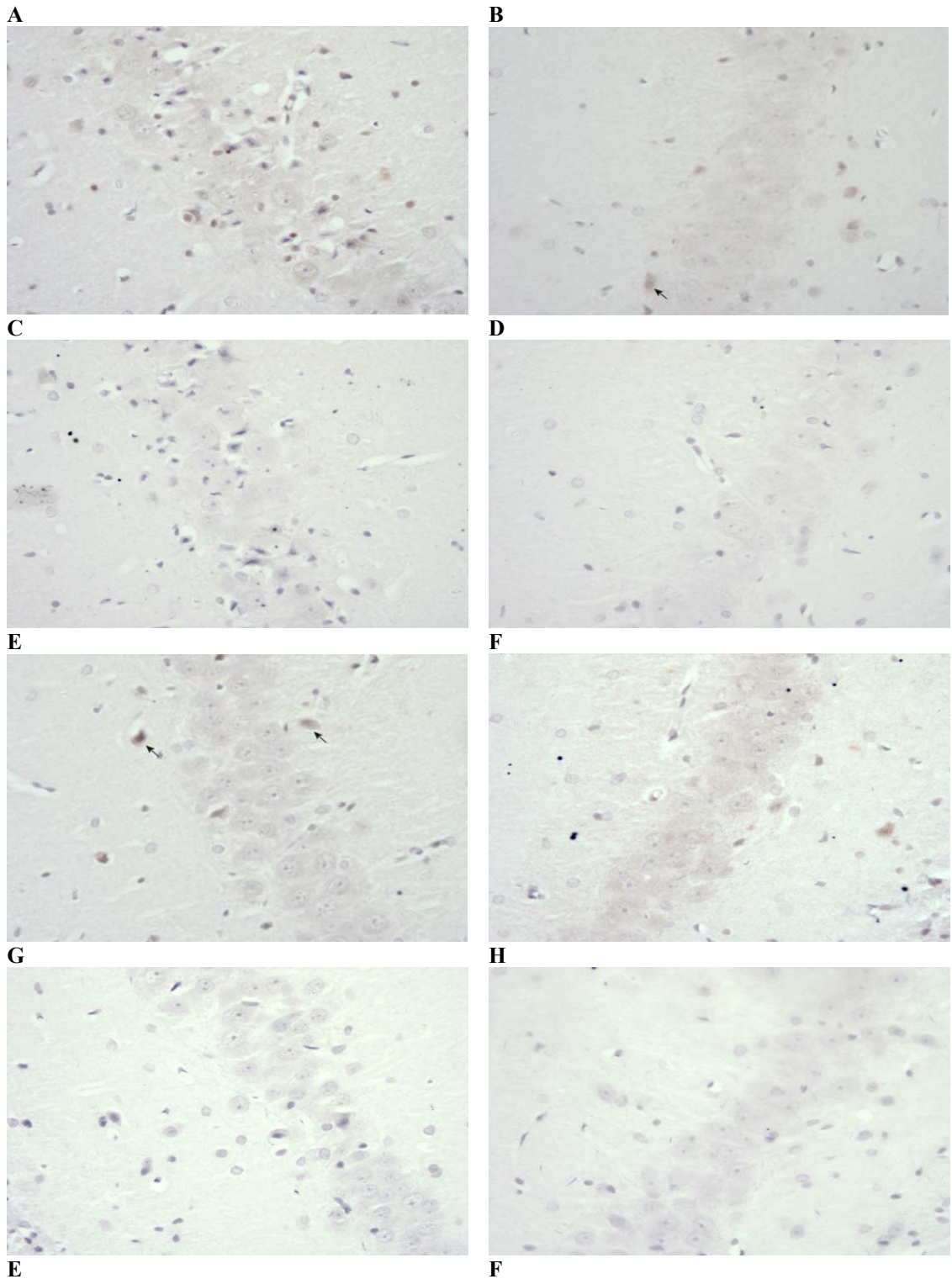


Figure 3.45. Immunohistochemistry for MCP-1/CCL2 vs. Control Sections. Hippocampus 9 Hours after Fluid Percussion Injury and Sham.

Ipsilateral (A, C, E, G) and contralateral hippocampus (B, D, F, H). Following FPI, MCP-1/CCL2 staining visualized by DAB (A&B) and with omission of primary (C&D). Following sham, MCP-1/CCL2 staining visualized by DAB (E&F) and with omission of primary (G&H). Immunohistochemical sections counterstained with haematoxylin. Paraformaldehyde fixed tissue. All x200.

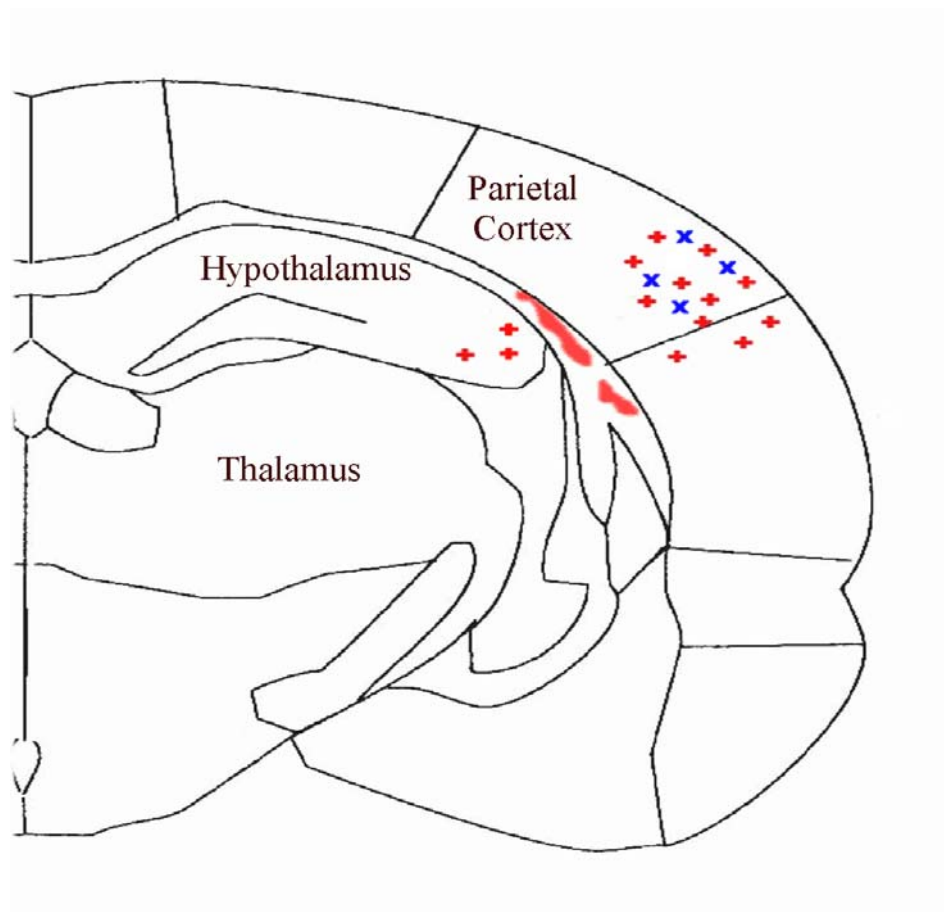


Figure 3.46. The Distribution of Increased MCP-1/CCL2 Staining after Lateral Fluid Percussion Injury.

MCP-1/CCL2 positive cells (x) 9 hours after injury co-distributed with acidophilic neurons (+).

		Ipsilateral Cortex				Contralateral Cortex			
Number		Deep		Outer		Deep		Outer	
		Staining	Count	Staining	Count	Staining	Count	Staining	Count
1	Trauma	+	4 (4-5)	++	9 (2-13)	+	0 (0-2)	+	5 (2-10)
2	Trauma	++	6 (3-11)	++	6 (5-8)	+	0 (0-2)	+	2 (0-3)
3	Trauma	+	6 (2-12)	++	22 (20-24)	+	4 (2-6)	+	12 (7-16)
4	Sham	+	0 (0-3)	+	9 (3-10)	+	0 (0-3)	+	3 (0-7)
5	Sham	+	3 (0-5)	+	5 (3-13)	+	3 (2-4)	+	6 (3-9)

Table 3.3. The Distribution and Intensity of MCP-1/CCL2 Staining in Fixed Tissue after Lateral Fluid Percussion Injury.

Positive cells were rated for intensity and counted. Staining intensity was scored as absent (-), light (+) and moderate (++). The number of cells and the intensity of MCP-1/CCL2 staining increased in the injured outer cortex whilst an increase in the number of positive cells was seen in the deep cortex. The numbers represent the median (range) of 3 slides at x200.

3.5.5.4. Identification of Cells Staining for Chemokine Proteins by Double Staining for Cell Specific Markers

Staining of tissue sections with cell type specific antibodies was performed to help identify chemokine positive cells. Antibodies to NeuN and GFAP were used to stain neurones and astrocytes respectively in fixed tissue sections from the animals used for the MIP-2/CXCL2 and MCP-1/CCL2 immunohistochemistry. An anti-CD11b antibody was used to identify monocytic cells but this is not compatible with paraformaldehyde wax embedded tissue and so frozen cryostat sections were used.

For MCP-1/CCL2 the low level of signal seen in the contralateral hemisphere and sham tissue, the absence of positive cells on some sections and a shortage of suitable tissue prevented the double staining being performed reliably in undamaged tissue.

3.5.5.4.1. NeuN and MIP-2/CXCL2 or MCP-1/CCL2 Double Staining

NeuN staining in the contralateral undamaged tissue colocalised with MIP-2/CXCL2 positive cells with neuronal morphology in both the cortex and hippocampus (Figure 3.47, 3.48, 3.49). Within the contusion the NeuN staining was less intense and disappeared in the centre of the lesion. Despite this, co-distribution of MIP-2/CXCL2 positive cells with the NeuN staining was visible towards the margins of the contusion (Figure 3.48 vs. 3.47). In the ipsilateral hippocampus co-distribution of the MIP-2/CXCL2 positive shrunken cells with the NeuN could not be demonstrated consistently (Figure 3.49).

For MCP-1/CCL2 the greatest immunohistochemical staining was seen in the contused cortex. However like the MIP-2/CXCL2 sections the NeuN staining was very weak and co-distribution of NeuN and MCP-1/CCL2 could not be reliably demonstrated (Figure 3.50 & 3.51).

3.5.5.4.2. *GFAP and MIP-2/CXCL2 or MCP-1/CCL2 Double Staining*

On sections previously stained for MIP-2/CXCL2 GFAP immunoreactivity was present in the contralateral cortex and hippocampus (Figure 3.52, 3.53 & 3.54) and ipsilateral contused cortex and hippocampus after FPI (Figure 3.52, 3.53 & 3.54). GFAP positive cells were also seen in the ipsilateral contused cortex of sections stained for MCP-1/CCL2 (Figure 3.55 & 3.56). However GFAP staining did not co-distribute with either MIP-2/CXCL2 or MCP-1/CCL2 positive cells.

3.5.5.4.3. *CD11b and MIP-2/CXCL2 Double Staining*

Frozen tissue sections from animals sacrificed at 4 hours after FPI (2 injury and 1 sham) and stained for MIP-2/CXCL2 were subsequently stained with an anti-CD11b antibody in order to identify reactive microglia and infiltrating monocytes, with frozen spleen used as a positive control. However no strongly CD11b positive cells were visible at this time point (Figures 3.58-3.59).

MCP-1/CCL2 staining could not be reliably detected in frozen tissue (3.5.5.3.5) and so double staining was not attempted in tissue from 9 hours after injury.

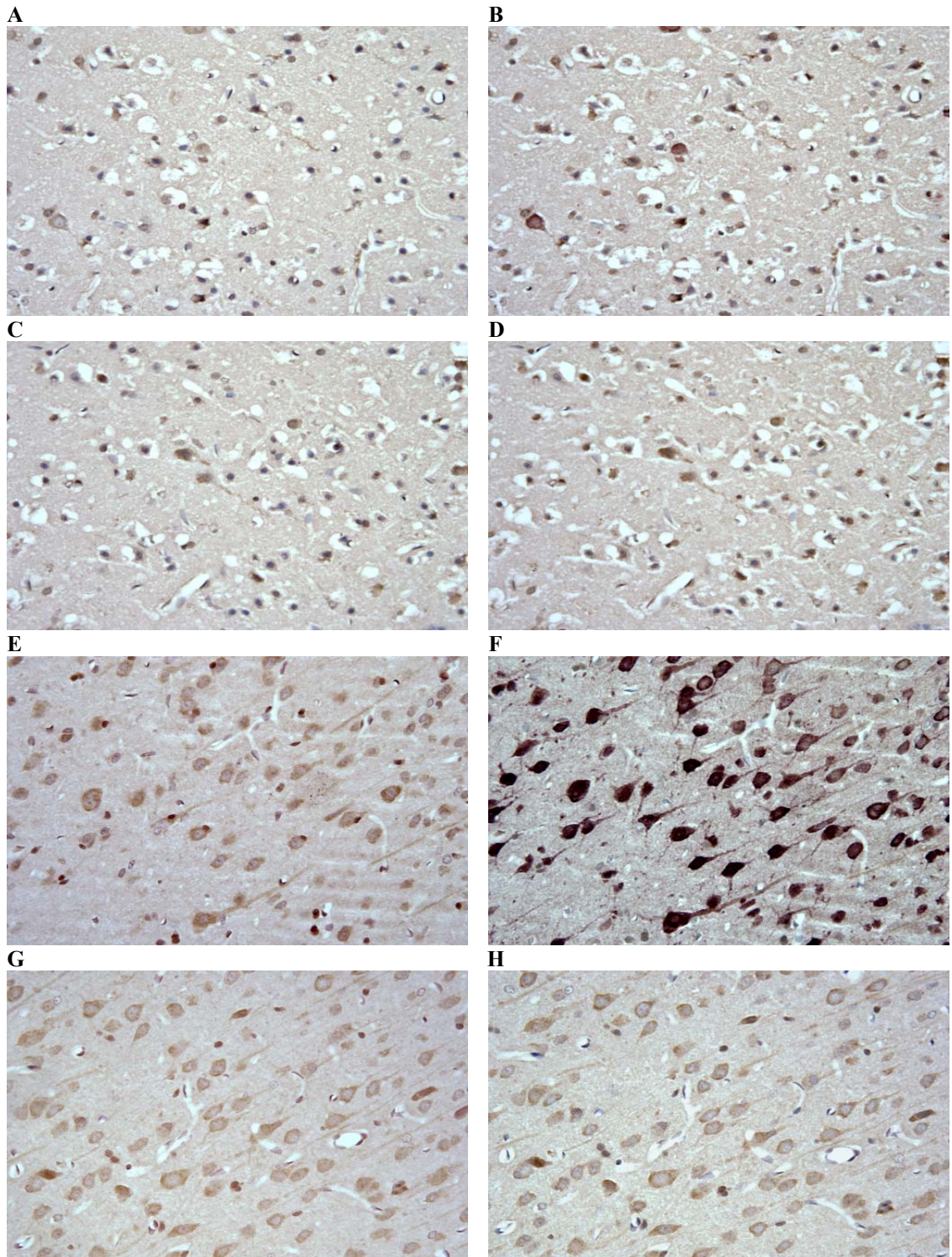


Figure 3.47. MIP-2/CXCL2 and NeuN Staining in the Deep Cortex 4 Hours after Lateral Fluid Percussion Injury.

(A) MIP-2/CXCL2 staining in the ipsilateral and (E) contralateral deep cortex, then (B&F) after double staining for NeuN. (C&G) Respective consecutive MIP-2/CXCL2 stained sections before and (D&H) after staining with the omission of the NeuN primary. MIP-2/CXCL2 visualised with DAB (Brown) and NeuN visualized with VIP (Purple). Counterstained with haematoxylin. Paraformaldehyde fixed tissue. All x200.

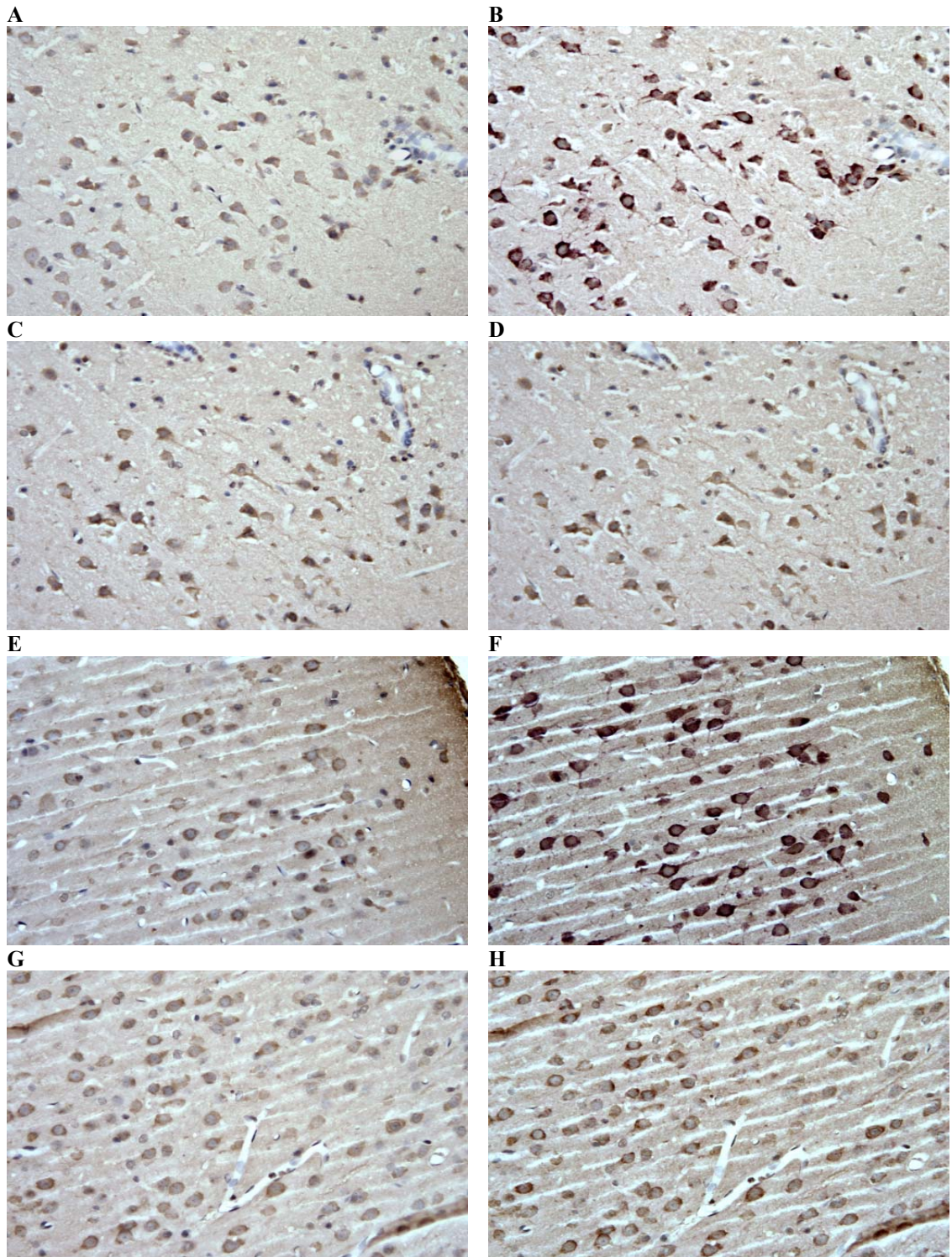


Figure 3.48. MIP-2/CXCL2 and NeuN Staining in the Outer Cortex 4 Hours after Lateral Fluid Percussion Injury.

(A) MIP-2/CXCL2 staining in the ipsilateral and (E) contralateral outer cortex, then (B&F) after double staining for NeuN. (C&G) Respective consecutive MIP-2/CXCL2 stained sections before and (D&H) after staining with the omission of the NeuN primary. MIP-2/CXCL2 visualised with DAB (Brown) and NeuN visualized with VIP (Purple). Counterstained with haematoxylin. Paraformaldehyde fixed tissue. All x200.

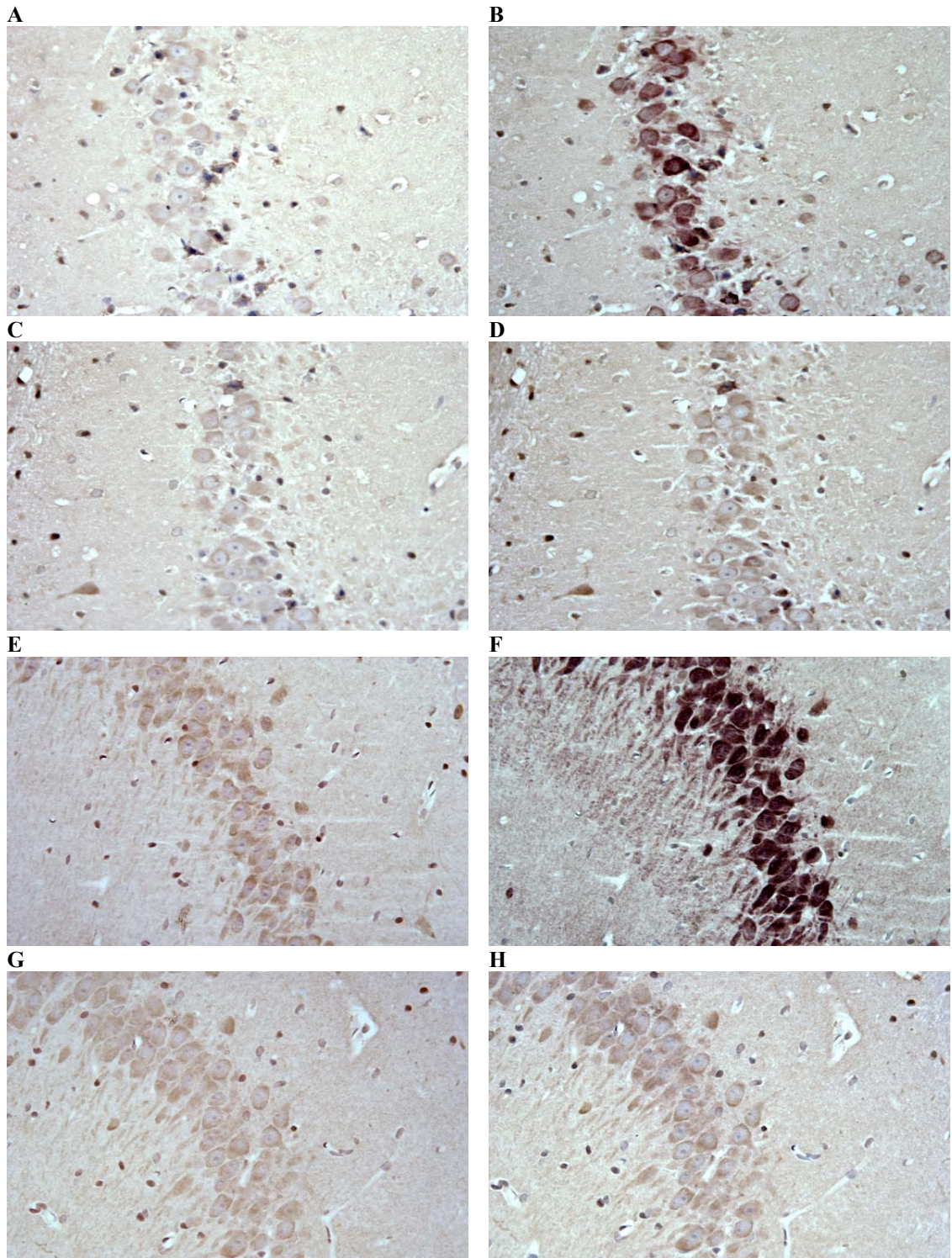


Figure 3.49. MIP-2/CXCL2 and NeuN Staining in the Hippocampus 4 Hours after Lateral Fluid Percussion Injury.

(A) MIP-2/CXCL2 staining in the ipsilateral and (E) contralateral hippocampus, then (B&F) after double staining for NeuN. (C&G) Respective consecutive MIP-2/CXCL2 stained sections before and (D&H) after staining with the omission of the NeuN primary. MIP-2/CXCL2 visualised with DAB (Brown) and NeuN visualized with VIP (Purple). Counterstained with haematoxylin. Paraformaldehyde fixed tissue. All x200

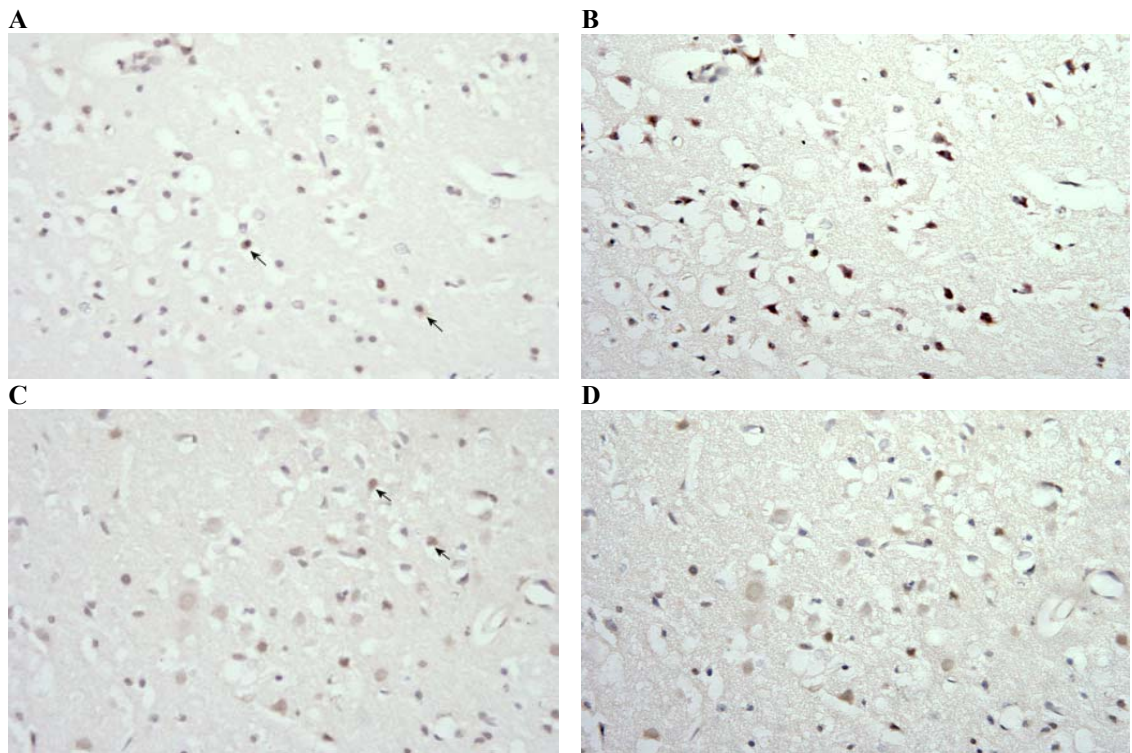


Figure 3.50. MCP-1/CCL2 and NeuN Staining in the Ipsilateral Deep Cortex 9 Hours after Lateral Fluid Percussion Injury.

(A) MCP-1/CCL2 staining in the ipsilateral deep cortex and (B) after double staining for NeuN. (C) Consecutive MCP-1/CCL2 stained section before and (D) after staining with the omission of the NeuN primary. MCP-1/CCL2 visualised with DAB (Brown) and NeuN visualized with VIP (Purple). Counterstained with haematoxylin. MCP-1/CCL2 positive cells (arrows). Paraformaldehyde fixed tissue. All x200.

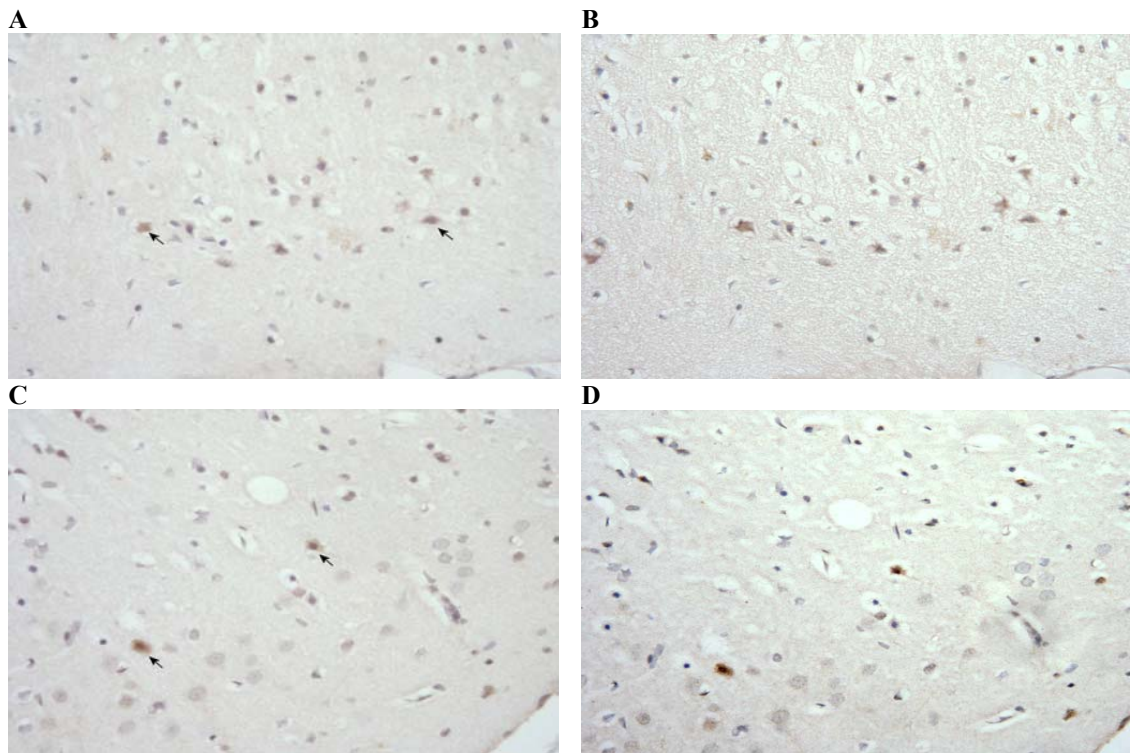


Figure 3.51. MCP-1/CCL2 and NeuN Staining in the Ipsilateral Outer Cortex 9 Hours after Lateral Fluid Percussion Injury.

(A) MCP-1/CCL2 staining in the ipsilateral outer cortex and (B) after double staining for NeuN. (C) Consecutive MCP-1/CCL2 stained section before and (D) after staining with the omission of the NeuN primary. MCP-1/CCL2 visualised with DAB (Brown) and NeuN visualized with VIP (Purple). Counterstained with haematoxylin. MCP-1/CCL2 positive cells (arrows). Paraformaldehyde fixed tissue. All x200.

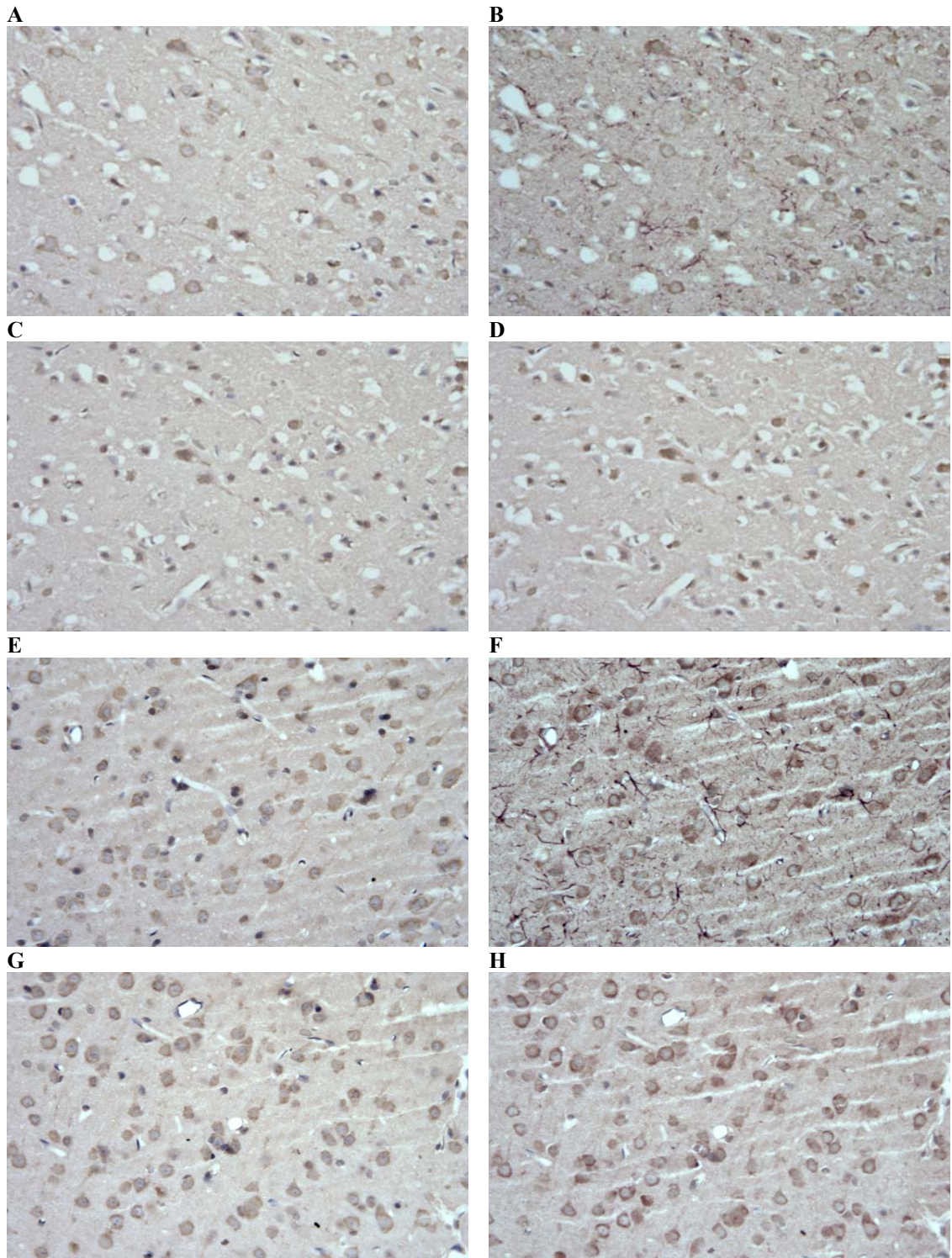


Figure 3.52. MIP-2/CXCL2 and GFAP Staining in the Deep Cortex 4 Hours after Lateral Fluid Percussion Injury.

(A) MIP-2/CXCL2 staining in the ipsilateral and (E) contralateral deep cortex, then (B&F) after double staining for GFAP. (C&G) Respective consecutive MIP-2/CXCL2 stained sections before and (D&H) after staining with the omission of the GFAP primary. MIP-2/CXCL2 visualised with DAB (Brown) and GFAP visualized with VIP (Purple). Counterstained with haematoxylin. Paraformaldehyde fixed tissue. All x200.

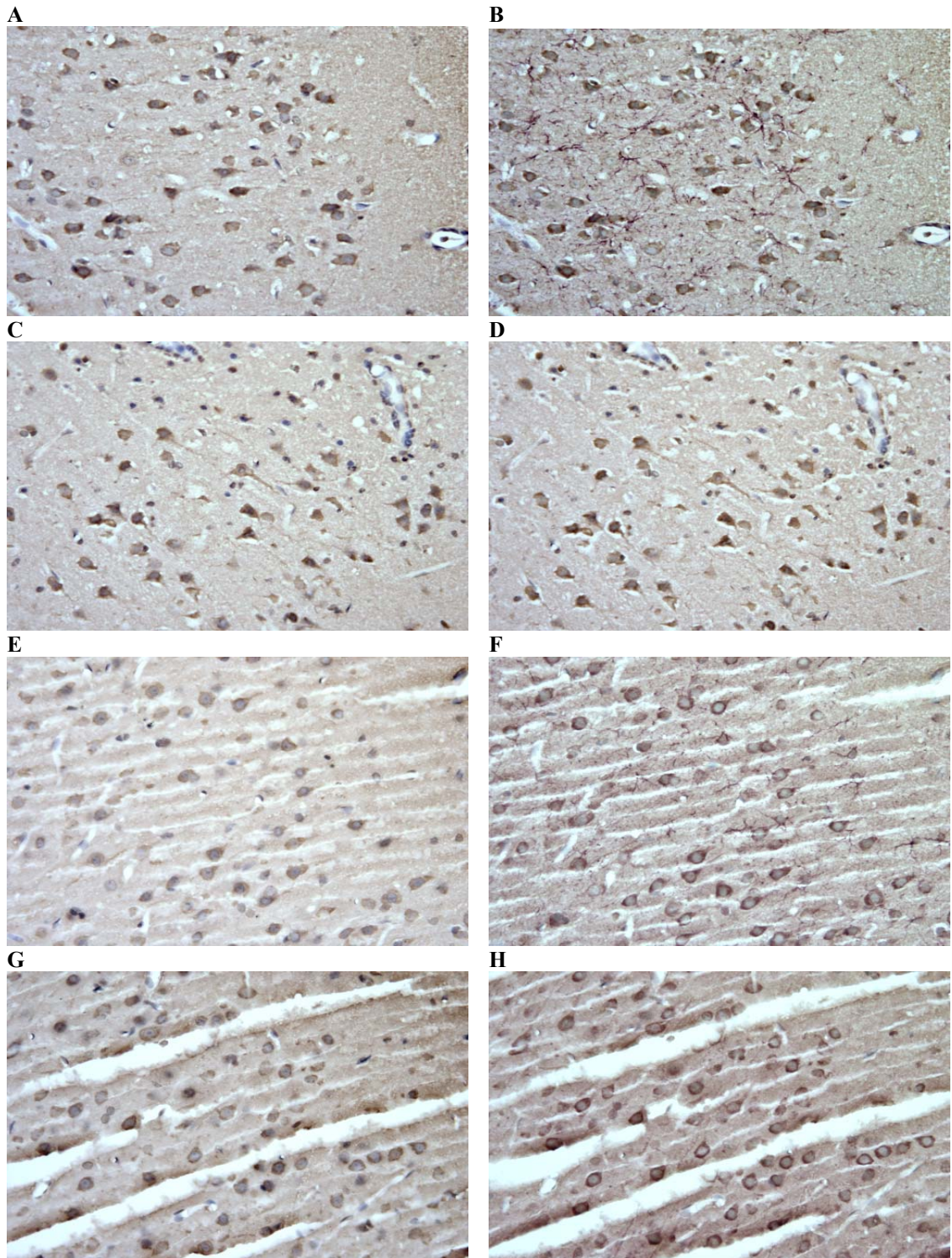


Figure 3.53. MIP-2/CXCL2 and GFAP Staining in the Outer Cortex 4 Hours after Lateral Fluid Percussion Injury.

(A) MIP-2/CXCL2 staining in the ipsilateral and (E) contralateral outer cortex, then (B&F) after double staining for GFAP. (C&G) Respective consecutive MIP-2/CXCL2 stained sections before and (D&H) after staining with the omission of the GFAP primary. MIP-2/CXCL2 visualised with DAB (Brown) and GFAP visualized with VIP (Purple). Counterstained with haematoxylin. Paraformaldehyde fixed tissue. All x200.

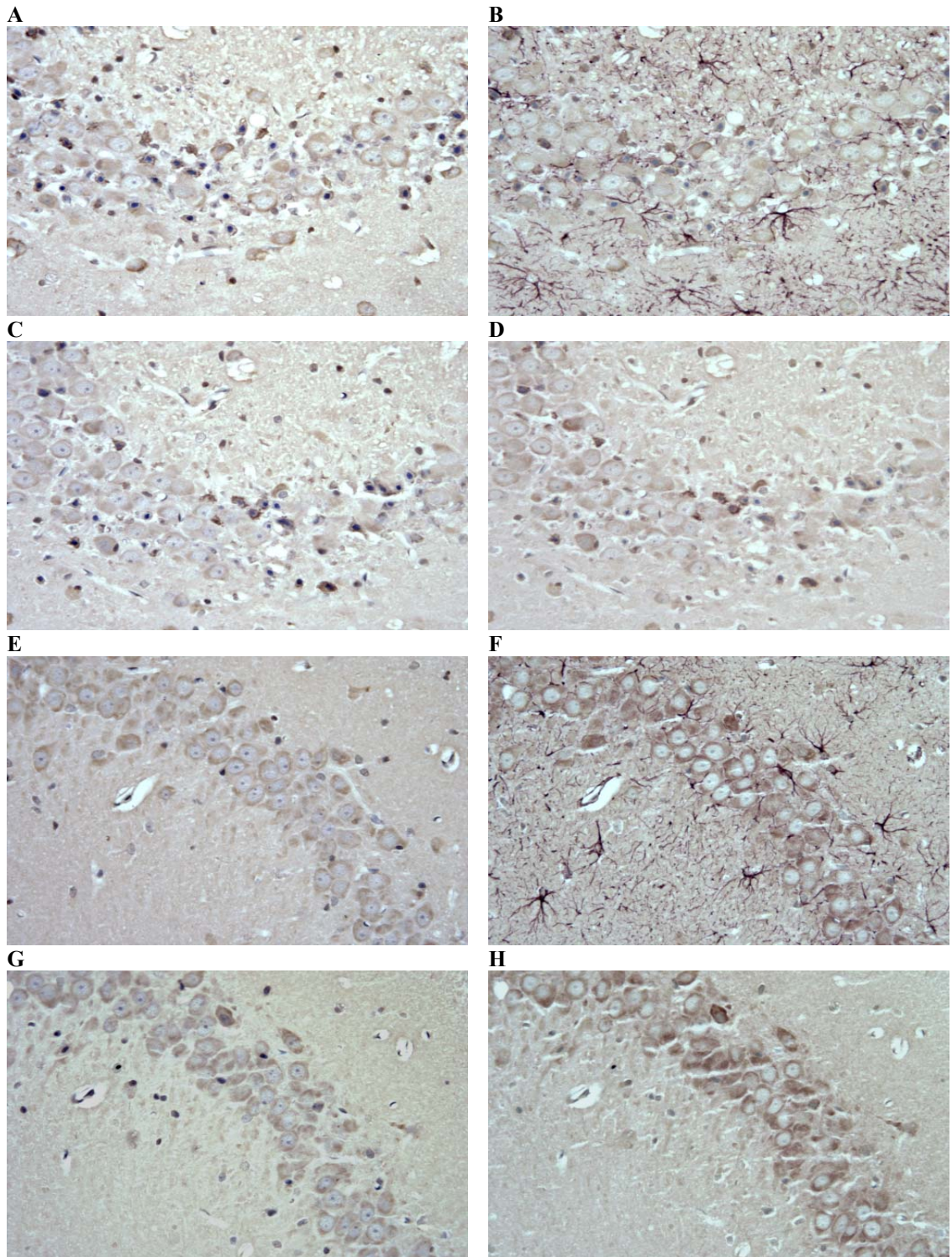


Figure 3.54. MIP-2/CXCL2 and GFAP Staining in the Hippocampus 4 Hours after Lateral Fluid Percussion Injury.

(A) MIP-2/CXCL2 staining in the ipsilateral and (E) contralateral hippocampus, then (B&F) after double staining for GFAP. (C&G) Respective consecutive MIP-2/CXCL2 stained sections before and (D&H) after staining with the omission of the GFAP primary. MIP-2/CXCL2 visualised with DAB (Brown) and GFAP visualized with VIP (Purple). Counterstained with haematoxylin. Paraformaldehyde fixed tissue. All x200.

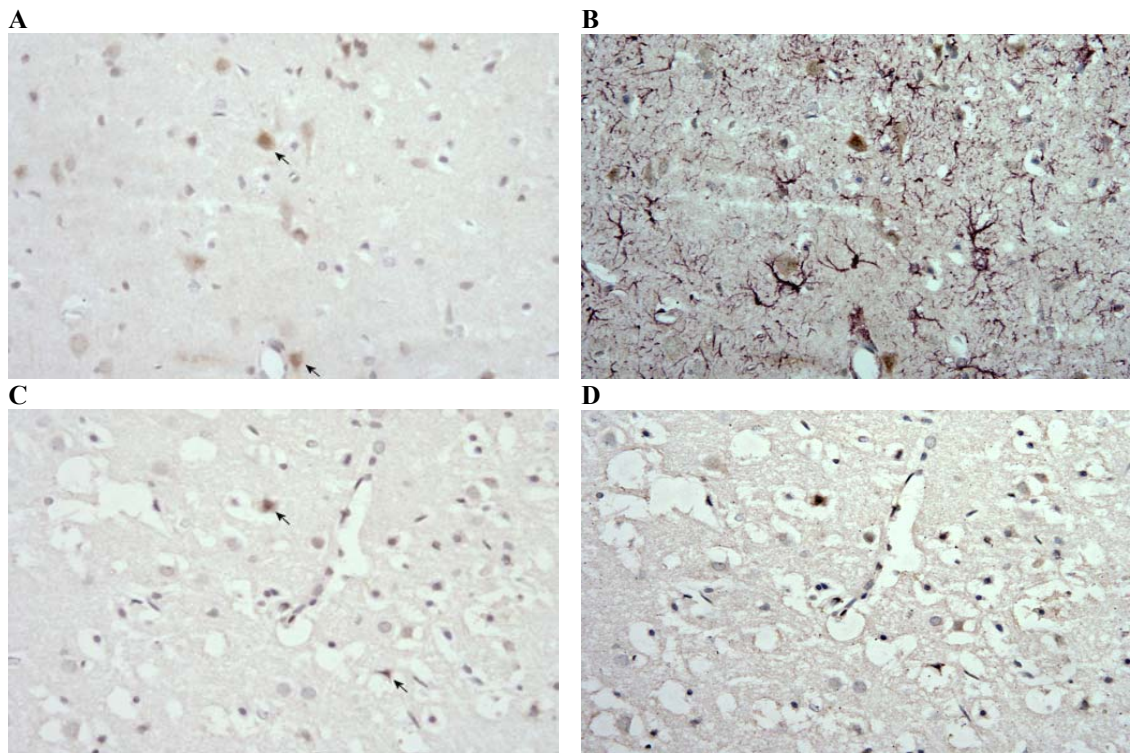


Figure 3.55. MCP-1/CCL2 and GFAP Staining in the Ipsilateral Deep Cortex 9 Hours after Lateral Fluid Percussion Injury.

(A) MCP-1/CCL2 staining in the ipsilateral deep cortex and (B) after double staining for GFAP. (C) Consecutive MCP-1/CCL2 stained section before and (D) after staining with the omission of the GFAP primary. MCP-1/CCL2 visualised with DAB (Brown) and GFAP visualized with VIP (Purple). Counterstained with haematoxylin. MCP-1/CCL2 positive cells (arrows). Paraformaldehyde fixed tissue. All x200.

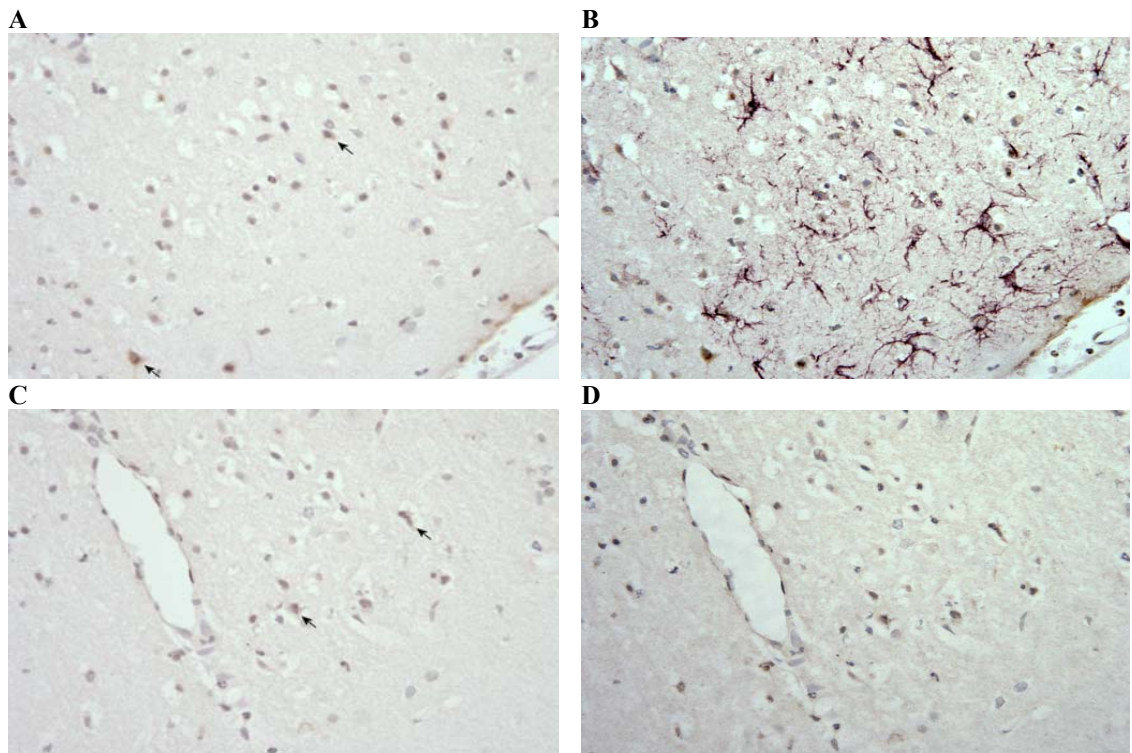


Figure 3.56. MCP-1/CCL2 and GFAP Staining in the Ipsilateral Outer Cortex 9 Hours after Lateral Fluid Percussion Injury.

(A) MCP-1/CCL2 staining in the ipsilateral outer cortex and (B) after double staining for GFAP. (C) Consecutive MCP-1/CCL2 stained section before and (D) after staining with the omission of the GFAP primary. MCP-1/CCL2 visualised with DAB (Brown) and GFAP visualized with VIP (Purple). Counterstained with haematoxylin. MCP-1/CCL2 positive cells (arrows). Paraformaldehyde fixed tissue. All x200.

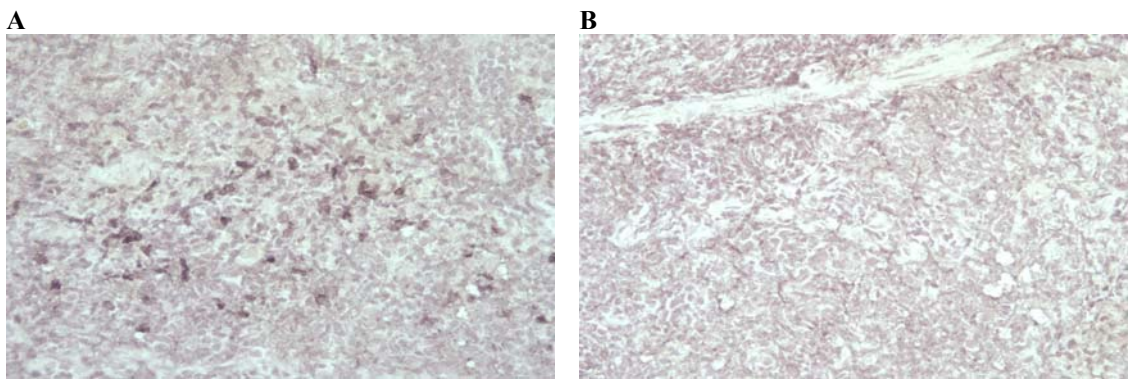


Figure 3.57. CD11b Staining in Sections of Rat Spleen.

(A) CD11b positive cells in rat spleen and (B) with the omission of the primary antibody. Visualized with VIP and counterstained with haematoxylin. Unfixed frozen tissue. All x200.

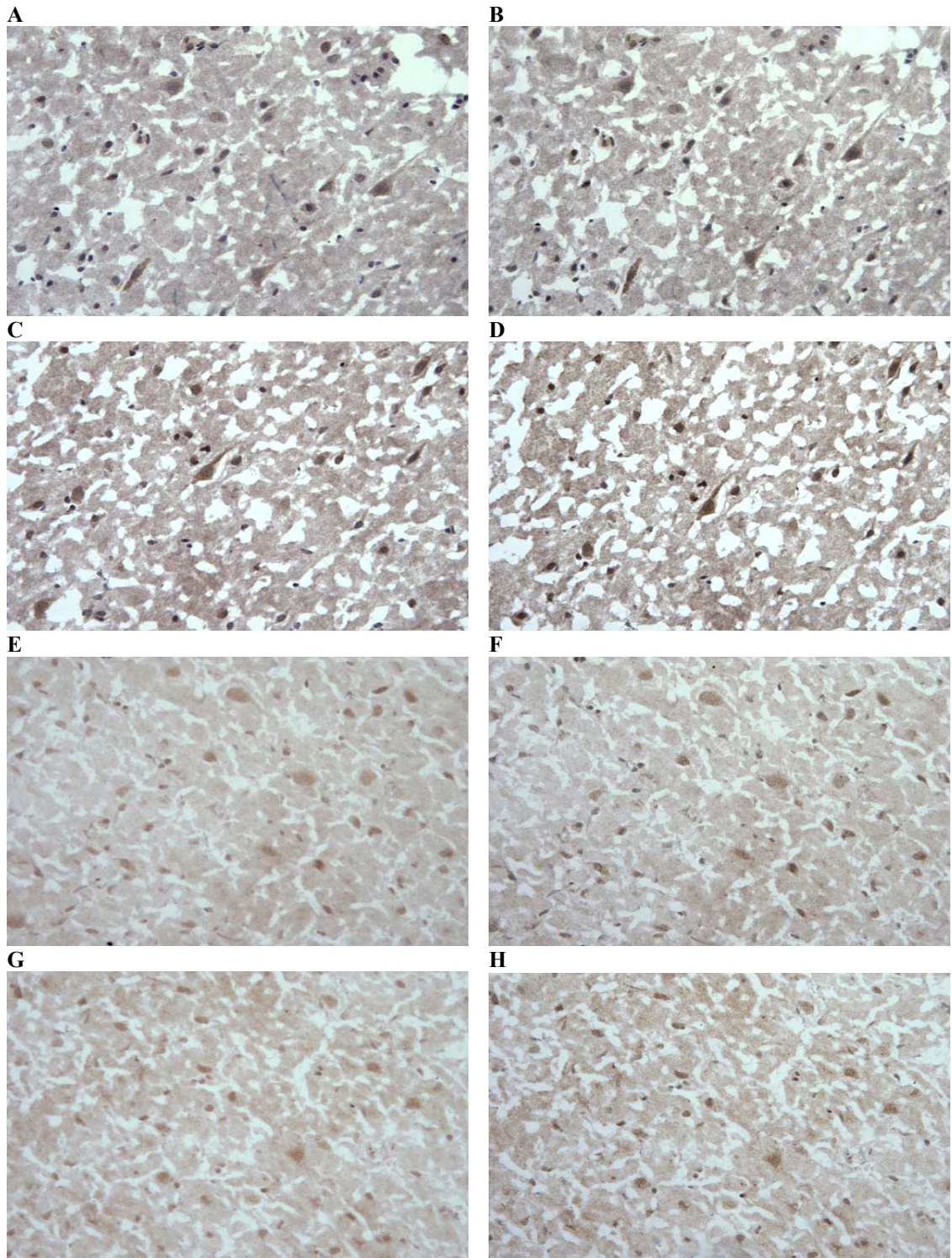


Figure 3.58. MIP-2/CXCL2 and CD11b Staining in the Cortex 4 Hours after Lateral Fluid Percussion Injury.

(A) MIP-2/CXCL2 staining in the ipsilateral and (E) contralateral cortex, then (B&F) after double staining for CD11b. (C&G) Respective consecutive MIP-2/CXCL2 stained sections before and (D&H) after staining with the omission of the CD11b primary. MIP-2/CXCL2 visualised with DAB (Brown) and CD11b visualized with VIP (Purple). Counterstained with haematoxylin. Unfixed frozen tissue. All x200.

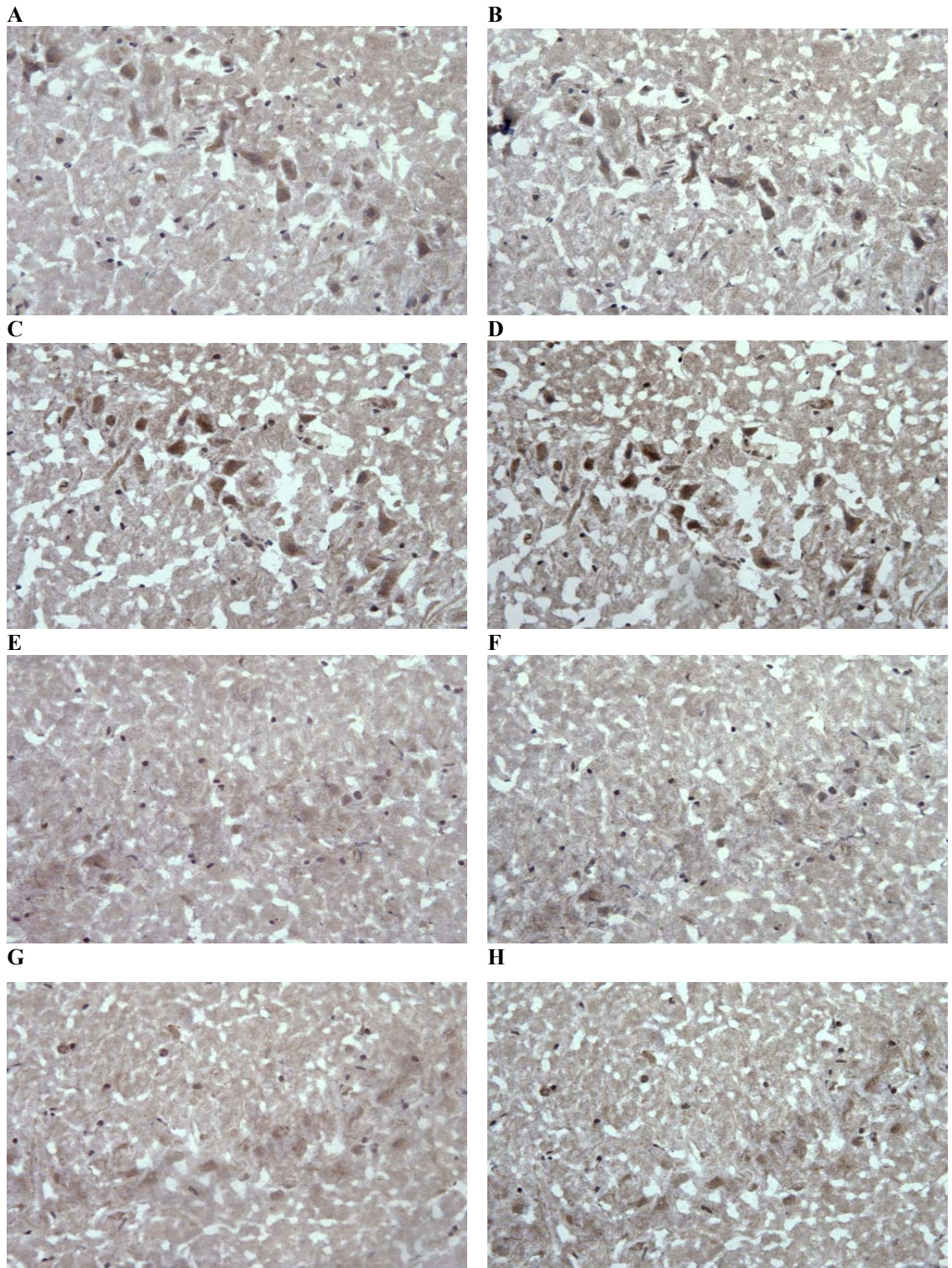


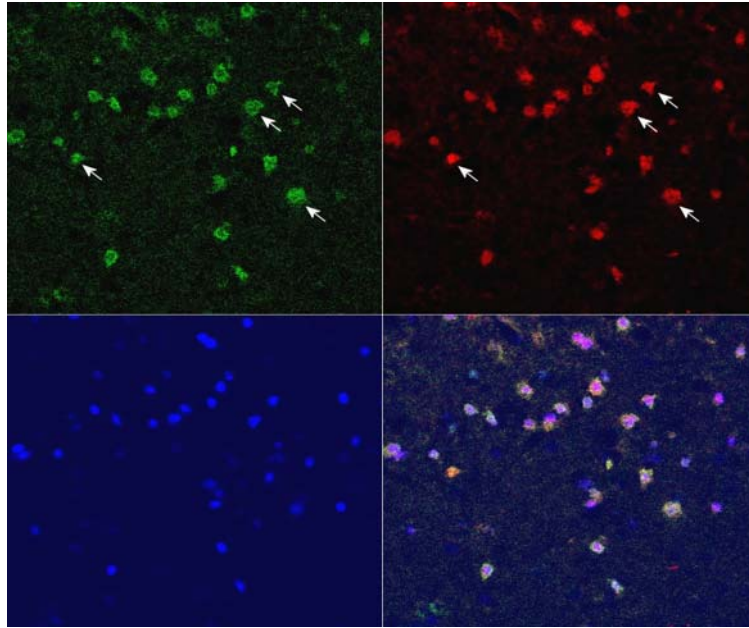
Figure 3.59. MIP-2/CXCL2 and CD11b Staining in the Hippocampus 4 Hours after Lateral Fluid Percussion Injury.

(A) MIP-2/CXCL2 staining in the ipsilateral and (E) contralateral hippocampus, then (B&F) after double staining for CD11b. (C&G) Respective consecutive MIP-2/CXCL2 stained sections before and (D&H) after staining with the omission of the CD11b primary. MIP-2/CXCL2 visualised with DAB (Brown) and CD11b visualized with VIP (Purple). Counterstained with haematoxylin. Unfixed frozen tissue. All x200.

3.5.5.4.4. Double Staining for Chemokines and NeuN; paraformaldehyde fixed tissue and visualisation by fluorescent secondary antibodies.

Although chemokine positive cells in contused tissue had the morphology of degenerating neurones, NeuN staining was poor in this region. This made it difficult to be satisfied that there was co-distribution of chemokine and cell marker in the ipsilateral deep cortex. In an attempt to test the hypothesis that the positive cells were neurones, I repeated the staining for MIP-2/CXCL2, MCP-1/CCL2 and NeuN. Visualisation was performed with fluorescent secondary antibodies using the refined immunohistochemistry protocol. I hoped that this might be a more sensitive technology for detecting the weak NeuN signal in damaged cells, whilst still being able to distinguish it from the chemokine signal. Two animals were sacrificed at 4 and 9 hours post lateral fluid percussion injury respectively. Co-localisation of MIP-2/CXCL2 and NeuN could be seen in the ipsilateral deep cortex (Figure 3.60). In the contralateral cortex similar co-localisation was also seen although the MIP-2/CXCL2 signal was less intense (Figure 3.61). In the ipsilateral deep cortex at 9 hours post injury NeuN and MCP-1/CCL2 signals also co-localised (Figure 3.62). However in the contralateral hemisphere the non-primary section also had a significant background signal and contained numerous artefacts. Considering this, no MCP-1/CCL2 signal above this background could be identified in the contralateral cortex (Figure 3.63).

A



B

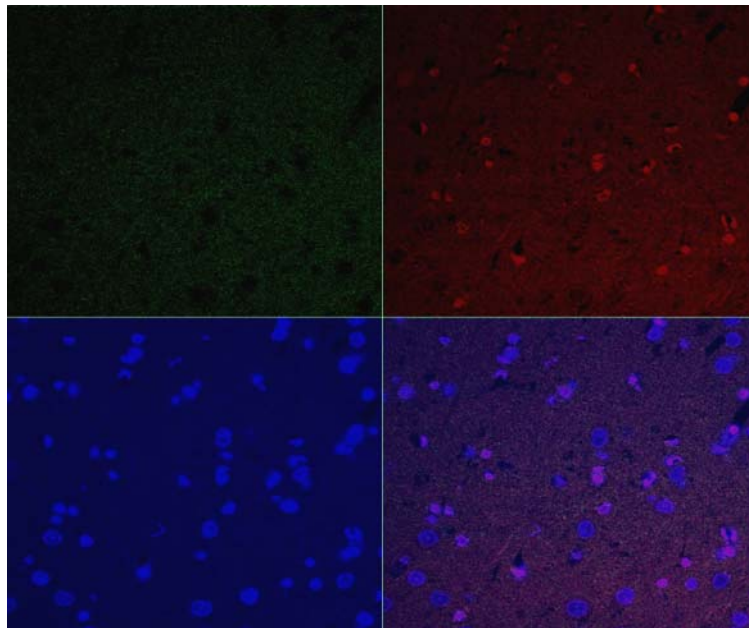
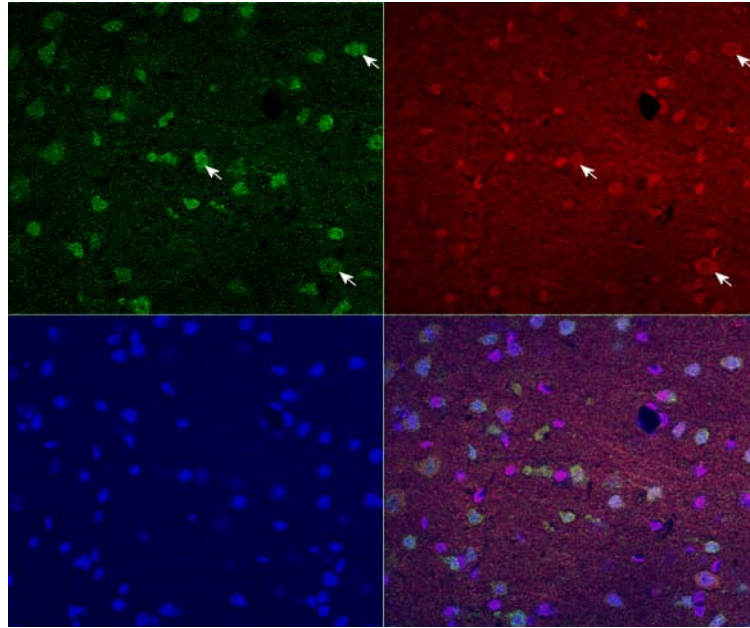


Figure 3.60. MIP-2/CXCL2 and NeuN Staining in the Ipsilateral Deep Cortex 4 Hours after Lateral Fluid Percussion Injury.

(Clockwise from bottom left) (A) TO-PRO®-3, NeuN, MIP-2/CXCL2 and merged image. Co-localising cells marked by white arrows. (B) Control section with omission of NeuN and MIP-2/CXCL2 primary antibodies. Visualisation with Alexa Fluro® 488 (Green) for NeuN and Alexa Fluro® 546 (Red) for MIP-2/CXCL2. Paraformaldehyde fixed tissue. All x400.

A



B

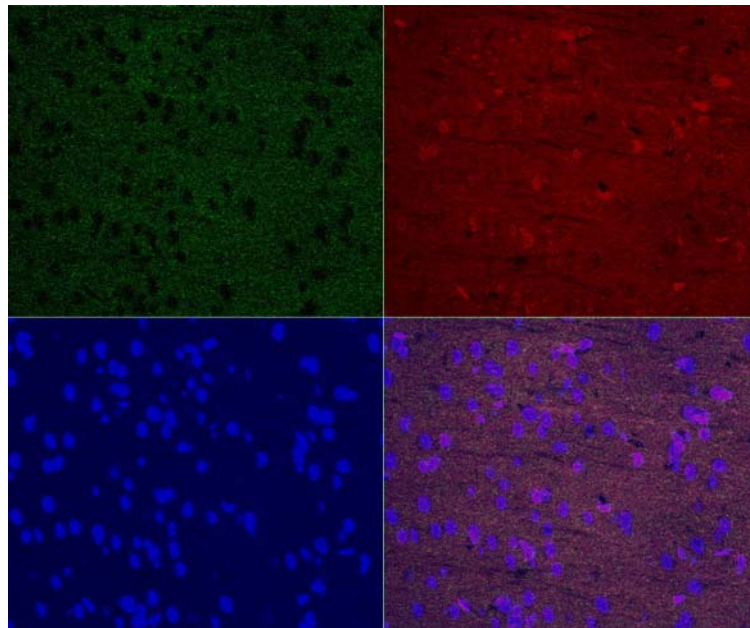
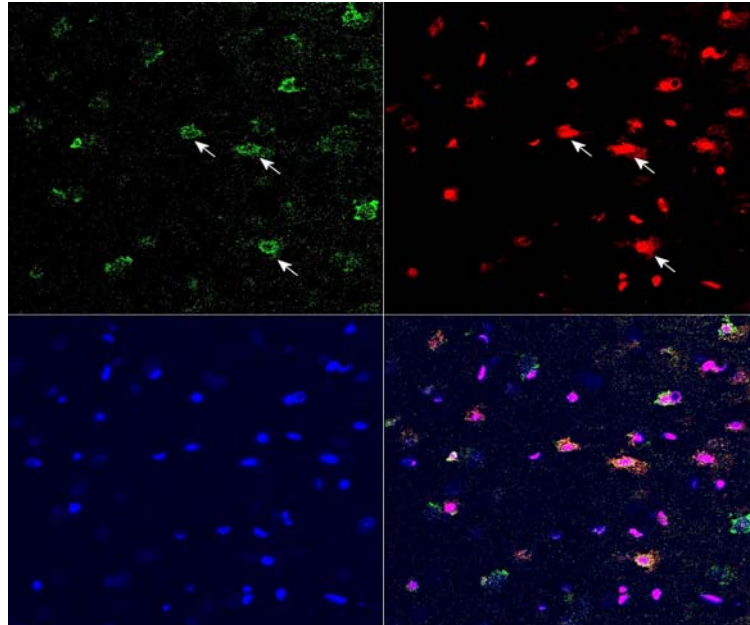


Figure 3.61. MIP-2/CXCL2 and NeuN Staining in the Contralateral Deep Cortex 4 Hours after Lateral Fluid Percussion Injury.

(Clockwise from bottom left) (A) TO-PRO®-3, NeuN, MIP-2/CXCL2 and merged image. Co-localising cells marked by white arrows. (B) Control section with omission of NeuN and MIP-2/CXCL2 primary antibodies. Visualisation with Alexa Fluro® 488 (Green) for NeuN and Alexa Fluro® 546 (Red) for MIP-2/CXCL2. Paraformaldehyde fixed tissue. All x400.

A



B

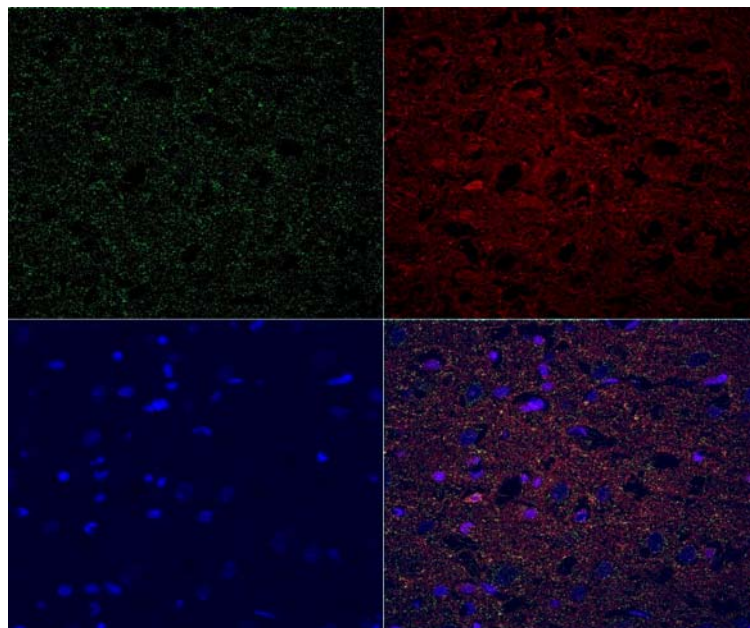
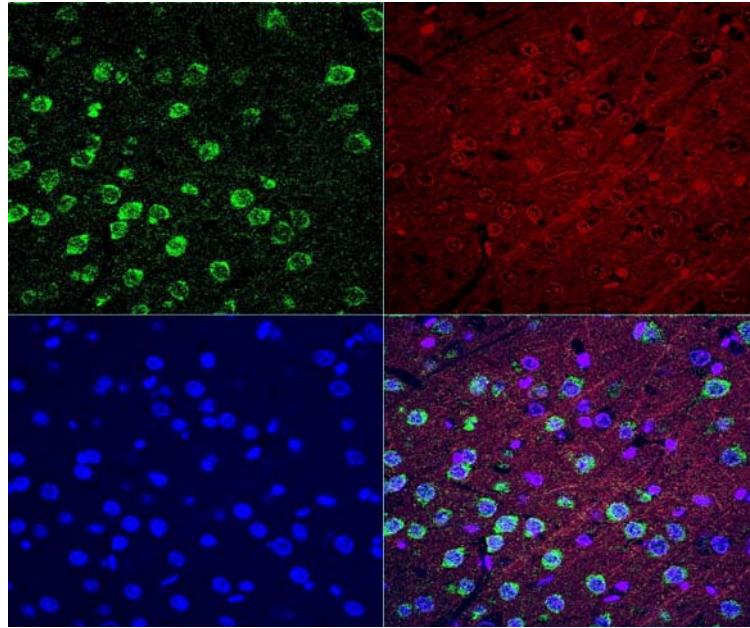


Figure 3.62. MCP-1/CCL2 and NeuN Staining in the Ipsilateral Deep Cortex 9 Hours after Lateral Fluid Percussion Injury.

(Clockwise from bottom left) (A) TO-PRO®-3, NeuN, MCP-1/CCL2 and merged image. Co-localising cells marked by white arrows. (B) Control section with omission of NeuN and MCP-1/CCL2 primary antibodies. Visualisation with Alexa Fluro® 488 (Green) for NeuN and Alexa Fluro® 546 (Red) for MCP-1/CCL2. Paraformaldehyde fixed tissue. All x400.

A



B

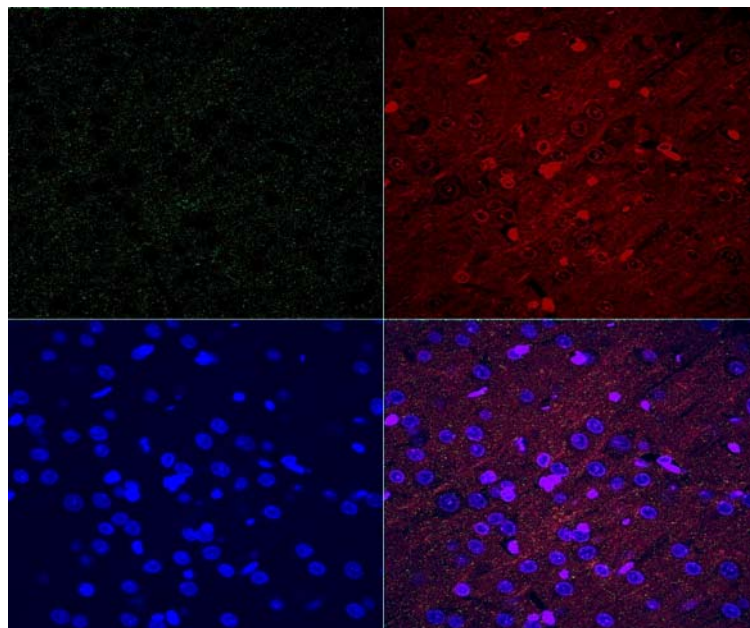


Figure 3.63. MCP-1/CCL2 and NeuN Staining in the Contralateral Deep Cortex 9 Hours after Lateral Fluid Percussion Injury.

(Clockwise from bottom left) (A) TO-PRO®-3, NeuN, MCP-1/CCL2 and merged image. (B) Control section with omission of NeuN and MCP-1/CCL2 primary antibodies. Visualisation with Alexa Fluro® 488 (Green) for NeuN and Alexa Fluro® 546 (Red) for MCP-1/CCL2. Paraformaldehyde fixed tissue. All x400.

3.6. Discussion

3.6.1. Summery of Achievements/Findings

- Established the LPFI model of TBI in Edinburgh.
- Developed an immunohistochemical protocol for visualising chemokine expression following LFPI.
- LFPI induces acute and transient expression of MIP-2/CXCL2 and MCP-1/CCL2 in the ipsilateral cortex.
- LFPI induces expression of MIP-2/CXCL2 in the ipsilateral hippocampus.
- In non-contused tissue MIP-2/CXCL2 is expressed in cells with the morphology of neurones and which co-distribute with the neuronal marker NeuN but not the astrocyte marker GFAP.
- Following trauma MIP-2/CXCL2 staining is increased in cells within the contused tissue which have the morphology of degenerating neurones.
- In non-contused tissue MCP-1/CCL2 is also expressed in cells with the morphology of neurones.
- Following trauma MCP-1/CCL2 staining is increased in cells within the contused tissue which have the morphology of degenerating neurones.
- MCP-1/CCL2 expression after LFPI but not MIP-2/CXCL2 is inhibited by dexamethasone. MIP-2/CXCL2 and MCP-1/CCL2 expression after LFPI are not inhibited by FK506.

3.6.2. Establishing the LFPI model of TBI

The LFPI model as described by McIntosh and colleges had not previously been used in the Britain prior to this study. Establishing the model was greatly facilitated by contacting Professor McIntosh and arranging to visit his facility in Philadelphia. This discussion was based on difficulties which had been encountered when another head trauma model, the Marmarou weight drop model of diffuse axonal injury (Marmarou et al., 1994), was introduced to the University. The first hand witnessing of the surgery and use of the model permitted a level of understanding of the technique that was not possible from published descriptions. Particularly useful was the observation of the pressure trace generated by the FPI device. This proved to be invaluable when the machine was initially assembled, filled and tested, as it was clear that the pressure trace produced was very different from what I had seen in Philadelphia. This was subsequently traced to a fine crack in the body of the FPI device. An additional benefit of first hand observation was that I was able to reassure the University veterinarians and the Home Office of the injury severity likely to be experienced, which greatly helped the granting of a project licence.

The initial non-recovery work produced a lesion in the parietal cortex with traumatic haemorrhage and histological evidence of neuronal damage in both the ipsilateral cortex and hippocampus consistent with the published literature (Cortez et al., 1989, Hicks et al., 1996). This was supported by the latter recovery studies of BBB disruption in the ipsilateral hemisphere, also consistent with the literature (Cortez et al., 1989, McIntosh et al., 1989), and the histology seen in the fixed tissue sections. Furthermore the recruitment of neutrophils and visualisation of monocytes post injury was as expected (Soares et al., 1995).

In order to successfully replicate the use of this model in Edinburgh I was very careful to use exactly the same materials and anaesthetic regime as was used in Philadelphia. The LFPI device and the specialist trephine were imported from the same suppliers used by Professor McIntosh's group. However, as described the mortality experienced initially was unacceptably high. This was reduced to approximately 5%, by removing the requirement for repeated administration of barbiturates and the use of pre-oxygenation prior to delivery of the injury. Despite these refinements some deaths still

occurred during surgery and after discussion with the veterinarians this was felt to be due to the low therapeutic index of barbiturate anaesthesia. In studies conducted subsequently with this model anaesthesia was induced and maintained with the volatile agent isoflurane which has the advantage of being titratable. The mortality rate associated with anaesthesia has now been reduced to zero.

3.6.3. Cerebral Chemokine Expression

I have demonstrated that after moderate LFPI in the rat there are early increases in both MIP-2/CXCL2 and MCP-1/CCL2 mRNA and protein in the damaged cortex (Figures 3.13 & 3.16). I have also shown that MIP-2/CXCL2 protein was significantly increased in the ipsilateral hippocampus following injury (Figure 3.17). The peaks in protein expression occur within 12 hours of injury, with MIP-2/CXCL2 preceding MCP-1/CCL2. These are also transient, returning to contralateral levels within 24 hours of injury. Similar increases in MIP-2/CXCL2 expression have been reported in other models of TBI. After closed head injury MIP-2/CXCL2 was significantly increased in the ipsilateral hemisphere of mice at 4 hours (Otto et al., 2001) and following cortical impact in rats peaked at 8 hours post injury (Valles et al., 2006). In both studies the concentration of MIP-2/CXCL2 had decreased by 24 hours. Elevated MCP-1/CCL2 concentration has also been described after experimental TBI (Rancan et al., 2001). This occurred in a diffuse axonal injury model. In keeping with the lack of neutrophil infiltration seen in this model (Csuka et al., 2000, Rancan et al., 2001) MIP-2/CXCL2 was not elevated after injury. Although MCP-1/CCL2 protein has been shown to be increased by cortical stab injury (Glabinski et al., 1996) and cortical resection (Muessel et al., 2000), to my knowledge this is the first report of an increase in MCP-1/CCL2 expression in a contusional injury.

3.6.4. Serum Chemokine Concentrations

I have also found that despite significantly increase cortical MIP-2/CXCL2 and MCP-1/CCL2 there was no increase in the serum concentrations of these chemokines associated with injury (Figure 3.18). Increases in the concentration of other inflammatory cytokines in blood or CSF after CNS injury have been described. Elevated levels of TNF, IL-1 and IL-6 (See section 1.4.1.1, table 1.1) have been

reported in patients following both elective neurosurgical procedures and TBI. Levels tend to be either raised on admission or peak within 24 hours of injury. In some studies analysis of both CSF and blood levels were performed simultaneously. TNF- α (Csuka et al., 1999, Morganti-Kossmann et al., 1997, Shiozaki et al., 2005), IL-1 β (Shiozaki et al., 2005) and IL-6 (Csuka et al., 1999, Hans et al., 1999, Kossmann et al., 1995, McClain et al., 1991, Morganti-Kossmann et al., 1997) were increased in both compartments. The CSF concentrations exceed those of the blood, suggesting that the injured CNS was the source of the cytokine expression.

The maximal increases in MIP-2/CXCL2 and MCP-1/CCL2 observed in the cortex after LFPI were in the order of 7 and 5 times that of baseline concentrations. The reason for the lack of increase in serum concentration with trauma is not clear. IL-8/CXCL8 is increased in serum following clinical TBI but at a level very much lower than that seen in the CSF (Hayakata et al., 2004, Kossmann et al., 1997, Morganti-Kossmann et al., 1997, Mussack et al., 2002, Otto et al., 2000, Whalen et al., 2000). In contrast to the clinical studies of patients with severe TBI, the LFPI model used in this study delivers a moderate level of injury (Reflected by a low mortality of less than 5% in spontaneously breathing animals). The absence of a measurable increase in serum chemokine concentration might therefore reflect the lower severity of injury in the experimental model vs. clinical TBI. Chemokines exert their chemotactic action at the endothelium where they are held on the endothelial surface to interact with rolling leucocytes. An increase in serum concentration is therefore not required for their function (Johnston & Butcher, 2002).

3.6.5. Inhibition of Chemokine Production by Dexamethasone

The administration of dexamethasone immediately after injury significantly reduced the expression of MCP-1/CCL2 but not MIP-2/CXCL2 across the range of doses employed (Figure 3.19). The inhibitory effect of the steroid on chemokine expression was studied at 4 hours, for MIP-2/CXCL2 and 9 hours, for MCP-1/CCL2, post injury. These time points corresponded to the peak expression of these chemokines in this model (Figure 3.16). FPI has previously been shown to induce IL-1 β mRNA and protein production in the injured cortex (Fan et al., 1995, Taupin et al., 1993). At both time points

in my study the tissue IL-1 β concentrations in the injured hemisphere were significantly decreased by dexamethasone (Figure 3.20). This finding would therefore imply that the failure to inhibit MIP-2/CXCL2 at 4 hours post injury was not due to insufficient penetration of dexamethasone in to the brain to inhibit inflammation.

The present studies (Chapter 2) demonstrated that in the rat astrocyte cell line CTX-TNA2, dexamethasone inhibited MIP-2/CXCL2 production (Figure 2.21). *In-vivo* dexamethasone also reduced MIP-2/CXCL2 production following the perfusion of lung tissue with LPS (Held et al., 2001) or the injection of bacterial enterotoxin into subcutaneous air pouches (Schramm & Thorlacius, 2003). The lack of inhibition of MIP-2/CXCL2 following trauma might be because the cells responsible for this expression are insensitive to dexamethasone. Although both IL-8/CXCL8 and MCP-1/CCL2 production can be inhibited by dexamethasone, in cultures of renal proximal tubular epithelial cells up to 10 μ M dexamethasone did not inhibit chemokine production (de Haij et al., 2002).

Pro-inflammatory cytokines such as TNF- α and IL-1 β have frequently been used as agonists to induced chemokine expression *in-vitro* (Aloisi et al., 1992, Barna et al., 1994, Calvo et al., 1996, Croitoru-Lamoury et al., 2003, Hayashi et al., 1995, Hua & Lee, 2000, Hurwitz et al., 1995, Janabi et al., 1999, Oh et al., 1999, Otto et al., 2000, Peterson et al., 1997). In chapter 2 I also demonstrated the significant increase in glial MIP-2/CXCL2 and MCP-1/CCL2 production with these cytokines (Figures 2.10 & 2.11). It is therefore interesting that despite the significant inhibition of cortical IL-1 β by dexamethasone, MIP-2/CXCL2 production was not inhibited. The expression of this chemokine occurred independently of IL- β . This suggests the possibility that *in-vivo* the production of MIP-2/CXCL2 after trauma is due to an inherent cellular response to trauma and not secondary to pro-inflammatory cytokine accumulation. Alternatively it could be secondary to the production of another pro-inflammatory cytokine such as TNF- α , the concentration of which I did not measure. However the latter would be counter-intuitive as TNF- α production has also been shown to be inhibited by glucocorticoid in both *in-vitro* and in *in-vivo* models of CNS injury (Buttini et al., 1997, Schwartz et al., 1998, Xu et al., 1998).

3.6.5.1. The Risk vs. Benefit of Steroid Administration after Brain Injury

In clinical practice steroids have been widely used to treat cerebral oedema associated with brain tumours, postoperative brain swelling (GALICICH & FRENCH, 1961) and then raised intracranial pressure generally (Alderson & Roberts, 2000). Despite over 30 years of use to treat traumatic brain injury, this has been largely empirical. Prior to 2004, despite nearly 20 clinical trials, neither moderate benefits nor harmful effects of steroid administration to patients with TBI could be excluded (Alderson & Roberts, 2000). However the recently published corticosteroid randomisation after significant head injury (CRASH) trial, a large multicentre randomised placebo controlled study of steroid treatment in over 10,000 patients with TBI, found that the use of steroids was associated with excess mortality leading to termination of the trial and early publication (Roberts et al., 2004). In spinal cord injury two clinical studies of high dose methylprednisolone demonstrated improvement in motor function (Bracken et al., 1990, Bracken et al., 1997) and the administration of methylprednisolone to patients with acute spinal cord injury has been adopted in many centres. However these results were based on subgroup analysis and multiple comparisons of secondary end points. The methodology of this work has been severely criticised (Coleman et al., 2000). Furthermore these results have not been reproduced (Pointillart et al., 2000). Similarly following acute ischaemic stroke no beneficial effect of steroid treatment on mortality has been demonstrated (Qizilbash et al., 2002).

Conversely animal models of acute CNS injury have frequently demonstrated that steroid treatment is associated with reduction in lesion volume, reduced cerebral oedema, improvement in function or reduced mortality (Barks et al., 1991, Bertorelli et al., 1998, Felszeghy et al., 2004, Hall, 1985, Harrison et al., 1973, Hoppe et al., 1974, Kobrine & Kempe, 1973, Limbourg et al., 2002, Pappius & McCann, 1969, Slivka & Murphy, 2001, Tuor & Del Bigio, 1996) (See section 2.2.2.2, table 2.2). These beneficial effects were associated with pre-insult administration or early treatment after injury. This would suggest that positive effect of steroid administration occurs through the modulation of processes that occur very early after injury. In the CRASH trial steroid treatment was administered within 1 hour of injury to only 27% of patients (Roberts et al., 2004).

Interestingly 2 studies of transient ischaemia appear to have conflicting results. In a study by Limbourg and colleagues dexamethasone treatment after transient ischaemia in the mouse reduced infarct volume assessed at 24 hours (Limbourg et al., 2002). However when an equivalent dose of methylprednisolone (McAuley, 2006) was administered repeatedly to rats after the same period of transient ischaemia no reduction infarct size was found at 72 hours after injury (Kim et al., 1995a) (See section 2.2.2.2.1, table 2.2). There are clearly differences in the species and drug employed in these studies but 2 potentially important ideas could also be considered. Firstly that in some injuries the administration of corticosteroids merely serves to modulate the evolution of the lesion but does not affect that final outcome. Secondly the possibility exists that large and sustained doses of steroid might in themselves be toxic.

The inconsistency between clinical and experimental results of steroid administration might therefore be in part due to delay in the administration of steroid relative to the injury and the subsequent expression of inflammatory mediators. The potential for a toxic effect of steroids on vulnerable neurones also exist.

3.6.5.1.1. The Effect of Glucocorticoids on Neuronal Survival

The overall effect of steroid administration might be complicated by several factors including heterogeneous responses of neuronal populations to glucocorticoid, neuronal endangerment, and modulation of growth factor expression.

3.6.5.1.1.1. Anatomical Selection

Glucocorticoids appear to influence neuronal survival or morphology and have been shown to be anatomically selective. The chronic elevation of corticosterone concentration by daily administration or repeated stress induces atrophy of the apical dendrites of CA3 pyramidal neurones, (Magarinos & McEwen, 1995a, Magarinos & McEwen, 1995b, Woolley et al., 1990). Conversely adrenalectomy results in massive cell death and shrinkage of the granule cells of the dentate gyrus, occurring over days (Gould et al., 1990, Sloviter et al., 1989).

3.6.5.1.1.2. *Neural Endangerment*

Acute administration of high doses of corticosterone alone does not harm neurones (Behl, 1998, Sapolsky, 1986). However such exposure does appear to compromise the ability of neurones to survive concurrent insults and therefore might be relevant in TBI (Gubba et al., 2000). Methylprednisolone and dexamethasone worsen quinolinic acid and N-methyl-D-aspartate (NMDA) induced striatal lesions (Supko & Johnston, 1994, Uhler et al., 1994) and in culture corticosterone exacerbates the effects of hypoxia-hypoglycaemia on rat hippocampal neurones (Tombaugh et al., 1992, Tombaugh & Sapolsky, 1993). These insults can potentially deplete energy supplies, either by increased metabolic demand or reduced substrate supply (Gubba et al., 2000). Glucose utilisation in hippocampal neurones has been shown to be impaired by glucocorticoids (Sapolsky, 1986) therefore promoting energy depletion. This becomes toxic when ATP reserves are low enough to cause neuronal depolarisation, resulting in glutamate excitotoxicity and lethal intracellular accumulation of calcium (Choi, 1994). Furthermore glucocorticoids inhibit glutamate uptake by astrocytes (Virgin, Jr. et al., 1991).

The potential protective effect of antagonising glucocorticoids has been investigated in TBI. Rats pre-treated for 48 hours with the glucocorticoid receptor antagonist mifepristone (RU486) (25mg/kg twice daily, SC) or vehicle were subjected to controlled cortical impact of moderate severity. At 24 hours post injury significant cell loss was seen in both CA1 and CA3 pyramidal hippocampal neurones. However pre-treatment with mifepristone significantly reduced the cell loss of the CA1 neurones (McCullers et al., 2002).

3.6.5.1.1.3. *Neurotrophic Factors*

Corticosterone exposure down regulates basal levels of brain derived neurotrophic factor (BDNF) mRNA in the hippocampus *in vivo* (Schaaf et al., 1997, Smith et al., 1995). BDNF has been shown to protect against glutamate induced neurotoxicity in the hippocampus (Mattson et al., 1995) and reduce the vulnerability of neurones to glucose deprivation (Cheng & Mattson, 1994). BDNF expression is also increased in the hippocampus after FPI. Adrenalectomy is associated with a further increase in

the expression of BDNF mRNA and this up regulation is prevented by corticosterone replacement (Grundy et al., 2000).

3.6.5.1.1.4. Inhibition of MCP-1/CCL2 Production by Dexamethasone

My finding that the cortical concentration of MCP-1/CCL2 but not MIP-2/CXCL2 is reduced by dexamethasone after LFPI suggests another mechanism by which steroid administration after TBI may be detrimental. Following focal TBI the pattern of leucocyte recruitment is characteristic with neutrophils predominating over the first 24-48 hours and then being replaced by infiltrating monocytes and reactive microglia (Aihara et al., 1995, Biagas et al., 1992, Clark et al., 1994, Hausmann et al., 1999, Holmin et al., 1997, Holmin et al., 1998, Soares et al., 1995). Macrophages phagocytose apoptotic neutrophils, in doing so preventing them undergoing secondary necrosis and releasing inflammatory proteases, thus helping to limit inflammation (Heasman et al., 2003, Savill et al., 1989). It has also been suggested that a negative feed back exists by which the activated macrophages ingesting apoptotic cells switch from a pro to an anti-inflammatory phenotype which secrete anti-inflammatory cytokines and further contribute to the resolution of inflammation (Duffield, 2003, Porcheray et al., 2005). Steroid administration could adversely modulate this system in two ways. Firstly glucocorticoid administration delays neutrophil apoptosis increasing the potential for protease release and free radical formation (Cox, 1995). Secondly my observation that dexamethasone reduces MCP-1/CCL2 concentration but not MIP-2/CXCL2 could potentially reduce monocyte/macrophage accumulation into contused tissue without reducing neutrophil recruitment. Neutrophil clearance could then be delayed and the resolution of inflammation inhibited, promoting further secondary brain injury.

3.6.6. Lack of Inhibition of Chemokine Production by FK506

The administration of FK506 prior to LFPI did not significantly diminish the increase in cortical MIP-2/CXCL2 or MCP-1/CCL2 concentrations at 4 or 9 hours after injury respectively (Figure 3.21). The cortical concentration of IL-1 β was not reduced by FK506 either (Figure 3.22). The dose route and timing of FK506 administration (1mg/kg IP 30 minutes prior to injury) has previously been shown to

reduce lesion volume following transient ischaemia (Butcher et al., 1997, McCarter et al., 2001). For MIP-2/CXCL2 and IL-1 β at 4 hours post LFPI, non-significant reductions in cortical concentrations of 23% and 18% respectively were found but the analysis was under powered due to the small sample size (n=3 per group). Conversely in cortical samples from FK506 treated animals at 9 hours post LFPI MCP-1/CCL2 and IL-1 β concentrations were increased. For MCP-1/CCL2 this was not significant at 7.6% but again the analysis was underpowered. However for IL- β concentration, the increase of 51%, was significant.

In-vivo FK506 has shown promise in pre-clinical studies, reducing both ischaemic lesion volume and ameliorating some features of TBI (See section 2.2.2.3.1, table 2.3). *In-vivo* microglial/macrophage and astrocyte activation following both permanent and transient ischaemia (Wakita et al., 1998, Zawadzka & Kaminska, 2005) along with cortical expression of IL-1 β , TNF- α and IL-6 mRNAs after transient ischaemia (Zawadzka & Kaminska, 2005) were reduced by treatment with FK506. FK506 inhibits the generation of superoxide radical by neutrophils (Nishinaka et al., 1993). The inhibition of neutrophil/leucocyte infiltration by FK506 has also been reported in following ischaemia in tissues including liver (Garcia-Criado et al., 1997, Suzuki et al., 1993, Wakabayashi et al., 1994), gut (Kubes et al., 1991) and retina (Tsujikawa et al., 1998).

What has not been clear in previous studies is whether the reduction of inflammatory mediators and cellular infiltrate associated with FK506 is due to an anti-inflammatory effect of the drug or secondary to reduced lesion volume and cell death. Recently FK506 has been shown to reduce TNF- α , IL-1 β and IL-6 mRNA expression in astrocytes and inhibited LPS induced mRNA expression of these cytokines by microglia, suggesting a direct anti-inflammatory effect (Zawadzka & Kaminska, 2005). In contrast to this study I did not find that FK506 significantly inhibited TNF- α induced MIP-2/CXCL-2 or MCP-1/CCL-2 protein production in rat CTX-TNA2 astrocytes (Figure 2.23)(Small 12.9% reduction in MCP-1/CCL2 production but the study was underpowered). When the in-vitro and in-vivo data are considered together it would appear that the anti-inflammatory properties of FK506 are quite specific.

3.6.7. Development of an Immunohistochemical Protocol for Chemokine

Expression

At the start of this study there were no commercially available antibodies sold as being suitable for MIP-2/CXCL2 or MCP-1/CCL2 immunohistochemistry in the rat. However a capture antibody was available for use as part of a MIP-2/CXCL2 ELISA which had been used to identify MIP-2/CXCL2 positive cells *in-vitro* (Wang et al., 2000). This was used to stain TNF- α treated astrocytes which I had demonstrated produce MIP-2/CXCL2. When visualised with a fluorescent secondary antibody it appeared to differentiate the TNF- α treated cells from those cultured in control media. However when this protocol was used on cerebral tissue sections obtained from animals receiving LFPI MIP-2/CXCL2 positive cells could not be identified. In part this was because I did not know what to look for. The visualisation of fluorescent secondary antibodies is also more complex than conventional light microscopy. The images were not resolved in real time and surveying a tissue section was therefore slow and comparisons between ipsilateral and contralateral areas of cortex difficult to make. The fluorescent marker also faded under the laser light preventing prolonged observation. With the adoption of chemokine visualisation with a biotinylated secondary antibody the ipsilateral and contralateral tissue could be compared at length and differences noted. The immunohistochemical protocol was then refined, firstly in frozen tissue and then in fixed sections, greatly improving the quality of the staining. Subsequently once positive cells could be readily identified by biotinylated secondary antibodies, visualisation with fluorescent secondary antibodies was successful.

3.6.8. Cellular Expression of MIP-2/CXCL2 and MCP-1/CCL2

In-vitro studies have reported the expression of MIP-2/CXCL2 and MCP-1/CCL2 by glia (Table 2.1). However I observed that *in-vivo* the expression of chemokines were localised to neurones.

Immunohistochemistry for MIP-2/CXCL2 (Figures 3.33, 3.34, 3.36 & 3.37) and MCP-1/CCL2 (Figures 3.40, 3.41, 3.43 & 3.44) demonstrated low level expression in undamaged cortex. Staining for MIP-2/CXCL2 was also seen throughout the hippocampus (Figures 3.35 & 3.38) but only occasional MCP-1/CCL2 positive cells were present (Figures 3.42 & 3.45).

The increase in MCP-1/CCL2 staining with injury was not as great as that for MIP-2/CXCL2 and the change in MCP-1/CCL2 staining was quantified by counting positive cells and grading the intensity of staining. A major weakness of this quantification was the lack of blinding which would have made the method more robust. However the findings of an increase in the number of positive cells and some increase in staining intensity in the damaged cortex were supported by the ELISA data, in which MCP-1/CCL2 was increased in the ipsilateral cortex (Figure 3.16).

In undamaged tissue expression of these chemokines was localised to cells with neuronal morphology and, in the case of MIP-2/CXCL2, which double stained for the neuronal marker NeuN (Figures 3.47, 3.48, 3.49). Following injury MIP-2/CXCL2 (Figures 3.33 to 3.38) and MCP-1/CCL2 (Figures 3.40 & 3.41) expression was localised to shrunken neurons. Double staining with the NeuN marker was poor in the contused tissue although for MIP-2/CXCL2 some co-localisation could be seen in the edges of the lesion (Figure 3.48). This reduction in NeuN staining could be analogous to the loss of microtubule associated protein (MAP-2) staining seen after cortical injury (Manley et al., 2006, Rhodes et al., 2002, Saatman et al., 2001). Using fluorescent secondary antibodies co-localisation of MIP-2/CXCL2 and MCP-1/CCL2 with NeuN staining was confirmed (Figures 3.60 & 3.62).

GFAP astrocyte staining and either MIP-2/CXCL2 or MCP-1/CCL2 did not co-distribute (Figures 3.52-3.56). The CD11b antibody, used initially to identify leucocytes, in cryosections, was not compatible with paraformaldehyde fixed tissue. With more time staining with an alternative antibody could have been attempted. Both Berman and Yamagami identified macrophages with an antibody against rat ED-1 (CD68) in fixed tissue (Berman et al., 1996, Yamagami et al., 1999). Alternatively the CD11b staining could have been performed on tissue after a limited post fixation in paraformaldehyde (Aihara et al., 1995, Soares et al., 1995). I had planned to perform immunohistochemistry for both MIP-2/CXCL2 and MCP-1/CCL2 on frozen cryostat sections at 4 and 9 hours post injury respectively, prior to double staining for CD11b positive cells. The MIP-2/CXCL2 staining in the frozen tissue was comparable to that seen in the fixed tissue, although technically inferior (Figures 3.29-3.32). However the MCP-1/CCL2 staining was unsatisfactory, with

DAB colouring present when the primary antibody was omitted. Therefore CD11b double staining was only completed on tissue from 4 hours post injury stained for MIP-2/CXCL2. In these slides CD11b positive monocytic cells could not be seen (Figures 3.58 & 3.59). For completeness MCP-1/CCL2 in frozen CNS tissue could have been performed as described by Kim and colleagues (Kim et al., 1995b). However the polyclonal rabbit anti rat MCP-1 antibody used was developed by this group and may not have been available (Jones et al., 1992). That said, macrophage infiltration into the parenchyma after brain injury is not believed to occur before 12 hours after injury (Hausmann et al., 1999, Holmin et al., 1995, Soares et al., 1995), with microglial activation also occurring relatively slowly (Aihara et al., 1995). This would support my finding that chemokine expression was not localised to monocytes acutely after LFPI.

In-vitro I have demonstrated that astrocytic expression of both MIP-2/CXCL-2 and MCP-1/CCL2 is significantly inhibited by dexamethasone (Figure 2.21). The lack of MIP-2/CXCL2 inhibition *in-vivo* by dexamethasone is in conflict with the *in-vitro* data. This inconsistency could be explained by my finding that MIP-2/CXCL2 and MCP-1/CCL2 are expressed by neurones and not glia *in-vivo*. MCP-1/CCL2 has been colocalised to astrocytes (Glabinski et al., 1996), monocytes and endothelial cells (Berman et al., 1996) after cortical stab injury (Section 1.4.2.3.2.3., table 1.4). This would suggest that *in-vivo* expression is specific to nature of the trauma. Following ischaemic lesions MCP-1/CCL2 has been commonly localised to astrocytes, microglia and monocytes (Che et al., 2001, Gourmala et al., 1997, Kim et al., 1995b, Minami & Satoh, 2003). In one report MCP-1/CCL2 was also localised to neurones, following PMCAO in mice (Che et al., 2001). In this study the cells were reported to be in the core of the lesion. Considering the ischaemia data (Table 1.4) together with my observation of the early expression in neurones after TBI suggests the possible hypothesis that initially MCP-1/CCL2 is localised to neurones in the core of the lesion, followed by glial cells in the penumbra (Che et al., 2001, Gourmala et al., 1997, Minami & Satoh, 2003). Later over days following an ischaemic insult monocytic cells invade the core of the lesion and become a source of MCP-1/CCL2 (Gourmala et al., 1997). A similar process may be operating following the focal contusion of LFPI but time points greater than 9 hours after injury were not studied in this present work.

**4. Serum Interleukin-8, Monocyte
Chemoattractant Protein-1 and
Soluble Interleukin-6 Receptor
Concentrations in Patients with
Cerebral Contusions**

4.1. Chapter Aims

- To determine the concentration of IL-8/CXCL8, MCP-1/CCL2 and sIL-6R in the serum of patients with cerebral contusions admitted to intensive care after TBI.
- To determine if in patients with contusional brain injury a relationship exists between serum chemokine or sIL-6R concentrations and the enlargement of contusions over time.

4.2. Background

As discussed previously in chapter 1 (See sections 1.13 & 1.1.5) patients sustaining traumatic brain injury (TBI) are at risk of deteriorating despite appropriate hospital management. This is typified by the patient that “talks and deteriorates” implying preservation of higher function initially and suggests that with suitable intervention outcome could be improved (Reilly et al., 1975). Deterioration occurs due to the occurrence of secondary injuries including the development of cerebral hypoxia, intracranial haematomas, enlarging contusions and the inflammatory response. Enlargement of contusions can develop over days, contributing to intracranial hypertension, often necessitating resection.

4.2.1. Inflammation and Contusion Enlargement

Contused tissue typically contains large numbers of neutrophils and later monocytes (See section 1.4.2.2.1). The presence of inflammatory cytokines in contused brain tissue (Fan et al., 1995, Fan et al., 1996, Holmin et al., 1997, Knobloch et al., 1999, Shohami et al., 1994, Taupin et al., 1993, Zhu et al., 2004)(See section 1.4.1.2.1, table 1.2) together with the recruitment of leucocytes suggests that these contribute to the enlargement of contusions. This view is broadly supported by the experimental evidence that cytokines promote oedema formation (de Vries et al., 1996, Feuerstein et al., 1998, Hara et al., 1997, Holmin & Mathiesen, 2000, Maruo et al., 1992, Megyeri et al., 1992, Quagliarello et al., 1991, Relton et al., 1996, Shohami et al., 1996, Yamasaki et al., 1995)(See sections 7.2.1.2, 7.2.2.1, 7.2.3.1 and tables 7.2 & 7.3) and the inhibition of leucocyte accumulation after acute brain injury is associated with reduced oedema formation and lesion size (Matsuo et al., 1994a, Matsuo et al., 1994b, Shiga et al., 1991)(See section 1.4.2.4.3, table 1.6). Furthermore cerebral contusions are often not apparent when a patient first presents but develop over 24 to 48 hours (Bullock et al., 1991). This formation follows the increase in inflammatory mediator concentrations reported in experimental TBI. In chapter 3, I demonstrated that MIP-2/CXCL2 expression following LFPI peaks at 4 hours after injury. This is similar to other focal experimental models where peak expression occurs between 4 and 8 hours (Otto et al., 2001, Valles et al., 2006)(See section 1.4.2.3.2.3, table 1.4). Peak neutrophil

accumulation follows this chemokine increase in experimental models and would correspond with the timing of contusion enlargement seen clinically (Biagas et al., 1992, Clark et al., 1994, Holmin et al., 1995, Soares et al., 1995).

4.2.1.1. Clinical Studies of Chemokine Expression and Acute Brain Injury

Compared with other pro-inflammatory cytokines (See section 1.4.1.1, table 1.1) there have been relatively few clinical studies of chemokine expression after acute brain injury. The neutrophil chemokine interleukin-8 (IL-8/CXCL8) (Hayakata et al., 2004, Kossmann et al., 1997, Mussack et al., 2002, Whalen et al., 2000) has been detected in both the blood and cerebrospinal fluid (CSF) of patients sustaining TBI. The CSF levels of IL-8/CXCL8 were greater than serum, suggesting that it is released into the circulation from the brain (Hayakata et al., 2004, Kossmann et al., 1997). IL-8/CXCL8 peaked in both compartments within 24 hours of injury (Hayakata et al., 2004). MCP-1/CCL2 is increased in the CSF but not the plasma of patients following ischaemic stroke (Losy & Zaremba, 2001).

With the increased expression of chemokines after acute experimental and clinical brain trauma preceding the accumulation of leucocytes into the brain and the enlargement of cerebral contusions it would be reasonable to believe that these events are related. If this is so, those patients with enlarging contusions might have more chemokine “spillage” from the inflamed cerebral parenchyma into the systemic circulation. The measurement of circulating chemokine concentration might differentiate those patients at risk of deteriorating due to contusion enlargement from those that would remain stable.

4.2.1.2. Soluble Interleukin-6 Receptor

Following acute injury to the brain the role of elevated tissue IL-6 is not yet clear. Neurotrophic (Hama et al., 1989, Hama et al., 1991, Kushima et al., 1992), neuroprotective (Ali et al., 2000, Loddick et al., 1998, Toulmond et al., 1992) and both pro (Biswas et al., 1998, Calvo et al., 1996, de Vries et al., 1996, Klein et al., 1997, Maruo et al., 1992, Modur et al., 1997, Penkowa et al., 1999,

Romano et al., 1997, Shafer et al., 2002, Watson et al., 1996, Wung et al., 2005) and anti- (Aderka et al., 1989, Benveniste et al., 1995, Jordan et al., 1995, Tilg et al., 1994, Xing et al., 1998) inflammatory actions have been described (See appendix section 7.2.3).

Evidence is emerging that IL-6 and its soluble receptor (Section 2.2.2.1.1) may contribute to the control of leucocyte recruitment. In 2001 Hurst and colleagues reported that in acute inflammation the release of sIL-6R from infiltrating neutrophils was responsible for the IL-6/sIL-6R mediated inhibition of neutrophil chemoattractant expression (Hurst et al., 2001)(Section 2.2.2.1.2).

The serum sIL-6R concentration after TBI has been measured in one clinical study. The median concentration of the receptor was not elevated above the normal range. However the majority of these patients had diffuse injuries (Hans et al., 1999). Diffuse injury is not associated with significant leucocyte recruitment (Csuka et al., 2000). Focusing on patients with contusional injuries, sIL-6R might be expected to be increased in the circulation of those patients who remain stable, compared with those with enlarging contusions, consistent with an anti-inflammatory role.

4.2.1.3. Pro and Anti-inflammatory Cytokines in Contusion Enlargement

I propose that an unfavourable balance of pro and anti-inflammatory cytokines may underpin the process of contusion enlargement and that this might be evident in the serum levels of these cytokines. Contusion enlargement resulting from inflammatory cell infiltration and leading to clinical deterioration would be associated with elevated serum levels of IL-8/CXCL8 and MCP-1/CCL2 compared to stable patients. Furthermore the sIL-6R concentration would be greater in stable patients compared to those with contusion enlargement, supporting an anti-inflammatory effect of IL-6. I have therefore investigated serum IL-8/CXCL8, MCP-1/CCL2 and sIL-6R concentrations in patients with severe contusional TBI requiring admission to intensive care.

4.3. Materials and Methods

4.3.1. Patients

Between November 2002 and June 2005 patients with severe traumatic head injury admitted to the Western General Hospital intensive care unit (ICU) were recruited into the study cohort, serum samples taken and considered for inclusion in the analysis of serum inflammatory mediator concentrations. Local ethics committee approval had been obtained prior to commencing the study. The Western General is a tertiary referral centre for neurosurgical patients covering the South East of Scotland. Age, sex, and best post resuscitation Glasgow coma scale (GCS) score (Range 3 to 15, 3 =deep coma and 15=normal) were recorded along with a summary of the head computerised tomography (CT) findings.

Patients were sedated, intubated and ventilated and then managed in accordance with established guidelines (Bullock et al., 1996) to maintain cerebral perfusion pressure (≥ 70 mmHg) and limit ICP (≤ 20 mmHg). This included vasopressors, moderate hyperventilation, paralysis and mannitol. Pyrexia $>38^{\circ}\text{C}$ was managed with paracetamol and cooling fans. Following a sustained elevation of ICP, refractory to further osmotherapy, a repeat CT scan of the head was performed and where necessary contusions resected.

The presence of cerebral contusion on the initial CT was required to be the predominant abnormality for patients in the study cohort to undergo analysis of serum inflammatory mediators. Patients in whom contusions were absent or minor features of the CT but subsequently deteriorated due to contusion enlargement were also included. Patients with significant extra-cranial injuries including pulmonary contusion, abdominal trauma or long bone fracture were excluded from the study. Similarly patients with chronic inflammatory disease were not included.

4.3.2. Sampling

From admission (Day 1) and for up to 11 days, blood was withdrawn from an arterial cannula into a non-heparinised collection tube (Monovette-sarstedt 03.1397.001, Nümbrecht, Germany) and allowed to stand at room temperature for 30 minutes to coagulate. This was then centrifuged for 15 minutes at 1000G. The serum fraction was then decanted into a fresh collection tube and stored at -20°C.

4.3.3. IL-8/CXCL8, MCP-1/CCL2 and sIL-6R Assay

Cytokine concentrations in the serum samples were determined using commercially available ELISA kits to measure human IL-8/CXCL8 (KHC0082), MCP-1/CCL2 (KHC1012), and sIL-6R (KHR0062) (Biosource International, Nivelles, Belgium). Each serum sample was measured in duplicate and the average value used.

Due to the limited volume of the extracellular fluid contained in the microvials (approximately 100µl) only IL-8/CXCL8 was measured in the microdialysis samples and this was performed once per sample.

4.3.4. Data and Statistics

The values for optical density obtained from the cytokine ELISA standards were entered into a commercially available graphing package (Sigma Plot 8.0, Systat Software Inc, London, UK) and a 4 parameter logistic regression curve plotted. These parameters were used to derive the cytokine concentrations from the optical densities of the samples tested. The lower limit of detection of the IL-8/CXCL8 ELISA was 5pg/ml. The data for IL-8/CXCL8 is plotted substituting this value for those samples in which IL-8/CXCL8 was not detected.

Inspection of the serum values revealed that they did not conform to a normal distribution and therefore the values are presented as median values with the ranges in parenthesis. Changes in serum levels over time were analysed by Kruskal-Wallis analysis of variance on ranks. Differences between

the subsets of data were compared with the Mann-Whitney rank sum test. Differences were considered statistically significant at $p < 0.05$.

4.4. Results

4.4.1. Patients

30 patients admitted to the ICU were recruited into the study cohort. Of these 18 patients (11 male and 7 female) with predominantly contusional brain injuries were identified (Table 4.1). Their mean age was 43 (Range 19 to 67). Four of these patients were initially managed outwith the ICU but suffered significant reductions in GCS between days 2 and 4 post injury, associated with contusion enlargement (Patients 1, 2, 4 & 5). One of these patients underwent a temporal lobectomy to remove a contusion. Four other patients (7, 12, 14 & 17) developed refractory intracranial hypertension whilst in ICU due to enlarging contusions, between days 1 and 3. Three of these underwent surgical lobectomy to remove the contusions. Three patients died whilst in intensive care (1, 7 & 14).

12 patients were not included in the serum analysis. The predominant CT abnormalities in these patients were subdural haematoma (7), extradural haematoma (2) and diffuse axonal injury (1). One patient with contusions had multiple long bone fractures and was not included. One patient had a skull fracture but the cause of the reduced GCS was probably excessive alcohol consumption.

The survivors were discharged to the neurosurgical high dependency unit (HDU) for further management (Observation, tracheostomy care, continuous positive airway pressure (CPAP) support and physiotherapy) and rehabilitation.

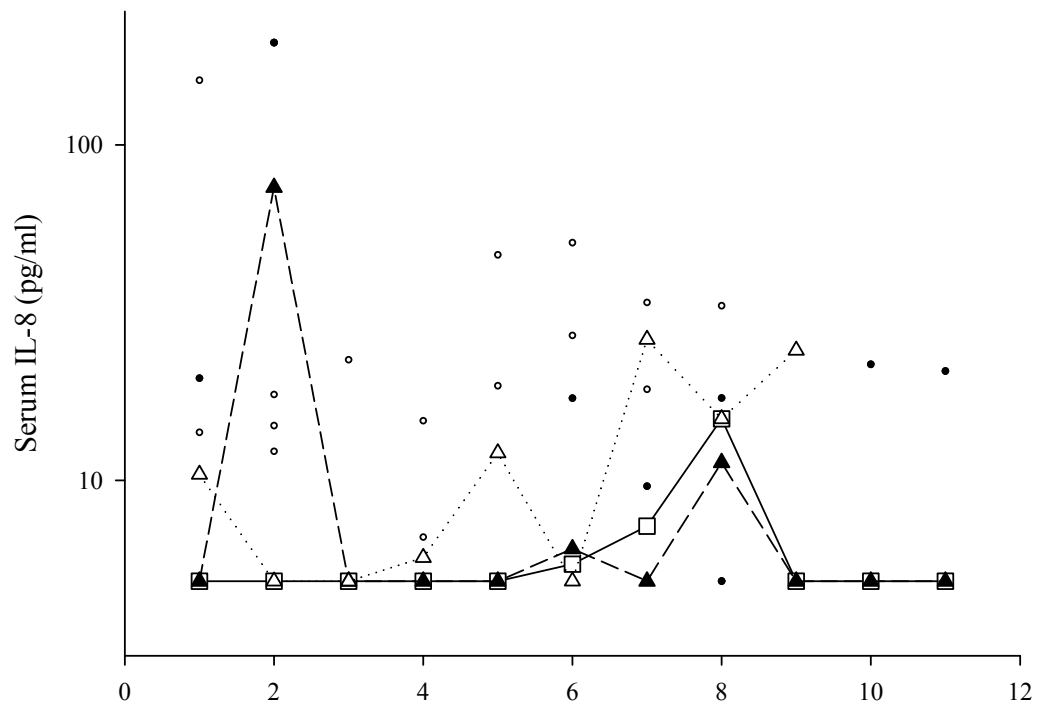
Patient	Age	Sex	GCS	Computerised Tomography Findings		Deterioration	Surgery	Outcome
1	45	F	12	L frontotemporal & R temporal contusions	D4	GCS dropped to 8		Died
2	47	F	12	Bilateral frontal & R temporal contusions	D2	GCS dropped to 9.		HDU
3	53	M	6	Bilateral frontal contusions & SDH, fronto-occipital skull #				HDU
4	25	M	13	Bilateral frontal & L temporal contusions+ R temporoparietal #	D4	GCS dropped to 10	Temporal lobectomy	HDU
5	66	M	14	L frontotemporal contusion, L temporo-occipital skull #	D3	GCS dropped to 9		HDU
6	20	M	9	L frontotemporal contusion, fronto-occipital #				HDU
7	39	M	3	R temporoparietal & frontal contusions, depressed skull #	D2	Increased ICP		Died
8	43	M	3	R temporal contusion, SDH, BOS #				HDU
9	40	F	6	R frontal contusion				HDU
10	62	M	7	L temporoparietal contusion, SDH	D1		Evacuation SDH	HDU
11	32	M	11	R frontoparietal contusion				HDU
12	67	F	9	R frontal contusion, SDH	D1	Increased ICP	R frontal & temporal lobectomy	HDU
13	37	M	7	Bitemporal contusions, depressed skull #, temporal & occipital #				HDU
14	45	F	3	Bifrontal contusions, BOS #	D2	Increased ICP	R frontal & temporal lobectomy	Died
15	19	M	10	Bilateral frontal contusions				HDU
16	35	M	7	L parietal contusions, facial #s				HDU
17	46	F	7	L temporal contusions, SDH	D3	Increased ICP	L temporal lobectomy	HDU
18	46	F	8	R frontal contusion, hydrocephalus secondary to traumatic SAH	D1		Ventricular access device	HDU

Table 4.1. Demographics of the Study Population and Outcome.

Base of Skull (BOS), day (D), fracture (#), Glasgow coma scale (GCS), high dependency unit (HDU), intracranial pressure (ICP), subarachnoid haemorrhage (SAH), subdural haematoma (SDH).

4.4.2. Serum Assays

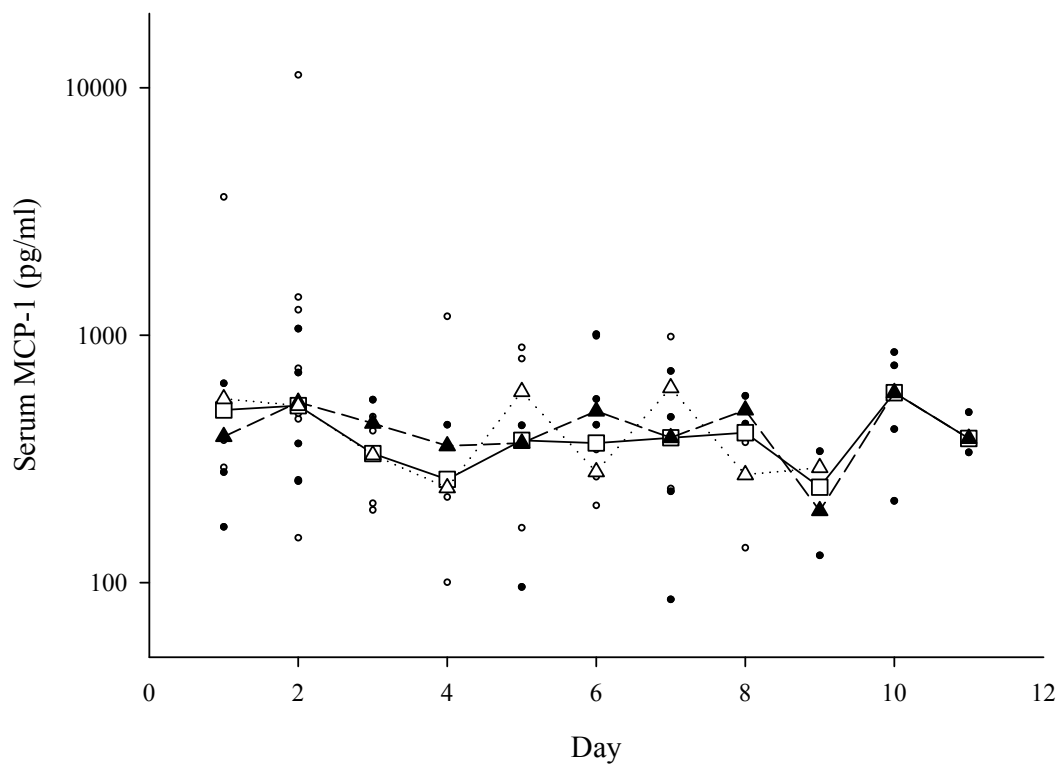
77 serum samples were available from the 18 patients distributed over 11 days. IL-8/CXCL8 was undetectable in 61% of the serum samples, median 0 (0-202) pg/ml. MCP-1/CCL2 and sIL-6R were detected in all samples, median 380 (86-11289) pg/ml and 72 (30-222) ng/ml respectively. Chemokine and receptor concentrations did not change significantly over time for the group of patients as a whole. Nor was there a change in IL-8/CXCL8, MCP-1/CCL2 or sIL-6R concentration over time in the patients that deteriorated clinically and/or who required surgical resection of contusions for elevated ICP (Figure 4.1, Figure 4.2 & Figure 4.3). Similarly when the temporal data were pooled, patients that deteriorated clinically and/or required lobectomy could not be distinguished from those that did not (0 (0-202) vs. 0 (0-157) pg/ml for IL-8/CXCL8, 385 (86-1062) vs. 372 (100-11289) pg/ml for MCP-1/CCL2 and 68 (37-179) vs. 73 (30-222) ng/ml for sIL-6R deteriorated/surgical resection vs. stable respectively (Figure 4.4). However in the 3 patients that died before leaving intensive care IL-8/CXCL8, MCP-1/CCL2 and sIL-6R were each significantly increased compared to those patients discharged to HDU (18 (0-202) vs. 0 (0-156) pg/ml for IL-8/CXCL8, 498 (339-1063) pg/ml vs. 368 (86-11289) pg/ml for MCP-1/CCL2 and 109 (37-179) ng/ml vs. 69 (30-222) ng/ml for sIL-6R died vs. discharged respectively) (Figure 4.5).



Day	1	2	3	4	5	6	7	8	9	10	11
Deteriorated	4	4	2	3	4	4	5	3	3	4	3
Stable	5	9	5	4	4	5	2	3	1	0	0

Figure 4.1. Serum IL-8/CXCL8 vs. Time after Contusional Brain Injury

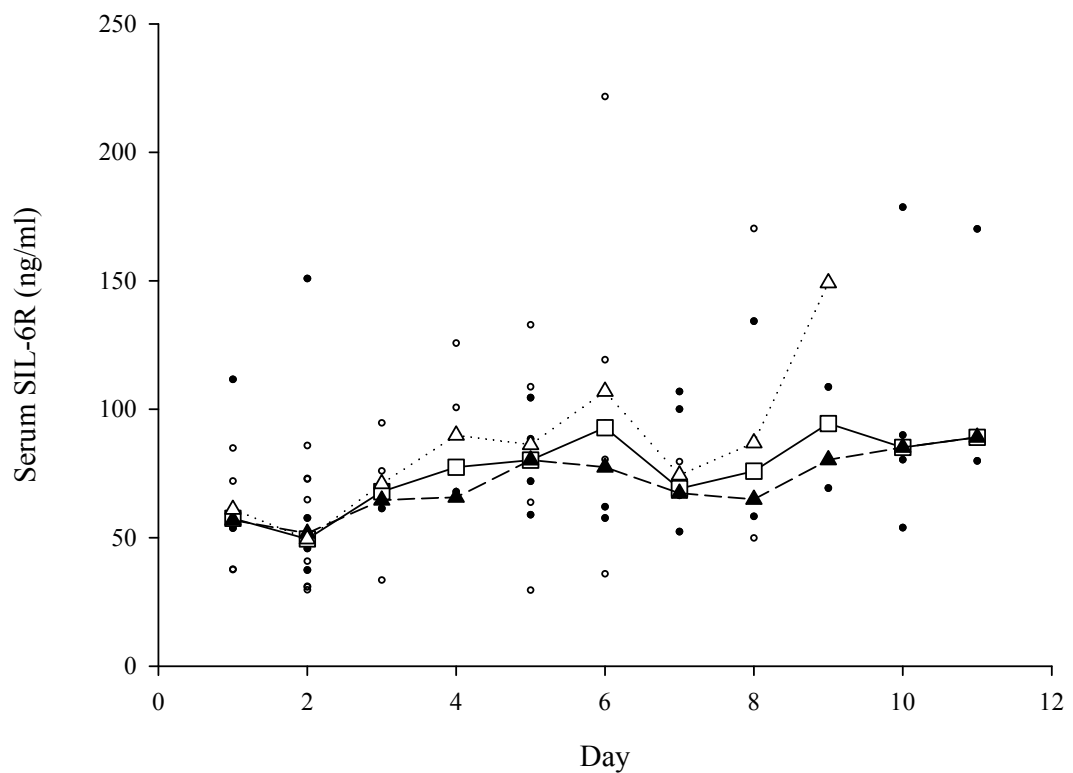
Data from 18 patients with contusional traumatic brain injury sampled in intensive care for up to 11 days after injury. Median chemokine concentration of the whole cohort over time ($\square$ solid line). Median chemokine concentration of patients with enlarging contusions ($\blacktriangle$ dashed line) and those that remained stable ($\triangle$ dotted line) over time. Individual sample concentrations are also shown for patients with contusion enlargement ($\bullet$) vs. those that remained stable ($\circ$). The number of patients available in the group with contusion enlargement vs. the number of patients available in the group that remained stable, each day is shown in the lower table. The lower limit of detection of the IL-8/CXCL8 ELISA was 5pg/ml and for clarity this value was substituted for those samples in which IL-8/CXCL8 was not detected.



Day	1	2	3	4	5	6	7	8	9	10	11
Deteriorated	4	4	2	3	4	4	5	3	3	4	3
Stable	5	9	5	4	4	5	2	3	1	0	0

Figure 4.2. Serum MCP-1/CCL2 vs. Time after Contusional Brain Injury

Data from 18 patients with contusional traumatic brain injury sampled in intensive care for up to 11 days after injury. Median chemokine concentration of the whole cohort over time ($\square$ solid line). Median chemokine concentration of patients with enlarging contusions ($\blacktriangle$ dashed line) and those that remained stable ($\triangle$ dotted line) over time. Individual sample concentrations are also shown for patients with contusion enlargement ($\bullet$) vs. those that remained stable ($\circ$). The number of patients available in the group with contusion enlargement vs. the number of patients available in the group that remained stable, each day is shown in the lower table.



Day	1	2	3	4	5	6	7	8	9	10	11
Deteriorated	4	4	2	3	4	4	5	3	3	4	3
Stable	5	9	5	4	4	5	2	3	1	0	0

Figure 4.3. Serum SIL-6R vs. Time after Contusional Brain Injury

Data from 18 patients with contusional traumatic brain injury sampled in intensive care for up to 11 days after injury. Median receptor concentration of the whole cohort over time ($\square$ solid line). Median receptor concentration of patients with enlarging contusions ($\blacktriangle$ dashed line) and those that remained stable ($\triangle$ dotted line) over time. Individual sample concentrations are also shown for patients with contusion enlargement ($\bullet$) vs. those that remained stable ($\circ$). The number of patients available in the group with contusion enlargement vs. the number of patients available in the group that remained stable, each day is shown in the lower table.

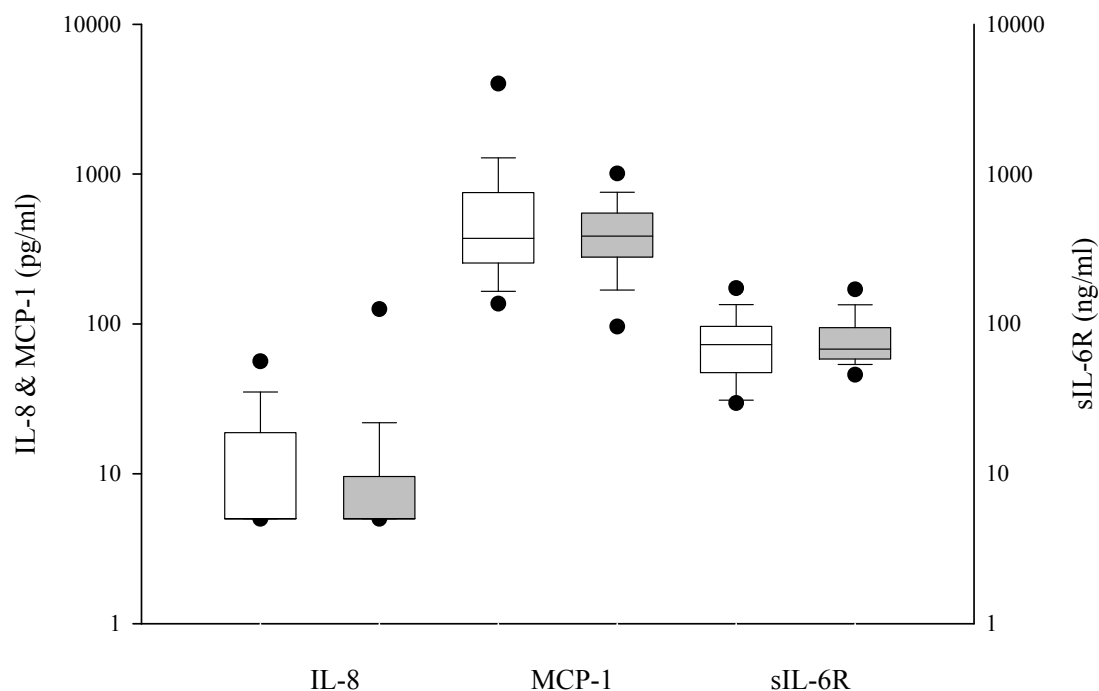


Figure 4.4. Serum IL-8/CXCL8, MCP-1/CCL2 and sIL-6R Concentrations Pooled as Deterioration vs. Stable

Data from 18 patients with contusional traumatic brain injury sampled in intensive care for up to 11 days after injury. Patients experiencing clinical deterioration and/or requiring resection of contusion (grey boxes) vs. stable patients (white boxes). The lower limit of detection of the IL-8/CXCL8 ELISA was 5pg/ml and for clarity this value was substituted for those samples in which IL-8/CXCL8 was not detected. Box plots depict median, 25th and 75th percentiles. Whiskers indicate 5th and 95th percentiles. Individual outliers are also shown.

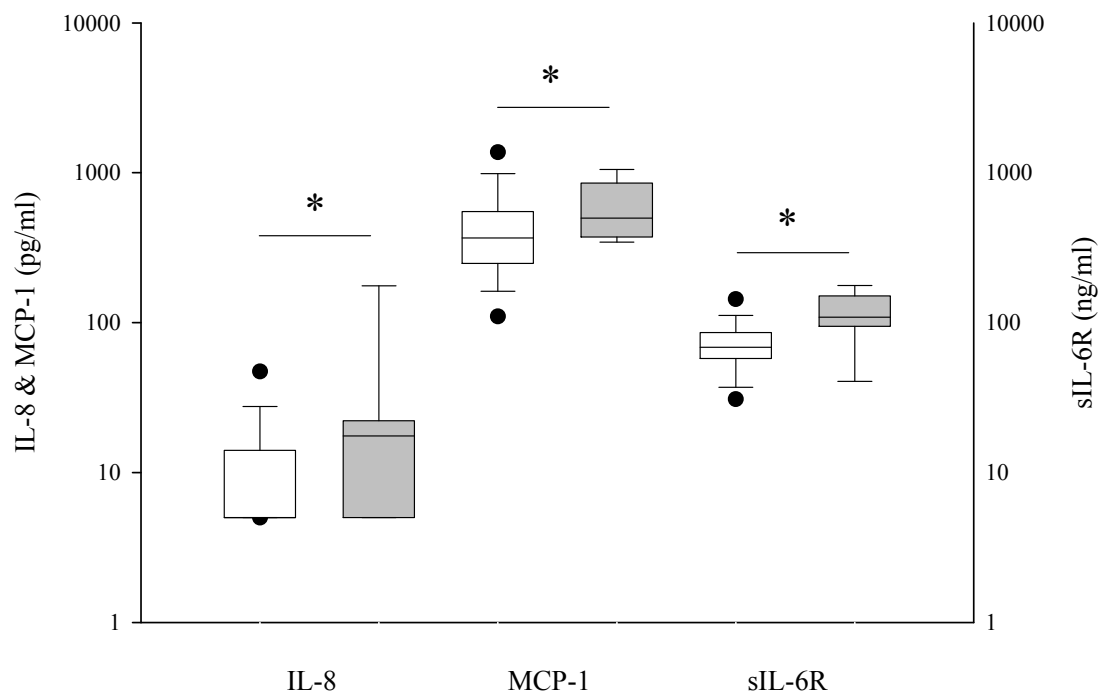


Figure 4.5. Serum IL-8/CXCL8, MCP-1/CCL2 and sIL-6R Concentrations Pooled as Died vs. Survived.

Data from 18 patients with contusional traumatic brain injury sampled in intensive care for up to 11 days after injury. Patients who died during the intensive care admission (grey boxes) vs. patients discharged from intensive care (white boxes). *, $p < 0.05$, died in intensive care vs. discharged. The lower limit of detection of the IL-8/CXCL8 ELISA was 5pg/ml and for clarity this value was substituted for those samples in which IL-8/CXCL8 was not detected. Box plots depict median, 25th and 75th percentiles. Whiskers indicate 5th and 95th percentiles. Individual outliers are also shown.

4.5. Discussion

4.5.1. Summary of Findings

- IL-8/CXCL8, MCP-1/CCL2 and sIL-6R are detectable in the serum of patients with predominantly contusional brain injuries.
- The measurement of these cytokines/cytokine receptor does not identify those patients with enlarging cerebral contusions.
- In patients with predominantly contusional brain injuries, IL-8/CXCL8, MCP-1/CCL2 and sIL-6R concentrations are significantly greater in those that die whilst in intensive care than in survivors.

4.5.2. Serum Samples

I hypothesised that an unfavourable balance of pro and anti-inflammatory cytokines would underpin the enlargement of cerebral contusions. The measurement of serum inflammatory mediators would therefore enable the patients that deteriorated due to contusion enlargement to be distinguished from the stable patients. I studied IL-8/CXCL8, MCP-1/CCL2 and sIL-6R concentrations in 18 patients with traumatic brain injury in which cerebral contusions were the predominant abnormality on computerised tomography.

In the patients that died whilst in intensive care IL-8, MCP-1 and sIL-6R were significantly greater than in those patients discharged (Figure 4.5). However contrary to the hypothesis, IL-8/CXCL8, MCP-1/CCL2 and sIL-6R concentrations were not significantly different in those patients with enlarging contusions compared with those with a more stable clinical course (Figures 4.1-4.4). Serum IL-8/CXCL8 concentrations after TBI have been described previously. Controls levels have ranged from 0-31 pg/ml (Kossmann et al., 1997, Mussack et al., 2001, Mussack et al., 2002). The concentrations of IL-8/CXCL8 measured in my study were above this range (0-202 pg/ml) but less than those reported in populations of patients with TBI by other workers. These have quoted wider ranges of 20-517 pg/ml (Mussack et al., 2002) and 0-2400pg/ml (Kossmann et al., 1997). Five patients in my study population underwent significant surgical procedures. In one, a subdural haematoma was evacuated whilst in 4 lobectomies were performed to remove contused tissue. In the studies by Kossmann (4 of 14) and Mussack (15 of 20) patients also underwent surgical procedures. These were either to evacuate haematomas or decompress the brain by opening the dura. It is therefore unlikely that surgery alone could account for the difference in the range of IL-8/CXCL8 concentrations reported. It is possible however that the process of removing contused tissue and therefore the source of the chemokine prevented the serum concentrations from increasing further. Alternatively a more severe initial injury could also have been responsible for the greater serum IL-8/CXCL8 concentrations, as in the previous studies all the patients included had a GCS <9 (Kossmann et al., 1997, Mussack et al., 2002).

For MCP-1/CCL2 there has been only one study in clinical brain injury. The serum chemokine concentration was measured 24 hours following ischaemic stroke (Losy & Zaremba, 2001). Serum values from controls were 239 ± 141 pg/ml (mean $\pm$ SD) and following stroke the mean MCP-1/CCL2 was 288 pg/ml, not significantly elevated. In the present study of patients with TBI, serum MCP-1/CCL2 was found to be greater with a median of 380pg/ml.

The normal range of serum sIL-6R reported by Hans was 19.2-54.8 ng/ml (Hans et al., 1999). In my group of patients the median was increased at 72 ng/ml (range 30-222 ng/ml). Interestingly in the Hans study of patients with TBI the median sIL-6R concentration was not increased but remained within the control range for the duration of the study, up to three weeks post injury. The majority of these patients had diffuse brain injuries. This has been shown experimentally to produce a different inflammatory response to focal brain injury, leucocyte recruitment not being a predominant feature (Csuka et al., 2000). The increase in sIL-6R in patients with contusional injuries would be consistent with the infiltration of neutrophils in to the brain and subsequent release of sIL-6R (See section 2.2.2.1.2).

Inflammation occurring after TBI is accepted as an important secondary injury mechanism. The best evidence for this comes from experimental studies in which antagonism of inflammatory cytokines or leucocyte infiltration in ischaemic, excitotoxic or traumatic injuries is associated with reduced lesion volume and/or improved functional outcome (Section 7.2.1, table 7.2, section 7.2.2, table 7.3 & section 1.4.2.4, table 1.6). In clinical studies increased blood levels of TNF- α , IL-1 β or IL-6 are associated with poor outcome after ischaemic stroke (Castellanos et al., 2002, Fassbender et al., 1994, Smith et al., 2004) and TBI (McClain et al., 1991). My finding that significantly greater serum IL-8/CXCL8, MCP-1/CCL2 and sIL-6R concentrations were present in the patients that died in intensive care is in keeping with the literature. In fact Mussack and colleagues reported that serum IL-8/CXC8 concentration 12 hours post TBI was predictive of poor outcome at 1 year (Mussack et al., 2002).

I originally hypothesised that the serum concentrations of the inflammatory mediators studied would be significantly different in patients with enlarging contusions. However the data do not support this

hypothesis. Several possible reasons for this should be considered. The first is the relatively small size of this study combined with small differences in the median serum concentrations of proteins measured between those patients that deteriorated due to contusion enlargement and/or underwent surgical resection of contusion and those that remained stable. The differences in serum concentration between the groups and the standard deviation of the data from this study can be used to estimate the sample size required for a future study. For MCP-1/CCL2 and sIL-6R, for a power of 0.85 and $p=0.05$ 400,000 and 2000 patients would be required respectively. Such a study is unlikely to be feasible. Furthermore even if these estimates could be supported and the measurement of these mediators used as the basis of a test to differentiate unstable from stable patients, the small differences in mediator concentrations combined with high variability would mean there would be a large overlap in values between the groups. Therefore sensitivity of the test would only be possible at the expense of specificity and such a test would have little clinical value. Secondly some patients with expanding contusions may not have had increases in ICP sufficient to necessitate repeat CT scanning and possible surgery and therefore would not have been identified. Thirdly it has been reported previously that IL-8/CXCL8 (Hayakata et al., 2004, Kossmann et al., 1997), MCP-1/CCL2 (Losy & Zaremba, 2001), sIL-6R (Hans et al., 1999) are each increased in the CSF more than in the serum after brain injury. In my clinical practice the insertion of ventricular catheters is uncommon as intracranial pressure is measured via parenchymal systems. However, since serum concentrations of these mediators are lower than those found in the CSF, blood sampling is likely to be a less sensitive method of determining the inflammatory state of the CNS. This is also in keeping with the site of action of the chemokines which are bound to the vascular endothelial surface in sites of inflammation (Johnston & Butcher, 2002). The release of chemokines into the circulation represents a “spillage” of factors rather than a direct signal driving inflammation and leucocyte recruitment.

An alternative approach could be the use of microdialysis catheters, placed into areas of contusion, to sample cerebral parenchymal fluid. Because of the proximity of the sampling site to the source of the cytokines, microdialysis could potentially offer a means of sensitively detecting relative changes in inflammatory mediator concentrations with experimental treatments or within the same patient as a

lesion enlarges or improves. Not only is the signal likely to be greater in the microdialysis samples but there may also be less “noise” due to systemic inflammatory events, than serum sampling.

5. Conclusions

5.1. Overview

Traumatic brain injury is a major cause of morbidity and mortality throughout the world. Greater understanding of the significance of secondary brain injury and its contribution to poor outcome has lead to guidelines directing resuscitation, transport and care of the brain injured patient (Bullock et al., 1996, Maas et al., 1997). Indeed there has been a reduction in mortality over the last 20 years (Lu et al., 2005) which is likely to be due, at least in part, to improved clinical management based on these guidelines. However despite many promising pre-clinical studies there has been a failure to introduce a specific therapeutic intervention to further improve outcome after brain injury (Maas et al., 2005, Maas et al., 2006).

Inflammation with cytokine release and leucocyte infiltration is a significant secondary injury processes. Antagonism of cytokines such as IL-1 β and TNF- α has produced significant reductions in injury severity in experimental models of traumatic brain injury (Knoblauch et al., 1999, Shohami et al., 1996, Toulmond & Rothwell, 1995)(Appendix tables 7.2 & 7.3). Similarly the inhibition of leucocyte recruitment has also been shown, particularly following ischaemic injuries, to improve outcome, maintain cerebral blood flow and reduced pathology (Table 1.6). Despite this experimental evidence supporting the inhibition of leucocyte recruitment into the injured brain the induction of neutropenia or the inhibition of adhesion molecule function is not feasible in clinical practice. Critically ill patients with acute brain injury are vulnerable to systemic sepsis, due to the requirement for artificial ventilation, indwelling cannulae and frequent concurrent infection. Any benefit of treatment would therefore be likely to be offset by infective complications. A greater understanding of the expression and regulation of chemotactic factors for leucocytes may reveal alternative means of regulating leucocyte entry into the CNS after injury.

5.2. General Conclusions

Rat glia stimulated with pro-inflammatory cytokines produce the chemokines MIP-2/CXCL2 and MCP-1/CCL2 with a response that was qualitatively similar to that of human derived cells. This supported the *in-vivo* use of a rat model of human disease in which to study glial chemokine

expression. Of note was the observation that the greatest chemokine response in rat derived cells followed treatment with TNF- α whereas in the human derived cell line the greatest response was with IL-1 β . The failure to obtain suitable microglial cultures to complete the inter species comparison was disappointing. In part this was compensated for by reference to the available literature. I found that neonatal rat microglia produce MIP-2/CXCL2 following incubation with TNF- α whereas it has been reported that human foetal microglia produce IL-8/CXCL8 (Ehrlich et al., 1998). Similarly I found that rat neonatal microglia do not significantly increase MCP-1/CCL2 with pro-inflammatory cytokines (although these studies were underpowered), which is consistent with Peterson and colleagues who reported that human foetal microglia do not significantly increase the concentration of MCP-1/CCL2 in supernatants after culture with TNF- α or IL-1 β (Peterson et al., 1997). The lack of a stable microglial cell line limited the number of experiments that could be performed. Primary neonatal cultures are relatively time consuming to establish and have a limited yield of cells compared with the immortalised lines. The human CHME5 line did not produce chemokines and no material for primary human cultures became available during the study. Further attempts to culture the rat HAPI microglial cell line would have been useful. Our principle cell culture technician, Ms Joyce McLuckie strongly suspected that the cells, shipped frozen, had become defrosted during transit. A new supply of these cells, if cultured successfully, would have allowed investigation of the inhibition of MIP-2/CXCL2 by dexamethasone and FK506 in microglia. However, the major source of chemokine expression in the rat derived cells studied was from astrocytes and so the modulation of microglial production would seem to be less significant.

The LFPI device was assembled, installed and shown to function as had been witnessed in Philadelphia. This model generated consistent contusions with gross pathology and histology that was in keeping with the published literature (Cortez et al., 1989, Hicks et al., 1996, McIntosh et al., 1989, Soares et al., 1995). The visit to Professor McIntosh's laboratory the University of Pennsylvania was invaluable in facilitating this process. Following LFPI, MIP-2/CXCL2 and MCP-1/CCL2 were found to increase in the ipsilateral hemisphere acutely, MIP-2/CXCL2 preceding that of MCP-1/CCL2. For MIP-2/CXCL2 this finding was consistent with the studies of Otto and colleagues in a weight drop model (Otto et al., 2001) and Vallès and colleagues (Valles et al., 2006) in the controlled cortical

impact model, both of which are also focal injury models. In the undamaged brain low levels of chemokine expression were localised to neurones in undamaged brain. For MIP-2/CXCL2 this could be seen morphologically and was supported by double staining for a neuronal antigen. For MCP-1/CCL2 only sufficient tissue was available for morphological localisation. In contused tissue increased MIP-2/CXCL2 and MCP-1/CCL2 staining was visible in cells with the morphology of degenerating neurones. The loss of the neuronal marker NeuN in the damaged cells prevented confirmation by double staining on light microscopy. However a very limited study using fluorescence secondary antibodies supported the morphological identification of these cells as neurones.

The immunohistochemical staining for MCP-1/CCL2 was not as clear as it was for MIP-2/CXCL2. Although immunohistochemistry is qualitative and not quantitative technique, this was consistent with the difference in concentrations of these chemokines in cortical samples after injury. Given more time the MCP-1/CCL2 protocol might have been better optimised, perhaps by using a wider range of antibody concentrations. Because the increase in MCP-1/CCL2 staining was not as great as for MIP-2/CXCL2 the change in expression was quantified by counting positive cells and grading the intensity of staining. A major weakness of this quantification was the lack of blinding which would have made the method more robust. However again the findings of an increase in the number of positive cells and some increase in staining intensity in the damaged cortex were supported by the ELISA data, in which the MCP-1/CCL2 concentration was increased in the ipsilateral cortex.

In keeping with the general anti-inflammatory effects of glucocorticoids, dexamethasone significantly inhibited MIP-2/CXCL2 and MCP-1/CCL2 expression in a rat astrocyte derived cell line. However *in-vivo* only MCP-1/CCL2 expression after injury is reduced by dexamethasone treatment. This difference is consistent with astrocytes not being a major source of these chemokines after LFPI *in-vivo*. Although multiple laboratory studies have suggested potential benefits, the use of steroids molecules in acutely brain injured patients cannot be supported based on the current clinical literature (Roberts et al., 2004). Whilst the Corticosteroid Randomisation after Significant Head Injury (CRASH trial)(Roberts et al., 2004) did not provide a explanation as to why there was an excess of

deaths in the steroid treated group potentially harmful effects of steroids on neuronal survival are described. The finding that dexamethasone treatment after LFPI reduced cortical MCP-1/CCL2 but not MIP-2/CXCL2 suggests a mechanism by which glucocorticoid treatment could prolong the inflammatory response and adversely affect outcome.

In contrast to dexamethasone the immunomodulatory agent FK506 did not inhibit MIP-2/CXCL2 or MCP-1/CCL2 expression either *in-vitro* or *in-vivo*. Studies were limited to a single concentration/dose of this agent. These were based on either a supramaximal concentration *in-vitro* or a dose, route and timing that had been shown to reduce injury *in-vivo* (Butcher et al., 1997, McCarter et al., 2001). A more thorough approach would have been to conduct a preliminary study in each case to investigate the effect of a range of treatment concentrations/doses. Furthermore greater consideration could have been given to the effect of small sample sizes on the power of the studies. As many of the analyses were underpowered small but significant changes in chemokine concentrations could have been missed.

Clinically the measurement of serum IL-8/CXCL8, MCP-1/CCL2 or sIL-6R did not distinguish TBI patients that deteriorated due to contusion enlargement. However these inflammatory mediators may be predictive of a poor outcome. This observation could be validated in a new cohort of patients, but the direct study of the CNS compartment could be more powerful.

Further study of the inflammatory response after TBI is warranted. Establishing the temporal profile of chemokine expression in patients with severe brain injury, perhaps using microdialysis would be important as it could support the relevance of the laboratory studies conducted to-date. Another important area to understand is the regulation of the expression of MIP-2/CXCL2 after TBI, determining if this can be modulated and whether this is sufficient to reduce the inflammatory response in the brain.

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7. Appendix

7.1. Cellular Expression of Pro-Inflammatory Cytokines

Local synthesis of cytokines by resident cells of the CNS, both glial and neuronal, has been described in a growing number of reports both *in-vivo* and *in-vitro*.

7.1.1. In-vivo Tumour Necrosis Factor

The publications describing the localisation of the constitutive or sham expression of TNF- α have been quite variable in their findings, perhaps reflecting the different sensitivities of the methods used. When detected expression appears to be predominantly neuronal (Knoblauch et al., 1999, Ohtaki et al., 2004). Lesser expression in meningeal (Holmin et al., 1997) and ependymal cells (Buttini et al., 1996, Holmin et al., 1997), the choroid plexus (Buttini et al., 1996), astrocytes (Knoblauch et al., 1999) and in association with vessels (Holmin et al., 1997, Knoblauch et al., 1999) is described. Early increased expression after injury is seen in neurones (Botchkina et al., 1997, Knoblauch et al., 1999, Liu et al., 1994, Ohtaki et al., 2004, Zawadzka & Kaminska, 2005) monocyctic cells (Botchkina et al., 1997, Buttini et al., 1996, Ohtaki et al., 2004, Uno et al., 1997, Zawadzka & Kaminska, 2005) astrocytes, neutrophils, endothelial and choroid plexus cells (Botchkina et al., 1997). Later expression, over days, is described for monocyctic cells (Buttini et al., 1996, Liu et al., 1994, Uno et al., 1997) and astrocytes (Holmin et al., 1997, Uno et al., 1997) (Table 1.7).

7.1.2. In-vivo Interleukin-1 β

The reports of constitutive and sham expression of IL-1 β are more consistent. IL-1 β is observed predominantly in neurones (Bandtlow et al., 1990, Breder et al., 1988, Lechan et al., 1990). Following ischaemic injury early expression was identified in microglia (Buttini et al., 1994, Yabuuchi et al., 1994, Zawadzka & Kaminska, 2005) and astrocytes (Zawadzka & Kaminska, 2005), whilst later expression after trauma was seen in monocytes and astrocytes (Holmin et al., 1997) (Table 1.7).

7.1.3. In-vivo Interleukin-6

The constitutive expression of IL-6 has also been localised to neurones (Gadient & Otten, 1994, Hans et al., 1999, Schobitz et al., 1993). Brain injury increased expression in neurones (Block et al., 2000, Hans et al., 1999), microglia (Atreya et al., 2000) and astrocytes (Maeda et al., 1994)(Table 1.7).

Injury	Species	Cytokine	mRNA/Protein	Method	Timing	Cell Type	Reference
Weight Drop	Rat	TNF- α	Protein	IHC/Double labelling	4 to 6 days	Predominantly astrocytes, rare mononuclear cells in cortex	Holmin 1997
					Constitutive/sham	Occasional meningeal, ependymal and perivascular cells	
LFPI	Rat	TNF- α	Protein	IHC/Double labelling	1 to 4 hours	Predominantly neurones of the cortex and less frequently the striatum, pyramidal cell layer and dentate gyrus of the hippocampus	Knoblauch 1999
					Sham	Less intense cortical neuronal expression, occasional astrocytes and microvessels	
PMCAO	Rat	TNF- α	Protein	IHC/Double labelling	24 hours	Nerve fibres of cortex, corpus callosum and striatum	Liu 1994
				IHC	5 days	Monocytes in the infarct	
					Sham	No staining	
PMCAO	Rat	TNF- α	Protein	IHC/Double labelling	30 minutes to 6 days	Microglia in cortex and caudate, macrophages in meninges adjacent to infarct	Buttini 1996
				IHC	Constitutive/sham	Ependymal cells and choroid plexus epithelial	
PMCAO	Rat	TNF- α	Protein	IHC	6 to 24 hours	Neurones, astrocytes, microglia, neutrophils endothelial cells, choroid plexus cells	Botchkina 1997
BTCCAO	Mouse	TNF- α	Protein	IHC, consecutive sections for cell markers/double staining for GFAP	Early	Microglia in hippocampal white matter	Uno 1997
					3 days	Microglia in pyramidal cell layer and astrocytes in the white matter of the hippocampus	
					Sham	Negligible staining	
TMCAO	Mouse	TNF- α	Protein	IHC/Double labelling	24 hours	Neurones, oligodendrocytes and occasional monocytes of cortex, not astrocytes	Ohtaki 2004
				IHC	Constitutive/sham	Dendrites and axons in the cortex	
TMCAO	Rat	TNF- α	Protein	IHC/Double labelling	24 hours	Microglia of cortex and striatum, neurones in the striatum	Zawadska 2005

Table 7.1. Cellular Localisation of Pro-Inflammatory Cytokines Constitutively and After Traumatic or Ischaemic/hypoxic Brain Injuries.

Bilateral transient common carotid artery occlusion (BTCCAO), immunohistochemistry (IHC), lateral fluid percussion injury (LFPI), messenger ribonucleic acid (mRNA), permanent middle cerebral artery occlusion (PMCAO), transient middle cerebral artery occlusion (TMCAO), tumour necrosis factor- α (TNF- α).

Injury	Species	Cytokine	mRNA/Protein	Method	Timing	Cell Type	Reference
None	Rat	IL-1 β	mRNA	ISH	Constitutive	Neurones of the pyramidal cell layer of the hippocampus, granular cells of the dentate gyrus and cerebellum, olfactory bulb, cortex and hypothalamus Possibly microglia in the striatum and septum	Bandtow 1990
None	Rat	IL-1 β	Protein	IHC	Constitutive	Neurones of the pyramidal cell layer of the hippocampus, neuronal processes in the hilus of the dentate gyrus, neurones of the hypothalamus, neurones of the olfactory tubercle	Lechan 1990
Weight Drop	Rat	IL-1 β	Protein	IHC/Double labelling	4 to 6 days Constitutive/sham	Predominantly mononuclear cells and astrocytes in cortex Occasional meningeal, ependymal and perivascular cells	Holmin 1997
PMCAO	Rat	IL-1 β	mRNA	ISH	2 days Constitutive/sham	Morphology suggests macrophage/microglia in cortex, striatum, hippocampus, thalamus and amygdala and adjacent meninges No staining	Buttini 1994
Transient Forebrain Ischaemia	Rat	IL-1 β	mRNA	ISH	30 minutes Sham	Morphologically glia in olfactory bulb, cortex, hippocampus, striatum, thalamus, amygdala. Probably microglia Purkinje cell layer of cerebellum and hypothalamus	Yabuuchi 1994
TMCAO	Rat	IL-1 β	Protein	IHC	24 hours	Microglia of the cortex and striatum, astrocytes of the corpus callosum	Zawadska 2005

Table 7.1 Continued. Cellular Localisation of Pro-Inflammatory Cytokines Constitutively and After Traumatic or Ischaemic/hypoxic Brain Injuries.

Immunohistochemistry (IHC), in-situ hybridization (ISH), interleukin-1 β (IL-1 β), messenger ribonucleic acid (mRNA), permanent middle cerebral artery occlusion (PMCAO), transient middle cerebral artery occlusion (TMCAO).

Injury	Species	Cytokine	mRNA/Protein	Method	Timing	Cell Type	Reference
None	Rat	IL-6	mRNA	ISH	Constitutive	Neurones of cortex, pyramidal neurones of hippocampus, granular neurones of the dentate gyrus, neurones of the thalamus and hypothalamus, granular cells of the cerebellum Ependymal cells of anterior lateral ventricle, glia of the internal capsule	Schobitz 1993
None	Rat	IL-6	mRNA	ISH	Constitutive	Neurones of pyramidal cell layer of the hippocampus, granular cell layer of the dentate gyrus, granular cells and purkinje cells of the cerebellum	Gadient 1994
Weight Drop	Rat	IL-6	mRNA	ISH	6 days	Mononuclear cells in the lesion	Holmin 1997
DAI	Rat	IL-6	Protein	IHC for IL-6 & cell markers on consecutive sections	1 to 24 hours Constitutive	Increased neuronal staining in cortex, CA1 and CA3 regions of the pyramidal cell layer of the hippocampus, basal ganglia and thalamus. A few cells associated with blood vessels in the basal cortex were possibly macrophages. Neurons of cortex, hippocampus, basal ganglia and thalamus	Hans 1999
BTCCAO	Gerbil	IL-6	Protein	IHC/Double labelling	1 to 4 days	Astrocytes of the cortex	Maeda 1994
TMCAO	Rat	IL-6	Protein	IHC/Double labelling	2 to 14 days Sham	Microglia and neurones (identified morphologically) of the penumbra No staining	Block 2000

Table 7.1 Continued. Cellular Localisation of Pro-Inflammatory Cytokines Constitutively and After Traumatic or Ischaemic/hypoxic Brain Injuries.

Bilateral transient common carotid artery occlusion (BTCCAO), diffuse axonal injury (DAI), immunohistochemistry (IHC), in-situ hybridization (ISH), interleukin-6 (IL-6), lateral fluid percussion injury (LFPI), messenger ribonucleic acid (mRNA), permanent middle cerebral artery occlusion (PMCAO), transient middle cerebral artery occlusion (TMCAO).

7.1.4. In-vitro Cytokine Expression

Cell culture experiments have demonstrated that lipopolysaccharide (LPS) activated rodent and human primary microglia express TNF- α (Frei et al., 1987, Lee et al., 1993b, Sawada et al., 1989), IL-1 β (Giulian et al., 1986, Lee et al., 1993b), and IL-6 (Lee et al., 1993b, Maeda et al., 1994, Sawada et al., 1992). Immortalised mouse microglia produce TNF- α , IL-1 and IL-6 after incubation with LPS (Righi et al., 1989). TNF- α also induced IL-1 β production by human primary microglia (Lee et al., 1993b).

Primary rodent astrocytes treated with LPS or substance P produce TNF (Chung & Benveniste, 1990, Lieberman et al., 1989, Martin et al., 1992, Sawada et al., 1989) and IL-1 (Martin et al., 1992). IL-1 β has been shown to stimulate production of TNF- α by rat (Chung & Benveniste, 1990) and human (Lee et al., 1993b) primary astrocytes. LPS, TNF- α , IL-1 β or hypoxia/reoxygenation also increases production of IL-6 by rodent primary astrocytes (Benveniste et al., 1990, Maeda et al., 1994, Sawada et al., 1992). In human primary astrocyte culture TNF- α and IL-1 β increase IL-6 (Aloisi et al., 1992, Lee et al., 1993b). In the human astrocytoma line U373, IL-6 is produced in response to IL-1 β , substance P and hypoxia/reoxygenation (Cadman et al., 1994, Gitter et al., 1994, Maeda et al., 1994). The response to hypoxia in rat primary astrocytes has been shown to be mediated via the generation of oxygen radicals (Maeda et al., 1994). Mechanical injury in the form of fluid percussion trauma applied to human primary astrocyte cultures also increases IL-6 protein production (Hariri et al., 1994).

IL-1 β mRNA has also been identified in rat primary cerebellar granule neurones (Bandtlow et al., 1990). TNF- α and IL-1 β mRNAs are expressed in human immunodeficiency virus infected human neuroblastoma cell line (Obregon et al., 1999). IL-6 is also produced by mouse primary neurones after incubation with TNF- α and IL-1 β (Ringheim et al., 1995).

TNF- α (Barna et al., 1990) and IL-1 (Giulian & Lachman, 1985) cause the proliferation of human and rat primary astrocytes, respectively, in culture. In mixed cultures of mouse primary astrocytes and microglia, TNF- α and IL-1 β increase the proliferation of the microglia. This effect is blocked by

antibody to colony stimulating factor-1, which is produced by astrocytes incubated with TNF- α or IL-1 β (Thery & Mallat, 1993).

7.2. Significance of Cytokine Expression

The cytokines constitute a large group of polypeptides with diverse local effects including the control of cell growth and differentiation, the inflammatory response and migration of leucocytes, the control of cytotoxic and phagocytic cells, wound healing and tissue remodelling (Woodroffe, 1995). They are also important mediators of the whole body metabolic response to trauma (Dinarello, 1985) and infection (Dinarello, 1991). Such systemic activities include the induction of fever, adrenocorticotrophic hormone (ACTH) release, muscle catabolism, neutrophilia and the production of acute phase reactants such as C reactive protein and the decreased synthesis of albumin (McClain et al., 1987, Sheeran & Hall, 1997).

The appreciation of the significance of the pro-inflammatory cytokine response is hampered by the often conflicting reports of detrimental and beneficial effects of each mediator. However perhaps the best evidence for a net deleterious effect on outcome after brain injury comes from the multiple studies in which the administration of a specific cytokine antagonist is associated with reduced injury. These are summarised in tables 7.2 and 7.3.

7.2.1. Tumour Necrosis Factor- α

The role of TNF- α in brain injury as a whole is controversial. Harmful effects of TNF include induction of inflammatory mediators, break down of the blood brain barrier, and exacerbation of ischaemic insults. However protective effects against EAA mediated damaged and oxidative stresses have been observed. Furthermore TNF- α may have a significant role in recovery from brain trauma.

7.2.1.1. Potentially Toxic Effects

In-vitro studies of TNF- α function have generated conflicting results, some studies suggesting harmful and others beneficial effects. Some of the observed differences may be occurring because of differences in the maturity of cells used in culture, the concentration of TNF- α administered or the timing of the TNF- α administration.

In cultured foetal neurones the addition of TNF- α potentiates glutamate toxicity and is associated with reduced glutamate metabolism and uptake (Chao & Hu, 1994). TNF- α also induces necrotic changes in oligodendrocytes and degeneration of myelin (Merrill et al., 1993, Selmaj & Raine, 1988a, Selmaj & Raine, 1988b).

Activated microglia produce NO, which is increased by TNF- α and associated with oligodendrocyte cytotoxicity in culture. The addition of an anti-TNF- α antibody or NO synthase inhibitors, both of which inhibit NO production, reduced the cytotoxicity (Merrill et al., 1993). Similarly TNF- α causes direct damage to cerebral endothelial cells with altered morphology and cytotoxicity. This can be inhibited by dexamethasone. TNF- α also increases NO and endothelin production in culture. This would suggest a possible role for TNF- α in the altered cerebrovascular responsiveness after acute brain injury (Estrada et al., 1995). Endothelial cells and astrocytes cultured with TNF- α also express intracellular adhesion molecule-1 (ICAM-1) (Otto et al., 2000).

Conversely pre-treatment of embryonic primary neurones with TNF- α is protective against glucose deprivation and EAAs, inhibiting the increase in intracellular calcium that occurs with glucose deprivation (Cheng et al., 1994). TNF- α pre-treatment also protects neuronal cultures from oxidative stress (Barger et al., 1995, Mattson et al., 1997). Similarly TNF- α prolongs cultured hippocampal neurone survival and protects organotypic hippocampal slice cultures against kainic acid exposure. These effects were absent in TNF receptor knockouts (Bruce et al., 1996). TNF- α induces manganese superoxide dismutase (Mn-SOD) expression. By preventing $\cdot\text{O}_2^-$ accumulation (1.3.4), Mn-SOD could inhibit mitochondrial peroxynitrite formation and lipid peroxidation (Mattson et al., 1997). TNF- α

also induces the proliferation of astrocytes in culture and has been implicated in gliosis associated with brain injury (Bruce et al., 1996, Selmaj et al., 1990).

7.2.1.2. Blood Brain Barrier Function and Leukocyte Accumulation

In-vitro exposure of cerebral endothelial cell monolayers to TNF results in increased permeability which, like IL-1 β , is abolished by cyclo-oxygenase inhibition (de Vries et al., 1996). Intracisternal TNF- α administration in rodents resulted in CSF leucocyte and protein accumulation (Kim et al., 1992, Ramilo et al., 1990) and increased BBB permeability to fluorescein and vasoconstriction of pial microvessels in pigs (Megyeri et al., 1992). Similarly intra-cortical injection of TNF- α in rats induced vasogenic oedema formation (Feuerstein et al., 1998, Holmin & Mathiesen, 2000) and leucocyte margination in capillaries (Feuerstein et al., 1998) but intraparenchymal accumulation of neutrophils or mononuclear cells were not reported (Holmin & Mathiesen, 2000). The intra-striatal injection of a larger dose of TNF- α , also in rats, was not associated with breakdown of the BBB at the injection site (Schnell et al., 1999) but leucocyte recruitment was detected, which was mainly monocytic (Blond et al., 2002, Schnell et al., 1999). Injection of TNF- α into the dorsal hippocampus of mice resulted in neutrophil margination in vessels but no parenchymal infiltration of either neutrophils nor monocytes (Andersson et al., 1992). The response to the injection of TNF- α therefore appears to depend on the region of the brain studied.

7.2.1.3. Traumatic Brain Injury

Acute expression of TNF- α in trauma models appears to be harmful (Table 7.2). In a closed head injury model, both prevention of TNF production by pentoxifylline (PTX) (An inhibitor at the transcriptional level) and the administration of TNF receptor (TNF-R) binding protein (TBP) (soluble fragment of cell surface TNF-R) have been used to reduce the activity of TNF- α . Following a closed head injury intravenous (IV) PTX and TBP significantly lessened peak oedema formation at 24 hours and facilitated the recovery of motor function over the first 14 days. TBP also attenuated disruption of the blood-brain barrier and reduced damage to hippocampal pyramidal cells (Shohami et al., 1996). In severe lateral FPI the intracerebroventricular (ICV) administration of a selective TNF antagonist prior

to and 1 hour following injury did not improved performance in a series of standardized motor tasks 24 hours after injury, but did when assessed at 7 and 14 days after injury (Knoblauch et al., 1999).

In animals chronically deficient in TNF the benefits of a lack of TNF at the time of the injury are also seen. However TNF may also have an important role in recovery from injury. Mice genetically deficient in TNF- α and subjected to controlled cortical impact had significantly less severe neurological deficits when compared to brain injured wild-types at 48 hours post-injury. However at 7 days post-injury there was no difference in motor scores between the knockouts and the wild-types and by 2-3 weeks the wild-type motor score had recovered to baseline whilst the TNF deficient mice had persistent significant deficit. Histology showed that initially there was no difference in the cortical injury cavity volume at 1 week post-injury between TNF deficient and wild-type animals. However the injury volume was significantly greater in the TNF deficient mice by 4 weeks post-injury (Scherbel et al., 1999). Similar results were obtained in mice deficient in both TNF- α and lymphotoxin- α (LT- α also known as TNF- β) using the closed head injury weight drop model. In the 24 hours after injury no significant differences in the degree of BBB dysfunction, infiltration of neutrophils or cell death were found between the deficient and wild-type animals. However in TNF deficient animals, mortality was significantly increased at 1 week compared to wild types (Stahel et al., 2000). One interpretation of this data from knockout animals is that the acute expression of TNF- α following TBI is detrimental but it is required for the resolution of inflammation and repair to occur normally.

7.2.1.4. Ischaemic Brain Injury

The role of TNF- α in stroke has been widely investigated (Table 7.2). Pre-treatment of rats with TNF- α ICV, 24 hours before either permanent or transient middle cerebral artery occlusion (PMCAO, TMCAO), significantly increased infarct size assessed 24 hours after the onset of ischaemia or reperfusion (Barone et al., 1997). Blocking endogenous TNF- α with repeated ICV administration of either a monoclonal antibody to TNF- α or with soluble TNF-receptor 1 (TNF-R1, p55, CD120a), commenced prior to injury, reduced focal ischaemic injury 24 hours after permanent

middle cerebral artery occlusion (PMCAO) (Barone et al., 1997). Intracortical injection of anti-TNF- α antibody (Meistrell et al., 1997) or the administration of a TNF-R construct (dimeric form of TNF-R1 linked to polyethylene glycol) either topically or systemically (Nawashiro et al., 1997a) also significantly reduced lesion size after PMCAO.

Mice subjected to TMCAO demonstrated a significantly reduced infarct volume when treated with ICV anti-TNF- α antibody (Yang et al., 1998a). Pre-treatment with an IV anti-TNF- α antibody has also been shown to reduce cortical damage and improved outcome at 24 hours after TMCAO (Lavine et al., 1998).

Conversely evidence that TNF- α can be supportive also exists. As mentioned pre-treatment of rats with ICV TNF- α 24 hours prior to PMCAO increased the ischaemic injury (Barone et al., 1997). However a relatively greater dose given ICV to mice 48 hours prior to permanent ischemia reduced the infarct volume (Nawashiro et al., 1997b). In animals deficient in TNF receptors (TNFR-KO, both p55 (TNF-R1, CD120a) and p75 (TNF-R2, CD120b)) and subjected to TMCAO the infarct area was increased. Similarly the injection of kainic acid into the dorsal hippocampus produced more hippocampal neuronal loss compared to wild types. Evidence of lipid peroxidation was increased and staining for the anti-oxidant enzyme Mn-SOD reduced in hippocampal neurones, suggesting that TNF protects neurones via antioxidant pathways. Interestingly, in TNFR-KO mice microglial reactivity was reduced (Bruce et al., 1996). This protective effect of the TNF receptors appears to be dependant on the presence of the p55 receptor. After TMCAO or kainic acid injection in to the dorsal hippocampus infarct volume and loss of CA3 hippocampal neurones was increased in the animals lacking the p55 but not the p75 subtype (Gary et al., 1998).

Injury	Species	Antagonist	Route	Timing	Result	Assessed	Reference
CHI	Rat	Pentoxifylline	IV	Immediately post	Reduced oedema Reduced motor deficit	24 hours 1 to 14 days	Shohami 1996
		TNF BP	IV	Within 5 minutes	Reduced oedema	24 hours	
					Reduced motor deficit	1 to 14 days	
					Reduced BBB dysfunction Reduced hippocampal	24 hours 14 days	
LFPI	Rat	TNF-R:Fc	ICV	15 minutes prior & 1 hour post	Reduced motor deficit	7 to 14 days	Knobloch 1999
			IV	15 minutes post	No improvement in motor deficit	Up to 14 days	
CCI	Mouse	TNF KO	-	-	Decreased neurological deficit acutely	48 hours	Scherbel 1999
					Less recovery of function	2 to 4 weeks	
					Increased lesion volume	2 to 4 weeks	
CHI	Mouse	TNF- α & LT- α KO	-	-	Increased mortality	Over 7 days	Stahel 2000

Table 7.2. The Antagonism of Tumour Necrosis Factor in Acute Brain Injury.

Closed head injury (CHI), controlled cortical impact (CCI), intracerebroventricular (ICV), intravenous (IV), knockout (KO), lateral fluid percussion injury (LFPI), tumour necrosis factor- α (TNF- α) tumour necrosis factor binding protein (TNF BP), tumour necrosis factor receptor (TNF-R).

Injury	Duration	Species	Antagonist	Route	Timing	Result	Assessed	Reference
PMCAO		Rat	Anti TNF- α antibody TNF-R	ICV	30 minutes prior 3 & 6 hours post onset	Reduced infarct volume	24 hours	Barone 1997
PMCAO		Rat	Anti TNF- α antibody CNI 1493 TNF synthesis inhibitor	Intracortical IV	1 hour pre or 2 hours post onset 1 hour pre onset 1 hour post onset 2 hours post onset	Reduced infarct volume Reduced infarct volume	24 hours	Meistrell 1997
PMCAO		Mouse	TNF-R:polyethylene glycol	Cerebral Surface (Topical) IP IV	At onset	Reduced infarct volume	24 hours	Nawashiro 1997
TMCAO	1 hour	Mouse	TNF-R (p55 & p75) KO	-	-	Increased infarct area	24 hours	Bruce 1996
TMCAO	1 hour	Mouse	Anti-TNF- α antibody	ICV	At reperfusion	Reduced lesion volume Reduced ICAM-1 positive vessels	24 hours	Yang 1998
TMCAO	1 hour	Rat	Anti TNF- α antibody	IV	1 hour pre onset	Increased CBF Reduced lesion volume	30 to 60 minutes of 24 hours	Lavine 1998
TMCAO Kainic acid injection to dorsal hippocampus	1 hour	Mice	TNF-R (p55 & p75 or p55) KO	-	-	Increased lesion volume Increased CA3 neuronal damage	24 hours	Gary 1998

Table 7.2 Continued. The Antagonism of Tumour Necrosis Factor in Acute Brain Injury.

Intracerebroventricular (ICV), intravenous (IV), knockout (KO), permanent middle cerebral artery occlusion (PMCAO), transient middle cerebral artery occlusion (TMCAO), tumour necrosis factor- α (TNF- α), tumour necrosis factor receptor (TNF-R).

7.2.2. Interleukin-1 β

Mature human 17.5kDa IL-1 β is generated by the cleavage of 31kDa pro-IL-1 β by interleukin-1 β converting enzyme (ICE), a member of the caspase family, also known as caspase-1 (Kostura et al., 1989, Rothwell, 1999). IL-1 appears to act as an endogenous mediator of the host responses to injury inducing fever, anorexia, sickness behaviour, slow wave sleep and alterations in neuroendocrine, cardiovascular and immune systems. These actions are mediated mainly through the hypothalamus and can be blocked by intra-parenchymal injection of interleukin-1 receptor antagonist (IL-1ra) or antibodies to IL-1 β (Rothwell, 1999).

IL-1 can induce a wide variety of responses, many of which could exacerbate neuronal damage. These include blood brain barrier dysfunction, adhesion molecule induction, recruitment of immune cells, damage to the cerebral vasculature, and the release of substances such as eicosanoids, β amyloid precursor protein, corticotrophin releasing factor, nitric oxide, free radicals and activated complement components. Growth factor and neurotrophin induction as well as modulation of calcium currents have also been reported and might be beneficial (Rothwell, 1999).

7.2.2.1. Blood-Brain Barrier Function and Leukocyte Accumulation

Exposure of a monolayer of rat cerebral endothelial cells to IL-1 β induces a decline in the trans-endothelial resistance suggesting increased permeability (de Vries et al., 1996). This can be completely abolished by cyclo-oxygenase inhibition or the introduction of the endogenous inhibitor of IL-1, IL-1ra. Intracisternal injection of IL-1 in rodents induces leucocyte and protein accumulation in the CSF and significantly increased BBB permeability to labelled albumin within hours of injection (Quagliarello et al., 1991, Ramilo et al., 1990). Intracortical (Holmin & Mathiesen, 2000) and intrastriatal (Blond et al., 2002, Schnell et al., 1999) IL-1 β injection in rats was associated with neutrophil accumulation which was greater than that of mononuclear phagocytes (Holmin & Mathiesen, 2000, Schnell et al., 1999). The micro injection of IL-1 β into the dorsal hippocampus of mice caused the margination of neutrophils in vessels close to the injection site but no parenchymal infiltration by either neutrophils or monocytes (Andersson et al., 1992). IL-1 β but not TNF- α was

associated with apoptotic changes in neurones at 24 hours, as evidenced by deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) and Bax staining (Holmin & Mathiesen, 2000). Holmin and Mathiesen reported that intra-cortical injection of IL-1 β could also induce vasogenic oedema (Holmin & Mathiesen, 2000). In contrast Schnell found that the intra-striatal injection of a lower dose of IL-1 β was not associated with breakdown of the BBB. Like TNF- α (See section 7.2.1.2) the exact response to parenchymal cytokine injection could depend on the cytokine administered, the region of the brain studied and the dose administered. In the spinal cord these cytokines produced a much greater leucocyte reaction and marked BBB break down (Schnell et al., 1999).

7.2.2.2. Prostaglandins and Nitric Oxide

Cultured astrocytes release eicosanoids, predominantly prostaglandin-E and Thromboxane-B2 in response to IL-1 β (Hartung et al., 1989). IL-1 β also induces the production of nitric oxide, by astrocytes in culture (Lee et al., 1993a), which has been implicated in the generation of free radicals after acute brain injury (See section 1.3.4).

7.2.2.3. Cytokine Interactions

A possible explanation for some of the inconsistency in the *in-vitro* results presented could be due to the limitations of studying the effects of single agonists on a single cell type, cultured in isolation. The inflammatory response clearly involves multiple mediators acting simultaneously.

In human foetal mixed cultures consisting predominantly of neurons and astrocytes, neither IL-1 β nor TNF- α were toxic individually. However in combination they caused marked neuronal injury and substantial amounts of nitric oxide were produced (Chao et al., 1995). IL-1 β induces nitric oxide production in cultured astrocytes which is augmented by the addition of TNF- α (Lee et al., 1993a). However, the near complete blockade of nitric oxide production was accompanied by only a partial reduction in neuronal injury. The addition of NMDA receptor antagonists to the cell cultures also reduced neuronal injury to IL-1 β and TNF- α , implying that the NMDA receptor was also involved in

neuronal toxicity. Treatment of brain cell cultures with IL-1 β plus TNF- α was reported to inhibit glutamate uptake and astrocyte glutamine synthase activity, two major pathways involved in protection from excitotoxicity (Chao et al., 1995). Taken together these findings provide some indication of how multiple mediators might interact in disease. The combined administration of cytokines activates the inter-related pathways of free radical generation and NMDA receptor mediated toxicity to damage neurones (Gilgun-Sherki et al., 2002, Love, 1999)(1.3.4).

The addition of IL-1 β to predominantly neuronal cultures does not result in overt cell death until high concentrations are reached (μ M) (Strijbos & Rothwell, 1995). Pre-incubation of these cultures with lower concentrations (nM) of IL-1 β was protective against EAA induced degeneration which was inhibited by IL-1ra. This was possibly mediated in part by nerve growth factor (NGF) as the addition of an anti-NGF antibody reduced the protection. It was suggested that IL-1 β causes the release of growth factor from glia (Strijbos & Rothwell, 1995). Conversely when cells are subject to additional stress IL-1 β can also contribute to mechanisms which lead to cell death. *In-vitro* cells subjected to hypoxia, incubation with TNF- α or the withdrawal of media containing nerve growth factor (NGF) undergo apoptosis. This can be reduced by blocking interleukin-1 β converting enzyme (ICE) or inhibiting IL-1 β mediated receptor function (Friedlander et al., 1996, Troy et al., 1996). Pre-incubation of cells with IL-1 β prior to an apoptotic stimulus reduces cell death by down regulating the IL-1 receptor, preventing the induction of apoptosis (Friedlander et al., 1996). Similarly *in-vivo* if combined with another injurious event such as ischaemia or excitotoxicity IL-1 β exacerbates neuronal damage. The ICV injection of IL-1 β either prior to or following PMCAO increases the infarct volume and was associated with a significant elevation in temperature (Loddick & Rothwell, 1996, Relton & Rothwell, 1992). Intrastratial injection of IL-1 β also increased infarct volume (Stroemer & Rothwell, 1998). Following 60 minutes of TMCAO ICV IL-1 β significantly increased oedema over that caused by ischaemia alone, at 24 hours after the insult. This was also associated with a significant increase in infarction size and neutrophil accumulation (Yamasaki et al., 1995). The intrastratial co-administration of IL-1 β and AMPA increased the resultant cortical damage over that of AMPA alone. Similarly intrastratial IL-1 β increased cortical damage due to the cortical administration of NMDA or AMPA (Lawrence et al., 1998).

7.2.2.4. Traumatic Brain Injury

Further evidence for a pathological role of IL-1 β comes from experiments using specific inhibitors, such as IL-1ra, an endogenous inhibitor, expressed in the hippocampus, hypothalamus and cerebellum of normal brain (Licinio et al., 1991).

In TBI, the repeated administration of IL-1ra ICV immediately following fluid percussion injury and continuing for 48 hours reduced cortical lesion volume by 44%. When the initial administration was delayed until 4 hours after injury the reduction in lesion volume remained statistically significant, at 28% (Toulmond & Rothwell, 1995)(Table 7.3)

7.2.2.5. Ischaemic Brain Injury

Following TMCAO the ICV administration of an anti-IL-1 β antibody or zinc protoporphyrin, an IL-1 blocker, reduced infarction size, neutrophil number and brain water content (Yamasaki et al., 1995). The ICV injection of an adenovirus vector containing IL-1ra cDNA in the days prior to PMCAO lead to increased IL-1ra expression which was associated with reduced lesion volume (Betz et al., 1995) and neutrophil accumulation into the ischaemic hemisphere (Yang et al., 1998b). Similarly ICV IL-1ra administered 30 minutes prior to and 10 minutes after induction of PMCAO resulted in a 50-70% reduction in infarction volume at 24 hours without significantly altering physiological parameters such as body temperature (Loddick & Rothwell, 1996, Relton & Rothwell, 1992). Excitotoxic damage caused by striatal infusion of NMDA/AMPA receptor agonists was similarly inhibited by IL-1ra co-administered into the striatum (Lawrence et al., 1998, Relton & Rothwell, 1992). When IL-1ra administration was delayed until 30 minutes after the onset of PMCAO a smaller but statistically significant reduction of 29% in cortical lesion volume was observed (Loddick & Rothwell, 1996). ICV IL-1ra administered at the onset of 60 minutes of TMCAO and again at reperfusion reduced infarct volume by 50-60% at 24 and 48 hours. Delay in administration of a single dose of IL-1ra to 3 hours after the onset of transient ischaemia sustained a significant reduction in lesion volume by 46% at 24 hours (Mulcahy et al., 2003).

Intravenous administration of IL-1ra at the onset of PMCAO and continued as regular subcutaneous injections has also been shown to be protective. Over 7 days after the onset of ischaemia significant reductions in weight loss, neuronal necrosis, lesion size and leucocyte recruitment in the ischaemic hemisphere were seen. Neurological function in the treated animals also improved significantly with time (Garcia et al., 1995). The systemic administration of IL-1ra also attenuated neuronal damage after PMCAO and 2 hours of hypoxia in rat pups. This treatment protocol remained effective when delayed until 1 hour, but not 3 hours, after the hypoxic insult (Martin et al., 1994). Similarly peripheral IL-1ra given immediately following PMCAO in adult rats also reduced infarct volume and oedema formation at 24 hours. However in this study delayed peripheral administration was not beneficial (Relton et al., 1996)(Table 7.3).

After 3 hours of TMCAO in mice genetically ICE deficient IL-1 β levels were 77% lower than in the wild-type littermates, 30 minutes into the reperfusion. In the knockout mice cerebral infarcts and brain swelling were reduced by over 40%. Neurological deficit and DNA fragmentation, a marker of apoptosis, were also decreased (Hara et al., 1997). A similar benefit was observed in neonatal mice undergoing transient focal ischaemia. The ICE deficient animals displayed similar significant reductions in ischaemic damage after 70 minutes of hypoxia/ischaemia. However with a more severe 120 minute injury no benefit was observed (Liu et al., 1999).

There is evidence that the actions of IL-1 β and IL-1ra are site specific and can affect remote regions. IL-1 β injected into the striatum exacerbates both striatal and cortical damage caused by ischaemic injury. However IL-1 β did not increase the injury when administered into the cortex (Stroemer & Rothwell, 1998). Similarly injection of IL-1ra into the striatum reduces damage to the striatum and cortex, but injection into the cortex had no protective effect on either area (Stroemer & Rothwell, 1997). Similarly in an excitotoxic model the intra-cortical injection of IL-1 β with NMDA or AMPA receptor agonists did not increase the cortical lesion volume over that of excitatory agonist alone and the co-infusion of IL-1ra with these toxins, into the cortex, did not reduce cortical damage. Conversely, whilst the addition of IL-1 β to NMDA or AMPA receptor agonists did not increase the striatal lesion volume over that of agonist alone the co-infusion of IL-1ra into the striatum reduced the

striatal lesion volume produced by either agonist. Furthermore intra-striatal IL-1 β and AMPA receptor agonist markedly increased the volume of cortical damage over that of AMPA receptor agonist alone and intra-striatal IL-1 β increased the cortical damage produced by the intra-cortical administration of NMDA and AMPA receptor agonists (Lawrence et al., 1998). Thus IL-1 β does not appear to mediate a neurotoxic effect via an action in the cortex. However in the striatum the modulation of IL- β can alter lesion size due to insults occurring either locally or remotely. .

7.2.2.6. Potentially Beneficial Effects of Interleukin-1 β

IL-1 β expressing cells are found in brain regions that also synthesize NGF mRNA. It has been speculated that IL-1 β might play a regulatory role in the synthesis of NGF in the CNS (Bandtlow et al., 1990)(See section 7.2.2.3). The depression of inward calcium currents in cultured guinea pig hippocampal neurones has been observed after treatment with IL-1 β (Plata-Salaman & Ffrench-Mullen, 1992) and IL-1 β applied *in-vitro* to rat dentate gyrus also inhibited NMDA receptor mediated transmission (Coogan & O'Connor, 1997). In culture it also stimulates the proliferation of astrocytes (Giulian & Lachman, 1985) and when injected into the cerebral cortex leads to astrogliosis and new blood vessel growth. This suggests that IL-1 secreting inflammatory cells may mediate wound healing in the CNS (Giulian et al., 1988).

Injury	Duration	Species	Antagonist	Dose	Route	Timing	Result	Assessed	Reference
LFPI		Rat	IL-1ra	10µg	ICV	15 minutes, 2, 4, 6, 8, 24 & 48 hours post	Reduced lesion volume	3 and 7 days	Toulmond 1995
						4, 6, 8, 24 & 48 hours post		3 days	
PMCAO		Rat	IL-1ra	10µg	ICV	30 minutes pre & 10 minutes post onset	Reduced lesion volume	24 hours	Relton 1992
PCAO & 7.5% O ₂	2 hours	Neonatal Rat	IL-1ra	100mg/kg	SC	Commenced 1 hour pre, onset or 1 hour post onset, then at onset, 1 3, 5, 9, 20 & 28 hours post to complete the series	Reduced neuronal loss	14 days	Martin 1994
PMCAO		Rat	IL-1ra cDNA vector	-	ICV	5 days pre onset	Increased IL-1ra Reduced lesion volume	24 hours	Betz 1995
PMCAO		Mouse	IL-1ra cDNA vector	-	ICV	5 days pre onset	Reduced neutrophil accumulation	24 hours	Yang 1998
PMCAO		Rat	IL-1ra	100mg/kg	IV then SC	Onset then 3 times a day SC	Decreased lesion area Reduced leucocyte recruitment Reduced neurological deficit	1 and 7 days 1 and 7 days Over 7 days	Garcia 1995
PMCAO			IL-1ra	10µg	ICV	30 minutes pre and 10 minutes post onset 30 minutes pre, onset or 30 minutes post onset Onset	Reduced lesion volume Reduced lesion volume Reduced lesion volume	24 hours 24 hours 7 days	Loddick 1996

Table 7.3. The Antagonism of Interleukin-1β in Acute Brain Injury.

Interleukin-1β (IL-1β), interleukin-1 receptor antagonist (IL-1ra), intracerebroventricular (ICV), intravenous (IV), lateral fluid percussion injury (LFPI), permanent carotid artery occlusion (PCAO), permanent middle cerebral artery occlusion (PMCAO), subcutaneous (SC).

Injury	Duration	Species	Antagonist	Dose	Route	Timing	Result	Assessed	Reference
PMCAO		Rat	IL-1ra	100mg/kg	SC	0, 4, 8, 12 & 18 hours post onset 1 hour post onset then 4, 8, 12 & 18 hours later	Reduced lesion volume Reduced oedema No reduced in lesion volume No reduction in oedema	24 hours	Relton 1996
PMCAO		Rat	IL-1ra	5µg	Striatal Cortex	Immediately	Reduced striatal & cortical lesion volume No reduction in cortical or striatal injury	24 hours	Stroemer 1997
TMCAO	1 hour	Rat	IL-1ra	10µg 20µg	ICV	Onset and reperfusion 1, 2 or 3 hours post onset	Reduced lesion volume	24 and 48 hours 24 hours	Mulcahy 2003
TMCAO	1 hour	Rat	Anti IL-1β antibody		ICV	At reperfusion	Reduced lesion area Reduced oedema	24 hours	Yamasaki 1995
PCAO & 10% O ₂	70 minutes	Neonatal Mouse	ICE KO	-	-	-	Reduced lesion volume	5 and 21 days	Liu 1999
	3 hours						Reduced lesion volume & oedema	24 hours	
TMCAO	2 hours	Mouse	ICE KO	-	-	-	Reduced hemispheric IL-1β Reduced DNA fragmentation	30 minutes 6 and 24 hours	Hara 1997
Striatal excitotoxic lesion		Rat	IL-1ra	5µg	Striatal	Immediately	Reduced lesion volume	24 hours	Relton 1992
Striatal excitotoxic lesion		Rat	IL-1ra	5µg	Striatal	Immediately	Reduced lesion volume	24 to 48 hours	Lawrence 1998
Cortical excitotoxic lesion		Rat	IL-1ra	5µg	Cortex		No reduction in lesion volume		

Table 7.3 Continued. The Antagonism of Interleukin-1β in Acute Brain Injury.

Interleukin-1β (IL-1β), interleukin-1β converting enzyme (ICE), interleukin-1 receptor antagonist (IL-1ra), intracerebroventricular (ICV), knockout (KO), permanent carotid artery occlusion (PCAO), permanent middle cerebral artery occlusion (PMCAO), subcutaneous (SC), transient middle cerebral artery occlusion (TMCAO). For transient models the times represent from reperfusion, unless stated

7.2.3. Interleukin-6

IL-6 is a multifunctional cytokine active in many different tissues and on many different cell types. Commonly described actions include the differentiation of B and T lymphocytes, the stimulation of haematopoietic progenitor cells and the induction of acute phase protein synthesis by hepatocytes (Kishimoto et al., 1995). Central effects of IL-6 include reduction of food intake, induction of fever, and stimulation of adrenocorticotrophic hormone (ACTH) synthesis (LeMay et al., 1990, Naitoh et al., 1988, Plata-Salaman, 1989). The precise role of IL-6 in the response of the brain to injury remains unclear.

7.2.3.1. Acute Inflammatory Effects

IL-6 increases the permeability of endothelial monolayers derived from both the cerebral vasculature (de Vries et al., 1996) and the systemic blood vessels (Maruo et al., 1992) suggesting a role in opening the BBB. It also induces the expression of adhesion molecules in endothelial cell cultures (Modur et al., 1997, Romano et al., 1997, Watson et al., 1996, Wung et al., 2005).

IL-6 treatment leads to morphological change, increases phagocytic activity and class II major histocompatibility complex (MHCII) expression in the BV2 microglial cell line (Shafer et al., 2002).

IL-6 also induces the expression of the chemokines interleukin-8 (IL-8/CXCL8) and monocyte chemoattractant protein-1 (MCP-1/CCL2) (Modur et al., 1997, Romano et al., 1997) by vascular endothelial cells and MCP-1/CCL2 by microglia (Calvo et al., 1996) and peripheral monocytes (Biswas et al., 1998).

Further suggestion of a pro-inflammatory role for IL-6 comes from the observation that in IL-6 deficient mice, glial activation following facial nerve axotomy (Klein et al., 1997) or focal cryo-injury to the frontoparietal cortex (Penkowa et al., 1999) was reduced. However in IL-6 deficient mice after focal weight drop injury there were no differences in neurological outcome, mortality (assessed up to 7 days post-injury), BBB dysfunction, neutrophil infiltration or cell death compared to wild-type

animals (Stahel et al., 2000). Similarly following MCAO neither infarct volumes or neurological function differed between wild-type and IL-6 deficient animals (Clark et al., 2000). Clearly the comparison of the effect of cytokine deficiency is very difficult when different end points are used in different models and this might explain the apparent conflict in the results obtained. Inhibition of only one element of the inflammatory cascade might be insufficient to significantly alter the pathological process in a particular model.

7.2.3.2. Chronic Over Expression

In transgenic models over expression of IL-6 is associated with glial activation. The production of IL-6 in astrocytes, under the control of the glial fibrillary acid protein (GFAP) gene promoter, leads to the development of neurological signs including ataxia, weakness, tremors and seizures and a severe neuropathology characterised by failure of the blood brain barrier (BBB) to develop, neuronal loss and dendritic degeneration in the hippocampus and cerebellum, mononuclear infiltration, astrocytosis and angiogenesis (Brett et al., 1995, Campbell et al., 1993). This is often cited as evidence that over expression of IL-6 is harmful. However neuronal over expression of IL-6, by linking the IL-6 gene to the neuron specific enolase promoter, also leads to astrocytosis and an increase in the number of ramified microglia but no neurodegeneration (Fattori et al., 1995). Similarly elevated serum IL-6 and sIL-6R in an IL-6/soluble interleukin-6 receptor (sIL-6R) double transgenic mouse (IL-6 driven by the liver specific murine metallothionein promoter and sIL-6R driven by the mouse phosphoenolpyruvate carboxykinase promoter, specific for liver and kidney (Peters et al., 1997)) was associated with abnormal gait, weakness, tremor and marked astrogliosis. However in this variant neither BBB breakdown nor neuronal damage were present (Brunello et al., 2000). Taken together this would suggest that effects of chronic over expression of IL-6 are site specific and greatest when targeted to astrocytes (Brunello et al., 2000).

7.2.3.3. Neurotrophic and Neuroprotective Actions

IL-6 has been shown to have a neurotrophic action, supporting the survival of catecholaminergic and cholinergic neurones in culture (Hama et al., 1989, Hama et al., 1991, Kushima et al., 1992), reducing

excitotoxic NMDA induced neuronal necrosis (Ali et al., 2000) and increasing the survival of neurones subjected to hypoxia/reoxygenation (Maeda et al., 1994). The CSF of patients following TBI contains an increased concentration of IL-6 and this has been shown to induce NGF production in astrocytes (Kossmann et al., 1996).

The infusion of IL-6 into the rat striatum reduced the loss of cholinergic neurons produced by the co-infusion of NMDA into the same area (Toulmond et al., 1992). PMCAO increases cortical IL-6 production and ICV IL-6 administration significantly reduced ischaemic damage (Loddick et al., 1998) whilst nerve regeneration was promoted in transgenic mice over-expressing IL-6 after trauma (Hirota et al., 1996).

7.2.3.4. Anti-inflammatory Effects

Potentially important anti-inflammatory effects of IL-6 have also been described including the inhibition of TNF- α expression by monocyte and astrocyte cultures (Aderka et al., 1989, Benveniste et al., 1995) and increasing the expression of IL-1ra (Licinio et al., 1991) by cultured macrophages and neutrophils (Jordan et al., 1995, Tilg et al., 1994). Furthermore, *in-vivo*, the levels of TNF- α , macrophage inflammatory protein-2 (MIP-2/CXCL2) and neutrophils were significantly greater following lung or peritoneal administration of endotoxin to IL-6 deficient animals, than wild-types (Xing et al., 1998).

7.3. Appendix References

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