Magnesium polyethylene glycol: a novel therapeutic agent for traumatic brain injury

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Declaration

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Dedication

This thesis is dedicated to my mother Tophers Sabiiti, who has always believed in me, encouraged and supported me in every way possible.

Publications, presentations and awards

The following articles have been published or accepted for publication or presentation during the period of my Masters candidature, and sections of these articles have been included in the present thesis.

Book Chapter

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Award

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ABBREVIATIONS

APP	Amyloid Precursor Protein
ATP	Adenosine Triphosphate
AQP	Aquaporin
BBB	Blood Brain Barrier
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
DAB	Diaminobenzidine tetrahydrochloride
CGRP	Calcitonin-gene Related Peptide
CNS	Central Nervous System
CPP	Cerebral Perfusion Pressure
CSF	Cerebrospinal fluid
DAI	Diffuse Axonal Injury
EB	Evans Blue
GCS	Glasgow Coma Scale
H&E	Haematoxylin and Eosin

ICP	Intracranial Pressure
IV	Intravenous
Mg^{2+}	Magnesium
NHS	Normal Horse Serum
NMDA	N-methyl-D-aspartate
NO	Nitric Oxide
PBS	Phosphate Buffered Solution
rpm	Revolutions per minute
SD	Standard Deviation
SEM	Standard Error of Measurement
SP	Substance P
SPC	Streptavidin Peroxidase Conjugate
TBI	Traumatic Brain Injury

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ABSTRACT

A number of experimental studies have shown that decline in intracellular free magnesium is a ubiquitous feature of traumatic brain injury (TBI), and that restoration of magnesium homeostasis improves both cognitive and motor outcome. However, a recent large, randomized clinical trial of magnesium in TBI failed, in part because of poor central penetration of the magnesium salt. Subsequent experimental studies in spinal cord injury have shown that magnesium penetration into the CNS can be facilitated if the magnesium salt is administered in a solution containing polyethylene glycol (PEG), a polymer that facilitates transport across the blood brain barrier and throughout the extracellular space. Accordingly, the current study characterised the therapeutic potential of high and low dose magnesium chloride, either alone or in combination with PEG, on oedema, blood brain barrier permeability, brain histology and functional outcome following moderate diffuse TBI in rats.

Adult male Sprague Dawley rats (350-380 g) exposed to moderate diffuse TBI induced using the impact acceleration injury model, were administered intravenous magnesium polyethylene glycol (Mg PEG) (254 μmoles/kg $MgCl_2$ in 1g/kg PEG), the same concentration (optimal dose) of $MgCl_2$ or PEG alone, or equal volume vehicle at 30 min postinjury. A separate group of surgically prepared animals were neither injured or treated and served as shams. All animals were subsequently assessed for oedema, blood barrier permeability, brain histology and functional outcome for up to 1 week after trauma. Administration of either Mg PEG or optimal dose $MgCl_2$ alone significantly improved all outcome parameters compared to vehicle treated or PEG treated controls with no significant difference between the magnesium treatment groups. Indeed,

magnesium treatment restored all parameters to sham levels. However, intravenous administration of one-tenth the magnesium concentration (25.4 μmoles/kg; low dose) had no beneficial effect on any of the outcome parameters whereas one-tenth the magnesium concentration in PEG (25.4 μmoles/kg $MgCl_2$ in 1g/kg PEG) had the same beneficial effects as optimal dose $MgCl_2$. We conclude that PEG facilitates movement of the magnesium salt across the blood brain barrier following TBI and that the combination of low dose magnesium in PEG significantly attenuates oedema, blood brain barrier permeability and improves motor and cognitive outcome following TBI.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 Traumatic Brain Injury.

1.1.1 Overview

Traumatic Brain Injury (TBI) is a global epidemic and has been reported as a leading cause of morbidity and mortality (Myburgh et al., 2008). It is defined as an insult to the brain that occurs as a result of an external mechanical force and is associated with an altered state of consciousness that may lead to permanent or temporary impairment of physical, psychosocial or cognitive functions (Langlois et al., 2006, Selassie et al., 2008). TBI may be classified as mild, moderate or severe depending on the Glasgow Comma Scale (GCS) score (Andriessen et al., 2011), which is scored out of 15 where eye opening (4), motor response (6) and verbal response (5) are assessed. Severe TBI is scored 3-8, moderate TBI 9-12 whereas mild TBI is scored 13-15 (Andriessen et al., 2011).

Leon-Carrion et al (2005) reported the incidence of TBI at 150-250 per 100,000 people per year in Australia. In the same study, an overwhelming 57.2% of the patients that sustained TBI were classified as severe (GCS: 3-8). This incidence exerts a significant socio-economic burden on the community (Langlois et al., 2006, Rapoport, 2012, Shivaji.T. et al., 2012). Thurman (1999) reported that in the United States alone, the cost of caring for people with disabilities following TBI was estimated at $37.8 billion in 1985.

Currently there is no therapy for TBI, with the only available strategies providing symptomatic management of intracranial pressure (ICP) and maintenance of blood pressure and arterial gases.

These do not address the underlying secondary injury mechanisms that have been associated with the development of neurological deficits (Bullock et al., 1999). Of the known secondary injury factors, Vink and Cernak (2000) have reported that magnesium decline is a ubiquitous factor in both focal and diffuse TBI, and that administration of magnesium salts acts as a multipotential therapy that attenuates a number of different secondary injury processes following experimental TBI. Accordingly, they proposed that magnesium salts might be a novel therapeutic intervention for use in clinical TBI. However, some subsequent clinical trials with magnesium have been unsuccessful due to poor central penetration, insufficient dosages and/or inappropriate dosing intervals (Bullock et al., 1999). Therefore an effective dose of a centrally acting magnesium formulation with the correct dosing interval is paramount in order to achieve significant magnesium-induced reduction in cell death and an improvement in functional outcome.

Polyethylene glycol (PEG) is a polymer that facilitates ion transport across the blood brain barrier and throughout the extracellular space, and has been successfully used in combination with magnesium salts in the treatment of experimental spinal cord injury (Kwon et al., 2009). Whether this approach would facilitate the CNS penetration of magnesium salts administered after TBI is unknown. The current thesis therefore characterises the therapeutic potential of high and low dose magnesium chloride, either alone or in combination with PEG, on oedema, blood brain barrier permeability, brain histology and functional outcome following moderate diffuse TBI in rats.

1.1.2 Epidemiology

TBI is a leading cause of death and disability in people under the age of 40 years (Fleminger and Ponsford, 2005), with the worldwide incidence estimated to be 150-250 per 100,000 population every year (Leon-Carrion et al., 2005). A study carried out in South Australia at the Royal Adelaide Hospital reported that the prevalence of TBI stood at 322 per 100,000 per year, which was higher than those reported in the United States and the United Kingdom (Hillier et al., 1997, Shivaji.T. et al., 2012). A recent study also carried out in Australia (Myburg et al., 2008) showed that 61.4% of the patients sustained trauma due to vehicular accidents and 24.9% due to falls, especially among the elderly. In the same study, using the initial GCS, 24.7% were classified as mild injury with a GCS 13-15, 18% moderate injury (GCS 9-12) and an overwhelming 57.2% with severe traumatic brain injury (GCS 3-8). One third of the patients did not survive beyond twelve months. Although a higher incidence of TBI was found in young men, younger age (less than 30) was also predictive of better outcomes (Hillier et al., 1997, Myburgh et al., 2008). Sadly, the study also reported that unfavorable outcomes and admission to the intensive care unit is still as high as it was 15 years ago, as were the pre-hospital secondary brain insults. Ultimately it is important to minimize secondary brain insults to improve outcomes from TBI (Myburgh et al., 2008).

1.2 Primary and Secondary Injury

1.2.1 Primary TBI

Primary TBI refers to the mechanical damage to the cerebral tissues caused by the kinetic energy delivered at the time of the head trauma (Kurland et al., 2012), and occurs at the time of the

trauma. The kinetic energy at the moment of impact causes shearing of the tissues and may lead to rupture of neurons, astrocytes, oligodendrocytes and blood vessels in either a focal or diffuse manner (John et al., 2001, Kurland et al., 2012). Common focal injuries include cerebral contusion, cerebral laceration, epidural hemorrhage, subdural hemorrhage, intra-cerebral hemorrhage and inter-ventricular hemorrhage (Bullock et al., 1999, Vos, 2011). Diffuse injuries are more widespread and are characterized by axonal separation (Cernak et al., 2004).

Rupture of micro vessels, extravasation of blood and diminished blood supply to the brain compromises oxygen and nutrient delivery and may lead to ischemia (Marshall, 2000, Nortje. J. and Menon. D, 2004, Werner. C. and K., 2007, Kurland et al., 2012). Moroever, the extravasated blood is toxic the central nervous system cells and may also incite secondary injury responses in the hours to days following TBI. While primary injury cannot be attenuated after TBI, it may be prevented through the use of helmets, airbags and seatbelts.

1.2.2 Secondary Injury

Secondary injury involves release of neurotransmitters, inflammatory factors, alterations in ion homeostasis, free radical formation, energy failure and oxidative stress, amongst others, in response to a primary event (Vink et al., 1988a, Bareyre et al., 1999), Donkin et al., 2007). It occurs within minutes to days following TBI, thereby providing a therapeutic window for intervention to prevent further injury and improve outcome. Indeed, it is thought that the secondary injury processes account for much of the high morbidity and mortality following TBI (McIntosh et al., 1996).

As mentioned above, one of the secondary injury processes occurring after TBI involves the release of neurotransmitters. Specifically, the release of glutamate has been implicated in excitotoxicity following TBI (Tihomir and Jutta, 1997), where the excitotoxins are usually acidic amino acids that react with specialized receptors in the brain leading to destruction of certain types of neurons. Glutamate concentration in the extracellular fluid is normally kept between 8 to 12μm. When the concentration of glutamate rises above normal values, neurons begin to fire abnormally, and as the concentration further increases, neurons are excited to death; hence the term excitotoxicity. Glutamate mediates it's actions through the N-Methyl-D-Aspartate (NMDA), amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate receptors (Arango and Mejia-Mantilla, 2006). The NMDA and AMPA receptors have distinct physiological properties and often coexist within the same synapse. In order to potentiate its actions at the NMDA receptor, glutamate binds with a co-agonist glycine. It should be noted that at resting membrane potential or if the membrane is slightly depolarized, very few or no ions flow through the NMDA channel as it is blocked by magnesium (Orrego and Villanueva, 1993). Therefore if a stimulus is not strong, binding of glutamate causes ions to flow through the AMPA receptors that are not blocked by magnesium. This causes an influx of Na^+ and an efflux of K^+. Further binding of glutamate to each AMPA receptor subunit causes additional opening of the channel, depolarizing the postsynaptic membrane even further. When glutamate binds to the NMDA NR2A receptor subunit, the clamshell shaped ligand domain of S1 and S2 closes. This closure causes tension in the receptor which flips open the channel to a partially open position. The co-agonist glycine then binds to the NR1 subunit and when the agonists occupy all the subunits, this induces the NMDA channel to full open. The fully open channel cannot yet permit influx of cations due to the voltage dependent block by magnesium (Orrego and Villanueva, 1993, Arango and Bainbridge, 2008).

When the post synaptic membrane depolarizes to -50mV through AMPA channel activity, the affinity of magnesium for its binding site decreases causing the magnesium ion to move out of the channel, thereby opening it fully. This causes an influx of Ca^{2+} and Na^{+} into the cell and an efflux of K^{+} into the extracellular space.

This kind of excessive stimulation occurs under pathological conditions such as TBI. The net influx of Na^{+} facilitates movement of water by osmosis into the cell leading to swelling (cytotoxic oedema) (Donkin et al., 2007). Similarly, the glia swell because they take up the K^{+} ions that accumulate in the extracellular fluid (Reilly, 2001). The influx of calcium ions through the NMDA channel leads to activation of several calcium-mediated enzymatic cascades (Donkin et al., 2007, Gurkoff et al., 2013) eventually leading to cell death (Tihomir and Jutta, 1997, Xiong et al., 1997, Vespa et al., 1998). Specifically, the activation of calcium dependent enzymes activates proteolysis and production of free radicals which triggers neurotoxic cascades such as uncoupling mitochondrial electron transfer from ATP synthesis (Vink and Cernak, 2000). Calcium binding to calmodulin forms calcium calmodulin complexes that activate several protein kinases including calcium calmodulin dependent kinase (CAM kinase), which phosphorylate the existing AMPA receptors thereby increasing their permeability to sodium. CAM kinase also promotes movement of AMPA receptors from intracellular stores to the postsynaptic membrane increasing their number and therefore the response to stimuli. Other calcium dependent enzymes that are activated include nitric oxide synthase, phospholipase A_2, calpains and protein kinase C (Arundine and Tymianski, 2004, Arango and Mejia-Mantilla, 2006).

Nitric oxide synthase is an enzyme involved in the formation of nitric oxide from its precursor L-arginine (Moncada and Higgs, 1993) and has been associated with several functions including formation of memory, vasodilatation, inhibition of platelet aggregation and inhibition of smooth

muscle proliferation (Radomski et al., 1990, Garthwaite, 1991, Schmidt and Walter, 1994). Nonetheless, excess production of nitric oxide is thought to be cytotoxic and cytostatic thereby causing cell damage (Moncada and Higgs, 1993). Nitric oxide interacts with oxygen derived radicals to form cytotoxic molecules (Beckman et al., 1990) including peroxynitrite, a highly toxic anion. Peroxynitrite causes single-strand breaks in DNA and therefore activates DNA repair protein poly (ADP ribose) polymerase (PARP) to catalyze the cleavage at the expense of NAD^+ (Love, 1999). Depletion of NAD^+ an important source of energy for many cellular processes accelerates brain injury.

Calcium-mediated activation of phospholipase A_2 (Siesjö, 1993) converts phospholipid to arachidonic acid, which is converted to hydroxyperoxyecosatetranoic acid (HPETE) with the help of lipoxygenase. HPETE is metabolised to leukotriene A_2 which is further converted to leukotriene C_3 then to leukotriene C_4 and finally to leukotriene E_4. Additionally, arachidonic acid is converted to prostaglandin H_2 through a second pathway via prostaglandin H_2 synthase. Prostaglandin H_2 is then converted to thromboxane, prostacyclin and prostaglandin D_2 and E_2. The formation of highly reactive prostaglandins, leukotrienes, thromboxanes and superoxide anions leads to neuronal damage by lipid peroxidation (Kontos and Wei, 1986, Yoshihiro, 2007).

Calpains, which are neutral cysteine proteases (Farr and Berger, 2010), are involved in cell signaling, cytoskeletal remodeling, regulation of gene expression, apoptosis and cell cycle regulation (Farr and Berger, 2010). While their regulation is complex and not properly understood (Farr and Berger, 2010), they have catalytic domains DI and DII (Sorimachi et al., 2011) where D1 is a central helix flanked on three faces by a cluster of alpha helices, while DII has two 3-stranded anti-parallel beta sheets and alpha helices (Moldoveanu et al., 2002). The relative orientation of DI and DII is defined by 2 calcium ions, and its activation is associated

with neuronal cell death. Finally, calcium-mediated increases in protein kinase C activity (Scott and Pawson, 2000, Bright and Mochly-Rosen, 2005) results in the phosphorylation of many metabolic and structural enzymes resulting in, amongst other things, the formation of free radicals (Bright and Mochly-Rosen, 2005) that initiate cell death pathways.

Other secondary events that may occur after TBI include a reduction in blood flow and decrease in tissue oxygen levels (ischemia), depletion of ATP and falls in pH. These ischemic events result in failure of the Na^+ / K^+ ATPase pump resulting in intracellular sodium accumulation and potassium efflux. The accumulation of intracellular Na^+ results in net water influx and cytotoxic oedema formation (Biber et al., 2009, Cook et al., 2009, Badaut and Fukuda, 2012). In response to increased intracellular Na^+, the Na^+ / Ca^{2+} exchanger subsequently reverses moving sodium out of the cell and calcium into the cell, thereby further activating the previously described calcium-mediated secondary injury pathways.

Apoptosis is programmed cell death (Villapol et al., 2014) and is one of the two mechanisms of cell death, the other being necrosis, which involves loss of cell membrane integrity and an uncontrolled release of cell death products into the extracellular space. Apoptosis can be initiated by a variety of stimuli such as heat, radiation, and an increase in intracellular Ca^{2+} (Bonfoco et al., 1995). Conti et al (1998) showed that cell death within the first 24hrs following TBI is mainly via necrosis whereas cell death beyond 24hrs is primarily apoptotic. However, Cernak et al (2000) demonstrated that apoptosis can begin as early as 4hrs after diffuse TBI and continue for at least 5 days, most likely in response to induced oxidative damage (Bonfoco et al., 1995, Nicotera et al., 1997, Villapol et al., 2014). As previously mentioned, Ca^{2+} influx leads to the activation of various enzymes including those involved with oxidative injury (Love, 1999). Activation of these enzymes leads to production of free radicals (Bonfoco et al., 1995) which

creates pores in the mitochondrial membrane, thereby increasing it's permeability and reducing the membrane potential. The increased permeability of the outer mitochondrial membrane releases cytochrome c into the cytoplasm, which is a trigger for apoptosis, whereas the reduction of the membrane potential results in diminished ATP production and mitochondrial swelling (Reed, 2000). Cytochrome c binds and activates cytosolic proteins, including pro-apoptotic protease activating factor (Apaf-1) (Reed, 2000), which itself activates caspase-9. Caspase-9 activates caspase-3, a proteolytic enzyme which is synthesized as an inactive pro-enzyme which is subsequently cleaved to generate two active units of 17kDa and 12kDa (Reed, 2000). Caspase-3 causes extensive protein cleavage (Reed, 2000, Zhang et al., 2005) leading to cell death. The apoptotic cells display marker molecules on their surfaces to enable recognition and uptake by macrophages (Borisenko et al., 2003, Liou et al., 2003). This is made possible by flipping of the phosphatidylserine from the inner cytoplasmic face to the extracellular face (Reed, 2000, Zhang et al., 2005). However, Denecker et al (2000) demonstrated that phosphatidylserine exposure occurs well before cytochrome c release and decrease in mitochondrial membrane potential. The apoptotic cells are taken up by macrophages without release of pro-inflammatory mediators and disappear without a trace (Clark et al., 1997, Clark et al., 2001).

1.3 Oedema and the Blood Brain Barrier

The central nervous system has a number of barriers that regulate movement of substances between the brain parenchyma, the blood and the cerebral spinal fluid spaces (Abbott et al., 2006). With respect to the interfaces with the CSF, there is the barrier formed between the brain and the interstitial fluid, the choroid plexus epithelium which is between the blood and the ventricular CSF, and the arachnoid epithelium lying between the blood and subarachnoid CSF

(Ballabh et al., 2004, Lossinsky and Shivers, 2004, Hawkins and Davis, 2005). There is also the blood brain barrier (BBB) that separates the brain parenchyma from the blood, and whose increased permeability after TBI is considered a major secondary injury factor.

1.3.1 The Blood Brain Barrier

The BBB consists of vascular endothelial cells, thick basement membrane and astrocytic end feet (Boulard et al., 2003, Franke et al., 2012, Verkhratsky et al., 2014), which together separate circulating blood from the brain extracellular fluid (Fig. 1.1). The endothelial cell layer contains tight junctions that do not exist in normal circulation, thus restricting movement of microscopic and hydrophilic molecules into the brain tissue while still allowing the diffusion of oxygen and carbon dioxide. Large molecules such as glucose and amino acids are transported across these endothelial cells with the help of carrier proteins (Boulard et al., 2003).

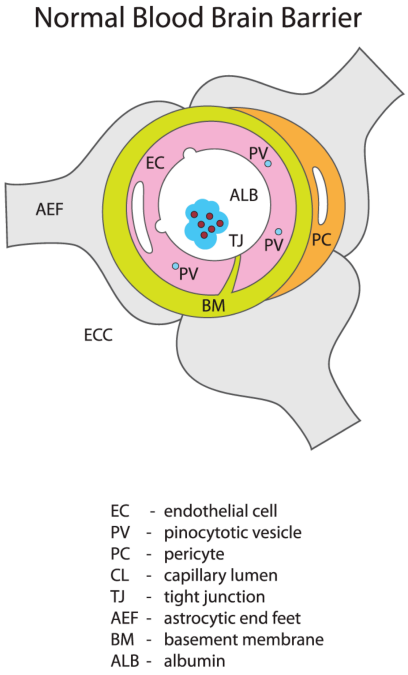


Figure 1.1. Structure of the normal blood brain barrier.

The tight junctions have a number of specialised proteins within them including occludin, claudin and junctional adhesion molecules (JAM). Occludin is a 60-65-kDA protein with four trans membrane domains. On the cytoplasmic side are the carboxyl (COOH) and amino (NH_2) terminals, while the other two domains are extracellular loops. These constitute the paracellular barrier of the tight junction (Hirase et al., 1997, Sonoda et al., 1999, Papadopoulous et al., 2001). The occludins appear to be involved in tight junction regulation. Claudins are 22 kDa phosphoproteins with four transmembrane domains. The claudin protein on one epithelial cell binds to other claudins on neighbouring cells to form the primary seal of the tight junction (Furuse et al., 1998, Liebner et al., 2000). Claudins 1 and 2 form an integral part of the tight junction and contribute to the trans-endothelial electrical resistance (Abbott et al., 2006), whereas claudin 11 is found within CNS myelin (Morita et al., 1999). The JAM molecules regulate the selectivity of the tight junctions and restrict passage of solutes.

These tight junctions hold the endothelial cells firmly together and regulate the movement of substances across the BBB (Butt et al., 1990). The endothelial cells are also supported by the basement membrane and the end feet of the astrocyte cell (Boulard et al., 2003).

1.3.1.1 Transport across the blood brain barrier

The BBB allows only limited entry of substances compared to the peripheral endothelium, largely because of the high electrical resistance generated by the presence of tight junctions, specifically 1000 Ωcm^2 in the brain endothelium compared to 2-20 Ωcm^2 in the peripheral capillaries (Butt et al., 1990). Substances move across the cell either through the tight junctions (paracellular) or across the cell membrane (transcellular) (Abbott et al., 2006). Smaller and

lipophilic substances including O_2, CO_2, barbiturates and ethanol can diffuse directly across the lipid cell membrane (Abbott et al., 2006, Persidsky et al., 2006). Larger molecules such as glucose and amino acids require specific carriers (Abbott et al., 2006, Ohtsuki and Terasaki, 2007). Glucose transporter 1 (GLUT1) is the bidirectional glucose carrier across the brain endothelial cells (Simpson et al., 2001) and may be present on both the luminal and abluminal sides of the endothelial cell (Lefauconnier, 1989, Abbott et al., 2006). Amino acids are carried across the endothelial cell by the L-type amino acid transporter-1 (LAT1) (Bradbury, 1985). There is also a Na^+/K^+ ATPase pump located on the abluminal side of the endothelial cell, which creates a Na^+ gradient as it operates (Lefauconnier and Hauw, 1884, Abbott et al., 2006). This gradient facilitates movement of some compounds that need to be moved across a concentration gradient from the blood into the interstitial fluid surrounding the brain (Abbott et al., 2006).

1.3.1.2 Functions of the Blood Brain Barrier

The BBB allows influx of nutrients like O_2 and glucose into the CSF and efflux of waste products like CO_2 out of the brain (Ballabh et al., 2004). The BBB also regulates the concentration of ions such as potassium, calcium and magnesium in the interstitial fluid thereby providing an optimal environment for neuronal function, and separates the neurotransmitters in the central nervous system from those in the periphery thereby minimizing chances of miscommunication (Abbott et al., 2006, Persidsky et al., 2006). For example, glutamate is taken up by excitatory amino acid transporters (EAAT1-3), however their entry is limited in the presence of a large opposing concentration gradient (Abbott et al., 2006). The BBB also prevents inflammatory cells such as macrophages, pathogens such as bacteria and viruses from moving from the blood into the brain (Dore-Duffy et al., 2000). Finally, CSF production occurs at the choroid plexus (the blood-CSF barrier) and is important given CSFs role as a cushion or buffer for the brain.

1.3.1.3 Astrocytes

Astrocytes are haemostatic, neuroglial star-shaped cells present in high numbers in the brain and spinal cord. They perform numerous functions including biochemical support of the endothelial cells that form the BBB, structural organization of the gray matter, neuron feeding, extracellular ion balance, water redistribution, as well as repair and fibrosis following trauma. They have also been found to potentiate memory and learning in the hippocampus (Sykova et al., 2002). Astrocytes have end feet, which are specialized foot processes that are apposed to the outer surface of blood vessels and the smooth muscle of arterioles, and thereby contribute to the control of blood vessel diameter through secretion of prostaglandins, nitric oxide and arachidonic acid (Abbott et al., 2006, Wolf and Kirchhoff, 2008, Attwell et al., 2010). With the ability to store glycogen, they are capable of glycogenesis, with the release of glycogen during periods of glycogen shortage acting as a glycogen fuel reserve buffer. Astrocytes also express plasma membrane transporters for several neurotransmitters such as glutamate, glycine and GABA, thereby contributing to the regulation of neurotransmitter uptake and release. These neurotransmitters are recycled by the astrocytes. Glutamate recycling, for example, involves transmitter uptake through excitatory amino acid transporter (EAATI-3) proteins. This transport is Na^+ dependent and is accompanied by an inward flow of water. The glutamate is then converted to glutamine within the astrocyte and recycled to the neurons (Amiry-Moghaddam and Ottersen, 2003, Abbott et al., 2006).

Astrocytes are also involved in regulation of ion concentration and cell volume at the BBB via uptake of K^+, regulation of the bicarbonate transporter and via the Na^+/H^+ exchanger (Parpura and Verkhratsky, 2012, Pellerin and Magistretti, 2012). Potassium uptake takes place through transporters such as the Na^+/K^+ ATPase, accompanied by a slight osmotic water gain and cell

swelling. Astrocytes distribute this water through aquaporin 4 (AQP4) water channels that are found in high density on their perivascular end feet (Kimelberg and Nedergaard, 2010).

Finally, astrocytes have been shown to play a role in modulation of synaptic transmission involving complex signaling between cells in the neurovascular unit including microglia and oligodendrocytes. An increase in intracellular calcium via receptor activation in the brain endothelium and astrocytes creates a wave of depolarization, which is spread through the astrocytic syncytium. This receptor activation and wave generation is triggered by glutamate, serotonin and even mechanical stimulation and propagates at a rate of about 100μm/sec (Pasti et al., 1997, Blomstrand, 1999).

1.3.1.4 Pericytes

Pericytes are flat, undifferentiated contractile cells derived from smooth muscle found in the central nervous system surrounding the endothelial cell layers of the capillary network. These cells are part of the neurovascular unit, which includes endothelial cells, astrocytes and neurons. They are classified as either granular and agranular (Farrell et al., 1987) and have an oval cell body and a prominent round nucleus with up to 90 ramifications (Nag, 2003). Pericytes possess the capacity to modify their structure as well as their gene expression, and in so doing, they regulate capillary permeability through capillary contraction and by cytokine and nitric oxide secretion (Peppiatt et al., 2006, Armulik et al., 2010, Bell et al., 2010, Daneman et al., 2010). Their ability to mimic astrocytes is important in maintaining structural integrity of the BBB and maintain barrier tightness. Additionally, they differentiate into cells that phagocytoze proteins and present antigen (Williams et al., 2001). Dore-Duffy et al (2000) found that pericytes migrate

away from brain micro vessels following trauma therefore increasing brain barrier permeability (Dore-Duffy et al., 2000).

1.3.2 Cerebral oedema

Cerebral oedema is the abnormal accumulation of water into the intracellular and extracellular compartments of the brain (Klatzo, 1987, Payen et al., 2003). As a major cause of death and disability following TBI, it is considered a major secondary injury factor and highlights the need for effective treatments to counter its occurrence. Under physiological conditions, the concentration of extracellular fluids on either side of the blood brain barrier is equal (Boulard et al., 2003). However, after an insult to the brain, this balance is disrupted and water accumulates on the parenchymal side of the BBB. Two major types of cerebral oedema have been described, namely cytotoxic and vasogenic oedema, while other subclassifications include interstitial, hyperemic and osmotic oedema (Boulard et al., 2003). Any increase in water content in the brain leads to increased ICP, which may cause brain herniation and death.

According to Starling's equation, fluid movement across the capillary membrane occurs as a result of diffusion, filtration or pinocytosis. Therefore, the movement of plasma proteins, ions and water into the interstitium increases if there is a positive driving force, or if the permeability is enhanced by chemical mediators, membrane disruption or a delayed inflammatory response (Payen et al., 2003). Inflammatory changes lead to alterations in BBB permeability and upregulation of AQP4 water channels on the perivascular end feet of the astrocytes; these channels facilitate fluid movement during oedema formation. Indeed, a study carried out by Verkman et al (2000) showed that aquaporin 4 knockout mice were protected against ischemic brain oedema (Verkman, 2000), suggesting that AQP water channels are critical participants in

the development of cytotoxic oedema and a therapeutic target to prevent brain swelling (Manley et al., 2000). There are actually 13 aquaporins known to exist in mammals of which AQPs 1, 4, and 9 are highly expressed in the human brain (Schneider et al., 1992, Badaut et al., 2002). AQP4 is predominantly expressed in the astrocytic end foot processes and at the ventricular interface (Badaut et al., 2002). AQP9 is co-expressed with AQP4 in the astrocytic foot processes (Badaut et al., 2002), while AQP1 is expressed in the choroid plexus and in ganglionic sensory neurons (Badaut et al., 2002). While inhibition of AQP4 expression is associated with a reduction in cytotoxic oedema, infarct area and an improvement in functional outcome (Schneider et al., 1992, fazzina et al., 2009, Kikuchi et al., 2009), vasogenic oedema was exacerbated in AQP4 knockout mice suggesting that AQP4 is also essential for resolution of this kind of oedema (Reese and Karnovsky, 1967, Betz et al., 1989, Papadopoulous et al., 2004). In experimental TBI, and during the early phase of vasogenic oedema formation, an increase in AQP4 expression in the glia limitans is observed accompanied by a downregulation of perivascular AQP4 (Ghabriel et al., 2006).

1.3.2.1 Cytotoxic oedema

The BBB is intact in the early stages of cytotoxic oedema, which occurs as a result of malfunctioning of the Na^+/K^+ ATPase pump, resulting in intracellular Na^+ accumulation. This leads to an influx of water from the extracellular compartment to the intracellular compartment with associated cell swelling (Boulard et al., 2003, Payen et al., 2003). In essence, cytotoxic oedema is a water compartment shift with no change in tissue water content or volume (Donkin and Vink, 2010). Accordingly, it is not a contributor to increased ICP compared to vasogenic oedema. However, cytotoxic oedema does adversely impact on cellular function by altering

intracellular metabolite concentration. The interstitial space also becomes rich in electrolytes but does not contain additional proteins (Schneider et al., 1992).

1.3.2.2 Vasogenic oedema

Vasogenic oedema occurs as a result of increased permeability of the BBB, either as a consequence of increased transcellular protein transport or breakdown of tight endothelial junctions (Boulard et al., 2003). The normally excluded intravascular proteins (Barzó et al., 1996, O'Connor et al., 2006) then enter the cerebral parenchymal extracellular space, with subsequent movement of water from the vasculature to the extracellular space in response to the osmotic gradient generated by the vascular proteins (Donkin and Vink, 2010). Accordingly, vasogenic oedema increases tissue water content leading to an increase in interstitial space (Donkin and Vink, 2010) with no cell swelling (Reese and Karnovsky, 1967, Betz et al., 1989). The increased permeability of the BBB after TBI is thought to occur through two processes, increase in hydrostatic pressure and/or increase in membrane permeability. Additionally, the BBB does not simply close after the initial opening but closes gradually, with smaller vascular components being permeable for up to 7 days following injury. The resultant vasogenic oedema has been shown to develop in the first few hours following TBI compared to the cytotoxic oedema that develops more slowly over the following days and persisting for up to 2 weeks (Hanstock et al., 1994, Barzó et al., 1997). In the initial phases following TBI, vasogenic oedema is thought to be the major contributor to oedema and the associated increase in ICP (Reulen et al., 1977).

When the ICP rises, according to the Monroe-Kellie hypothesis where cerebral perfusion pressure (CPP) = mean arterial blood pressure (MABP) – ICP, the body's response to a fall in CPP is to raise systemic blood pressure and dilate cerebral blood vessels. Increase in MABP beyond

170mmHg will exceed the upper limit of autoregulation causing extravasation of solutes and water into the brain tissue and thus creating a vicious cycle (Olivecrona et al., 2007). Notably with CNS injury, the influence of chemical mediators such as kinins, histamine, nitric oxide, arachidonic acid and free radicals is to breach the physical integrity of the BBB and increase its permeability. This kind of scenario usually occurs in response to trauma, tumors, and late stages of cerebral ischemia (Boulard et al., 2003, Payen et al., 2003)

1.3.2.3 Interstitial oedema

Interstitial oedema is usually caused by obstructive hydrocephalus. The increase in ICP leads to rupture of the CSF barrier and trans-ependymal flow of CSF into the brain and spread into the extracellular spaces and white matter. Unlike vasogenic oedema, the CSF in interstitial cerebral oedema contains no protein (Payen et al., 2003).

1.3.2.4 Osmotic oedema

Under physiological conditions, CSF and brain extracellular fluid osmolality is slightly lower than that of plasma. Therefore, conditions that lead to lowering of the plasma osmolality such as dilution by excessive water intake, cause the pressure gradient to change towards the brain resulting in osmotic oedema (Payen et al., 2003). The consequences of osmotic oedema are significant and like all forms of oedema, may be detrimental if the oedema is not effectively managed.

1.3.3 Management of oedema

In 1985, the cost of caring for people with disabilities following TBI was estimated at $37.8 billion dollars, in part because few pharmacological regimens are available for patients to improve outcome (Thurman et al., 1999, Hoane, 2005). To date, blanket prophylaxis is still used for patients regardless of the mechanism of injury or the extent of injury (Bullock et al., 1999). The current management of oedema is mainly symptomatic and includes the use of osmotic agents (mannitol, hypertonic saline solution), decompressive craniectomy and sedation. Despite their widespread use, these strategies all have adverse side effects and disadvantages.

1.3.3.1 Hypertonic Saline

Hypertonic saline is used to control intracranial hypertension and improve neurologic recovery following severe TBI (Roquilly et al., 2011). By drawing fluid down an osmotic gradient from the interstitial space to the vasculature, hypertonic saline solution improves intracranial compliance and decreases ICP. However, hypertonic saline solution and other osmolytes such as mannitol pose a risk of rebound intracranial hypertension (Roquilly et al., 2011). It should be noted that the osmotic process associated with dehydrating brain tissue require a reasonably functional BBB. A permeable BBB may allow the osmotic agent to enter the brain parenchyma and thus draw water into the interstitial space. Furthermore, hypertonic saline solution has been found to induce severe hypernatremia (osmotic demyelination syndrome) or cerebral pontine myelinosis, renal failure, dehydration, hypotension and phlebitis if the dose is not carefully titrated. Cerebral pontine myelinosis is characterized by osmolar induced pons demyelination that presents with a decline in the level of consciousness, motor deficits and quadriparesis (Roquilly

et al., 2011). Rising hypernatremia poses risk of mental confusion, lethargy, delirium, seizures, and thrombosis.

1.3.3.2 Sedation

In severe TBI, secondary injuries are sometimes attenuated by decreasing the metabolic demands of the brain with benzodiazepines or barbiturates such as midazolam and fentanyl. Body temperature is maintained between 36°C and 37°C, while arterial blood pressure and oxygen saturation are kept in the normal range (Roquilly et al., 2011).

1.3.3.3 Decompressive craniectomy

Decompressive craniectomy evolved from a primitive form of surgery known as trephining and involves the neurosurgical removal of part of the skull to provide room for a swelling brain to expand (Ban et al., 2010). The optimal timing to perform this procedure is still controversial and its efficacy to promote neurological recovery has not been agreed. While the procedure has been found to lower ICP to levels below 20mmHg in 80% of cases, mortality rates still remain significantly high. Morbidity and mortality have been linked to the late complications following the procedure, which include expansion of hemorrhagic contusions, infection, subdural hygroma, and hydrocephalus (Honeybul and Ho, 2014).

Expansion of hemorrhagic contusions may occur due to loss of tamponade effect following bone removal, therefore facilitating extension of the hematoma (Sedney et al., 2014). Secondly, external cerebral herniation, which is protrusion of swollen brain tissue through the craniectomy, may occur. External cerebral herniation can lead to laceration of the cortical veins at the craniectomy edge causing ischemia and necrosis of the hernia contents. Yang et al (2008)

reported this complication in 27.8% of the patients that had a decompressive craniectomy. Aarabi et al (2009) reported subdural hygromas due to accumulation of CSF in the subdural space in 57.4% of decompressive craniectomies (Aarabi et al., 2009). They also reported that most subdural hygromas resolved spontaneously, especially if they formed on the ipsilateral side. In terms of infection, an increased risk of infection at the time of replacement of the bone flap (cranioplasty) has been reported (Sedney et al., 2014). The average time between craniectomy and cranioplasty is 6 months, with patients undergoing cranioplasty within 3 months of craniectomy having a lower complication rate (Chang et al., 2010). Post-traumatic hydrocephalus, which is characterized by accumulation of CSF due to disturbances in the fluid dynamics (Sedney et al., 2014), has also been reported.

Syndrome of the trephined, which is characterized by headaches, dizziness, irritability, difficulty concentrating, as well as memory and mood disturbances, has been reported following the procedure. It is postulated that the sinking of the scalp overlying the decompressive craniectomy transmits atmospheric pressure directly onto the exposed brain surface and contributes to the syndrome (Sedney et al., 2014). Moreover, drainage of CSF through the decompressive craniectomy may also create a negative pressure in the spinal canal, which coupled with the effects of gravity and atmospheric pressure on the exposed brain, potentially shifts the infratentorial compartment resulting in herniation and brain stem compression. This is known as delayed paradoxical herniation (Vilela, 2008).

1.3.3.4 Hyperventilation

High CO_2 levels or low blood O_2 levels cause cerebral blood vessels to dilate increasing volume of blood in the brain and contributing to a rise in ICP. Additionally, inadequate oxygenation

forces the brain to resort to anaerobic metabolism that produces lactic acid and lowers the pH, facilitating vasodilatation and perpetuating the problem. It should be noted, however, that blood vessels constrict when CO_2 levels are below normal; hyperventilation of a patient with high ICP therefore reduces that ICP (Bor-Seng-Shu et al., 2012). However, the negative impact of this approach is that vasoconstriction will reduce oxygen perfusion.

1.3.3.5 Corticosteroids

These are a class of chemicals that include steroid hormones produced by the adrenal cortex in vertebrates. They are involved in numerous physiologic processes that include stress and immune response, regulation of inflammation, carbohydrate and protein metabolism (Asehnoune et al., 2011). The drug class includes prednisolone, betamethasone, cortisone, dexamethasone, hydrocortisone, methylprednisolone, prednisone and triamcinolone and has been found in some studies to reduce the risk of death by 1-2% (Czekajlo and Milbrandt, 2005). However, the more recent CRASH trial incorporating over 10,000 patients demonstrated that they had no significant effect on ICP, blood flow, herniation, clinical outcomes or mortality following TBI (Denker et al., 2000).

1.3.3.6 Hypothermia

Hypothermia lowers a patient's body temperature thus reducing metabolic rate and the risk of tissue injury in case of insufficient blood flow. In addition to slowing cellular metabolism and lowering the body's need for oxygen, hypothermia decreases cell membrane permeability thereby reducing ion influx and resultant autodestructive cascades. It has also been reported to reduce reperfusion injury associated with oxidative stress following restoration of blood flow to tissues after ischemia (Ji-yao and Xiang-feng, 2007). It is usually achieved by surface cooling using

cooling blankets, and requires about 4 to 8hrs to achieve a target temperature of 33°C to 35°C. Alternatively, intravenous administration of iced 4°C crystalloid solution can be used to rapidly lower body temperature by 2°C over 30 minutes (Ji-yao and Xiang-feng, 2007), noting that temperatures lower than 32°C increase the risk of infection. Consistent with intracranial hypertension and brain oedema persisting for longer than 7 days following severe TBI, prolonged periods of hypothermia have been found to be more effective than shorter periods of 48 hours (Ji-yao and Xiang-feng, 2007). Following severe TBI, it has been shown to reduce the risk of death by 19% and the risk of poor neurological outcome by 22% compared to normothermia. Timing is paramount as it is important to initiate cooling within 4 hours of injury (Ji-yao and Xiang-feng, 2007). Similarly, rate of and timing of rewarming is a critical factor given the increased risk of pneumonia with rewarming, and its association with increased adverse outcomes.

None of the above interventions are widely accepted as improving outcome following TBI, which is unsurprising considering that they do not target the mechanisms associated with oedema formation and development of increased ICP. Recent evidence has shown that the neuropeptide substance P is critically involved in oedema formation and may offer a potential therapeutic target after TBI.

1.4 Substance P

In 1931, Von Euler and Gaddum isolated a substance from horse brain and gut that caused hypotension and named it preparation P. Four years later, Gaddum changed its name to substance P. Since then, a number of related substances have been isolated and named neurokinin A and B

(Kimura et al 1983), and more recently, neuropeptide K and Y. The five peptides are collectively known as tachykinins due to their effect on smooth muscle contraction.

Substance P (SP) is a neuropeptide that functions as a neurotransmitter and as a neuromodulator. It is localized to sensory nerve fibers and has been shown to play a role in inflammation (Parker et al., 1998), pain, depression, memory and learning (Huston and Hasenöhrl, 1995), induction of immediate early genes (Bereiter et al., 1997), affective behavior, neurodegenerative diseases and emesis (Rupniak and Kramer, 1999, Chappa et al., 2006). The amino terminus group of SP has been found to promote memory whereas the carboxyl terminus group is involved in reinforcement (Donkin et al., 2007). Its release from sensory nerve fibres is associated with formation of vasogenic oedema and increased vascular permeability (Walsh D. T. et al., 1995). Recently, SP release following TBI has been linked to increased BBB permeability and formation of vasogenic oedema (Vink and Nimmo, 2002). Inhibition of SP binding using a SP antagonist after TBI not only reduced BBB permeability and oedema, it also increased brain free magnesium concentration (Vink et al., 2004), reduced axonal injury and improved motor and cognitive outcome (Donkin et al., 2007) (Donkin et al., 2009, Donkin et al., 2011).

1.4.1 Synthesis

Substance P is derived from pre-propeptides, which are synthesized on the membrane bound ribosomes in the cell body of the peptidergic neuron. After cleavage of the hydrophobic N-terminal signal peptide and protein folding in the lumen of the endoplasmic reticulum, the pro sequence guides the peptide into the Golgi apparatus where it is packaged into vesicles that are

transported along the axon to the nerve terminal and released by exocytosis (Donkin et al., 2007). Substance P is widely distributed in the rhinencephalon, telencephalon, basal ganglia, hippocampus, amygdala, septal areas, diencephalon, hypothalamus, mesencephalon, met encephalon, pons, myelencephalon and spinal cord.

1.4.2 Functions of Substance P

SP is thought to enhance plasma protein extravasation (vascular permeability) and leucocyte adhesion to post capillary venules (Newbold and Brain, 1995, Donkin et al., 2011), and is secreted together with calcitonin gene related peptide (CGRP), which is the primary vasodilator. Following brain injury, receptor-binding sites for SP have been found to increase on the glia (Lin, 1995, Turner et al., 2011) and several studies have confirmed that astrocytes become reactive as a result of SP following injury (Marriott et al., 1991, Palma et al., 1997). By promoting astrocyte reactivity, SP upregulates the production of several soluble mediators such as cytokines, prostaglandins, thromboxane derivatives as well as reactive oxygen species, that exacerbate the injury process (Vink et al., 1987, Vink et al., 1988b, Carthew et al., 2012). SP also induces endothelial cells to produce nitric oxide and primes polymorphonuclear cells for oxidative metabolism (Hafstrom et al., 1989, Persson et al., 1991). In study of neurogenesis following diffuse TBI, Carthew et al (2012) characterised the action of SP on neurogenesis. Their results showed that SP release promoted microglial proliferation 48hrs post-TBI that in turn suppressed neurogenesis. Moreover, administration of the SP, NK1 antagonist n-acetyl tryptophan (NAT) decreased microglial proliferation post injury but did not increase neurogenesis (Carthew et al., 2012). Their study also showed that SP infusion increased microglial proliferation 48hrs post injury, clearly supporting a role for SP in microglial proliferation following TBI (Carthew et al.,

2012). The NK1 tachykinin receptor antagonists have also been shown to reduce perivascular inflammatory infiltration, circulating histamine, prostaglandin E2 (PGE2), and lipid peroxidation (Kramer et al., 1997). SP binding to its NK1 receptor in the CNS has been shown to directly induce a non-apoptotic form of programmed cell death in the hippocampal, striatal and cortical neurons. This is independent of caspase activation although it requires gene expression (Castro-Obregon et al., 2002). Finally, Donkin et al (2011) demonstrated that administration of the NK1 antagonists was neuroprotective after TBI even when administered up to 12 h after injury, although only highly lipid soluble forms of the antagonist were effective, suggesting centrally mediated effects of the compounds conferred neuroprotection (Donkin et al., 2011).

1.5 Magnesium

Magnesium decline has been identified as a ubiquitous secondary injury factor leading to neurological deficits following TBI (Vink et al., 1987, Vink et al., 1988b, Heath and vink, 1999c). While extensive supportive evidence for a neuroprotective role for magnesium in experimental TBI has been reported, this has not translated to the clinical setting (Lee et al., 1976, Vink, 1991, Vink et al., 2003, van den Heuvel and Vink, 2004, Temkin et al., 2007, Vink et al., 2009, Sen and Gulati, 2010). Most of the experimental TBI studies have been limited to focal models of mild to moderate severity and administered different magnesium salts, namely magnesium chloride and magnesium sulphate (Hicks et al., 1995, Park et al., 1996). Those studies that have used diffuse models of TBI have reported that magnesium improves neurological outcome following TBI (Heath and vink, 1999c). Specifically, administration of 254μmoles/kg of magnesium salts to rats after TBI significantly improved both motor and

cognitive outcome over a one week period following injury (Heath and vink, 1999c). These authors postulated that, amongst other mechanisms, magnesium had a beneficial effect on the ability of a cell to repair itself by enhancing ATP production and cytosolic phosphorylation ratio (Heath and vink, 1999c). The clear implication is that administered magnesium required central penetration to confer neuroprotective effects.

1.5.1 Distribution of Magnesium in the human body

Magnesium is distributed between bone (53%), intracellular muscle compartments (27%) and soft tissues (19%). In the human body, less than 1% of total body magnesium is found in serum and red blood cells (Fawcett et al., 1999). Serum magnesium is present in 3 states namely free (ionized) 62%, protein bound (33%) and bound to anions such as citrate and phosphate (5%) (Fawcett et al., 1999). 90% of the intracellular magnesium is bound to organic matrices (Fawcett et al., 1999).

1.5.2 Role of magnesium

Magnesium is the fourth most common cation in the body and the second most common intracellular cation only after potassium (Fawcett et al., 1999). It is an essential cofactor for various kinases in the body and all energy requiring processes including cellular respiration, oxidative phosphorylation and glycolysis (Buchachenko et al., 2012). It is also involved in protein synthesis, the cell cycle as well as neuronal functioning, and has been identified as a physiological calcium blocker (Fawcett et al., 1999). In part, the ability to reduce calcium entry is through its effects on the NMDA receptor (Mcdonald et al., 1990). Specifically, the NMDA receptor exhibits a voltage dependent magnesium block following TBI thereby regulating the

influx of calcium ions that trigger excitotoxic cascades. (Heath and vink, 1999c). Magnesium also stabilizes the cell membrane and maintains membrane integrity (Cook et al., 2009). By so doing, magnesium is thought to maintain the integrity of the BBB thereby reducing vasogenic oedema (Ghabriel et al., 2006). By relaxing smooth muscle, magnesium decreases vasospastic episodes and maintains blood flow reducing ischemia, formation of reactive oxygen species and lactic acid production (Cook et al., 2009).

1.5.3 Magnesium administration after CNS injury

A number of studies have now demonstrated the neuroprotective effects of magnesium sulphate in moderate to severe brain injury (Vink et al., 1988a, McIntosh et al., 1989). Heath and Vink also demonstrated the therapeutic dose response of magnesium salts in severe TBI with dose being dependent on the parenteral route of administration and not the salt used. (Heath and vink, 1997). While a number of motor outcome tests have been used to assess efficacy of magnesium administration in rodent TBI, the rotarod tests have been the mostly widely used given their utility in both focal and diffuse models of rodent TBI (Vink et al., 1996). Using the rotarod test, animals typically demonstrated a 60% decline by day 1 following injury and their performance improved over the next 6 days with repeated exposure to the test. Animals treated with magnesium sulphate at 30 min after the insult only showed a 25% decline in motor function, with almost complete recovery to the pre- injury values in motor function by day 7 after injury. It is possible to utilize either the magnesium chloride or the magnesium sulphate salt in treatment of TBI. However, Durlach et al (2007) have highlighted that while magnesium sulphate is more commonly used than magnesium chloride, the latter has some clinical, safety and pharmacologic advantages than the former (Durlach et al., 2007).

1.5.3.1 Magnesium Chloride

Early studies of hepatic ischemia showed that administration of ATP-magnesium chloride complexes was effective in the treatment of the condition (Ohkawa et al., 1983). Accordingly, McIntosh et al (1989) investigated the efficacy of high and low dose magnesium chloride and compared it with ATP-magnesium chloride complexes and ATP alone in TBI (McIntosh et al., 1989). The study showed that at 24 hours and one week post injury, the saline treated animals demonstrated severe neurological impairment and continued to exhibit moderate-severe neurological deficits up until 4 weeks post injury (McIntosh et al., 1989). Animals that were treated with low dose magnesium chloride (12.5 μmoles) showed a small improvement in composite neurological score although animals that received high dose magnesium chloride (125 μmoles) showed a significant improvement in neurological score compared to the saline treated controls (McIntosh et al., 1989). In the same study, animals that were treated with ATP-magnesium chloride (low dose) or ATP alone showed a slight improvement in neurological scores, but these scores were not significantly better than those exhibited by saline treated animals (McIntosh et al., 1989). They concluded that magnesium chloride may have been effective as a treatment by limiting excitotoxicity, since the ion plays an important regulatory role in the gating of the NMDA channel (Nowak et al., 1984). They did not exclude the possibility that magnesium may have had positive effects on TBI outcome by reducing arachidonic acid and prostanoid synthesis (Nigam et al., 1986) or by reducing cerebral vascular tone and maintaining perfusion of injured tissues following TBI (Seelig et al., 1983). In contrast, they concluded that the improvement in neurological outcome was unrelated to competitive inhibition of voltage dependent calcium channels since calcium channel blockers do not appear to be effective in TBI (McIntosh et al., 1989).

1.5.3.2 Magnesium Polyethylene glycol (Mg PEG)

Polyethylene glycol ($HO\text{-}CH_2\text{-}(CH_2\text{-}O\text{-}CH_2\text{-})_n\text{-}CH_2\text{-}OH$), also known as polyethylene oxide (PEO) or polyoxide ethylene (POE) depending on the molecular weight, is a hydrophilic polymer that attaches to various protein medications increasing their membrane permeability, decreasing rate of clearance and facilitating longer acting medicinal effects, longer dosing intervals and reducing toxicity. While a limited number of early studies have utilised PEG in the treatment of TBI, none have used the compound to facilitate BBB penetration. In contrast, early studies have used combined magnesium and PEG in traumatic spinal cord injury (Lee et al., 1976). Subsequent to these early studies, Ditor et al (2007) demonstrated that magnesium sulphate in a PEG formulation resulted in improved neuroprotection around the injury site and greater locomotor recovery in spinal cord trauma compared to administration of magnesium sulphate alone (Ditor et al., 2007). Later, Kwon et al (2009) established the optimal dose for magnesium in a PEG formulation to help guide the treatment parameters for a clinical trial of a magnesium salt in PEG formulation in acute human spinal cord injury (Kwon et al., 2009).

Magnesium sulphate in a PEG formation was more effective in reducing lesion size at 42 days post injury (to 51% compared to saline vehicle) than either magnesium sulphate alone (33%) or PEG alone (20%) (Kwon et al., 2009). Additionally, animals that were treated with magnesium chloride in PEG (127 μmoles/kg) every 8hrs totaling either 2, 4 or 6 infusions had lesion volumes of $3.16\pm0.43mm^3$, $2.68\pm0.27mm^3$, and $2.18\pm0.28mm^3$, respectively, compared to the saline controls that were at $4.58\pm0.56mm^3$, corresponding to a percentage reduction in lesion volume of 31%, 42% and 53%, respectively. (Kwon et al., 2009). Using the 12 point Ferguson transformation, these Mg PEG treated animals had locomotor scores of 8.6±0.68, 8.5±0.45 and 9.4±0.51, when administered 2, 4 or 6 injections, respectively, compared to the saline control

locomotor score that was at 7.2±0.54 (Kwon et al., 2009). The authors then went on to compare the effects of magnesium chloride dose at either 127 or 254 μmoles/kg. Two infusions administered 8hrs apart resulted in lesion volumes of 3.16±0.43mm^3 and 1.86±0.31mm^3, representing reductions of lesion volumes of 31% and 60% compared to saline controls (Kwon et al., 2009). The corresponding locomotor scores at 6 weeks post injury for the 254 μmoles/kg and 127μ/kg doses were 10.0±0.71 and 8.6±0.68 compared to 7.2±1.6 in the saline control animals (Kwon et al., 2009). Clearly, the higher dose was more effective. Finally, the treatment was more effective when repeated every 6 hrs as opposed to every 8 hrs, with best results achieved when treatment is started as soon as possible after trauma.

The comparison between magnesium sulphate and magnesium chloride in PEG showed no statistical difference between the two salts but the authors noted that the magnesium chloride treated group appeared to have slightly better locomotor recovery early on compared to the magnesium sulphate treated group (Kwon et al., 2009). They also concluded that 4 to 6 doses of magnesium salts in a polyethylene glycol solution provided greater reductions in lesion volume and better locomotor recovery than 2 doses (Kwon et al., 2009). Finally, the efficacy of magnesium chloride in a PEG solution was found to be greater than that of methylprednisolone and that this efficacy was not negatively or positively influenced by the concomitant administration of methylprednisolone (Kwon et al., 2009).

1.6 Models of Injury

In order to identify mechanisms of injury and develop novel neuroprotective therapies, animal models are used to replicate the features of clinical TBI. A number of different models have been

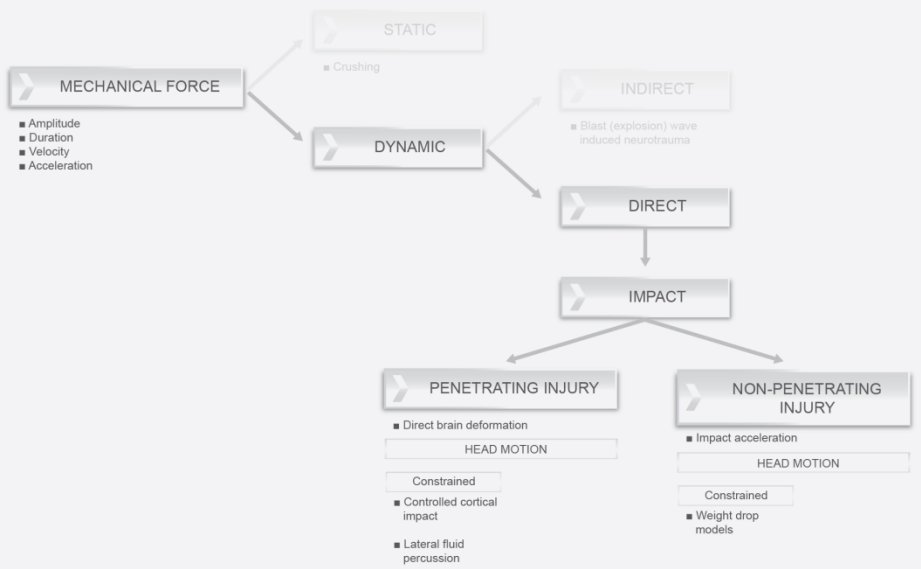


Figure 1.2: Models of injury

developed (Fig. 1.2), all of which attempt to reproduce one or more of the following types of primary injury: 1) direct contusion of the brain through the skull; 2) brain contusion caused by coup and contra coup movement; 3) diffuse axonal injury caused by shearing forces; and 4) bleeding from ruptured blood vessels, especially the bridging veins with formation of hematomas (Cernak et al., 2000). Despite having a lissencephalic brain compared to the human cortex, rodents remain the most commonly used animals for modeling human TBI.

The mechanical force used to induce the primary event in these experimental models inflicts either static or dynamic injury, with the former depending more on the amplitude and duration of the force rather than the velocity and acceleration. These static models are used to illustrate morphological and functional processes as in, for example, direct crushing of a cranial nerve with forceps. Dynamic injury can be direct or indirect (Fig 1.2), with indirect injury occurring as a result of, for example, a blast wave where the kinetic energy of the oscillating pressure waves

traverse the body and impart their effects on brain tissue, thereby inducing neurotrauma (Cernak et al., 2000). More common are the direct, dynamic brain injuries which are subdivided into impact and non-impact models, both of which are then further sub-classified depending on whether there is direct brain deformation (penetrating injury) or indirect brain deformation which occurs with non-penetrating injury (Cernak et al., 2000).

1.6.1 Fluid Percussion injury model

The fluid percussion model is an example of a dynamic impact model with direct brain deformation, which has been widely used for the study of pathology, physiology and pharmacology of TBI. In this model, a craniotomy is made either centrally or laterally at or adjacent to the midline between bregma and lambda. A fluid pressure pulse is then directed onto the intact dura at the site of the craniotomy using a fluid percussion injury device. The device consists of a Plexiglas cylinder-shaped reservoir that contains sterile isotonic saline. One end of the reservoir is then connected via a tube to the Luer-loc cemented on the animal's skull at the site of the craniotomy. The other end Plexiglas cylinder has a pendulum which is released to impact a rubber covered bung in contact with the fluid within the reservoir thus generating a pressure pulse which is transmitted to the exposed dura of the animal. Severity of injury is directly proportional to the height from which the pendulum is released, and thus the pulse generated within the cylinder (Vink and McIntosh, 1990, Li et al., 2011). This model produces petechial hemorrhage in the brain parenchyma, subarachnoid hemorrhages and tears in the brain parenchyma, behavioral and cognitive deficits as well as herniation of the brain stem and respiratory depression (McIntosh et al., 1989, Ibolja, Morales et al., 2005). The degree of brain stem herniation and respiratory depression is much more severe in the midline model as opposed to the lateral model. In this respect, craniotomy position is paramount in determining the type of

injury. Even within the lateral model, a craniotomy that is positioned less than 3.5mm from the sagittal suture will produce damage in both ipsilateral and contralateral cortices, whereas a craniotomy made at greater than 3.5mm from the sagittal suture will not produce any contralateral damage. Nonetheless, the model still remains one of the most frequently used models in the world as it produces both focal and some diffuse injury (Morales et al., 2005, Morganti-Kossmann et al., 2010), even though many consider the open (exposed dura) nature of the injury is a pre-treatment against development of elevated ICP.

1.6.2 Controlled cortical impact model

In the controlled cortical impact (CCI) model, the impact is delivered by a compressed air driven metallic piston over an intact dura to the brain parenchyma (Manvelyan, 2006). The ability to control piston velocity, depth of penetration and dwell time is considered to allow better control over the impact severity (Morales et al., 2005). Accordingly, the model has been referred to as the rigid percussion model. This model consists of a pneumatically controlled, metal cylinder usually with a 4 to 5cm stroke and having an impact velocity of between 0.5 to 10 m/s. This impact velocity is directly proportional to the air pressure that drives the impactor. Vertical adjustment of the crossbar holding the cylinder varies the depth of cortical deformation, usually between 1 and 3mm, whereas the impact dwell time varies between 25 and 250ms (Cernak et al., 2000, O'Connor et al., 2011). The CCI model has been found to mimic many aspects of clinical TBI including decreased cerebral perfusion pressure, increased ICP, neuroendocrine, metabolic changes and coma. This model has also been associated with acute subdural hematoma formation, axonal injury, concussion, and derangement of the BBB (Morales et al., 2005). The

advantage of this model is the ability to control the injury parameters such as time, velocity and depth of impact whereas its disadvantage are the extensive bleeding and associated ischemia at the site of impact, the lack of brain stem deformation, and the presence of a pressure relieving craniotomy (Morales et al., 2005).

1.6.3 High velocity missile injury

This model is mainly used to study the pathophysiology of a missile wound to the brain. In the model developed by Carey et al (1990), the animals are anaesthetised and placed in a stereotaxic frame. The sloping right frontal wall is surgically removed, thereby allowing the missile to penetrate through the vertically intact posterior sinus wall. This model involves firing a 2mm, 3mg steel sphere from a distance of 80cm at 220 m/s or 280m/s, with the missile usually traversing through the right hemisphere from front to back with a delivered energy between 0.9 and 1.4J. The alternative Finnie model, Finnie (2001), involves use of a 0.22m firearm to inflict brain injury in physically restrained sheep, with the bullet fired at a 3m range and traversing through the temporal lobes of the sheep from right to left. The pathology produced by these models includes hyperglycemia, increased ICP, decreased cerebral perfusion pressure and an increased hematocrit.

1.6.4 Controlled contusion model

The injuries generated by the controlled contusion model are mostly focal in nature as opposed to the aforementioned models that also cause varying degrees of diffuse axonal injury. This method is therefore well suited for studies focusing on cerebral contusions and oedema as opposed to

research on molecular and cellular mechanisms of post traumatic neuronal cell death (Tornheim and McLaurin, 1981). The original model described by Tornheim and McLaurin (1981) used a Remington humane stunner to deliver an oblique lateral impact or a blow directly to the coronal suture in cats (Tornheim and McLaurin, 1981). The rat model developed by Goldman et al (Goldman et al., 1991) applied a pendulum force to a point approximately 9mm from the coronal suture, with the angle of impact adjusted using a normogram. This model generated early increase in cerebral permeability and increased ICP. While the physiological and morphological changes generated in the focal contusion are comparable to those sustained in some forms of human TBI, the inability to produce diffuse axonal injury has made these models less popular.

1.6.5 Impact acceleration models

Of the impact acceleration models, Marmarou's weight drop model (Marmarou and Foda, 1994) is the most commonly used. In this model, a rat is placed on a 10cm deep foam bed directly under the trauma device. The device consists of a 450 g brass weight contained within a Plexiglas tube , with the weight falling freely from a pre-determined height onto a stainless steel disc (10mm diameter by 3mm depth) fixed with dental cement to the rodents' skull centrally between the lambda and bregma fissures. The stainless steel disc acts as a helmet that reduces the incidence of skull fractures. When the weight is dropped from a 2 m height, this model is associated with a 12.5% incidence of skull fracture and a mortality rate of less than 20%. Pathology includes massive diffuse axonal injury in the corpus callosum, internal capsule, optic tracts, cerebral and cerebellar peduncles and the long tracts in the brain stem. Motor and cognitive deficits that persist for several weeks to months are readily apparent. Model characterization by Vink et al (1995) confirmed that there is a significant reduction in free magnesium concentration and cytosolic phosphorylation (Heath and vink, 1995), while others have shown the production of

pro-inflammatory mediators, induction of calpains and caspases, as well as induced release of cytochrome c and pro apoptotic Bcl-2 from the mitochondria into the cytosol (Cernak et al., 2004). Cernak et al (2004) argued that the impact acceleration model is at risk of rapid repeat injury as the weight rebounds from the animal's skull and results in a second impact (Cernak et al., 2004). Accordingly, they modified the model to utilize an air driven impactor that contacts a steel disc cemented onto the rodent skull, with the velocity and dwell time under user control. They also incorporated a gel-filled base of known compressibility rather than a foam bed. Using this model, it was noted that the systolic blood pressure, brain oedema and BBB permeability increased immediately, and that it replicated many of the biochemical and neurological changes that take place following human TBI (Morales et al., 2005). One drawback to the Cernak modification is that there is no rotational injury in the model, reducing the degree of axonal injury and the associated motor and cognitive deficits. Accordingly, the Marmarou model is still considered the most relevant to produce diffuse axonal injury as observed in most clinical situations.

1.7 Synopsis

A considerable amount of experimental evidence has been reported supporting the use of magnesium salts in the treatment of TBI. However, a recent large, randomized clinical trial of magnesium in TBI failed, and while the precise reasons are unknown, it is thought that poor central penetration of the magnesium salt may be, in part, responsible. Recent experimental studies in spinal cord injury have shown that magnesium penetration into the CNS can be facilitated if the magnesium salt is administered in a solution containing polyethylene glycol (PEG), a hydrophilic polymer that facilitates transport across the blood brain barrier and

throughout the extracellular space. To our knowledge, there are no reports on the efficacy of magnesium PEG in TBI. Accordingly, the present study will examine the efficacy of combined magnesium PEG in TBI, focusing on BBB permeability, oedema formation and functional outcome. Moreover, given the recent evidence suggesting that substance P is integrally involved in development of increased BBB permeability and formation of oedema after TBI, this study will also assess the effects of magnesium PEG on SP immunoreactivity after TBI.

Hypothesis

That Mg PEG results in significant reductions in SP release, BBB permeability, oedema formation and neurological deficits after TBI.

Aims

1) To determine whether magnesium PEG reduces oedema and BBB permeability after TBI.

2) To determine whether administration of magnesium PEG improves neurological outcome following TBI.

3) To determine whether magnesium PEG attenuates SP immunoreactivity after TBI.

4) To determine whether lower concentrations of magnesium can be used in combination with PEG compared with magnesium alone and still result in improved BBB permeability, oedema formation and neurological outcome after TBI.

CHAPTER 2

METHODS

2. METHODS

2.1 Animal Care

2.1.1 Ethics

All experimental protocols were approved by the experimental ethics committee of the University of Adelaide (ethics number M-063-2013) as stated in the guidelines for the use of animals in experimental research by the Australian National Health and Medical Research Council.

2.1.2 General

Adult male Sprague-Dawley rats (350-380 g) were used in all experiments. Animals were obtained from the breeding colony of the Waite campus (University of Adelaide) at least 3 days before experimentation, and then housed locally in a conventional 24° rodent room on a 12-hour light-dark cycle, with water and food supplied *ad libitum*.

2.2 Experimental Procedures

2.2.1 Anesthesia

Animals were placed in a transparent plastic induction chamber and anaesthetized with 5% isoflurane in air via a calibrated vaporizer with a flow rate of 1.5L/min. Isoflurane was obtained as a volatile liquid and stored below 25°C away from direct heat and sunlight. When the pinched tail or pinched toe reflex was suppressed, animals were intubated and anesthesia maintained using 1.5-2% isoflurane in 1.5L air.

Lignocaine

Lignocaine was supplied as an aqueous solution and stored in a cabinet at room temperature. It was used as a local anesthetic where it was administered subcutaneous via a 25-gauge needle at a dose of 0.1ml per animal.

2.2.2 Induction of TBI

TBI was induced in all animals using the impact acceleration model developed by Mamarou and colleagues (Marmarou and Foda, 1994). Anaesthetised animals were placed on a thermostatically controlled heating pad, their heads shaved and a midline incision made on the dorsal surface between the bregma and lambda sutures. The skin was retracted and a steel disc was fixed to the skull with polyacrylamide adhesive, which was allowed to set for 20 seconds. Injury was then induced by releasing a 450gm brass weight from a height of 2m onto the steel disc. The animal and 10 cm foam bed was immediately removed after impact, thus preventing any potential second impact with the weight rebound. The animals were then removed from the foam bed and the intubation tube reconnected to the ventilator. Any animals that sustained skull fractures were euthanised with an overdose of sodium pentobarbital (60mg/kg) and excluded from the study. The steel disc was removed from the skull and midline incision closed using surgical clips (9mm Auto wound clips, Benton Dickinson, USA). Animals were maintained on the ventilator for at least 10 min, checked for spontaneous respiration periodically and then weaned off the ventilator. After recovery from the effects of anesthesia in a heated recovery box, they were returned to their home cages.

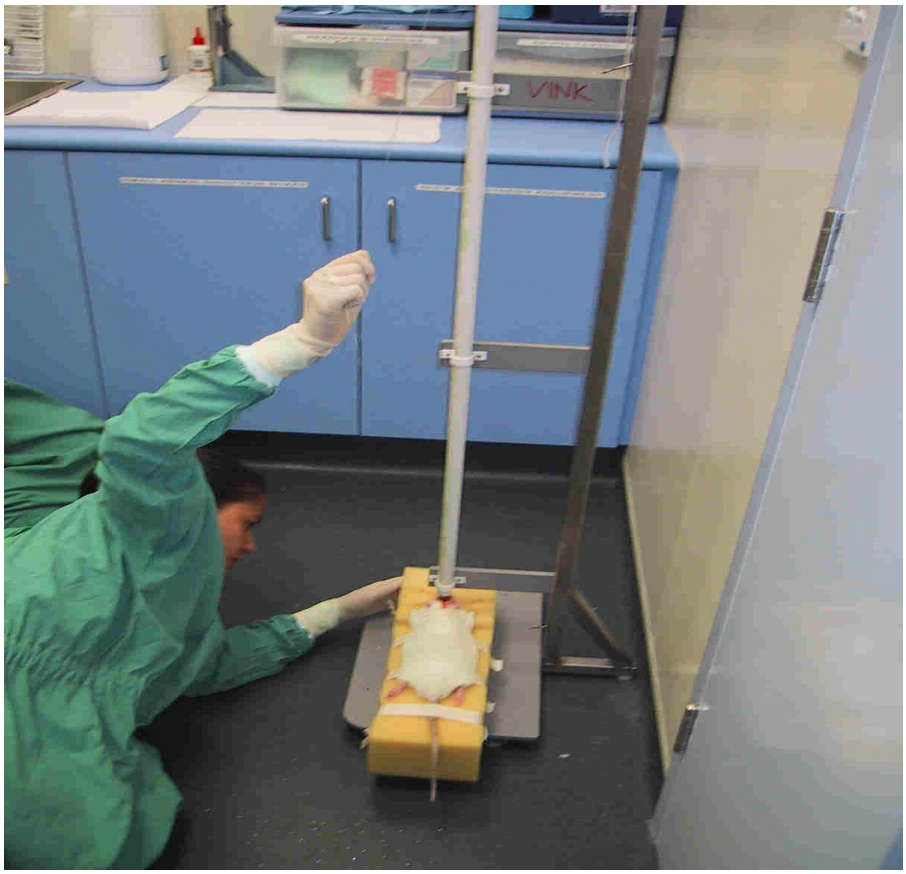


Figure 2.1. The impact acceleration weight drop model. Note that the weight was carefully aligned to centrally strike the steel disc attached to the animal's skull. The animal was fixed to the foam bed using adhesive tape.

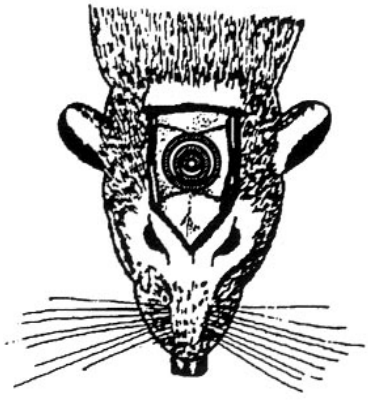


Figure 2.2. Animal with stainless steel disc attached.

2.2.3 Perfusion

Normal saline was used for perfusions involving Evan's blue, whereas 10% buffered formalin was used for all other perfusions. At the preselected time points of 5hr, 3day and 7day, subgroups of animals were anesthetised with isoflurane and placed in the supine position on a wide rack suspended above a stainless steel sink. A bilateral thoracotomy was performed to expose the heart, and a blunt 19 gauge, 37mm needle inserted through the apex of the left ventricle and into the ascending aorta. Heparin (5000IU in 1 ml of saline) was injected slowly into the aorta over 30 seconds and the right atrium incised to permit vascular flushing. Perfusion is complete when the fluid leaving the aorta becomes clear. The brains were removed for Evan's Blue determination or stored in 10% buffered formalin for histology.

2.3 Drug Treatments

At 30mins after injury, animals were administered with an intravenous dose of either $MgCl_2$ (254 μmoles/kg; optimal dose), Mg PEG (254 μmoles/kg $MgCl_2$ in 1g/kg PEG), PEG alone (1g/kg), an equal volume of saline (vehicle), 1/10 $MgCl_2$ (25 μmoles/kg), or1/10 $MgCl_2$ in PEG (1g/kg). The optimal dose of $MgCl_2$ was taken from previous work by our laboratory (Health and Vink, 1999d). All injections were via the tail vein with all agents being stored at room temperature prior to administration. Treatments were performed blinded.

2.4 Functional Tests

2.4.1 Motor function

The rotarod device was used to assess motor function in all animals given that it has been shown to be the most sensitive test of motor function in rodents following TBI (Hamm, 1994). The rotarod device consists of motorized rotating assembly of 18 rods of 1mm in diameter (Fig. 2.3). The animals are placed on the rods and are required to grip them as they rotate thus introducing a grip test component. The speed of the device is increased from 0 to 30 revolutions per minute (rpm) at intervals of 3 rpm every 10 seconds as shown in table 2.1 below.

Time (secs)	Rotational speed (rpm)
10	0
20	3
30	6
40	9
50	12
60	15
70	18
80	21
90	24
100	27
110	30
120	30

Table 2.1. Time (secs) and rotational speed of the rotarod device.

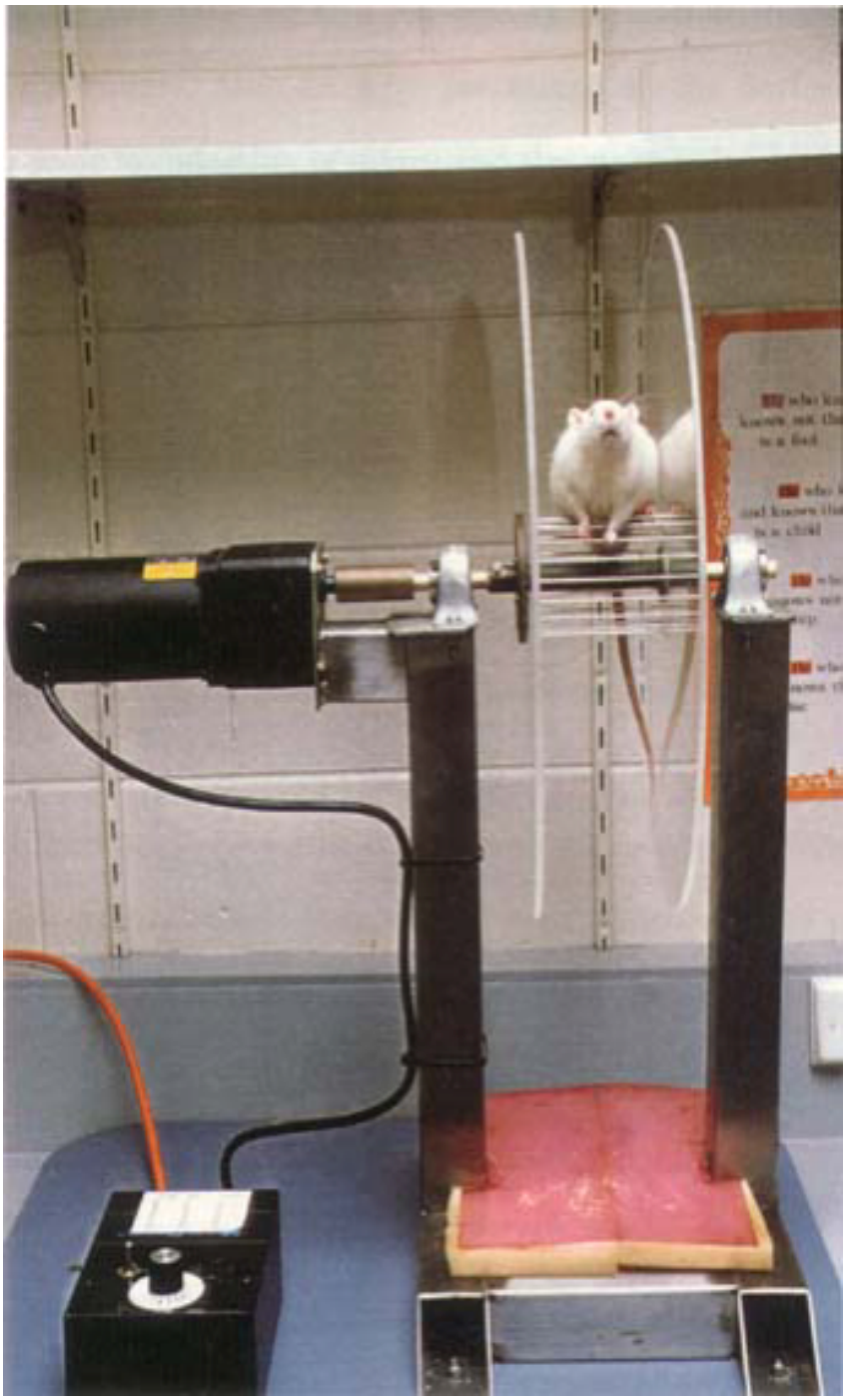


Fig 2.3. The rotarod device for motor function evaluation

2.4.2 Cognitive outcome

The object recognition test was used to determine cognitive outcome. This test utilises an open box (80cm long, 80cm wide and 80cm deep) in which objects are placed. The objects are made of a biologically neutral material such as glass, plastic or metal and weighted so that the animals cannot displace them. To familiarize them to the environment, animals were placed in the empty box for up to 5 sessions of 10mins exposure before the test itself was carried out. This test consists of two phases, a sample phase (3 min) and a choice phase (3min) with 15min retention interval between the two phases. In the sample phase, two identical objects are placed in the back corners of the open box, 10cm away from the sidewall. The animal is placed in the open box facing away from the objects. The total time spent in exploring the 2 objects is recorded. After 3mins of exploration the animal is removed from the open box and returned to its cage. After a 15min break the rat is reintroduced to the open box for a further 3mins: the choice phase. In this phase, the objects are in the same locations that were occupied by the previous sample objects, although only one of two objects is identical to the objects seen in the sample phase while the other is a new, novel object. Time spent exploring each object, where the rat touches the object with its nose or the rats nose is directed toward an object at a distance less than or equal to 2cm, is recorded. Turning around or sitting on an object is not considered as exploratory behavior.

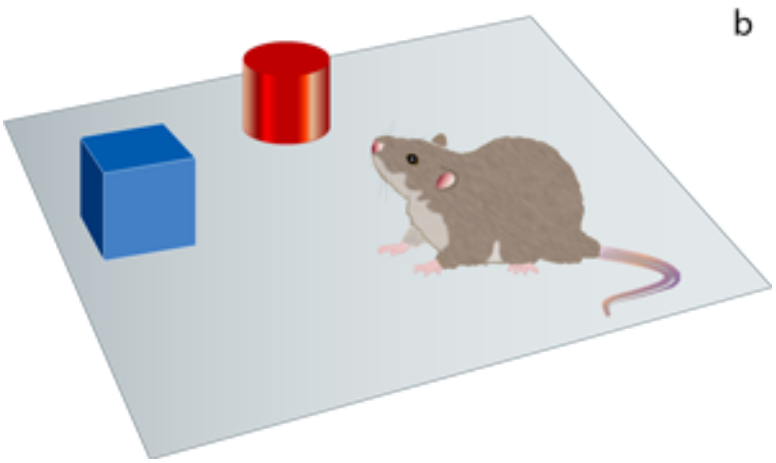


Fig. 2.4. Object recognition test: choice phase.

2.5 Oedema measurement

2.5.1 Brain water content

The amount of brain water content was calculated on the basis of wet weight versus dry weight using a Mettler Toledo halogen moisture analyzer (Fig. 2.5). At the preselected time points, brains were rapidly removed and sectioned into left and right hemispheres, which were placed on separate slides. Another slide was placed on top of the tissue on each slide and the tissue was then crushed into a paste between the two slides. The slides are placed into the moisture analyzer in which they are weighed before being rapidly heated to 180° for 10 minutes and subsequently reweighed. The final % water reading is displayed automatically at the conclusion of the assessment. The entire process is done as quickly as possible to avoid evaporation of the moisture from the extracted brain tissue.

Fig 2.5. Mettler Toledo halogen moisture analyzer

2.6 Blood Brain Barrier Permeability

2.6.1 Quantitative Evaluation

Evan's blue (EB) dye (Sigma-Aldrich, Sydney, Australia) was used to assess BBB permeability. This dye binds to albumin and can therefore be used as a marker of albumin extravasation into the brain parenchyma during periods of BBB disruption. A 4% solution of EB dye (2.0 ml/kg) was administered intravenously into the tail vein at 4.5 hours following TBI. After a 30mins period to allow for dye circulation, the animal was re-anesthetized with isoflurane, a thoracotomy performed and the animal perfusion flushed with saline to remove vascular dye. The animals were then decapitated, their brains removed and separated into right and left hemispheres. The right and left hemispheres were individually weighed for quantitative determination of EB albumin extravasation(Adelson et al., 1998). Samples were homogenized in 7.5ml phosphate buffered saline (PBS) and 2.5ml of trichloroacetic acid solution (TCA) added (Sigma-Aldrich, Sydney, Australia). Samples were then cooled overnight and centrifuged for 30 min at 1000g the

following day. The supernatants were removed and the amount of EB was measured at 610nm using a spectrophotometer. EB is expressed as μg/mg of brain tissue against a previously obtained standard curve for EB absorbance.

2.7 Tissue Processing

2.7.1 Blocking and Embedding

All brains for histology and immunohistochemistry were processed and blocked in paraffin wax. Briefly, removed brains were placed in a Kopf rodent blocker and cut into 2mm sections. Each section was then placed in a cassette, cushioned on both sides with foam and secured with a metallic cover. The tissue was then processed for 20 minutes in each graded ethanol bath (50%, 70%, 80%, 95% and two x 100%), followed by two xylene baths for 1.5hrs each, and finally four paraffin baths of increasing time (30,60,60 and finally 90 minutes).

2.7.2 Microtome sectioning

The paraffin embedded blocks were mounted onto the block holder of a standard microtome (Microm, Walldorf Germany) and cut into 5μm sections. The sections were then floated on a water bath and placed on microscope slides coated with APT (Cat. No. A-3648, 500ml; Sigma-Aldrich, Sydney, Australia) before being left in the oven overnight at 37^0C to dry.

2.8 Histology and Immunohistochemistry

2.8.1 Haematoxylin and Eosin staining

At 5hrs, 3days and 7days after TBI, brains of animal subsets were removed, processed and paraffin embedded for histological assessment as described above. Prior to staining, slides were placed in an oven at 80^0C to melt the wax, and rehydrated with xylene (2 baths at 1 min each), and alcohol (100% and 90% at 1 min each). The slides were then rinsed in running tap water for 1 min and placed in haematoxylin (Lillie Mayers alum) for 5mins. The slides were taken out and rinsed in running tap water again and then placed in a differentiator bath of acid alcohol, rinsed in running tap water, placed in saturated aqueous lithium carbonate and in running tap water yet again. The slides were subsequently placed in eosin (eosin 15g, erythrosin 5mg, calcium chloride 5g, water 2L) for 3mins. The slides were then dehydrated with ethanol (90% for 15secs, then 2 baths 100% ethanol for 15secs each) and xylene (2 baths of 2 minutes) before being coverslipped using Gurr's Depex mountant. Slides were then dried, excess resin removed and scanned with a Hamamatsu nanozoomer.

2.8.2 Substance P Immunohistochemistry

At 5hrs, 3days and 7days after TBI, brains of animal subsets were removed, processed and paraffin embedded for histological assessment as described above. Slides were deparaffinised in xylene (3 baths for 5mins each), dehydrated with 100% ethanol (2 baths for 5mins each) and rinsed in distilled water before being immunolabelled with substance P (SP) primary antibody (1:2000 in NHS; polyclonal Santa Cruz Cat No.SC-9758) by overnight incubation at room temperature. After washing in PBS, slides were incubated with an anti-goat IgG-HRP conjugated

secondary antibody (1:250 in NHS; Sigma-Aldrich) for a minimum of 30mins at room temperature. Following this, slides were incubated in the tertiary streptavidin peroxidase conjugate (SPC) (1:1000 in NHS; Pierce) for at least 1hr at room temperature, and the subsequent immunocomplex visualized using diaminobenzidine tetrahydrchloride (DAB) as a chromogen in a peroxidase reaction (Sigma-Aldrich, Sydney, Australia). Slides were digitally scanned using a Hamamatsu nanozoomer.

2.8.3 Albumin

At 5hrs, 3days and 7days after TBI, brains of animal subsets were removed, processed and paraffin embedded for histological assessment as described above. Slides were deparaffinised in xylene (3 baths for 5mins each), dehydrated with 100% ethanol (2 baths for 5mins each) and rinsed in distilled water before being incubated in 3% hydrogen peroxide in methanol for 15mins to block endogenous peroxidase activity. They were subsequently rinsed in PBS for 2mins before being incubated with primary antibody (rabbit anti-albumin diluted 1:2000 in PBS) overnight at room temperature and thereafter rinsed in PBS (3 baths, 5mins each). Sections were then incubated with HRP-streptavidin reagent diluted 1:400 in PBS for 30mins at room temperature and rinsed in PBS (3 baths, 5mins each) before being incubated with DAB for 7mins and rinsed in PBS (2 baths, 5mins each). The sections were counterstained with hematoxylin, rinsed in distilled water, dehydrated through 95% ethanol for 5mins, 100% ethanol, cleared in histolene and a coverslip placed on the slide with mounting medium.

2.9 Statistical Analysis

Data are shown as mean +/- standard error of measurement (SEM). Repeated measures analysis of variance (ANOVA) followed by Bonferroni *t*-tests were used to analyze functional outcome. Brain water content and BBB permeability was analysed using one-way or two-way ANOVA, as appropriate, followed by individual Student Neuman Keuls tests. A p value of 0.05 was considered statistically significant. The Prism computer program (Graphpad™ Software, SanDiego, CA) was used for all analyses.

CHAPTER 3

RESULTS

3. RESULTS

3.1 Blood brain barrier permeability after TBI

BBB permeability after TBI is considered a precursor to vasogenic oedema formation, in itself a major contributor to secondary injury and eventual mortality and morbidity. This series of experiments characterises the effects of magnesium chloride, either alone or in combination with PEG, on BBB permeability after diffuse TBI in rats. The concentration of magnesium has also been varied in aspects of the current experiments to assess whether PEG may potentially facilitate magnesium's actions.

3.1.1 Experimental outline

Adult male Sprague rats (350-380g) were anaesthetized with isoflurane and injury induced by dropping a 450g brass weight from a height of 2m onto the stainless steel disc affixed centrally to the animals skull as described in detail in section 2.2.2 (Marmarou and Foda, 1994). At 30mins after injury, animals were administered with either an intravenous dose of $MgCl_2$ (254 μmoles/kg), Mg PEG (254 μmoles/kg $MgCl_2$ in 1g/kg PEG), PEG (1g/kg), an equal volume of saline (vehicle), 0.1 x $MgCl_2$ (25.4 μmoles/kg) or 0.1 x Mg PEG (25.4 μmoles/kg $MgCl_2$ in 1g/kg PEG).

The permeability of the BBB was assessed using Evan's blue dye, which binds to albumin, a vascular protein that is not normally able to cross the intact BBB. Briefly, 0.4ml of 4% EB dye was injected into the tail vein of animals at 4.5 hrs after injury (n=5-6/group). After 30mins of circulation time, the animals were saline perfused, decapitated, their brains removed and

homogenized with phosphate buffered solution (PBS) and trichloroacetic acid (TCA). The EB extravasation was measured using a spectrophotometer.

BBB permeability to plasma albumin was also determined using albumin immunohistochemistry. Animals (5/group) were killed and their brains fixed and paraffin blocked at 5hrs, 3days and 7days. Slides were then prepared and stained for albumin immunoreactivity as described in detail in section 2.8.3.

All data are expressed as mean +/- SEM. Statistical differences were determined using a repeated measure analysis of variance followed by individual student-Newman-Keuls post-hoc tests using Graphpad Prism® software. A P value of 0.05 was considered significant.

3.1.2 BBB permeability after TBI

3.1.2.1 Determination of BBB permeability to EB

In sham animals, the amount of Evan's blue in the brain parenchyma was negligible (3.4 ± 0.3 μg/g), consistent with the presence of an intact BBB (Nimmo et al., 2004). However, in animals that were subject to TBI and administered saline (vehicle) at 30 min post-injury, brain EB concentration was 52.2 ± 1.0 μg/g ($p<0.0001$ versus sham), reflecting increased BBB permeability (Fig 3.1). Treatment with Mg PEG or $MgCl_2$ reduced barrier permeability to near sham levels following TBI to 3.9 ± 0.4 and 4.8 ± -0.5, respectively, indicating a relatively intact BBB. On the other hand, treatment with PEG alone did not improve BBB permeability, with brain EB concentration at almost vehicle levels (50.5 ± 1.1 μg/g; $p<0.0001$). When the dose of the magnesium salt was reduced to $1/10^{th}$, brain EB concentration was 52.0 ± 0.6 μg/g, which was no different to the saline treated, vehicle animals. In contrast, when the reduced magnesium

concentration was administered in combination with PEG (0.1 x Mg PEG), brain Evans blue was again reduced to sham levels (3.8 ± 0.1 µg/g; $p < 0.0001$).

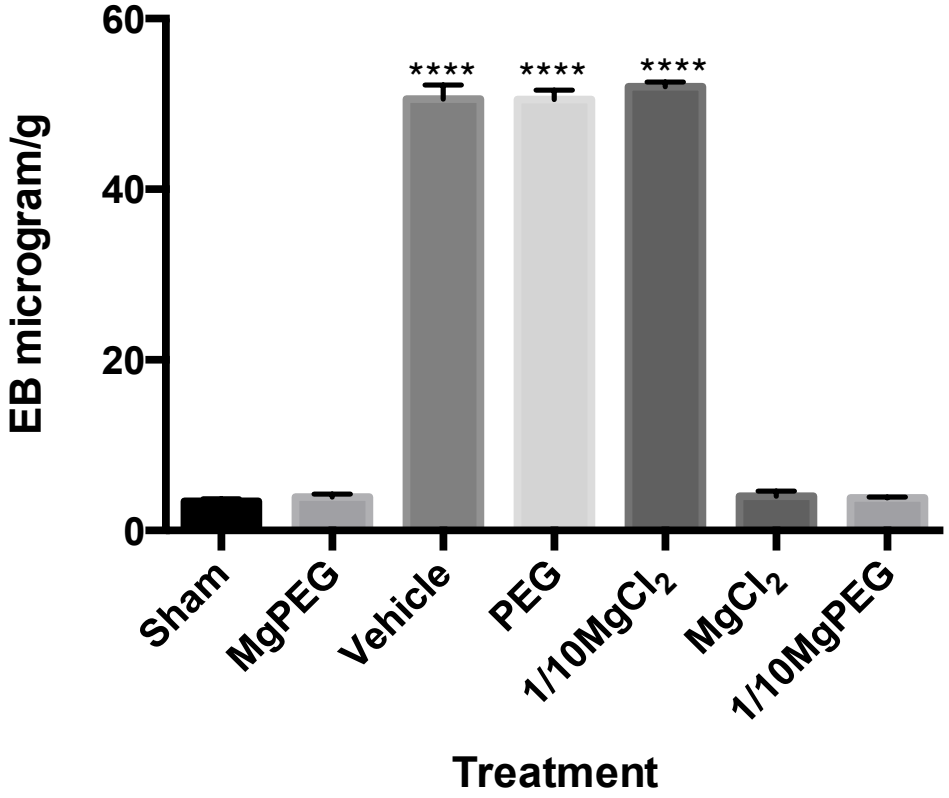


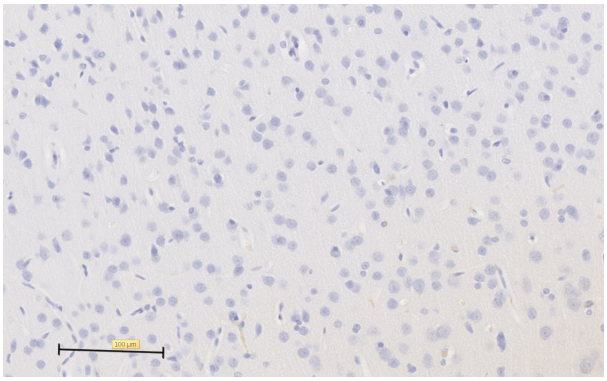
Fig. 3.1. BBB permeability in rats at 5hrs following severe diffuse TBI and subsequent treatment at 30mins with 254µmoles/kg $MgCl_2$, Mg PEG (254µmoles/kg $MgCl_2$ in 1g/kg PEG), PEG alone, an equal volume of vehicle (saline), $1/10^{th}$ Mg PEG and $1/10^{th}$ $MgCl_2$. **** = $p < 0.0001$ versus shams; n = 5 or 6/group.

3.1.2.2 BBB permeability by albumin immunoreactivity

Albumin immunohistochemistry was also performed to confirm the effects of the various treatments on BBB permeability, with maximal changes in albumin immunoreactivity noted at 5 h postinjury, declining by 3 days postinjury and being virtually absent by 7 days. Data is

therefore limited to this time point. Sham (uninjured) cortex (Fig 3.2 A) showed minimal albumin immunoreactivity confirming that the BBB was intact in this group of animals. There is increased albumin immunoreactivity perivascularly in vehicle treated animals (Fig 3.3B), indicating BBB opening at the 5hr time point after TBI, which is consistent with previous studies (Klatzo et al., 1980, Reulen et al., 1980) and with the EB results of the previous section. PEG administration did not significantly affect albumin immunoreactivity after TBI (Fig 3.2C), with intensity of staining qualitatively similar to the vehicle treated group. In contrast, administration of high dose $MgCl_2$ results in a marked reduction in albumin immunoreactivity when compared to vehicle (Fig 3.3D). Similarly, administration of high dose $MgCl_2$ in combination with PEG resulted in a marked reduction in immunoreactivity (Fig 3.3E), supporting the conclusion that magnesium is the active substance responsible for attenuation of BBB permeability after TBI. As expected, the results characterising BBB permeability after TBI by albumin immunoreactivity were consistent with the results obtained using EB dye.

Fig 3.2. Cortical albumin immunoreactivity at 5 hrs following TBI in rats. (A) Sham (uninjured) animals show minimal albumin immunoreactivity. (B) Vehicle treated animals show intense albumin immunoreactivity. (C) PEG treated animals show intense albumin staining similar to vehicle treated animals. (D) Markedly reduced albumin immunoreactivity is observed following treatment with 254 μmoles/kg $MgCl_2$ as compared to vehicle treated animals. (E) Combined $MgCl_2$ in combination with PEG also demonstrated marked reduction in albumin immunoreactivity compared to vehicle and PEG treated animals. Bar is 100μm.



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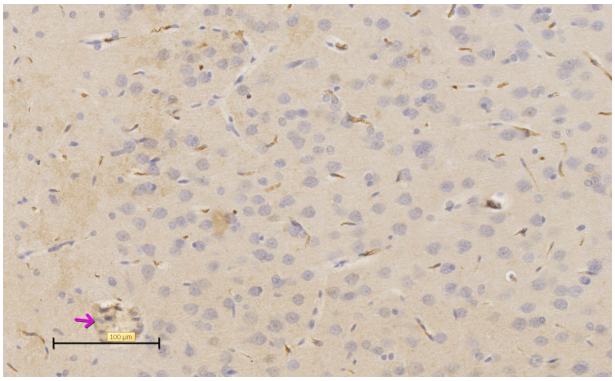

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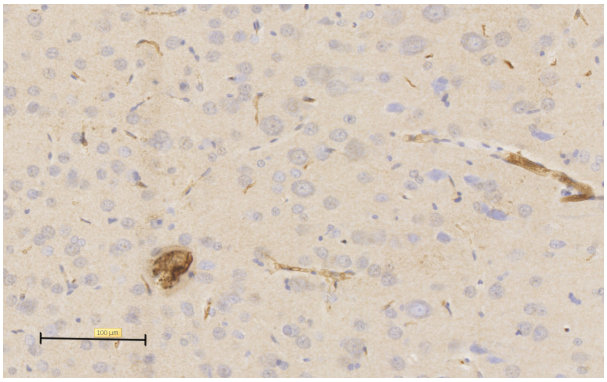

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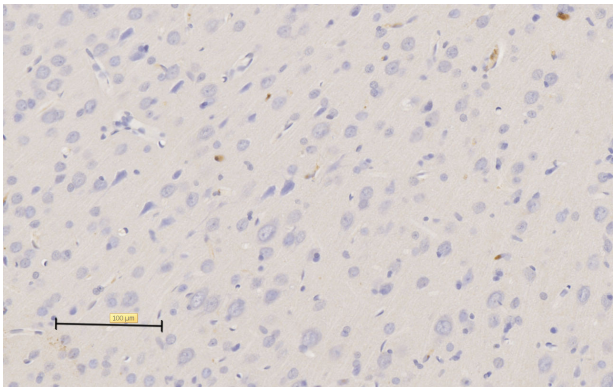

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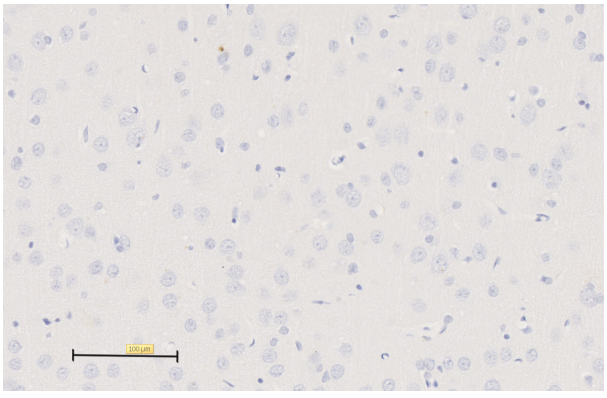


E

3.2 Magnesium and oedema, substance P release and cell injury

It has been well documented that magnesium is neuroprotective following experimental TBI (Heath and vink, 1997, 1999a, Cook et al., 2011), in part by reducing oedema formation. While the exact mechanisms associated with this reduction in oedema are unknown, Kramer et al (1997) showed that magnesium decline after ischemic myocardial injury increases substance P (SP) release. Whether a similar mechanism exists after TBI is unknown, although magnesium is known to similarly decline after TBI and recent studies with NK1 antagonists, which block SP receptor binding, have been shown to increase brain free magnesium after TBI (Vink et al., 2004). SP has also been shown to play a critical role in oedema formation, including following TBI (Vink et al., 2004, Cao et al., 2007, Donkin et al., 2011). The current study therefore characterises the effects of magnesium, either alone or in combination with PEG, on oedema formation, SP release following TBI as well as cell injury. The concentration of magnesium has also been varied in aspects of the current experiments to assess whether PEG may potentially facilitate magnesium's actions.

3.2.1 Experimental outline

The impact acceleration model was of diffuse TBI used to induce a brain injury as described in detail in Chapter 2. Adult male Sprague rats (350-380g) were anaesthetized with isoflurane and injury induced by dropping a 450g brass weight from a height of 2m onto the stainless steel disc affixed centrally to the animals skull as described in detail in section 2.2.2 (Marmarou and Foda, 1994). At 30mins after injury, animals were administered with either an intravenous dose of $MgCl_2$ (254 μmoles/kg), Mg PEG (254 μmoles/kg $MgCl_2$ in 1g/kg PEG), PEG (1g/kg), an equal

volume of saline (vehicle), 0.1 x $MgCl_2$ (25.4 μmoles/kg) or 0.1 x Mg PEG (25.4 μmoles/kg $MgCl_2$ in 1g/kg PEG).

At 5hr after TBI, animals were re-anaesthetized, their brains removed and oedema measured (n=5-6/group) by weight wet/dry weight analysis using a moisture analyzer as described in detail in section 2.5.1. For SP immunohistochemistry, animals (n=5/group) were killed and their brains fixed and paraffin blocked at 5hrs, 3days and 7days. Slides were then prepared and stained for SP immunoreactivity as described in detail in section 2.8.2. To assess cell damage, slides were stained with H&E as described in detail in section 2.8.1.

3.2.2 Oedema, substance P and neuronal cell injury after TBI

3.2.2.1 Oedema

Previous studies have established that maximal oedema in this model of trauma appears at 5 hrs after TBI (O'Connor et al., 2006), therefore oedema measurements in the present study focused on this time point. Sham or uninjured animals demonstrated a brain water content at 5 hrs posttrauma of 78.4 ± 0.2 %, which is consistent with values reported by others in rat experiments (Bareyre, 1997, Nimmo et al., 2004). After TBI, brain water content in saline treated (vehicle) animals was 79.13 ± 0.19 % ($p<0.001$), supporting the development of mild oedema, which is typical in this type of injury (Fig. 3.3). Treatment with PEG alone did not significantly reduce brain water content compared with saline treated vehicle animals, with a value of 79.08 ± 0.38 % ($p<0.001$) recorded at 5 hrs. The brain water content following administration of optimal doses of Mg PEG and $MgCl_2$ was 78.82 ± 0.14 and 78.83 ± 0.22, respectively, which is significantly less than vehicle and PEG treated controls ($p<0.001$) and similar to sham levels. Reducing the dose of

$MgCl_2$ to $1/10^{th}$ of the optimum dose negated the beneficial effects of magnesium administration, with brain water values of 79.16 ± 0.1 % recorded, which is similar to vehicle controls. In contrast, administration of $1/10^{th}$ $MgCl_2$ in PEG significantly reduced brain water to 78.61 ± 0.14, which is significantly less than PEG alone ($p<0.05$).

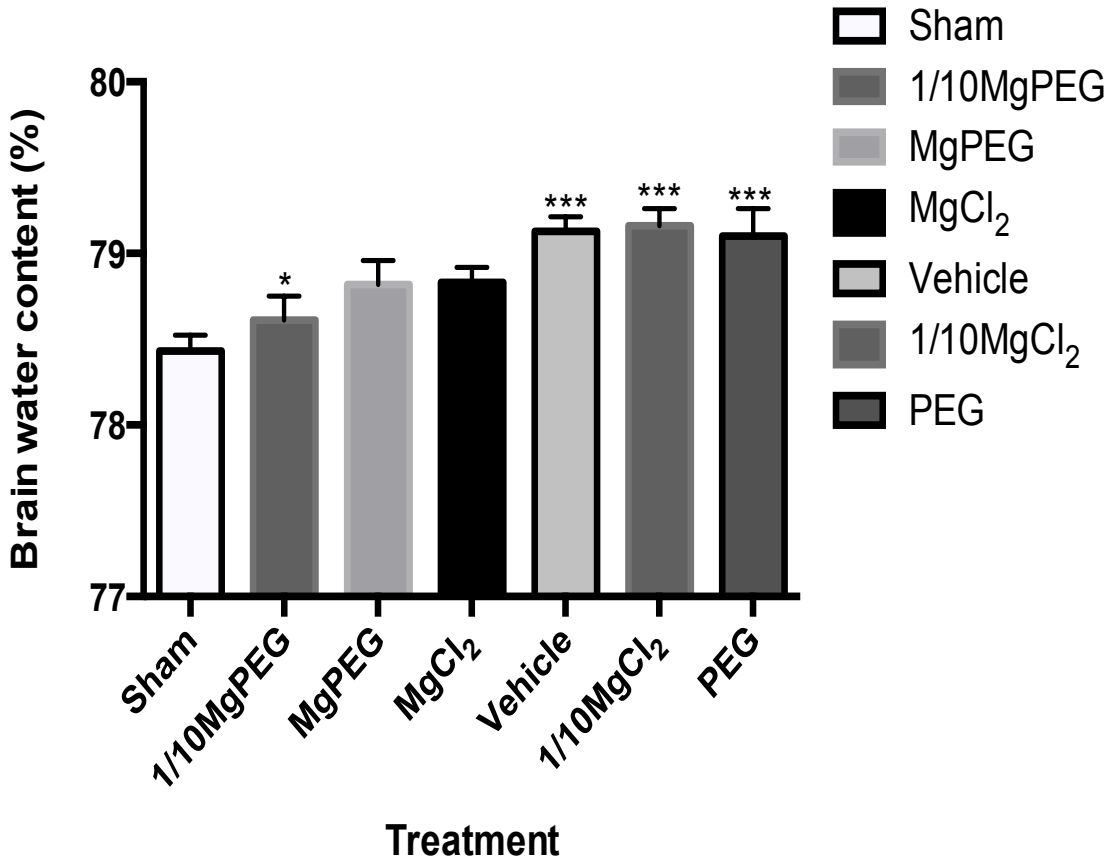
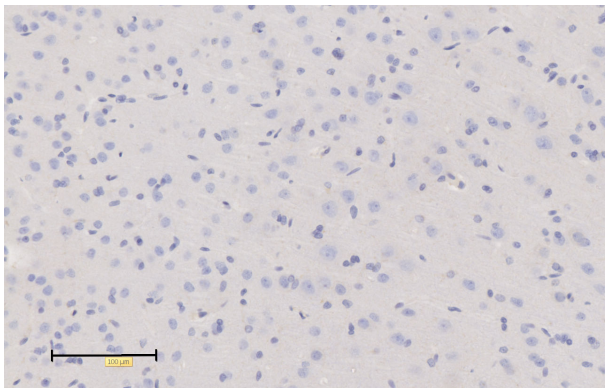


Fig. 3.3. Oedema at 5hrs after severe diffuse TBI in rats. Animals were treated at 30 min after TBI with 254µmoles/kg $MgCl_2$, Mg PEG (254µmoles/kg $MgCl_2$ in 1g/kg PEG), PEG alone, an equal volume of vehicle (saline), 0.1x Mg Cl_2 in PEG or 0.1x $MgCl_2$. ***=$p<0.001$ versus shams; * = $p < 0.05$ versus PEG alone, vehicle and 0.1x $MgCl_2$. n=5 or 6/group.

3.2.2.2 Substance P immunoreactivity

Substance P immunoreactivity after diffuse TBI was maximal at 5 hrs, decreased by 3 days and was largely absent by 7 days after TBI (results not shown). This is consistent with previous observations from our laboratory (Donkin et al., 2009) and accordingly, the data in the present study focused on therapy-induced changes at 5 hrs after TBI. Fig 3.4 shows the cortical SP immunoreactivity at 5hrs after diffuse TBI in rats. In sham animals, there was minimal SP immunoreactivity present. After TBI and with saline (vehicle) treatment, there was a marked upregulation of SP immunoreactivity consistent with SP release after trauma. This increase in SP intensity at 5 hrs was unaffected by PEG treatment which increased to levels similar to the saline treated animals. Treatment with the optimal dose of $MgCl_2$ reduced the SP immunoreactivity relative to the vehicle treated animals such that they appeared as shams. Similarly, the optimal dose of $MgCl_2$ administered with PEG (Mg PEG) significantly attenuated SP immunoreactivity after TBI relative to animals treated with PEG alone. Notably, the effects of the various treatment combinations on SP immunoreactivity was similar to their effects on oedema.

Fig 3.4. SP immunoreactivity at 5hrs after diffuse TBI in rats. (A) Minimal SP immunoreactivity is present in sham (uninjured) animals. (B) Intense SP immunoreactivity 5hrs after injury with vehicle treatment. (C) Intense SP immunoreactivity in the PEG treated animals after TBI. (D) Reduced SP immunoreactivity relative to vehicle treated animals is apparent following treatment with $MgCl_2$. (E) Reduced SP immunoreactivity relative to PEG treated animals is apparent following treatment with Mg PEG.



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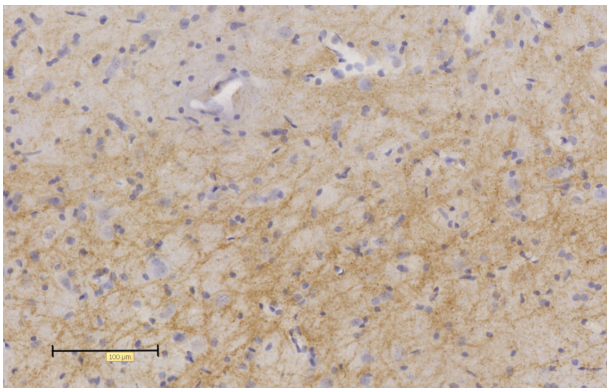

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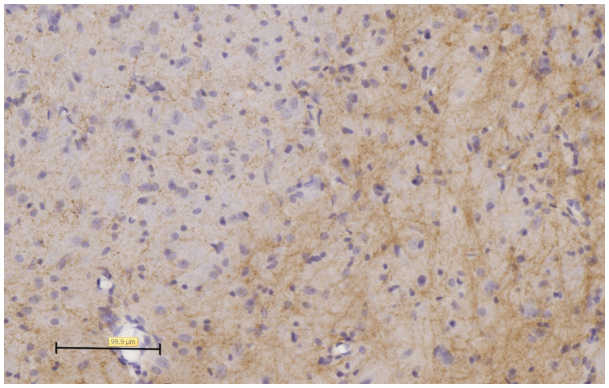

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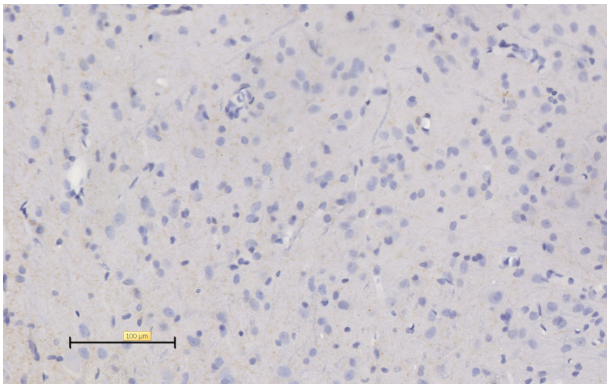

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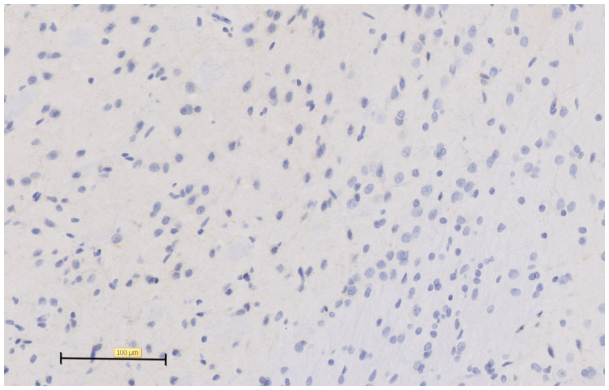

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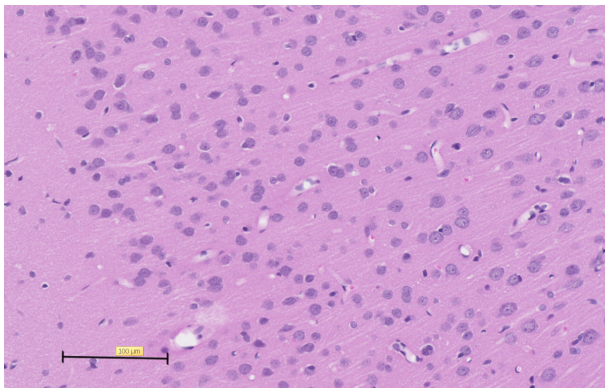
3.2.2.3 Haematoxylin and eosin staining

Haematoxylin and eosin was used to assess neuronal injury at 3days following TBI and treatment with the various drug interventions. The study focused on the cortex directly beneath the injury site, as well as the hippocampus. As expected the sham (uninjured) cortex demonstrated normal cell bodies and architecture (Fig. 3.5). TBI with subsequent saline (vehicle) treatment resulted in the appearance of abnormal neuronal cell bodies and particularly dark cell change reflecting cell stress. Treatment with PEG demonstrated similar abnormal cell bodies and dark cell change as noted in the saline (vehicle) treated animals. In contrast, treatment with optimal dose $MgCl_2$ resulted in a marked reduction in abnormal cells relative to vehicle treated animals and the increased presence of normal appearing neurons. Similarly, treatment with optimal dose $MgCl_2$ in PEG resulted in more healthy looking neurons being present with few abnormal cells compared to PEG alone.

Similar results were noted in the CA2 region of the hippocampus at 3 days following diffuse TBI in rats (Fig. 3.6). As expected, sham animals show normal neuronal cells and hippocampal architecture. Induction of TBI followed by treatment with saline vehicle resulted in the appearance of extensive abnormal dark cell bodies and loss of architecture. Similarly, PEG treatment did not improve the dark cell change and disruption of architecture noted in the vehicles. In contrast, treatment with the optimal dose of $MgCl_2$ resulted in the predominance of essentially normal cells and architecture, with minimal dark cell change being present. Similarly, injured animals administered Mg PEG demonstrated largely normal cells and CA2 architecture closely resembling sham animals. These treatment effects in the hippocampal CA2 persisted for 7 days following TBI (Fig. 3.7), even if the magnesium concentration in the PEG combination was reduced to $1/10^{th}$.

Fig 3.5. Cortical haematoxylin and eosin staining at 3days following diffuse TBI in rats. (A) Sham cortex showing largely normal cell bodies and architecture. (B) Saline treated (vehicle) animals demonstrating abnormal neuronal cell bodies with dark cell change. (C) PEG treated rats with marked abnormal cell bodies and dark cell change. (D) Animals treated with optimal dose $MgCl_2$ (254 μmoles/kg) show predominantly healthy neuronal cells, and minimal dark cell change. (E) Mg PEG treated rats demonstrate predominantly healthy neuronal cells at 3 days after TBI.



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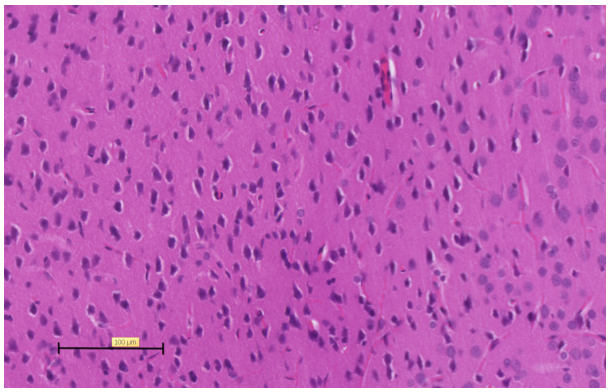

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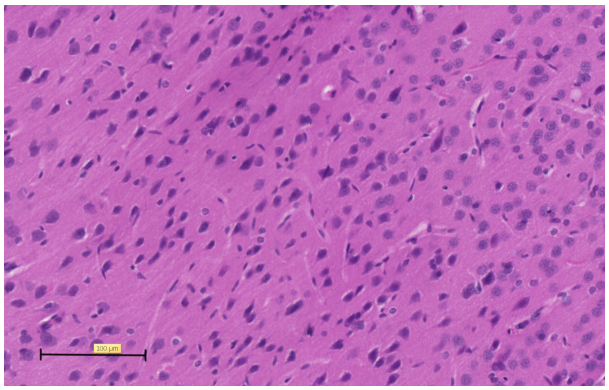

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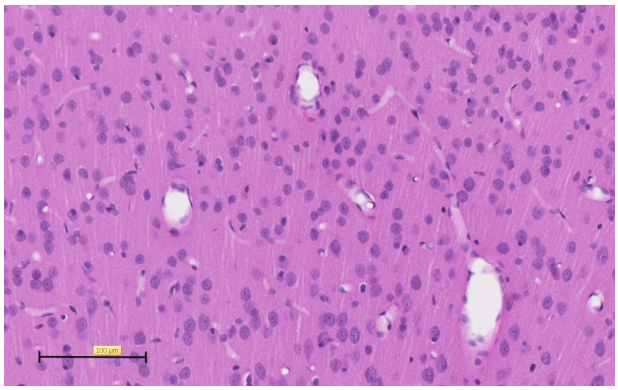

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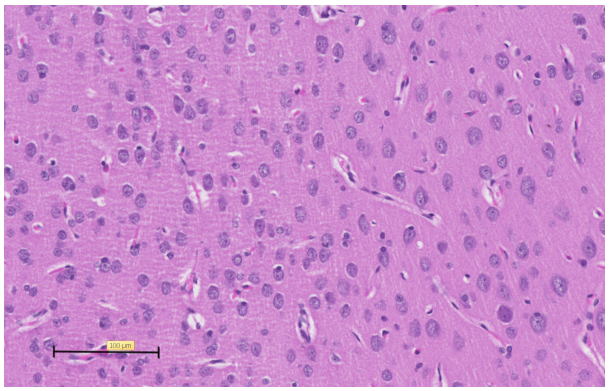

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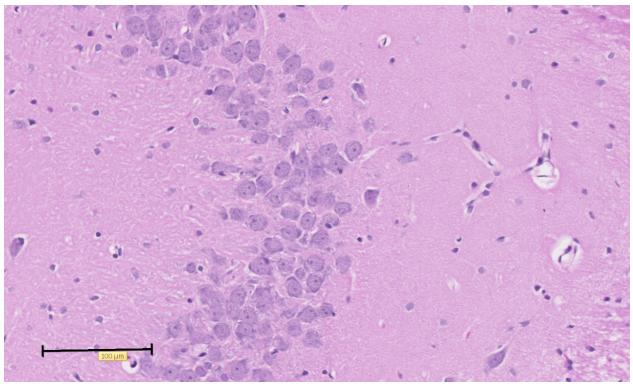
Fig 3.6. Haematoxylin and eosin staining in area CA2 of the hippocampus at 3 days following diffuse TBI in rats. (A) Sham animals show normal neuronal cells with normal architecture. (B) Vehicle treated animals demonstrate abnormal dark cell bodies and loss of architecture. (C) PEG treated animals demonstrate abnormal dark staining cells and disrupted architecture like the vehicle treated animals. (D) Optimal dose of $MgCl_2$ (254 μmoles/kg) resulted in essentially normal cells and architecture, with minimal dark cell change present. (E) Mg PEG treated animals with normal cells and architecture resembling sham animals.



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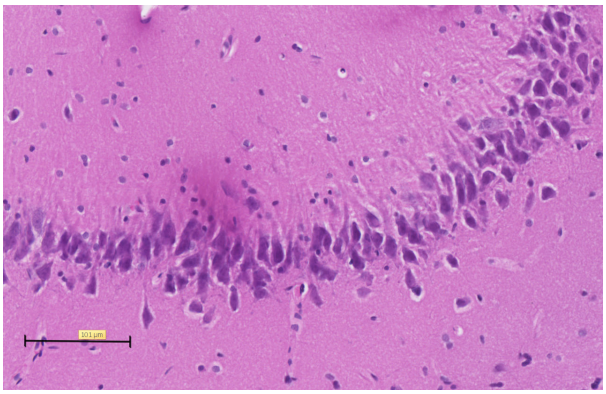

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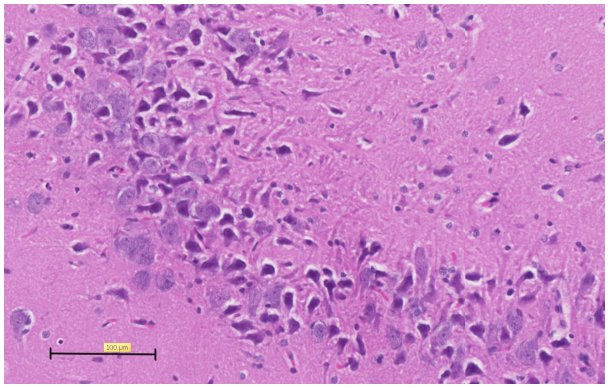

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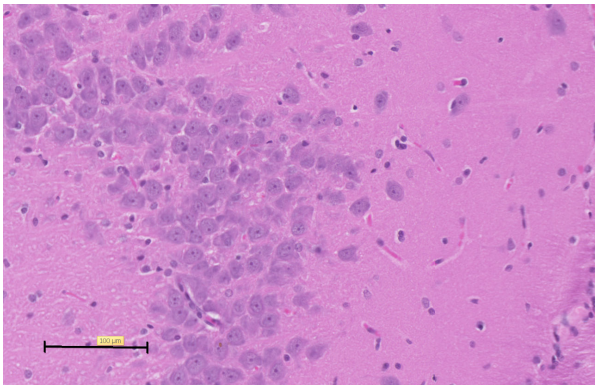

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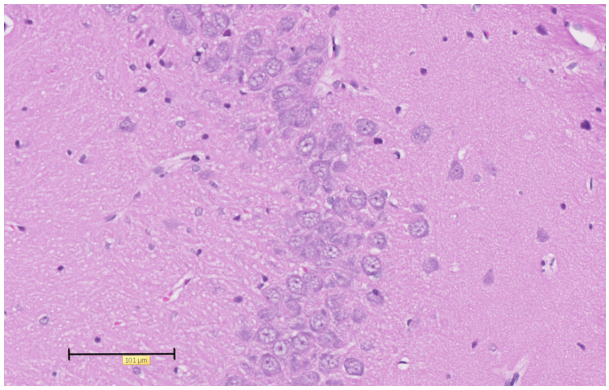

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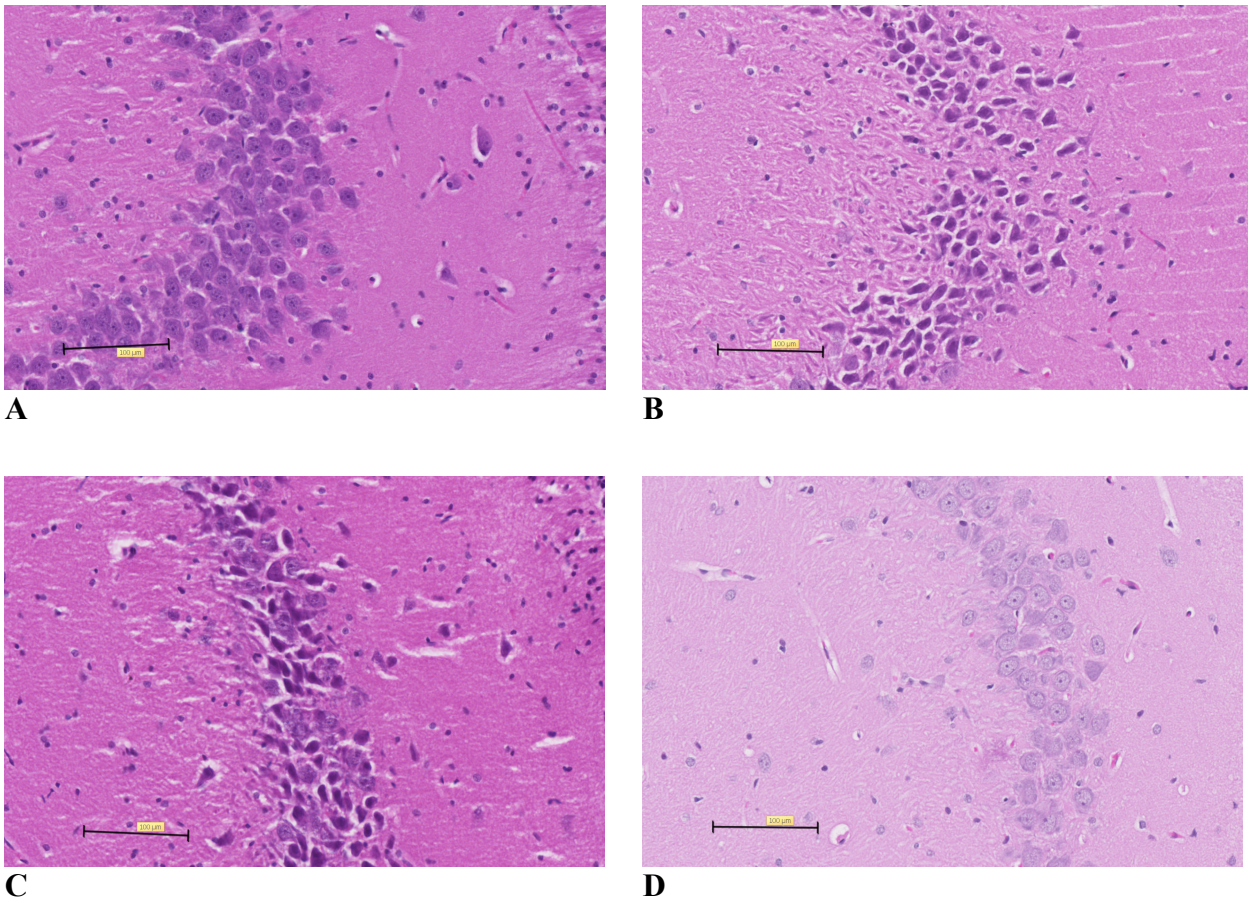

Fig 3.7. Haematoxylin and eosin staining in area CA2 of the hippocampus at 7 days following diffuse TBI in rats. (A) Sham animals demonstrating normal neuronal cell bodies and architecture. (B) Vehicle treated animals showing extensive dark cell change and abnormal hippocampal architecture. (C) Animals treated with 0.1x $MgCl_2$ (25 µmoles/kg) demonstrate extensive dark cell change and abnormal architecture similar to the vehicle (saline) treated animals. (D) Animals treated with 0.1x Mg (25 µmoles/kg) administered in PEG demonstrate essentially normal neuronal cell bodies and architecture, with minimal dark cell change.

3.4 Functional Outcome

Although the previous sections have demonstrated that combination magnesium and PEG may be neuroprotective following TBI, tests of functional outcome are required to substantiate that the positive effects on the others brain parameters translate into improved neurological outcome. In previous studies of rodent experimental TBI, the rotarod test has been found to be the most sensitive and reliable in the assessment of motor deficits (Hamm et al, 1994). For cognitive function, the object recognition test as described by Ennaceur and Delacour (1987) has been used successfully to characterise posttraumatic cognitive deficits and the effects of pharmacological intervention (Ennaceur and Delacour, 1987). Previous studies have also shown that magnesium administration following TBI improves functional outcome (Vink, 1991, Vink et al., 2003, Winn et al., 2007, Vink et al., 2009). Whether magnesium in PEG improves motor and cognitive function, even at low magnesium concentration, has yet to be shown. In the current series of experiments, the rotarod and open field tests have been used to assess the effects of magnesium, either in the presence or absence of PEG, on functional outcome following diffuse TBI in rats.

3.4.1 Experimental design

Animals (n=5-8/group) were subject to impact acceleration injury and administered either an intravenous dose of $MgCl_2$ (254 μmoles/kg), Mg PEG (254 μmoles/kg $MgCl_2$ in 1g/kg PEG), PEG (1g/kg), an equal volume of saline (vehicle), 0.1 x $MgCl_2$ (25.4 μmoles/kg) or 0.1 x Mg PEG (25.4 μmoles/kg $MgCl_2$ in 1g/kg PEG) at 30 posttrauma as described in detail in Chapter 2. Motor and cognitive outcome were then assessed for 7 days following TBI. Motor deficits were assessed using the rotarod test, which requires animals to walk on a motorized rotating assembly of 18 rods, each 1mm in diameter, for up to 2 min with the rotational speed of the assembly

increasing from 0 to 30 revolutions per minute (rpm) in intervals of 3 rpm every 10secs. The duration in seconds at the point at which the animal completed the 2 min task, fell from the rods, or gripped the rods and spun for 2 consecutive rather than actively walking was recorded as the score. All animals were exposed to rotarod training over a period of 5 days prior to injury to establish a performance baseline. Cognitive function was assessed using the object recognition test where objects are placed within a 80cm long, 80cm wide and 80cm high box. The objects to be discriminated are made of a biologically neutral material such as glass, plastic or metal and weighted so that the animals cannot displace them. The test consists of a sample phase (3 min) and a choice phase (3 min) with a 15 min interval between the two phases. In the sample phase two identical objects are placed in the box. The animal is then also placed in the box and the total time (up to 3 min) spent exploring each object is recorded. After being returned to its cage for 15 min, the rat is reintroduced to the box and a choice phase started for a further 3 min. In the choice phase, one of the objects is replaced with a new novel object and the time spent in exploring each object is again recorded.

Data are shown as mean ± standard error of measurement (SEM) and were analysed by repeated measures analysis of variance (ANOVA) followed by individual Bonferroni t-tests.

3.4.2 Functional outcome after TBI

3.4.2.1 Rotarod

Sham animals demonstrated consistently high rotarod scores averaging 120 secs over the 7-day assessment period. After diffuse TBI, the saline treated (vehicle), PEG treated and $1/10^{th}$ $MgCl_2$ (25 μmoles/kg) treated animals all demonstrated significant motor deficits ($p<0.001$) versus sham

animals recording rotarod scores of between 20 and 40secs for the entire 7-day assessment period. In contrast, animals treated with either optimal dose $MgCl_2$ (254 µmoles/kg), optimal dose $MgCl_2$ in PEG (Mg PEG) or low dose $MgCl_2$ (25 µmoles/kg) in PEG showed significantly higher rotarod scores than vehicle treated animals ($p< 0.0001$). Indeed, animals treated with both low and optimal dose magnesium in PEG recorded rotarod scores between days 4 to 7 following TBI that were comparable to the sham values.

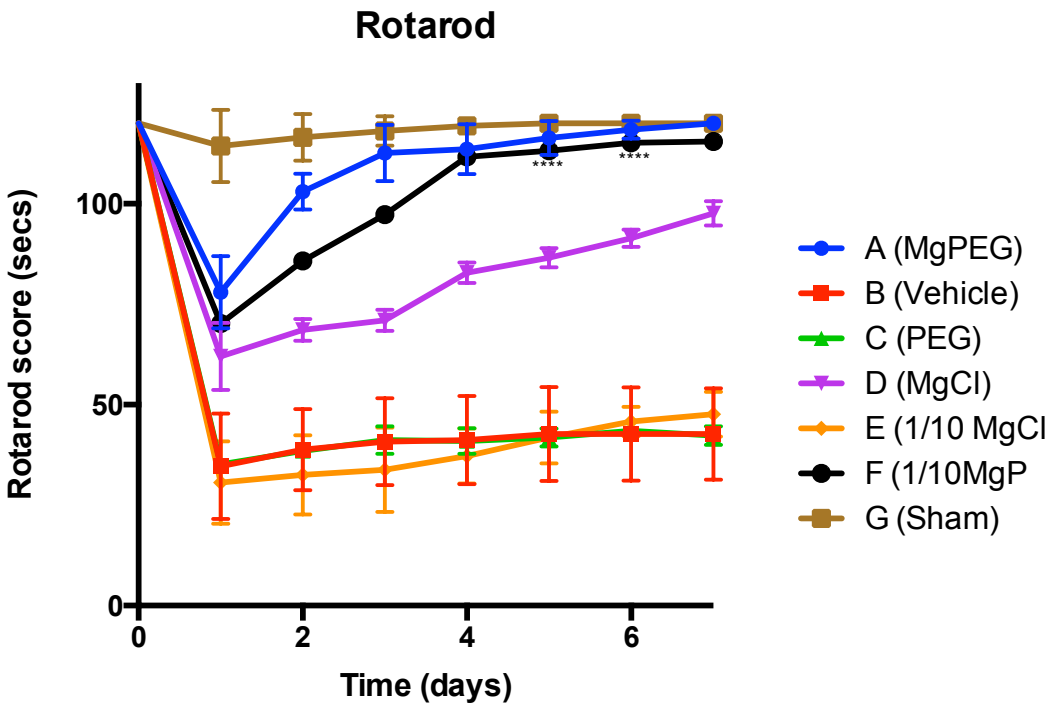

Fig. 3.8. Motor function over 7 days following TBI as assessed using the rotarod. Animals treated with optimal dose $MgCl_2$, Mg PEG (254µmoles/kg $MgCl_2$ in 1g/kg PEG) or 1/10 $MgCl_2$ (25 µmoles/kg) in PEG performed significantly better that vehicle PEG or 1/10 $MgCl_2$ (25 µmoles/kg) treated controls. Animals combining magnesium with PEG recorded the best rotarod outcomes. ****$p<0.0001$ compared to saline treated (vehicle) animals.

3.4.2.2 Object recognition

In the sample phase, all animals were exposed to two identical objects, which they equally explored. In the choice phase, saline treated (vehicle) animals also explored both objects equally, being unable to differentiate between the objects after TBI. This is in contrast with sham injured animals, which spent more time exploring the novel object. Similarly, PEG and $1/10^{th}$ $MgCl_2$ treated animals couldn't differentiate between the familiar and novel object in the choice phase, indicating significant cognitive deficits. In contrast, animals treated with optimal dose $MgCl_2$, optimal dose $MgCl_2$ in PEG and $1/10^{th}$ $MgCl_2$ in PEG were able to differentiate between the familiar and novel objects in the same manner as sham animals, which was significantly better than the performance of vehicle treated controls ($p < 0.0001$).

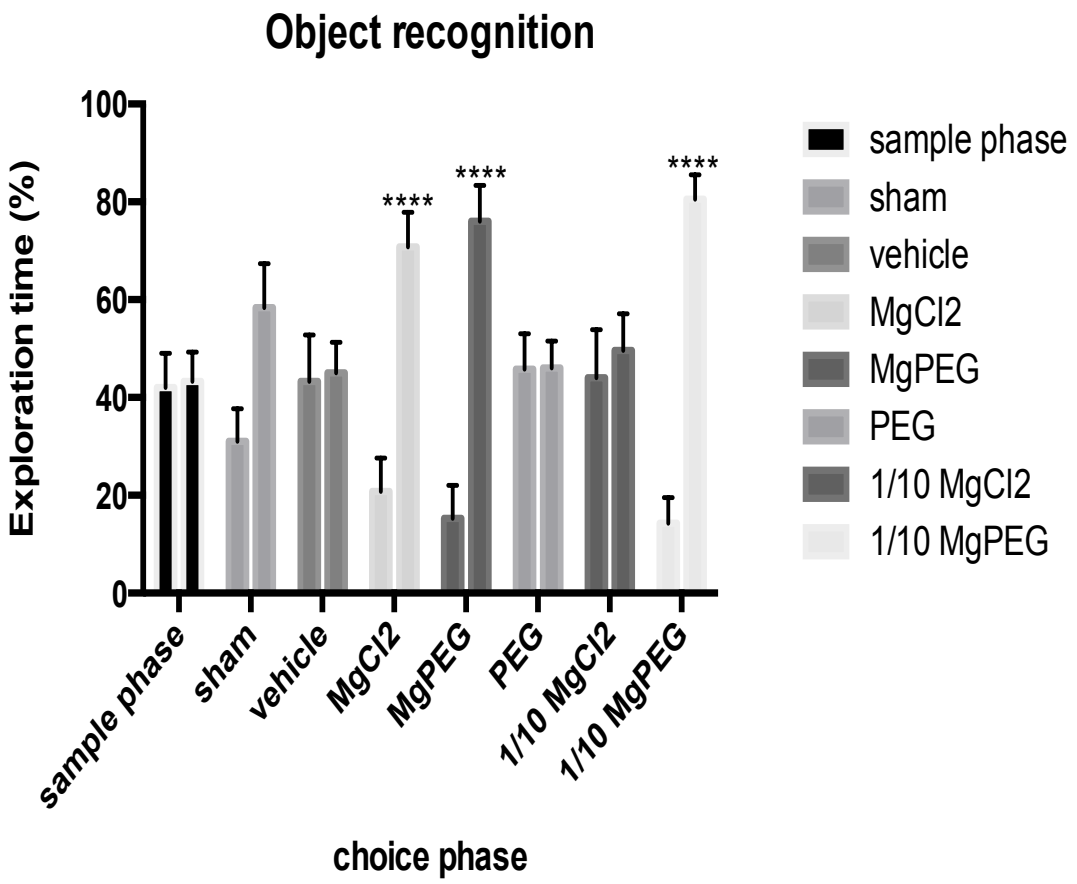

Fig. 3.9. Object recognition test outcomes after diffuse TBI in rats. Animals (n=5/group) were treated with 254μmoles/kg $MgCl_2$, Mg PEG (254μmoles/kg $MgCl_2$ in 1g/kg PEG), PEG alone, an equal volume of vehicle (saline), $1/10^{th}$ Mg in PEG or $1/10^{th}$ $MgCl_2$ at 30mins post injury. ****$p<0.0001$ compared to vehicle treated animals.

CHAPTER 4

DISCUSSION

4. DISCUSSION

The present thesis has demonstrated that administration of either optimal dose magnesium (254 μmoles/kg) in PEG (Mg PEG) or optimal dose $MgCl_2$ alone significantly improved all outcome parameters compared to vehicle treated or PEG treated controls with no significant difference between the magnesium treatment groups. Indeed, magnesium treatment restored some of the parameters to sham levels. However, intravenous administration of one-tenth the magnesium concentration (25 μmoles/kg; low dose) had no beneficial effect on any of the outcome parameters whereas one-tenth the magnesium concentration in PEG (25 μmoles/kg $MgCl_2$ in 1g/kg PEG) had the same beneficial effects as optimal dose $MgCl_2$. We conclude that PEG facilitates movement of the magnesium salt across the blood brain barrier following TBI and that the combination of low dose magnesium in PEG significantly attenuates oedema, blood brain barrier permeability and improves motor and cognitive outcome following TBI.

4.1 Blood brain barrier permeability and albumin immunohistochemistry.

Previous studies with magnesium salts have demonstrated magnesium's beneficial effects on the BBB (Heath and Vink, 1999e, Esen et al., 2003b). A number of studies reported that magnesium reduced BBB permeability by decreasing reactive oxygen species and increasing antioxidant levels by upregulating superoxide dismutase (Garcia et al., 1998, Cernak et al., 2000). Alves (2014) reported that the BBB consists of the endothelium supported by basement membrane, astrocytes, pericytes and neurons, which makes it selectively permeable especially to proteins (Alves, 2014). Albumin, one such protein is normally contained within the blood vessels. Adukauskiene et al (2007) reported that permeability to albumin increases following TBI (Adukauskiene et al., 2007). This albumin has been found to be responsible for the ensuing

microglial proliferation and increase in Ca^{2+} ions (Hooper et al., 2005). Albumin binds to Evan's blue dye, which was used in this study to assess BBB permeability.

In the current study, we used the 5 hr time point to determine effects of the various therapies on BBB permeability given that this time point has been shown to have maximal BBB permeability in previous studies of TBI (Vink et al., 2003b, Nimmo et al., 2004). The increase in BBB permeability also results in fluid compartment shift more precisely vasogenic oedema, which is followed by cytotoxic oedema (Vink et al., 2003b). Several studies reported that magnesium attenuates BBB permeability and oedema formation (Vink and McIntosh, 1990, Heath and Vink, 1999b, van den Heuvel and Vink, 2004). Our results demonstrate that the saline treated animals (vehicle) had an increase in uptake of the Evan's blue dye indicating increased BBB permeability compared to the sham animals ($p<0.0001$). The magnesium treated animals were not significantly different from the shams. Additionally, the Mg PEG treated animals and the 0.1x Mg PEG group were similar to the shams. Kwon et al (2009) reported that Mg PEG reduced lesion size and improved functional outcome in spinal cord injury. This is the first study to demonstrate the effects of Mg PEG on BBB following TBI.

Polyethylene glycol (PEG) is a non toxic molecule that can enter rat brain parenchyma (Koob, 2006). Therefore, we suggest that PEG enhanced central penetration of the magnesium salt because the 0.1x Mg PEG dose had the same beneficial effects as the optimal dose compared to the 0.1x $MgCl_2$ whose effect was consistent with that of the saline treated animals (vehicle). Additionally, PEG probably increases the bioavalability of magnesium for a period sufficient to improve neuronal survival. The current study further shows that Mg PEG reduced BBB permeability and that 0.1x of the optimal Mg PEG dose had similar beneficial effects. Previous studies reported that magnesium reduced BBB permeability by decreasing reactive oxygen

species and increasing antioxidant utilization such as superoxide dismutase (Garcia et al., 1998, Cernak et al., 2000).

Performing albumin immunohistochemistry confirmed our assessments of BBB permeability using EB. The saline treated (vehicle) animals showed intense albumin staining at 5hrs. Duvdedani et al (1995) demonstrated intense albumin staining and BBB permeability at 2hrs following frontal cortical contusion injury. Animals treated with Mg PEG closely resembled the shams suggesting that Mg PEG played a role in attenuation of BBB permeability. Previous studies with magnesium salts in TBI showed that magnesium reduced BBB permeability through a number of pathways including restoration of altered aquaporin 4 immunoreactivity, acting as an NMDA receptor antagonist, and by enhancing glycolysis and oxidative phosphorylation (Okiyama, 1995, Feldman et al., 1996, Esen et al., 2003b, Ghabriel et al., 2006). Additionally the decrease in BBB permeability could also be attributed to the increased availability of magnesium considering its concentration has been found to decline following TBI (Vink et al., 1987, Heath and Vink, 1996, 1999b). The current study suggests that PEG may have increased magnesium's availability for a period long enough to sustain neuronal survival, which shows that PEG might be a potential strategy for drug delivery. In terms of enhancing drug delivery, Alonso et al (2011) investigated the use of ultrasound to open the blood brain barrier as a drug delivery strategy. The side effects of such a procedure have not been examined. They showed that albumin extravasation was observed up to 24hrs following ultrasound guided BBB penetration, which was taken up by glia (Alonso et al., 2011). Whereas ultrasound guided BBB opening is expensive and requires trained personnel, PEG is cheap and easy to administer.

4.2 Oedema and substance P release

Oedema is a major secondary injury mechanisms following TBI and is characterized by neuronal inflammation involving expression of adhesion molecules, migration of cells to the injury site and secretion of inflammatory molecules (Morganti-Kossmann et al., 2002). This process occurs as a result of increased vascular permeability, ionic imbalances, oxidative stress, excitotoxicity and mitochondrial failure (Morganti-Kossmann et al., 2002). A number of studies have demonstrated the role of magnesium in attenuation of oedema following TBI (Demediuk et al., 1988, Bareyre et al., 1999, Ghabriel et al., 2006, Cook et al., 2011). A study carried out by Esen et al (2003) where $MgSO_4$ was administered intraperitoneally following severe diffuse TBI reported a significant reduction in brain water content in the left hemisphere. This reduction was attributed to the role of magnesium as an NMDA antagonist, as a co-factor for all energy processes, and in calcium regulation and transport. In a study by Feng and colleagues, $MgSO_4$ was administered intraperitoneally immediately after trauma; 24hrs later total brain water content was significantly lower in the magnesium treated animals compared to the trauma group (Feng, 2004). In the present study, $MgCl_2$ was the preferred salt (Durlach et al., 2007), administered intravenously at 30mins post trauma and oedema measured at 5hrs which is consistent with previous results that have found it to be maximal at that time point (O'Connor et al., 2006). A study carried out by Feldman and colleagues showed that magnesium administered 1hr after injury attenuated brain oedema in the contused hemisphere compared to the untreated rats (Feldman et al., 1996). Also there is a growing body of evidence suggesting that magnesium concentration declines following TBI (Vink et al., 1987, Vink et al., 1988a, Heath and Vink, 1996, Vink et al., 1996, Heath and Vink, 1998, 1999b, Vink and Cernak, 2000, Mendez et al., 2005), which correlates with motor and cognitive deficits.

To our knowledge, only a few studies have shown the effects of Mg PEG in spinal cord injury (Ditor et al., 2007, Kwon et al., 2009). In the study by Kwon et al (2009), animals received up to 3 infusions of Mg PEG, methylprednisolone and $MgCl_2$ at 2,4 and 6hrs whereas we had one infusion of Mg PEG, $MgCl_2$, PEG at 30mins post injury in our study (Kwon et al., 2009). Additionally 254μmole/kg of $MgCl_2$ was found to be superior to 127μmole/kg, which was the optimal dose for the current study. Previously, Ditor et al (2007) demonstrated the effects of $MgSO_4$ and PEG in spinal cord injury (Ditor et al., 2007). The animals received up to 2 doses at 15mins and 6hrs intravenously with $MgSO_4$ alone and $MgSO_4$ and PEG resulting in significant dorsal myelin sparing as well as reduction in lesion volume. However treatment with PEG alone showed some myelin sparing, which was attributed to its ability seal cell membranes and restore normal ion permeability (Ditor et al., 2007). Duerstock and Borges (2007) applied PEG to the injury site of guinea pig spinal cord immediately and 7hrs after trauma: they reported an increase in volume of intact spinal parenchyma and reduced cystic cavitation using three-dimensional morphometry. Previous studies with PEG showed that it promotes fusion of severed axons in mammals and guinea pig (Krause and Bittner, 1990, Shi and Borgens, 1999, Shi et al., 1999). Additionally Koob et al (2005) reported that PEG selectively labeled cells whose membrane was compromised. In their study PEG was administered 2hrs following injury and ethimide bromide (EB), 6hrs later. Ethimide bromide selectively labels cells whose plasma membrane has been compromised. The ethimide bromide labeling in the PEG treated animals was similar to the sham animals (Koob et al., 2005). Also PEG treated animals showed a reduction in horseradish peroxidase (HRP) uptake (Koob et al., 2005). They attributed their results to PEG's ability to seal off damaged cell membranes thereby potentiating action potential conduction and preventing

mixing of intracellular and extracellular compartments (Koob et al., 2005). Whether the cell membrane repair is complete after PEG treatment remains unknown (Borgens, 2003).

In another study of TBI, PEG was administered 2hrs, 4hrs and 6hrs after injury (Koob et al., 2008). The animals were examined for behavioral recovery using an open field test. The PEG treated animals showed significant improvement in their exploratory behavior compared to the untreated ones (Koob et al., 2008). Additionally, the 2 and 4hr time points for PEG administration were found to have better outcomes compared to the 6hr time point (Koob et al., 2008). Previously Koob et al (2006) demonstrated that PEG administration reduced APP accumulation in the thalamus but not in the medial longitudinal fasciculus following TBI (Koob, 2006). Taken together the studies show that PEG acts selectively on damaged cells and that this selective action is due to its ability to seal damaged membranes. In our study, PEG did not attenuate oedema nor did it reduce the release of substance P. This could be attributed to the fact that the vasogenic pathway is the main contributor to oedema and possibly the reconstitution of the cell membrane did not affect oedema formation, also because its resolution occurs through different mechanisms including reuptake by astrocytes through AQ4 water channels. These pathways are independent of PEG's ability to reconstitute cell membranes. Oedema is associated with substance P release (Donkin et al., 2009), which regulates inflammation, wound healing and the immune response (Fig 4.2). Furthermore, they showed that substance P concentration in the blood stream increased significantly post TBI and declined to near sham levels 5hrs later (Donkin et al., 2009). They postulated that this decline in brain free magnesium concentration was due to uptake by vascular proteases. Vink et al (2004) showed that substance P release is associated with decline in intracellular brain free magnesium concentration and that a substance P antagonist restores magnesium homeostasis. Indeed, they showed that magnesium concentration declined to

0.27±0.2mM in the saline treated (vehicle) animals whereas that of the NAT (substance P antagonist) treated animals was near sham levels (0.47±0.06mM). Furthermore, magnesium deficiency in rat myocardium was found to increase substance P-induced inflammation following ischemia (Kramer et al., 1997). In addition Turner et al (2011) demonstrated that a substance P antagonist attenuated oedema, BBB permeability and improved functional outcome after ischemic injury (Turner et al., 2011).

Our results show that optimal dose $MgCl_2$, Mg PEG and 0.1x Mg PEG attenuated oedema and substance P release. Whereas a number of studies have investigated the role substance P in oedema formation following TBI (Huston and Hasenöhrl, 1995, Cao et al., 2007, Donkin et al., 2007, Donkin et al., 2009, Donkin and Vink, 2010, Turner et al., 2011), only a few have demonstrated the effect of substance P release on brain free magnesium concentration (Vink et al., 2004). As such, this is the first study to show that Mg PEG and 0.1x Mg PEG reduce substance P release and hence oedema formation.

4.3 Histological H&E analysis

Staining with hematoxylin and eosin showed extensive neuronal dark cell change both in the cortex and hippocampus after TBI, which is consistent with previous studies (Turner et al., 2004, Harford-Wright et al., 2010). The injured neurons appear dark purple, and abnormally stretched with pyknotic nuclei. As expected this dark cell change was absent in the sham animals and clearly apparent in the vehicle and PEG treated animals. The optimal dose $MgCl_2$ and Mg PEG treated animals showed a significant reduction in dark cell change. Additionally 0.1x Mg PEG demonstrated the same beneficial effects as the full dose (254μmole/kg).

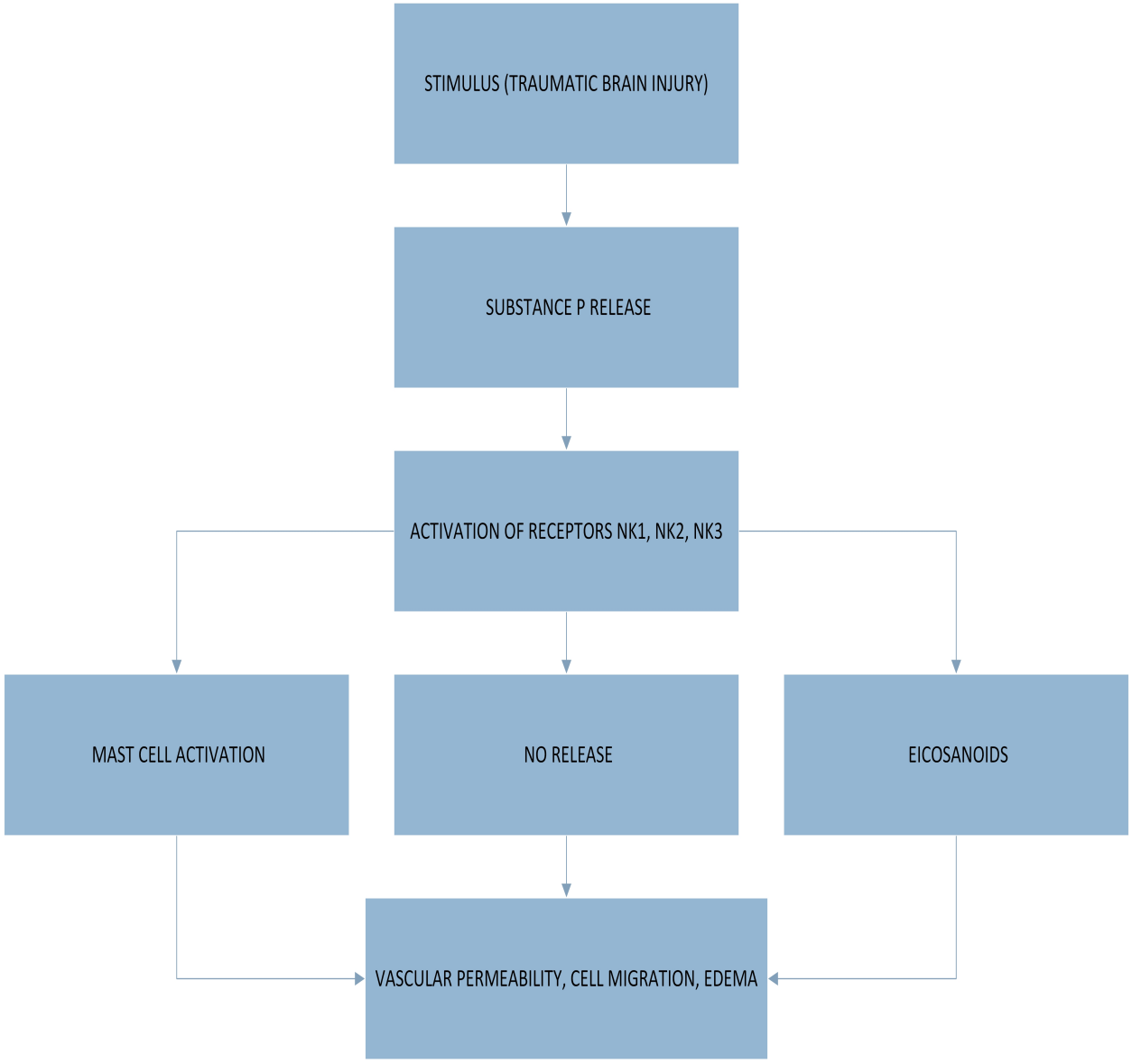

Fig 4.2. Substance P resulting in neurogenic inflammation following traumatic brain injury.

Our results show the beneficial effects of Mg PEG and its 0.1x Mg PEG dose as evidenced by the reduction in neuronal dark cell change in the treated animals compared to the saline treated (vehicle) animals. The neuronal dark cell change occurs as a result of an inflammatory response to trauma, which involves activation of astroglia and microglia (Block and Hong, 2005). The activated astroglia become hypertrophic leading to increased production of glial fibrillary protein forming scars that hinder regeneration (Block and Hong, 2005), whereas activated microglia fix complement molecules (Block and Hong, 2005).

Browne et al (2004) showed that magnesium salts offered long-term neuroprotection of hippocampal tissue and reduces its progressive loss following TBI (Browne et al., 2004). Previously Vink and his colleagues using phosphorous magnetic resonance spectroscopy following fluid percussion head injury demonstrated that decrease in intracellular free Mg^{2+} is associated with irreversible tissue injury (Vink et al., 1988a). Furthermore, in a study by Saatman et al (2001), magnesium deficiency prior to injury exacerbated neuronal cell death and cytoskeletal damage. Indeed animals that were fed on a magnesium deficient diet demonstrated significant neuronal cell death within the cortex and hippocampus when compared to the magnesium treated rats and those that were fed on a normal diet (Saatman et al., 2001). Moreover magnesium has been shown to attenuate induction of p53mRNA, which primes cells for death (Muir et al., 1999). In their study, animals were administered with either 125μmoles of $MgCl_2$ or an equal volume of saline. The sham animals did not express p53 6hrs post injury whereas the magnesium treated animals demonstrated significant reduction in the number of labeled cells in the cortex compared to the saline treated animals (Muir et al., 1999). Also Bareyre et al (2000) showed that magnesium ($MgCl_2$) when administered 15mins post injury significantly reduced cortical damage ($p<0.01$) following lateral fluid percussion injury (Bareyre et al., 2000). In the

hippocampus, magnesium administration reduced neuronal loss when administered before TBI (Enomoto et al., 2005). Experimental studies with magnesium in spinal cord injury showed that it significantly reduced neuronal loss (Kwon et al., 1976, Lee et al., 1976, Kwon et al., 2009).

Nonetheless, in a randomized clinical trial with magnesium sulphate where continuous high dose infusions were administered for 5days commencing within 8hrs of injury, magnesium offered no neuroprotection (Temkin et al., 2007). The same study reported that in fact magnesium had a detrimental effect on the brain injured patients (Temkin et al., 2007), most likely because of the higher doses used than in experimental studies. The higher doses were apparently required because of the poor penetration of the magnesium salts across the human BBB. The current study sought to address the underlying issues by administering $MgCl_2$ in a polyethylene glycol formulation (Mg PEG). Indeed staining with hematoxylin and eosin demonstrated significant reduction dark cell change (neuronal cell loss) in the Mg PEG treated animals. Importantly, 0.1xMg PEG showed the same beneficial results as the full dose (254μmole/kg) indicating that PEG facilitated its central penetration. Whilst a number of studies have investigated PEG's mechanisms of action including membrane reconstruction (Koob et al., 2008), repair of injured axons (Krause and Bittner, 1990), it's actions remain unknown pending further investigation.

Neuronal dark cell change has been referred to as an artifact due to inadequate perfusion and fixation (Cammermeyer, 1961, Brierley and Brown, 1981). However, in the current study the saline treated (vehicle) animals showed dark cell change, consistent with previous studies (Agardh et al., 1980, Auer et al., 1984, Marmarou and Foda, 1994), whereas the sham and optimal dose magnesium treated animals did not. Therefore, any dark cell change was not considered an artifact.

4.4 FUNCTIONAL OUTCOME

Our current results also showed that $MgCl_2$, Mg PEG and 0.1x Mg PEG attenuated functional deficits following severe diffuse TBI. This improvement was associated with reduced BBB permeability, reduced substance P release, reduced oedema formation, and reductions in cell death (Bara, 1984, Feldman et al., 1996, Bareyre et al., 2000, Saatman et al., 2001, Browne, 2004, Hoane, 2004, van den Heuvel and Vink, 2004). Potentially inhibition of the n-methyl D-aspartate channel (Mcdonald et al., 1990), increased glycolysis (Garfinkel, 1985) and oxidative phosphorylation (Fagian, 1986) all played a role in this neuroprotection.

Specifically, animals treated with Mg PEG and 0.1x Mg PEG showed significant improvement in motor function by day 4 as assessed by the rotarod compared to the saline treated (vehicle), 0.1x $MgCl_2$ and PEG alone. The rotarod was the preferred test for motor function because it has previously been shown to be the most sensitive after TBI (Hamm, 1994). Vink et al (2003) demonstrated the effect of magnesium on sensorimotor and anxiety performance. In their study, $MgSO_4$ (254 μmoles/kg) was administered 30mins post injury and animals tested for sensorimotor and anxiety performance (Vink et al., 2003). The magnesium treated animals reported very high learning rate with 13secs improvement per week similar to that observed in shams whereas the vehicle animals showed no improvement (Vink et al., 2003). Smith et al (1993) combined magnesium with ketamine, both non-competitive antagonists of the NMDA receptor. They showed that magnesium alone, ketamine or both combined improved spatial learning (Smith et al., 1993). However Bareyre at al (1999) demonstrated that magnesium administration post injury did not improve spatial learning although motor deficits were attenuated. Such a discrepancy could be attributed to use of different protocols. Heath et al

(1998), using phosphorus magnetic spectroscopy showed that brain free magnesium concentration was directly associated with neurologic motor function (Heath and Vink, 1998). Brain free magnesium concentration declines by about 50% following TBI and is associated with impaired ATP production and ion homeostasis across cell membranes (Heath and Vink, 1999b). In our study, magnesium was administered 30mins post injury but brain free magnesium concentration levels were not taken as they have previously been determined in our laboratory. In a focal model of brain injury, magnesium attenuated long-term behavioral impairments (Hoane, 2004). Furthermore McIntosh et al (1988) reported that magnesium deficient animals had higher mortality and neurological deficits and treatment significantly improved outcome (McIntosh et al., 1988). In 1989 they combined magnesium with ATP, which improved neurological dysfunction (McIntosh et al., 1989), the dose response (0.1x 125mumol) however did not show any positive outcomes. Similarly the current study examined the effects of 0.1x $MgCl_2$ on oedema, BBB permeability and functional outcome. The animals that received the one-tenth dose of $MgCl_2$ did not show any beneficial outcomes and in fact resembled those that received saline. Notably, combination treatment with PEG was necessary to observe any improvement in outcome, suggesting that central penetration is the key to magnesium's neuroprotective effects.

Hoane et al (2003) demonstrated the behavioral effects of magnesium therapy following bilateral medial cortical lesions in rats (Hoane et al., 2003). They administered $MgCl_2$ (1mmol or 2mmol), 15mins post injury, which improved working memory in the Morris water maze but did not improve reference memory (Hoane, 2004). This improvement in working memory was attributed to magnesium's ability to prevent p53 expression and degeneration of subcortical structures (Hoane, 2004). The higher dose (2mmol) was found to be more protective against neurodegeneration compared to the lower dose (1mmol) (Hoane, 2004), whereas Heath and Vink

(1995) showed that lower doses were better. Additionally administration of magnesium 24hrs post injury did not offer any neuroprotection indicating that its therapeutic window is between 15mins and 24hrs (McIntosh et al., 1989, Heath and Vink, 1999d, Hoane and Barth, 2002, Hoane, 2004). In the current study, magnesium was administered 30mins post injury as it has previously been found to be most efficacious at this time point (McIntosh et al., 1989).

The object recognition test was preferred for cognitive assessment and recognition memory in the current study because it is cheap, easy to administer and is reliable (Berlyne, 1950, Aggleton, 1985, Ennaceur and Delacour, 1988, Ennaceur and Aggleton, 1997, Donkin, 2006, Ennaceur, 2010, Gaskin, 2010, Hayser and Chemero, 2012). Donkin (2006) showed that administration of N-acetyl tryptophan (substance P antagonist) improved recognition memory using the object recognition test. This test reflects the use of learning and recognition memory and the choice to explore novel objects by magnesium treated rats indicates recovery. The current study shows that animals treated with $MgCl_2$ spent more time exploring the new object indicating recovery of recognition memory after TBI. Additionally treatment with Mg PEG and 0.1x Mg PEG reported even better results whereas those treated with PEG and 0.1x $MgCl_2$ spent less time exploring the novel object, which confirms the notion that PEG and 0.1x $MgCl_2$ did not attenuate cognitive deficits. The fact that 0.1x Mg PEG attenuated cognitive deficits shows that it could be a potential therapeutic agent for TBI.

4.5 Conclusion

This thesis demonstrates that administration of magnesium following moderate to severe diffuse TBI in rats attenuates oedema, BBB permeability, substance P release, neuronal cell death and improves functional outcome. Treatment with magnesium resulted in an improvement in both motor (rotarod test) and cognitive (object recognition) outcome. The current study also shows for the first time that magnesium in a polyethylene glycol formulation (Mg PEG) improves all of these parameters, even at one-tenth the dose of magnesium, which in itself has no beneficial effect. These findings suggest that when used in combination with PEG, PEG facilitates central penetration of the magnesium salt after TBI and that accordingly, lower doses of magnesium may be used thereby reducing the possibility of any potential side effects from toxicity. Accordingly, low dose magnesium in combination with PEG may offer a novel therapeutic strategy for use in clinical TBI.

CHAPTER 5

REFERENCES

5. REFERENCES

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