AQUAPORINS: GATEKEEPERS OF OEDEMA IN TRAUMATIC BRAIN INJURY

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A thesis submitted to the University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy

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Prof. Robert Vink
Prof. Andrea Yool
DEclarations

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Joshua Luke Burton

2014
DEDICATION

"How much truth can a spirit endure; how much truth can it dare? This became for me more and more the actual test of value. Error (the belief in the ideal) is not blindness; error is cowardice. Every conquest, every step forward in knowledge is the outcome of courage, of hardness towards one’s self; of cleanliness towards one’s self." - Ecce Homo, Preface, Friedrich Nietzsche

This thesis is dedicated in memory of Lesley Gillian and John Rex Kimber who instilled in me the significance and value of study. For without them I would not have come this far.
ACKNOWLEDGEMENTS

From my earliest recollection I was blessed by Lesley and John by their imparting to me the true value of knowledge. Thus my decision to undertake a PhD was not one of if, but rather when. Indeed Plato's infamous Allegory of the Cave has remained a key part of my own ideology and in many respects has mirrored the early challenges I have so far faced throughout my life with an undying desire to seek the light.

There is also unquestionably a deep sense of sadness in closing this part of my studies, for not only am I taking a step away from a place of self-growth but so too the presence of those who have inspired me. My mentor and principle supervisor Prof. Robert Vink has not only guided me throughout my candidature academically, but so too has continually inspirited within me my thirst for learning by his vast research experience and unique teaching approach, epitomising the ideal of evolving academia. There is yet so much I could and dearly wish to learn from him in all aspects of my life and so I only hope that I am fortunate enough to one day be in a position to continue my tuition. Thank you Bob, you will always remain to me a pillar of insight.

Next I would like to thank my secondary supervisor Prof. Andrea Yool, for whom without the studies conducted within this thesis would simply not have been possible. With her immense understanding of physiology and great expanse of research to support her claims, she was singularly responsible for having developed in vitro the novel pharmaceutical agents used within this research.

I would also like to thank the various members of the Vink laboratory who have either assisted in experimental protocol or provided valued feedback on my work throughout my candidature. Indeed arguably the special maxim of the Vink team is one of comradely and so should be cherished as an example for other research groups to follow.
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<tr>
<td>ADC</td>
<td>Apparent diffusion coefficient</td>
</tr>
<tr>
<td>AMDA</td>
<td>A-amino-3-hydroxy-5-methyl-4-isoxazolpropionate</td>
</tr>
<tr>
<td>AQP</td>
<td>Aquaporin</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain-barrier</td>
</tr>
<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPP</td>
<td>Cerebral perfusion pressure</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DAI</td>
<td>Diffuse axonal injury</td>
</tr>
<tr>
<td>EAA</td>
<td>Excitatory amino acid</td>
</tr>
<tr>
<td>ECF</td>
<td>Extracellular fluid</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia-inducible factor 1-alpha</td>
</tr>
<tr>
<td>ICP</td>
<td>Intracranial pressure</td>
</tr>
<tr>
<td>ISF</td>
<td>Interstitial fluid</td>
</tr>
<tr>
<td>Kir4.1</td>
<td>Inward-rectifying potassium channel</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>Na+/K+-ATPase</td>
<td>Sodium-potassium adenosine triphosphatase pump</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>OAP</td>
<td>Orthogonal array of particles</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>TBI</td>
<td>Traumatic brain injury</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial cell growth factor</td>
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CHAPTER 1: INTRODUCTION

1 INTRODUCTION
Despite advances in neurosurgical techniques and critical care management, traumatic brain injury (TBI) continues to be a principal cause of death and chronic disability in people throughout the world (Finfer and Cohen 2001; Fleminger and Ponsford 2005; Le´on-Carri´on et al. 2005). Pathological swelling of the brain (cerebral oedema) arising from such injuries is of major significance to patient clinical outcomes (Miller et al. 1977; Guerra et al. 1999). Left untreated, brain oedema drives a severe rise in intracranial pressure (ICP), resulting in widespread hypoxia, decreased cerebral blood flow (CBF), ischaemia, brain herniation and ultimately death. However, as the precise mechanisms surrounding cerebral oedema formation remain poorly understood, treatment approaches for this life-threatening condition remain inadequate, targeting symptoms rather than causes (Bullock et al. 1999). Indeed so dire are the consequences of injury induced brain swelling that its manifestation accounts for up to half of all mortality and morbidity in victims of TBI (Feickert et al. 1999; Chiaretti et al. 2002). Recently, brain water channels known as aquaporins (AQP) have been identified as being integrally involved in the genesis and resolution of oedema following acute head injury (Ke et al. 2001; Papadopoulos et al. 2002; Papadopoulos et al. 2004; Saadoun et al. 2005; Ribeiro Mde et al. 2006; Zador et al. 2007; Kimura et al. 2010; Yool et al. 2010; Oliva et al. 2011; Fukuda et al. 2012; Katada et al. 2012; Verkman 2012). Yet given the complexity of fluid dynamics and altered water channel activity following TBI, the precise role of AQPs in brain tissue water transport remains unclear (Jablonski and Hughes 2006; Tait et al. 2008; Zador et al. 2009; Nesic et al. 2010; Saadoun and Papadopoulos 2010; Li et al. 2011; Iacovetta et al. 2012). Traditional classification of cerebral oedema into its cytotoxic (cellular), vasogenic, osmotic and hydrocephalic variants (Klatzo 1987) has withstood the ages. However, it is now widely accepted within neurosurgical settings that a continuum often exists from one form to the other during the pathological progression of oedema following TBI (Bramlett and Dietrich 2004; Kimelberg 2004; Pasantes-Morales and Cruz-
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Rangel 2010). Equally it has been well established that many of the physical deficits following acute head trauma develop progressively with time and involve multiple molecular pathways of neuropathology, forming a ‘secondary injury’ cascade (Norenberg et al. 2004; Werner and Engelhard 2007; Rowland et al. 2008; Greve and Zink 2009). Of these secondary factors that make up the injury cascade, it is the genesis of brain oedema that serves as a principle prognostic factor for neurological outcome. Indeed, given that eighty percent of the brain is water (Tait et al. 2008), it is clear that a far greater understanding of the channels which regulate cerebral fluid flow is required in order to improve clinical results.

The human intracranial volume is made up of the brain parenchyma, cerebrospinal fluid and intravascular compartments (Tait et al. 2008), and is composed of a highly complex architecture of neurons, supportive glia and integrated neuronal-vascular relationships. Not surprisingly, studies which have aimed at attenuating cerebral oedema via the blunt knockout of AQP activity have thus far yielded mixed results (Manley et al. 2000; Badaut et al. 2002; Papadopoulos et al. 2002; Amiry-Moghaddam et al. 2003; Sun et al. 2003; Papadopoulos et al. 2004; Solenov et al. 2004; Hirt et al. 2009; Amiry-Moghaddam et al. 2010; Brian et al. 2010; Kimura et al. 2010; Nesic et al. 2010; Saadoun and Papadopoulos 2010; Fukuda et al. 2012; Katada et al. 2012). The discovery and development of novel pharmaceutical agents specifically directed at modulating AQP4 and 1 channel activity by our laboratory therefore offers promise of a non-invasive, mechanistic approach that could potentially revolutionise critical care.

The introduction of this thesis will initially describe the known pathophysiology involved in diffuse TBI and its contribution to cerebral oedema, with a subsequent consideration of brain fluid dynamics and cellular controlled homeostasis along with the potential role of AQP water channels in the evolution and resolution of injury induced brain swelling.
CHAPTER 1: INTRODUCTION

1.1 Epidemiology

1.1.1 Australia

Severe TBI typically results in either death or chronic disability (AIHW 2008). For decades it has been clearly documented that such injuries are a leading cause of mortality and morbidity in people under the age of 45 years throughout the Western world (Bruns and Hauser 2003; Tagliaferri et al. 2006; Livecchi 2011; Asemota et al. 2012). Yet regardless of the severity and dire outcomes commonly associated with TBI, each year thousands of young Australians continue to be affected by this unresolved pandemic (AIHW 2008; AIHW 2009). In 2005 22,000 incidents of severe brain trauma were recorded in hospitals across the nation, resulting in approximately 980 deaths. Fall related injuries (9,233) were reported as being the most common cause of brain trauma during this period, although vehicular related road accidents closely followed attributing for 7,153 hospital admissions (AIHW 2008). Similarly in 2008, a government-funded report suggested that the leading cause of TBI in young adults aged between 20-24 years was road related accidents (Myburgh et al. 2008). However, with an ongoing exponential rise in the nation's aging population (ABS 2009), health authorities are now beginning to turn their attention towards acute brain injuries due to falls (AIHW 2008).

In 2008 it was reported that lifetime expenditure costs associated with nationally recorded incidences of TBI exceeded $184,000,000 each year (AIHW 2008). Yet despite such a high financial impost, it is the additional burden of long term and often incomplete neurological rehabilitation facing TBI victims which exacerbates the severity of such injuries.

1.1.2 Global

Throughout 23 European nations the principal cause of death and disability in people under 40 years of age is TBI. Each year over 1,600,000 hospitalisations are recorded as being acute
CHAPTER 1: INTRODUCTION

head injury related, with a further 66,000 recorded fatalities directly arising from such
injuries (Nelson et al. 2004). Nationally reported rates of TBI range from as high as 365 per
100,000 of the population in Western Sweden (Andersson et al. 2003), to 83 per 100,000 in
Glasgow (Kay and Teasdale 2001). Young adult males comprise the largest cohort of TBI
patients throughout Europe with principle causes of injury being motor vehicle accidents and
alcohol associated trauma (Boto et al. 2006). As in Australia, there is also a growing number
of fall related brain injuries within the elderly cohort across Europe (Kleiven et al. 2003;
Yates et al. 2006). In the United States over 2,000,000 TBI patients are hospitalised each
year, with approximately 50,000 mortalities and over 80,000 patients acquiring a permanent
neurological impairment (Faul et al. 2010). Of these road traffic accidents, falls and assaults
were seen to be the leading contributors to TBI across the nation (CDC 2006), reflecting a
common injury profile throughout much of the globe.

1.2 Neuropathology

Mechanistically, injuries arising from acute head trauma may be classified as either primary
or secondary events. Primary mechanical injury to the brain at the time of trauma can only be
targeted by preventative measures. Secondary events however, are triggered by the primary
insult and are thought to involve a complex cascade of neuropathological mechanisms, often
culminating in cerebral swelling. Typically characterised by either its cytotoxic, vasogenic or
interstitial variants (see Classification of brain oedema), the formation of cerebral oedema
following TBI frequently paves the way for further secondary injury. Small increases in the
volume of oedematous fluid may produce a negligible rise in ICP, as the lower pressures of
the CSF and intravenous compartments contract in order to serve as a volume buffer for
excess water (Saadoun and Papadopoulos 2010). However, once the CSF and venous reserves
are exhausted, even the slightest increase in oedema fluid volume results in a significant
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elevation in ICP (Saadoun and Papadopoulos 2010). The difference between ICP and mean arterial pressure (MAP) is termed cerebral perfusion pressure (CPP) and although it may vary between 60-150mmHg, CBF is physiologically auto regulated at a constant 50ml/100g/min when fluctuations occur between this range. Yet given that the formation of cerebral swelling will often cause a rise in ICP, reduced CPP quickly ensues and subsequently results in widespread brain ischaemia. With the onset of ischaemia, there often comes an elevation in the level of blood carbon dioxide (CO₂), which then causes ICP to rise even further. In even more dire scenarios the onset of significantly high ICP will consequently result in brain herniation due to a radical shift in the parenchyma within the cranium. The damage to the brain itself in herniation is self-evident, however it is specifically the compression of major cerebral arteries and the brainstem against the edge of the dura, tentorium and falx which leads to sudden patient death (Unterberg et al. 2004).

Although the control of brain water homeostasis is incompletely understood, a number of recent studies have produced compelling evidence suggesting a fundamental role of AQP4 and 1 in both the formation and resolution of brain oedema. However, as the precise function that the water channels serve in the secondary injury cascade would appear to be strongly contingent upon the type of injury, a far clearer understanding of AQPs is required in order to step closer towards a therapeutic therapy in this life threatening condition. Moreover, given the characteristic delayed nature of the secondary injury cascade, these water channels are set to be likely targets for pharmacological intervention in the amelioration of post traumatic cerebral swelling (Vink et al. 1991; Esen et al. 2003; Nimmo et al. 2004; Ghabriel et al. 2006; Vink and Nimmo 2009; Yool et al. 2010).
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1.2.1 Primary injury mechanisms

Primary TBIs arise as a direct result of mechanical forces exerted at the time of trauma. Injury events, such as the head striking an object, commonly result in focal cerebral contusions (brain against bone) or intracerebral haemorrhage (the shearing of cerebral vasculature). Conversely, acceleration/deceleration forces often produce profound movements of the brain within the cranium, typically leading to diffuse axonal injury (DAI) and widespread tissue damage. Therefore it is well understood that primary mechanical deformations to the brain following head trauma directly alter the structural and functional integrity of its vasculature, supportive glia, neurons and axons, in a typically combined diffuse, focal or multifocal pattern (Greve and Zink 2009).

1.2.1.1 Biomechanics of TBI

The biomechanics of brain injury begin with a mechanical force or load sets both it and the head in motion, with any abrupt alteration to this force producing both diffuse and focal cerebral damage (Besenski 2002). Several variants of this loading can arise during primary injury however, of these it is dynamic impulse which most typically predominates in human brain trauma (Graham et al. 2000). Once the head is set into motion, or an already moving head is stopped without striking an object, dynamic impulse results in generating a sole inertial force (Graham et al. 2000). The magnitude of such an applied force varies over time, with the extent of mechanical damage to brain tissue being directly dependent upon head velocity, rate of acceleration change, speed of motion and displacement (Stalhammar 1990). Consequently dynamic impulsive loading commonly results in three principle forms of head acceleration; rotational, translational and bending-stretching (Davis 2000). Rotational injuries occur when acceleration forces turn the head suddenly upon its axis, around its centre of gravity and place undue mechanical stress upon the connections surrounding the brain.
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(Grcevic 1988). Movements of this form within the skull consequently result in the rupturing of bridging veins and neuronal vasculature, causing axonal injury, swelling and bleeding. Translational acceleration injuries arise when extreme movement of the head occurs along its centre of gravity and lead to pronounced contusion (Stalhammar 1990). Whereas bending-stretching damages, due to abrupt changes in acceleration force, often lead to brain stem injury and associative respiratory depression (Stalhammar 1990; Davis 2000). Thus it is due to a combination of these widespread biomechanical events which produces many of secondary injuries known to arise post TBI and frequently culminate in severe cerebral oedema.

1.2.1.2 Focal and diffuse TBI

Structural damages in TBI are typically classified as either diffuse or focal events. Focal injuries, often caused by a direct localised mechanical force(s), are regions of focussed damage typically associated with symptoms related to the area of brain affected (Povlishock et al. 1994). Diffuse injuries however, or multifocal injuries are often caused by combined acceleration/deceleration forces and commonly result in widespread vascular and neuronal damage. Diffuse injuries also frequently produce large scale cerebral effects such as widespread ischaemia, hypoxia and associate brain swelling (Blumbergs et al. 2008; Saatman et al. 2008). Therefore it is typical in a majority of human brain trauma cases for patients to exhibit a combination of diffuse injury effects.

1.2.1.3 Respiratory depression, hypoxia and oedema

Respiratory depression is typically observed in both the clinical and experimental setting of severe head injury (Yamamoto et al. 1999), with the subsequent formation of hypoxia and ensuing oedema formation, often resulting in fatal complications. Indeed far prior clinical
studies have shown that up to 65.6% of human TBI cases demonstrate comorbidity with hypoxic interference (Becker et al. 1977; Chesnut 1995). Decreases in brain tissue oxygenation ($\text{PbtO}_2$) may arise due to a many number of primary and secondary injury events. Anoxia occurs when the brain is completely deprived of oxygen and is often associated with brainstem injury (Stalhammar 1990; Yan et al. 2011). Hypoxia however, is brought about by a decrease in the supply of oxygen to the brain and hence multiple secondary factors can and often do contribute to the genesis of cerebral hypoxia. Severe elevations in ICP and decreases in CBF can rapidly produce both local and diffuse ischaemia, which in depriving the brain of essential blood flow result in the formation of hypoxia events. Severe decreases in $\text{PbtO}_2$ can on its own result in producing severe cerebral impairment however, it is its exacerbation of oedema and its associative damages on brain tissue which typically determines patient neurological outcomes (Ishige et al. 1987; Hellewell et al. 2010; Yan et al. 2011). Indeed such pathologies are of particular importance when considering the role of AQPs in atypical cerebral fluid homoeostasis, as hypoxia, ischaemia, vascular leakage and their associated biochemical neuroinflammatory responses have all been reported to directly relate to alterations in brain water channel activity (see AQP4 and mediators of brain oedema).

Thus in view the heterogeneous nature of human TBI, it is critical for experimental animal models investigating clinically relevant cerebral swelling, to include both the widespread mechanical nature of diffuse brain injury with an associative induction and control of post injury hypoxia. Marmarou et al. (Marmarou et al. 1994) well established weight drop model of diffuse TBI closely replicates many of these factors described, by mimicking the characteristic blunt force acceleration/deceleration events so typically observed in human falls and motor vehicle accidents (see The impact acceleration model of diffuse TBI). Similarly this injury model is well known to reliably produce widespread and bilateral
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axonal, neuronal and microvasculature damages as well as extensive DAI. However, it is the ability of this model to produce both vasogenic and cytotoxic cerebral swelling (see Classification of brain oedema) which makes it ideal for investigating the role AQPs play in oedema formation post diffuse TBI. Moreover with the additional option to induce controlled hypoxic events via mechanical ventilation post head injury, this model can provide an appropriate platform from which to evaluate how cerebral water channels may play an integral part in traumatic brain oedema.

1.2.2 Secondary injury mechanisms

Secondary brain injuries occur within minutes to hours following primary head trauma and are thought to involve a myriad of biochemical mediators. Atypical interactions between these molecules then lead to the genesis of numerous pathological cascades, often culminating in the formation of cerebral swelling. Although not all of these secondary pathways may directly influence the activity of brain AQPs, many do play a role in the formation of brain oedema and as such are essential to introduce. Injury induced impairments to the regulation of CBF and cell metabolism (see Metabolic alterations and brain bioenergy) frequently result in hypoxic and ischaemic events throughout the brain and subsequently produce a build-up of lactic acid as a by-product of anaerobic glycolysis. However, with anaerobic metabolism being insufficient to sustain cellular energy requirements, adenosine-5'-triphosphate (ATP) reserves deplete and energy dependent membrane ionic pumps begin to fail. In addition to the bioenergetic failure, excitatory amino acids (EAA) such as glutamate and aspartate are released resulting in an over activation of N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolpropionate (AMPA) and voltage dependent calcium (Ca^{2+}) and sodium (Na^{+}) channels, resulting in terminal membrane depolarization. The influx of Ca^{2+} and Na^{+} then lead to numerous intracellular catabolic mechanisms. Excessive
intracellular Ca\(^{2+}\) concentrations activate proteases, atypical secondary messenger cascades, lipid peroxidases and phospholipases, which in turn increase internal levels of damaging free radicals and fatty acids. Degenerative structural changes then arise as a result of activated caspases, endonucleases and translocases which fragment DNA, inhibit nucleosomal repair and alter the function of biological membranes. Collectively these events result in the membrane degradation of cellular and vascular scaffolds to the extent of ultimately culminating in either programmed (apoptotic) or necrotic cell death.

1.2.2.1 Metabolic alterations and brain bioenergy

Cerebral energy state, or the so termed bioenergetic state, is reflected either directly by brain tissue concentrations of ATP and phosphocreatine or rather indirectly by the ratios of lactate to pyruvate. However, brain metabolism is more commonly represented by its measured consumption of both glucose and O\(_2\) (Diringer et al. 2000; Glenn et al. 2003). Following diffuse TBI, both of these factors are seen to swiftly decline with significant spatial and chronological heterogeneity (Cunningham et al. 2005), with the severity of primary insult having been shown to be directly correlated with the degree of metabolic and bioenergetic failure (Wu et al. 2004). Patient clinical outcomes are also often echoed in the measure of both factors with lower metabolic and energy cases typically resulting in poorer neurological results (Vink et al. 1994; Tavazzi et al. 2005).

Decreases in brain metabolism post TBI are principally thought to arise as a consequence of mitochondrial impairment, reduced ATP production, dysfunctional cellular respiratory rates and associative intramitochondrial Ca\(^{2+}\) influx (Clark et al. 1997; Verweij et al. 2000). As such if cerebral ATP concentrations are insufficient to sustain basic cellular energy requirements, bioenergetic failure occurs. In this event energy dependent brain ion pumps such as Na\(^+\)/K\(^+\)-ATPase fail and in turn disrupt ionic homeostasis (Simard et al. 2007), a
common prelude to the formation of cellular swelling (see Cytotoxic). Moreover, with the absence of sufficient ATP reserves, fundamental cellular processes such as internal phosphorylation cannot occur and thus notably impair a wide variety of typical metabolic processes integral to cell health. Therefore, given that widespread metabolic failure often directly impairs primary cell function, the decline in brain bioenergetics post TBI is of critical significance to the genesis of osmotic, ischaemic and even vasogenic oedema (Chen et al. 2004).

Peak alterations in brain energy levels often occur early post TBI, reaching their maximal state within the initial few hours to first full day following trauma. However, at three days following initial insult, TBI-induced hypometabolism and subsequent declines in brain energy levels typically return to pre-injury levels (Heath and Vink 1995). Despite the restoration of normal energy states during the chronic stages post brain injury, the acute secondary damage has often already taken place, leaving limited potential for neurological improvement with the return of metabolic supply.

1.2.2.2 Ionic homeostasis and electrochemical gradients

Traumatic brain injury induced alterations to ionic homeostasis frequently underpin the genesis of differing forms of cerebral oedema, as well as other types of ischaemic injury. Much of the fluid throughout the CNS is contained within the intracellular compartment, which when compared with cerebrospinal and interstitial fluids (ISF), has a far higher concentration of potassium (K\(^+\)) and lower levels of Na\(^+\) and Ca\(^{2+}\). Under physiological conditions these ions are transported against their electrochemical gradients via means of energy dependent pumps, integral to the homeostatic transport of cellular K\(^+\), Na\(^+\) and Ca\(^{2+}\). However, in the setting of post-traumatic metabolic deficit, the Na\(^+\)/K\(^+\)-ATPase pumps fail, resulting in an intracellular influx of Na\(^+\) and water, causing cells to swell. Thus
pathologically induced bioenergetic failure often equates to oncotic cell death. Moreover, essential electrochemical gradients required for secondary active and passive ionic transport processes are equally dependent on the functionality of the Na⁺/K⁺-ATPase. Hence any injury induced alterations to the processes which regulate the solute composition and volume of ISF are directly related to the genesis of cellular oedema.

Typically water is maintained at a thermodynamic equilibrium across the plasma membrane of many of the brain cells and as such the osmotic concentration of extra and intracellular fluid is equal under physiological conditions. Cellular membranes are freely permeable to water transport through their AQPs and hence alterations in the concentration of solutes will establish a transmembrane osmotic gradient, determining the directional flow of fluid into and out of cells. As such shifts in the intracellular solute content (isosmotic volume) or extracellular solute concentrations (anisosmotic volume) mediate the swelling or shrinkage of cells and are typically maintained in balance by the metabolically driven production and removal of osmotically active substances. Therefore in pathological conditions, which alter brain metabolism, net solute entry and exit will result in unmediated cellular swelling (hypotonicity) or shrinkage (hypertonicity).

Significant alterations to isosmotic cell volume are also of particular concern to brain function, as transmembrane ion fluxes across neuronal cell membranes alter during the propagation and generation of action potentials. Indeed neuronal activity typically results in changes to the extracellular levels of K⁺, Na⁺, Ca²⁺ and chloride (Cl⁻) and hence disturbances to this equilibrium can subsequently alter neuronal and glial cell volume (Iwasa et al. 1980). However, under physiological conditions brain cells are able to respond to such alterations in cell volume via the automated adjustment of intracellular concentrations of ions or solutes. Known as either regulatory volume decrease (RVD) or regulatory volume increase (RVI), energy sufficient cells adjust to ionic flux by automated changes in the activity and
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expression of ion channels and transporters (Strange 1992; McManus et al. 1995). Therefore changes in cell membrane transport and metabolism appropriately alter intracellular concentrations of solutes. Cell RVI counters hypertonicity and triggers the activation of Na\(^+-\)K\(^+-\)Cl\(^-\) sensitive cotransporters (NKCC1), resulting in an influx of Na\(^+\) and water (Lam et al. 2005; Kahle et al. 2008). Conversely, acute hypotonicity is countered by RVD via the activation of K\(^+-\)Cl\(^-\) cotransporters and K\(^+\) and Cl\(^-\) channels, resulting in a cellular efflux of K\(^+\) and Cl\(^-\). In the setting of ischaemic injury, inappropriate activation of the NKCC1 and other ion transporters in association with the failure of RVD, results in the swelling of cells. From a functional perspective, the hypotonicity of brain vascular endothelia (see Blood brain barrier) can also result in a decrease in CBF, furthering ischaemia and leading to cerebral infarct. Similarly, the swelling of AQP1 rich choroid plexus epithelia and/or the AQP4 prominent ventricular ependyma will often compromise their structural integrity, altering their permeability and potentially leading to further cerebral oedema.

1.2.2.3 Excitatory amino acids and oxidative stress

Under physiological conditions, amino acids such as glutamate and aspartate serve as principal EAAs throughout one half of all synapses within the brain (Lipton and Rosenberg 1994). Following TBI, enormous EAA efflux occurs and is well established as being a major cause for some of the observed deleterious effects on neurons and astrocytes (Floyd et al. 2005; Yi and Hazell 2006). At present there are no known direct correlations between injury induced excitotoxicity and brain AQPs, although it is of note that there is almost exclusive expression of EAA transporters (EAAT1/EAAT2) in AQP4 rich glia.

Over activation of metabotropic and ionotropic EAA receptors often triggers numerous catabolic processes including a loss of BBB integrity and thus vasogenic leakage. Under such conditions there is a physiological attempt to restore altered ionic gradients: however,
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increases in Na⁺/K⁺-ATPase activity elevates metabolic demand, resulting in a circuitous uncoupling between CBF and cell metabolism (Obrenovitch and Urenjak 1997; DeWitt and Prough 2003). Excessive activation of NMDA receptors also typically induces the release of pro inflammatory mediators, which also significantly contribute to the genesis of vasogenic oedema (Cuesta et al. 1999). Similarly, coactivation of metabotropic glutamate receptors and purinergic receptors on astrocytes following TBI often results in phospholipase C-dependent increases in astrocytic Ca²⁺ levels, activating Ca²⁺ sensitive phospholipase A₂ (PLA₂). Abundant accumulation of arachidonic acids then contributes to cerebral vasodilatation via cyclooxygenase-2 (COX)-dependent build-up of dilating agents such as prostaglandin E₂ (PGE₂)(Haydon and Carmignoto 2006).

Injury induced ischaemic events can also result in an enormous influx of Ca²⁺ into cells, which in addition to themselves activating a variety of damaging intracellular processes, equally contribute to atypical EAA release and subsequent excitotoxicity. Elevations in intracellular Ca²⁺ content are well known to contribute to the production of reactive oxygen species (ROS) and the activation of Ca²⁺ dependent protein calpains (Bevers and Neumar 2008; Vosler et al. 2008). Oxidative stress induces the generation of ROS such as O₂ free radicals and associative elements, including hydrogen peroxide (H₂O₂), nitric oxide (NO) and the subsequently produced peroxynitrite (ONOO⁻). Together excessive generation of ROS and the depletion of endogenous antioxidant systems, such as catalase and superoxide dismutase, promote the peroxidation of vascular and cellular structures, inhibit mitochondrial electron transport chains and induces protein oxidation as well as DNA cleavage (Bayir et al. 2005; Shao et al. 2006). Such pathological mechanisms are in of themselves more than adequate to promote cell death and impair neuronal function. However, it is often the case that during the progression of apoptotic programmes and inflammatory processes, atypical intracellular activity will trigger the activation of further damaging secondary signalling
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pathways (Chong et al. 2005). Indeed production of intracellular ROS are also seen to play a part in the regulation of AQP4 activity (Arima et al. 2003), where exposure to the reactive molecules upregulate the water channels expression. Alternatively, intracellular Ca\(^{2+}\) cascades as typically seen acutely post TBI, can stimulate production of protein kinase C (PKC), which promotes the internalisation of AQP4 (Haj-Yasein et al. 2011).

1.2.2.4 Apoptosis and necrosis

Two principle forms of cell death may arise after TBI, namely necrosis or apoptosis. Apoptosis is a form of cellular self-destruction, initiated via activation of an intrinsic cell suicide program whenever cells become redundant and/or are severely impaired due to ongoing pathology. Under normal physiological conditions, translocation of phospholipids such as phosphatidylserine generate minute although progressive disintegration and lysis of membranes, initiate fragmentation of DNA and condense chromatin (Sastry and Rao 2000). Redundant intracellular particles (apoptotic bodies) are then subsequently removed by exocytosis. Thus apoptosis plays an essential role in maintaining healthy physiology via controlled elimination of redundant cells (Kerr et al. 1972). Neurons undergoing apoptosis are at first morphologically sound, with sufficient ATP supply to maintain physiological membrane potential (Iwasa et al. 1980). During these initial stages of the apoptotic pathway, cells frequently do not release harmful intracellular substances into the surrounding tissue. Cellular organelles continue to remain intact and there is frequently no change in ionic pump activity (Sastry and Rao 2000). However, within hours to days following TBI, apoptosis may play a role in contributing to subsequent neuronal decay (Kerr et al. 1972; Sastry and Rao 2000). Recent research suggests that the formation of cerebral oedema post injury may lead to unwarranted apoptosis of neurons (Yang et al. 2005). Therefore, therapies that are able to alleviate brain swelling and/or reduce a rise in ICP, might contribute to ameliorating neuronal
apoptosis following TBI and potentially attenuate some of the typically observed functional deficits.

On the other hand, necrosis is a form of cell death in response to severe mechanical damage and/or ischaemic/hypoxic tissue decay, frequently seen in association with abundant EAA release and metabolic failure (Sastry and Rao 2000). During this process, proteases, lipid peroxidases and phospholipases typically begin to autolyse essential biological membranes. Morphological changes indicative of cellular decay are prominently observed as well as leakage of cell contents, usually provoking an inflammatory response in surrounding tissue. The resulting cellular detritus is then recognised as antigen and by being removed via inflammatory processes, typically result in scar formation (Saadoun et al. 2005). Similarly cellular deterioration of this form frequently involves the impairment of intracellular homeostasis, disruption to ionic pump activity, depletion of ATP reserves and subsequent rupturing of cell contents leading to cytotoxic swelling (Sastry and Rao 2000). Cellular organelles in these events are also often destroyed, inclusive of essential energy producing mitochondria. However, with Ca\(^{2+}\) influx often occurring in parallel with necrotic degradation of cell cytostructures, Ca\(^{2+}\) dependent proteases are typically activated and stimulate mitochondrial citrate cycle activity and the production of further damaging ROS.

**1.2.2.5 Astrogliosis**

Reactive astrogliosis (astrocytosis) within the brain following TBI is characterised by the hypertrophy of astrocytes with atypical morphological changes (Amaducci et al. 1981). Astrocytic responses of this form are often identifiable at 24 h post injury and are seen to peak at approximately three days following trauma. Alternative to their typical morphology, reactive astrocytes are distinguishable by their cytoplasmic enlargement, establishment of elongated cytoplasmic processes and increase in their production of intermediate filament
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glial fibrillary acidic protein (GFAP) (Baldwin and Scheff 1996; Werner and Engelhard 2007). Such ‘glial scarring’ produced by reactive astrocyte activity is believed to be an attempt by the CNS to isolate damaged neural tissue and restore homeostasis following injury (Fitch et al. 1999). Indeed, reactive glial cells have been shown to reduce excessive levels of extracellular EAAs post injury, restore and sustain normal ionic activity and provide neurotrophic support (Kimelberg et al. 1992; Muller et al. 1995). However, astrocytic activity of this form is also thought to be detrimental to neurological function as the modified glia interfere with typical axonal regeneration, subsequently impairing neuronal regrowth (Ridet et al. 1997).

Numerous studies have also shown that reactive astrocytes may have an interconnected relationship with AQP4 (Zador et al. 2009; Kinoshita et al. 2010; Li et al. 2011; Tourdias et al. 2011; Verkman 2012) given that the water channel is typically found at the end foot and subpial processes of the cell (see Aquaporins). Microglial activation and astrogliosis are well-established components of neuroinflammation following TBI (Nedergaard et al. 2003) and as such, AQP4 may potentially serve an additional role in the neuroinflammatory response as well as being a key player in cerebral oedema. Indeed, in predominantly cellular models of brain injury (Saadoun et al. 2005; Auguste et al. 2007) the observed absence of AQP4 in perivascular glia end feet may, in part, be responsible for decreased astrocyte hypertrophy as a result of reduced water entry and migration towards the site of injury. Alternatively, in principally vasogenic events (Fukuda et al. 2012), AQP4 would appear to be upregulated on astrocytes during the resolution phase of cerebral swelling. AQP4 has also been colocalised with the inward-rectifying potassium channel, Kir4.1 (Badaut et al. 2000; Badaut et al. 2000) found on astrocytic end feet. Such an association may be significant given that in mouse AQP4 knock out studies (Binder et al. 2006) a delayed K+ reuptake has been shown, suggesting a role for the water channel in K+ homeostasis via the facilitation of water
diffusion along $K^+$ concentration gradients (see Aquaporins, neuronal activity and Kir4.1). Therefore AQP4 may also serve in part to mediate the altered neural excitatory response of reactive migrating glia post TBI.

1.3 Brain fluid dynamics and homeostasis

1.3.1 Formation, function and resolution of CSF

1.3.1.1 CSF Function

The tissues of the CNS rely heavily upon the continuous circulation and turnover of CSF in order to maintain a feasible homeostatic environment. Similarly the brain itself, with its volume tightly enclosed within the confines of the cranium, depends on a highly efficient mechanism of production, removal and control of CSF secretion (Oreskovic and Klarica 2010). The CSF is generally ascribed to possess five primary mechanical and physiological functions (Bulat and Klarica 2011) namely, to transport nutrients and waste to and from the CNS, to supply a chemically viable environment conducive to neural signalling, to provide a neutrally buoyant space for the brain, to shelter the neural tissue from direct contact with either the cranium during external body loading and to protect the CNS from potential fluctuations arising with blood pressure.

1.3.1.2 CSF Formation

It has long been suggested that CSF is principally secreted via the choroid plexus into the ventricular space and is to a lesser extent derived from ECF (Brown et al. 2004), with AQPs 4 & 1 playing a key part in this process. However, more recent evidence has proposed that an additional bulk of CSF volume, along with brain ISF, is derived from water filtration via the arterial capillaries (Oreskovic and Klarica 2010). Indeed, given that CSF is primarily
composed of water, contemporary studies purport that this portion of it does not flow in a unidirectional manner along the CSF space, but is rather formed and reabsorbed locally via vascular osmosis (Bulat and Klarica 2011). In this model, the remaining macromolecular substances reside for far longer within CSF and ISF transit, to be progressively carried bidirectionally along the CSF space via diastolic-systolic displacement (Enzmann and Pelc 1992; Bulat et al. 2008).

Anatomically the choroid plexuses are branch-like structures comprised of villi, which project directly into the ventricles (Welch 1963). Ensheathing each villus is a singular epithelial cell layer, which in accordance with the traditional view is involved in a two part process in order to generate CSF. Initially plasma (a combination of ions and water) is driven via pressure gradients across the choroidal endothelia, and in so doing is passively filtered between choroid ISF and capillary blood (Redzic et al. 2005). The CSF is then subsequently secreted across a single epithelial layer, which is tightly regulated via an assortment of ion channels and epithelial transporters. Less is known about the precise channels and carriers directly involved in brain capillary CSF production however, it is clearly understood that amongst these mediators AQP1 (see Aquaporins) serves a key role in this process (Prat et al. 1998; Longatti et al. 2004; Zador et al. 2007). Indeed up to 70% of CSF is classically believed to be derived from the choroid plexi and approximately 30% from the ISF, originating from its transport across blood vessels (Hartman 2009). Other reports suggest that the BBB is involved in the partial generation of CSF fluid (Abbott 2004), although the suggested volume is not sufficient to have any effect on the traditional choroidal hypothesis.

1.3.1.3 CSF formation in TBI

Typically CSF formation rates are seen to vary with age and thus may drive a chronological change in cerebral CSF volume throughout life (May et al. 1990). Similarly alterations in
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CSF flow are reported to occur throughout circadian rhythms and might reflect a common, albeit minor change in rate flow. Indeed under typical physiological conditions rate of CSF secretion is known to slightly change, although such fluctuations are considered to be negligible (Bulat and Klarica 2011). In contrast to this is the rate at which CSF flow may change in neuropathology.

Classically CSF dynamics are not thought to alter following traumatic injury per se. However, trauma induced blood accumulation within the ventricular system and/or transtentorial herniation can frequently lead to cisternal block (Barnes and Hoff 1976) and the genesis of hydrocephalus. Similarly, profound elevations in ICP, most commonly observed to occur in association with severe CNS trauma, directly correlate with a notable decrease in CSF production (Barnes and Hoff 1976; Savva et al. 2003), most probably due to injury-induced reductions in choroidal or brain arterial blood flow. Likewise, rates of CSF formation are also thought to be modified by other pathophysiological functions directly affected by trauma. Metabolic and other pathophysiological processes such as changes to the osmotic pressure of blood, commonly occur following brain injury and have been shown to typically modify CSF production (McComb 1983; Brown et al. 2004; Bulat and Klarica 2011). Recent studies have also shown that numerous secondary injury mechanisms directly alter the constituents of CSF (Uzan et al. 2001; Conti et al. 2004). It is well established that acute brain injury typically results in the accumulation of neutrophils within the tissues parenchyma. However, contemporary research has demonstrated that these inflammatory mediators often migrate into in the CSF and contributing to the pleocytosis observed post TBI. Indeed injury provoked infiltration and accumulation of inflammatory mediators such as tumour necrosis factor alpha (TNF-α) and various interleukins in the CSF have been reported (Csuka et al. 1999; Singhal et al. 2002; Schuhmann et al. 2003; Hayakata et al. 2004).

Likewise elevations in NO metabolites appear within the CSF following head trauma (Uzan
et al. 2001). In such events the well characterised production of NO via astrocytes, neurons, and microglia (Feuerstein et al. 1998) leads to a peak in CSF NO concentration at approximately 24 h post TBI (Uzan et al. 2001). The precise role of these inflammatory mediators in the CSF following head injury is unclear. However, given that recent studies have shown an interaction between the accumulation of cytokines and the activity of brain AQPs following head injury (see Tumour necrosis factor-alpha), it is important to consider their appearance within the CSF when examining the role of water channels in brain oedema.

1.3.1.4 ICP and the formation of CSF in TBI

Classic CSF hypotheses would suggest that its formation arises by an active or dynamic nature and thus its rate of production should not be altered following changes in ICP. Indeed, earlier studies contentiously concluded that any fluctuation in ICP had a negligible effect on CSF formation (Heisey et al. 1962; Cutler et al. 1968; Sklar 1980; Artru 1988). However, in subsequent studies a linear correlation was demonstrated between the two factors, showing that an increase in ICP decreased the production of CSF (Frier et al. 1972; Martins et al. 1977; Weiss and Wertman 1978; Orešković et al. 2000; Oreskovic and Klarica 2010). It would be reasonable to suggest that CSF formation may instead arise as a result of a passive process rather than a dynamic one with CSF volume being, in part, regulated by hydrostatic pressure (Bulat et al. 2008; Oreskovic and Klarica 2010). Such a conclusion is significant when considering the formation of cerebral oedema and AQP activity post TBI, as in addition to the well-known rise in ICP following acute brain injury, increased hydrostatic pressure gradients between blood vessels and the parenchyma can result in the biphasic opening of the BBB (Shima 2003). Indeed given the essential role that AQPs play in the formation of CSF and potentially its reabsorption (Prat et al. 1998), it is critical to consider the temporal
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activity of water channels when attempting to understand cerebral oedema following brain injury.

1.3.1.5 CSF Resolution

CSF is believed to be cleared into the lymphatic and venous systems via several physiological mechanisms involving a variety of anatomical locations (Koh et al. 2005; Brodbelt and Stoodley 2007), with the AQP water channel family playing an integral role in this process. Classically the arachnoid granulations and villi of the skull were thought to be the sole sites for CSF reabsorption into the superior sagittal sinus and epidural veins (Bradbury and Cole 1980; Brodbelt and Stoodley 2007), although the cranial lymphatic drainage pathways were subsequently identified as playing a role (Brodbelt and Stoodley 2007). Whereas the cranial route of CSF clearance is principally via the cribiform plate into the cervical lymphatics, lymph reabsorption accounts for approximately 40% of CSF clearance (Abbott 2004) and is thought to be a passive process primarily driven via a pressure differential between the venous and subarachnoid systems (Oreskovic and Klarica 2010). Under physiological conditions the pressure difference between these two systems far exceeds its required capacity given that its function depends on the one-way valve properties of the arachnoid villi. When the pressure of the CSF is higher than that of the venous system, CSF can flow into the blood however, once this pressure gradient is reversed the valves collapse so as to ensure that blood cannot pass the other way. It has been suggested that at higher values of CSF pressure both the arachnoid villi and the lymphatic mechanisms work in tandem in the clearance of fluid, whilst at lower pressures the lymph routes predominate (Bradbury and Cole 1980; Koh et al. 2005; Bulat and Klarica 2011) (Abbott 2004).
As further described in detail later, two members of the AQP family are most abundantly expressed within the human brain. AQP4 is found to be richly expressed in brain astrocytes, with particularly high concentrations localised to perivascular and periventricular end foot processes (Badaut et al. 2002). These channels function in the movement of water between the brain parenchyma and CSF (Zador et al. 2009). The protein is also observed in glial adjacent to the glia limitans externa (Venero et al. 2001), where it is similarly believed to serve an essential part in the transport of fluid between the brain and arachnoid space. The AQP1 water channel is observed to be densely situated within the apical epithelial membrane of the choroid plexus lining the cerebral ventricles (Ghabriel et al. 2006), where it is integral to the rapid transport of CSF and water secretion into the ventricular space (Amiry-Moghaddam et al. 2003). With the distribution of these two channels at prominent brain tissue fluid interfaces, it is reasonable to suggest that the role of AQPs within the CNS is one of fluid regulation. However, with rodent AQP knockout studies so far yielding a variety of interpretations, the exact role of these protein channels in CNS fluid transport would appear complex.

1.3.2 Barriers of the CNS

Three principle molecular barriers exist between the tissues of the CNS, its fluid filled spaces and that of peripheral circulation. The BBB is primarily comprised of a series of cells which together form the neurovascular unit. Anatomically situated between the brains ISF and blood, the BBB principally serves as a mediator of molecular exchange between peripheral circulation and the tissues of the CNS (Dolman et al. 2005). Occupying both the ventricular system and the entirety of the subarachnoid space (between the pia and arachnoid mater surrounding the brain) is CSF (Brown et al. 2004). CSF is secreted via the choroid plexuses
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of the lateral, third and fourth ventricles and delineates the barriers between the choroid plexuses epithelium (between blood and ventricular CSF) and the arachnoid epithelium (between the subarachnoid CSF and blood) (Johanson et al. 2008). Thus given the critical locality of these barriers between brain tissue and its fluid filled interfaces, they are likely regions of investigation in the study of post-traumatic oedema.

1.3.2.1 Blood brain barrier

Structurally the BBB consists of three principal microvasculature elements, endothelial cells, pericytes and perivascular astrocytic end foot processes, which together with the surrounding neurons make up the 'neurovascular unit'. These cells serve to act as a selective ‘physical barrier’ to substances attempting to enter the brain via the circulation and as such its subsequent ‘breakdown’ in TBI leads to the genesis of vasogenic oedema. Further within this structural organisation is the suggestion of gliovascular units, in which individual astrocytes are seen to communicate directly with associative segments of the cerebral microvasculature, whilst also supporting the function of specific neuronal territories (Nedergaard et al. 2003).

Endothelia

Tight junction (TJ) proteins situated between adjacent cerebral endothelial cells are more complex within the brain, and unlike their peripheral counterparts, are composed of intramembranous particles which more effectively obstruct the intercellular cleft (Begley and Brightman 2003). Indeed so significant are the restrictions of brain endothelial TJs that they inhibit even the transport of smaller ions such as Cl⁻ and Na⁺, with typical cerebral transendothelial electrical resistance (TEER) beyond 1,000 ohm.cm² having been recorded (Butt et al. 1990). A number of molecules have been identified as being integral to the unique formation of brain TJ structure (Hawkins and Egleton 2006), although it is the presence of the transmembrane proteins claudin and occludin that are of particular importance to BBB
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permeability. The primary function of the protein occludin would appear to be one of TJ regulation, although it is also capable of linking with the zonula occludens protein 1 (ZO-1) (Yu et al. 2005). The claudin group of proteins are believed to contribute to the high TEER uniquely present within the brain (Wolburg and Lippoldt 2002). Junctional adhesion molecules (JAM-A, JAM-B and JAM-C) are also present in cerebral endothelia, which similar to the occludin family, are seen to be involved in the maintenance as well the formation of brain TJs (Yeung et al. 2008). It is in combination that these physical elements often force cerebral molecular movement via a transcellular route rather than transporting paracellularly between TJ routes.

In addition to inhibiting passage of certain substances, the BBB also serves to supply the brain with essential nutrients as well as mediating the efflux of certain waste by-products (Begley and Brightman 2003; Abbott 2005). Smaller molecules such as CO$_2$ and O$_2$ are capable of being transported freely through the BBB’s lipid membranes, which is also the entry route taken by synthetic lipophilic agents such as ethanol and barbiturates. Specific transport systems are also present on the abluminal and luminal membranes of brain endothelium that serve to regulate permissive transcellular transport of specific hydrophilic molecules across the barrier (Begley and Brightman 2003). Larger hydrophilic molecules, such as proteins and peptides are however, typically restricted in their transport, although important exceptions are sometimes made in instances of either adsorptive or receptor mediated transcytosis (Pardridge 2003). Ionic and fluid transitions from the circulation across the BBB are similarly highly selective, requiring specific ion channels and transporters (Abbott 2004). In comparison to blood plasma, ISF possess far lower protein, K$^+$ and Ca$^{2+}$ content and a far higher concentration of magnesium (Mg$^{2+}$). Cerebral endothelia additionally protect brain synaptic signalling from ionic fluctuations, commonly seen to arise in the systemic circulation. Furthermore, the BBB also assists in maintaining a separation between
neuroactive and neurotransmitter agents that often act differently within the periphery as compared to the CNS, therein permitting alternative usage between the two systems without crosstalk.

A number of extracellular and intracellular enzymes are reported to coexist along with BBB endothelia in order to provide a further means of metabolic barrier support (el-Bacha and Minn 1999). The importance of such barrier support is particularly evident when considering that intracellular enzymes such as the cytochrome group are capable of inactivating numerous toxic and neuroactive compounds, whilst ecto enzymes such as nucleotidases can metabolize essential ATP. Curiously, in contrast to the peripheral circulation, the AQP1 water channel is not generally observed in brain vascular endothelia (Dolman et al. 2005) although does appear in tumour cell capillaries not supported by astrocytic end feet. Observations such as these suggest an integral role of glia in signalling the down regulation of the water channel in adjacent brain endothelium.

**Astrocytes**

In investigating the mechanisms surrounding BBB transport, a great deal of information has been gained in understanding the unique contribution of the cerebral endothelium. However, equally of importance has been the identification of other cell types, which in combination with the microvasculature endothelium make up the BBB. Cerebral capillaries are surrounded by astrocytes, pericytes, microglia and neuronal processes and in larger vessels of the brain such as arteries, pericytes are replaced by a continuous smooth muscle cell layer (Iadecola 2004). Only recently has research begun to focus its attention on the essential function of perivascular astrocytic end feet, which amongst a host of other properties, have been shown to secrete chemical factors that modulate endothelial permeability, as well as mediate many of the unique features specific to the barrier morphology (Abbott 2002). Indeed, so intimate is
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the cell-to-cell communication between astrocytes and the cerebral endothelium that it would suggest that successful barrier maintenance hinges highly upon the maturational and localisation states of glia (Bauer and Bauer 2000). Evidence supporting such a symbiotic relationship between the two cell lines is particularly evident in cell culture studies where astrocytes are reported to be capable of up regulating endothelium TJ adhesion (Rubin et al. 1991), strengthening metabolic barrier efficiency (Haseloff et al. 2005) and increasing the expression of essential barrier transporters such as glucose transporter 1 (GLUT1) (McAllister et al. 2001). Astrocytes are also able to secrete a variety of chemical factors that are critical in supporting BBB cell functionality. Glial derived neurotrophic factor (GDNF) is known to enhance endothelial TJ adhesion and support neuronal survival (Igarashi et al. 1999), while astrocytic angiopoetin 1 (ANG1) is integral to successful vascular development and angiogenesis (Anderson and Nedergaard 2003).

Astrocytes are also reported to be key players in the transport of water across the BBB given that AQP4 (Agre 2006) is most prominently expressed on the end feet of glial processes. These glial end foot extensions tightly encircle the cerebral microvasculature forming a fine lamellae in close proximity to the exterior of endothelial surface (Kacem et al. 1998). Such positioning of the water channel, colocalised along with the Kir4.1, clearly implicates highly selective fluid transport and ionic regulation as a function of the astrocyte. Beyond the scope of the brain vascular barrier, astrocytic end feet adjacent to the glia limitans externa and the ventricular ependyma similarly express high concentrations of AQP4 (Badaut et al. 2002). Given the anatomical relationship of these cells and the complexity of chemical interactions between them, it is clear that multiple synergistic functions are involved in sustaining the BBB. Indeed, such is the bidirectional make-up of this relationship that studies have shown an endothelium induced differentiation of astrocytes via their expression of the leukaemia inhibitory factor (LIF) (Mi et al. 2001). In light of such a symbiotic relationship between the
barriers endothelium and the AQP4 rich glia, it is important to consider the role astrocytes play in the maintenance of barrier integrity and their part in the inhibition of traumatic oedema.

*Pericytes*

Pericytes are also considered as essential mediators of BBB permeability. Reciprocal molecular communications between endothelial cells and pericytes have been shown to mediate vascular development, remodelling and maturation (Levéen *et al.* 1994). Release of secretion factors by endothelia induces pericyte migration towards the endothelial cell wall and thus promotes vessel maturation. Endothelial produced platelet-derived growth factor (PDGF)-B is also seen to bind to its PDGFR-ß receptor on pericytes, which subsequently recruits additional cells near endothelia to promote vascular growth. Similarly, pericyte expressed ANG1 has been shown to bind to its endothelial tyrosine kinase Tie-2 receptor (Sundberg *et al.* 2002), therein promoting remodelling of the vasculature and prevention of vessel leakage via its action as an antipermeability factor (Thurston *et al.* 2000). The joint secretion of transforming growth factor beta 1 (TGF-ß1) from both pericytes and endothelium also inhibits proliferation and migration and hence contributes to barrier permeability function (Pepper 1997). Interestingly, studies have recently reported that pericytes often express cues that directly modulate protein attachment on astrocytic end feet (Armulik *et al.* 2010), thus suggesting that inhibition of pericytes may result in the abnormal distribution of AQP channels throughout glia.

1.3.2.2 *Blood CSF barrier*

As has been previously described, CSF is principally secreted via the choroid plexi into the ventricular space, as well as to a lesser extent derived from ECF produced by the cerebral vasculature communicating with the CSF through ependymal cells lining the ventricles
(Kimelberg 2004). The functional component of the choroid plexus is comprised of a number of brain capillaries ensheathed within a layer of differentiated ependymal epithelium, which unlike their BBB counterpart, are fenestrated and possess no TJs, thus permitting the transport of small molecules throughout (Laterra et al. 1999). In close proximity to this arrangement however, is a seamless layer of epithelial cells and TJs that link them, forming the blood CSF barrier. AQP1 is expressed in high concentrations on the apical membrane of this barrier, where microvilli face towards the CSF in the role of fluid secretion. Active transport, diffusion and facilitated diffusion into CSF are all known to occur in the choroid plexus (Zeuthen 1991; MacAulay and Zeuthen 2010), with some active transport of metabolites from the CSF into the blood. It is well known that the barrier’s epithelial layer is minimally hyperosmolar relative to its surrounding fluid. However, such a low permeability to water is principally believed to be driven by the basolateral membrane as its apical CSF face is localised by AQP1. Yet to make sense of how a region of high water transport can arise across an epithelium with low water permeability, a number of studies have shown the asymmetric localisation of ion channels, transporters and the Na⁺/K⁺-ATPase pump alongside AQP1 (MacAulay and Zeuthen 2010). Analogous to the joint relationship proposed to occur between AQP4 and the Kir4.1 in astrocytes, AQP1 in choroid epithelium is suggested to work in tandem with the K⁺/Cl⁻ cotransporter in order to drive water transport.(Kimelberg 2004)

The precise mechanism by which fluid is transported across the choroid plexus remains unknown. However, water fluxes across the apical membrane were reduced by up to 80% in mouse AQP1 knock-out studies with negligible decreases in overall CSF production (Oshio et al. 2005), suggesting that the water channel serves an integral role in CSF secretion. Given that recent research has demonstrated a critical association between CSF volume and ICP in
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TBI, it is relevant to consider the significant part AQPI may play in traumatic cerebral oedema.

1.4 Cerebral oedema

Although broadly recognised as a pathological condition of brain swelling, it was not until 1905 before a distinction was made between general cerebral swelling (brain oedema) and vascular derived oedema (Reichardt 1905). Brain oedema was then recognised as a complex life threatening condition, frequently resulting in an elevation of ICP, subsequent ischaemia, hypoxia, brain herniation and ultimately death. The neuropathologist Klatzo (Klatzo 1967) sought to further clarify brain oedema in his seminal work which defined two principle forms of brain swelling, namely vasogenic and cytotoxic. By definition, vasogenic oedema was proposed to occur in instances of injury to the cerebral vascular wall and therein lead to an “escape of water and plasma constituents” into the surrounding brain parenchyma as a result of BBB permeability. Cytotoxic (cellular) oedema was thought to arise as a consequence of “noxious factors” directly effecting brain tissue and thus result in intracellular swelling whilst the permeability of the vasculature principally remained undisturbed (Klatzo 1987). Critically however, Klatzo also suggested that although two very differing mechanisms were involved in the genesis of cerebral swelling, he believed that typically there was a coexistence of each following brain trauma. Research conducted since then has suggested that the generalised term cytotoxic oedema should be more accurately described as ‘cellular oedema’ as often intracellular K⁺ influx is the cause of widespread cell swelling (Walz 1992). Further refining this simple definition, more recent studies have identified that brain oedema is often influenced by a concert of highly intricate molecular, cellular and structural changes to BBB function, cell volume regulation and autodestructive mediators (Klatzo 1987; Betz et al. 1989), developing either independently or converging along shared pathological pathways.
Two decades after Klatzo’s initial, Fishman (Fishman 1975) went on to identify interstitial or hydrocephalic oedema, commonly observed in patients with hydrocephalus, while a fourth form of osmotic oedema was also defined, typically arising from osmotic substance imbalance driving an influx of water into cells (Klatzo 1994; Mongin and Kimelberg 2004). It is now widely recognised that there is a continuum from one form of oedema to another and as such, therapeutic interventions that only target a single aspect of this temporally evolving pathology are unlikely to be effective in eliminating brain swelling.

1.4.1 Classification of brain oedema

1.4.1.1 Vasogenic oedema and the role of AQP

The functional integrity of the BBB is of fundamental importance to the preservation of normal brain volume and cerebral fluid homeostasis. Hence structural and/or functional impairment to this barrier leads to the formation of vasogenic leakage. Damages to the BBB may arise either directly due to mechanical injury, indirectly via autodestructive mediators or through a combination of both (Klatzo 1994; Vink et al. 2003). Total net swelling of the brain must always involve a corresponding gain of water and as such will frequently lead to a rise in ICP once the limited capacities of the brain’s fluid compartments are no longer able to compensate for significant volume increases (Popp et al. 1996). Increased permeability of the BBB endothelial wall also permits the free transport of plasma proteins such as albumin into the parenchymal space. This protein movement is normally restricted because of intracellular structural components of the BBB such as endothelial TJs, transmembrane proteins claudin and occludin, as well as junctional adhesion molecules. Physiologically sustained hydrostatic pressure also contributes to the inhibited transport of excess solutes and fluid into the brain, with opposing osmotic forces between the differing compartments serving as additional means to drive water back into circulation (Rapoport and Thompson...
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1973). However, in the event of BBB breakdown this osmotic control is lost. Under these conditions, a permeable BBB results in the extravasation of intravascular fluid into the interstitial space, expanding the extracellular compartment.

The molecular disruption of the BBB has been shown to be mediated by a variety of substances such as vasoactive agents (Abbott 2000), endopeptidases, bradykinins (Plesnila et al. 2001), and more recently tachykinins such as SP (Vink et al. 2003). With the strategic localisation of AQP4 channels on perivascular astrocytic end feet, such BBB disruption is also thought to disrupt AQP4 channels, which have been implicated as playing a role in barrier integrity (Nico et al. 2001; Nicchia et al. 2004; Zhou et al. 2008). Molecular studies have shown that the water channels form via the regimented arrangement of intramembranous particles that collectively aggregate into structures known as orthogonal arrays (OAPs) (Rash et al. 2004). The formation of these arrays would appear to be contingent upon the presence of key proteins within the basal lamina such as alpha-1-syntrophin (α-syntrophin) and dystrophin protein complexes (Neely et al. 2001; Amiry-Moghaddam et al. 2003). Given the connection between the water channel and the proteins of the basal lamina, it would seem logical that AQP4 plays an essential role in the ability of astrocytes to maintain BBB integrity. Indeed Haj-Yasein and colleagues demonstrated, via the use of a GFAP promotor in mice targeted specifically to delete AQP4 from astrocytes, that transport of water across the BBB occurs exclusively through AQPs, with the deletion of the water channel compromising barrier permeability (Haj-Yasein et al. 2011). Recent research has also shown a correlation between the activity of AQP4, matrix metalloproteinase-9 (MMP-9) and hypoxia-inducible factor 1-alpha (HIF-1α), known mediators of BBB dysfunction (Rosenberg et al. 1998; Rosell et al. 2006; Feiler et al. 2011; Higashida et al. 2011). Their roles are nonetheless distinct. Pharmacological inhibition of HIF-1α and MMP-9 resulted in attenuation of barrier permeability and an associated
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reduction in brain swelling. However, suppression of AQP4 water channel activity in the setting of vasogenic leakage did not ameliorate brain oedema (Wang et al. 2012), suggesting that the water channel may actually play a part in resolving vasogenic oedema.

Cytokines such as TNF-α are also believed to directly increase BBB permeability via their direct action on brain endothelium (Abraham et al. 1996), as well as by triggering astrocytic release of interleukin-1 beta (IL-1β) and thus produce an immunoregulatory loop involving endothelin 1 (ET-1) and ZO-1 (Didier et al. 2003). Importantly however, elevated levels of TNF-α post TBI have been shown to correlate with an increased expression of astrocytic AQP4 (Ding et al. 2009; Zou et al. 2012), most probably via an interaction with HIF-1α. Although it would not seem to be the case that AQP4 directly mediates BBB integrity, given its close proximity to intercerebral vessels and interaction with molecular mediators of barrier permeability, it is quite possible that there is an indirect connection with barrier integrity.

In terms of vasogenic oedema, studies utilising a principally vasogenic cold lesion injury model have demonstrated that the presence of AQP4 is correlated with a resolution of existing vasogenic oedema (Papadopoulos et al. 2004). Similarly, studies using a middle cerebral artery occlusion (MCAO) model demonstrated a reduction in AQP4 expression within astrocytes of the ischaemic core, while in contrast, an elevation in AQP4 expression in glial end foot process was observed in surrounding penumbra (Frydenlund et al. 2006). Water channel expression patterns of this form suggest that AQP4 serves as a limiter to water influx in the cytotoxic ischaemic core during the period directly following artery occlusion, whereas it plays a role in ameliorating vasogenic oedema in the penumbra. It seems evident that the expression of AQP4 is regionally dependent on the type of oedema and that any pharmaceutical modulation of the channel should be mindful of its local role.
1.4.1.2 Cytotoxic oedema and the role of AQP

Cytotoxic or cellular oedema (Barzo et al. 1997), is characterised by sustained intracellular water accumulation irrespective of BBB permeability (Gaetz 2004; Amiry-Moghaddam et al. 2010). It results in the swelling of cells due to increased permeability of the cell membrane to Na\(^+\) and K\(^+\), bioenergetic failure, defective ionic pump activity and sustained uptake of osmotically active solutes (Klatzo 1987; Vink et al. 1994). Under physiological conditions, influx of osmotically active Na\(^+\) is counterbalanced by its active energy dependent elimination via the Na\(^+\)/K\(^+\)-ATPase, thus preventing intracellular accumulation of osmoles which would result in a flow of water into cells (Kimelberg 1995). However, in pathological settings as seen post TBI, shifts in the brains metabolism and consequential energy failure disturb cellular ionic pump equilibrium and thus increase the intracellular accumulation of Na\(^+\). Functional impairment of mitochondria in response to hypoxia and ischaemia further propagates the already ensuing ionic pump failure and subsequently aggravates the deleterious effects of cellular oedema. Sustained disruption of brain electrochemical stability early after TBI because of EAA release (Amara and Fontana 2002) also leads to excessive activation of metabotropic glutamate receptors, as well as glial and neuronal transporters, resulting in a significant influx of Na\(^+\), Ca\(^{2+}\), Cl\(^-\) and hydrogen (H\(^+\)) and thus contributing to the formation of glial and neuronal cell oedema (Amara and Fontana 2002). Moreover in the context of such pathological disruptions to the typical transport of EAAs and solutes, impairments to intracellular pathways are further comprised by the Ca\(^{2+}\) mediated activation of secondary messenger cascades and the increased production of damaging ROS.

Given the fundamental role of AQPs in the transmembrane transport of water, downregulation of the channels at a time of bioenergetic depletion and cellular swelling may serve to inhibit the early onset of intracellular fluid influx. Moreover, given the integral part
AQP4 rich astrocytes play in the reuptake of extracellular glutamate and K⁺ (Walz 1992; Walz 2000), maintenance of glial metabolism via the inhibition of cellular swelling post TBI is probable to serve a role in the amelioration of impaired neuronal activity. Indeed cerebral neurons are significantly outnumbered by glia 20:1 and can swell beyond five times their typical volume. Hence the attenuation of astrocytic swelling is likely to assist in reducing neural damage in the setting of cellular oedema (Kimelberg 1995). Recent studies have also suggested that AQP4, Na⁺/K⁺-ATPase and the metabotropic glutamate receptor 5 (mGluR5), assemble to form a macromolecular complex/transporting microdomain in astrocytes (Illarionova et al. 2010). Findings such as these are of considerable importance when contemplating the role of AQP4 in cytotoxic swelling, as the formation of such a microdomain would play a highly significant role in the regulation of cellular water volume and K⁺ homeostasis. Recent research would support such a notion, demonstrating that in a water intoxication mode of oedema, AQP4 knockout mice exhibit significantly reduced levels of atypical brain moisture content when compared to controls (Manley et al. 2000; Zador et al. 2007). Conversely, in studies that increased the expression of AQP4 in the setting of a principally cellular oedema, it was found that upregulation of the channel in cytotoxic events increased brain water content (Katada et al. 2012). Consistent with this observation, studies employing a transient middle cerebral artery occlusion (tMCAO) injury model demonstrated a distinct loss of AQP4 despite retained expression of astrocytic GFAP (Friedman et al. 2009). These results suggest that physiological downregulation of AQP4 in glia surrounding an ischaemic core occur as a defensive response to the onset of regionalised cellular oedema.

Thus the nature of brain AQP expression in pathological conditions would appear to be highly contingent upon injury form. Indeed as described prior, the oxygen sensor HIF-1α, often upregulated in pathological conditions such as hypoxia or ischaemia, has been associated with an increase in both MMP-9 and AQP4 (Mu et al. 2003; Kaur et al. 2006; Li
et al. 2008). Importantly, such studies employed the use of brain injury models that were well characterised in terms of exhibiting temporal profiles of both ischaemia and oedema. Taken together these results would therefore strengthen the view that downregulation of AQP4 activity attenuates cytotoxicity driven cellular swelling.

1.4.1.3 Osmotic or ionic oedema and the role of AQP

Neither cytotoxic or vasogenic by nature, osmotic or ionic oedema arises in the presence of an intact BBB when the osmolality of blood plasma drops below that of the brain, thus resulting in a flow of water from the vasculature into the ISF. Hypo-osmolar conditions of this form result in reduced serum Na\(^+\) concentrations, which allow water to enter the brain and distribute throughout the extracellular space. Cerebral swelling of this form may also occur when plasma osmolality is normal and brain tissue ionic levels are high, such as is the case in the core of lesions following infarct, haemorrhage or contusions (Milhorat 1992; Katayama and Kawamata 2003). In addition to frequently causing astrocytic swelling, osmotic brain oedema may also significantly increase ISF volume whilst spreading via bulk flow along the normal pathways, typically increasing the rate at which extracellular markers are cleared into the CSF (Wald et al. 1978). Thus the occurrence of osmotic oedema can trigger a notable rise in the rate of CSF formation (DiMattio et al. 1975) without any direct contribution from the choroid plexus.

Previous studies have shown that increases in total brain volume due to osmotic swelling are typically modest (Wasterlain and Torack 1968) as oedema of this form not only is vented swiftly but also fails to involve cytotoxic mechanisms. However, despite its common ease of resolution, the onset of osmotic oedema is reduced in correlation with a reduction in AQP4. \(\alpha\)-syntropin knockout studies in mice demonstrated a significant delay in the formation of oedema during induced hyponatremia (Amiry-Moghaddam et al. 2004), while prolonged
periods of brain osmotic oedema increases perivascular AQP4 immunoreactivity (Vajda et al. 2000; Yeung et al. 2009). Taken together, these results suggest that inhibition of perivascular AQP4 during the initial stages of osmotic swelling attenuates the net flow of fluid into the brain from blood plasma, whilst once ionic imbalance is reached the water channel is upregulated so as to facilitate ISF clearance.

1.4.1.4 Hydrocephalic or transependymal oedema and the role of AQP

Oedema of this form is most appropriately characterised by either noncommunicating hydrocephalus in which there is an inhibition of the typical flow of CSF within the ventricular space, or in its communicating variant where an obstruction arises distal to the ventricles, resulting in an impaired absorption of CSF into the subarachnoid compartment (Gjerris and Børgesen 2000). During such events elevations in intraventricular pressure lead to a migration of CSF through the ependyma into the periventricular white matter, raising the extracellular fluid volume (Milhorat et al. 1970; Milhorat et al. 1970). Rich in Na⁺ content the ensuing oedematous fluid damages the structural integrity of the periventricular regions such that there is diffuse separation of glia and axons. Remaining astrocytes subsequently swell and ultimately undergo a progressive atrophy (Milhorat et al. 1970). Chronic variations in hydrocephalus are reported to increase the hydrostatic pressure within the white matter, which damages axons and consequentially recruits a significant microglial response. Focal or diffuse destruction of the ependyma may also arise with distortion and collapse of periventricular capillaries (Bigio 1993). The precise aetiology of hydrocephalic oedema is not of direct relevance to this thesis, however, it is relevant to note such pathological changes when considering the role of AQPs in these settings. Few publications have studied the correlation between AQPs and hydrocephalic oedema formation, however, water channels are now being considered as a significant part of this process. Whole brain and cortex
examinations of hydrocephalic rats has demonstrated a significant decrease in the expression of AQP1 within the choroid plexus, whilst AQP4 was moderately elevated (Paul et al. 2009; Kalani et al. 2012; Castaneyra-Ruiz et al. 2013). Studies of transient hydrocephalic oedema have shown that expression of AQP4 & 1 are significantly altered by both region and time throughout the transforming face of the condition (Skjolding et al. 2010). It is therefore reasonable to suggest that AQP provides a modulatory effect on the stability of ependyma during the progression of hydrocephalus. The reduction of choroid AQP1 serves to decrease CSF production at a time of superfluous fluid influx, whilst simultaneously AQP4 is increased to facilitate water clearance, thus demonstrating a likely synergistic effect between the two channels in the attenuation of brain swelling.

1.4.1.5 Ischaemia in oedema and the role of AQP

The development of ischaemia following severe TBI is well known to involve a combination of cytotoxic and vasogenic events (Hossmann 1994; Papadopoulos et al. 2004; Tourdias et al. 2011; Shi et al. 2012). Brain oedema is commonly observed to occur when CBF goes beneath 10ml/100g/min, resulting in the consequential failure of essential ionic pump activity. It is nonetheless difficult to generally characterise the temporal profile of AQP expression in these events. Metabolic deficit, a prelude to cytotoxicity and potentially caused by ischaemic events, is seen to be attenuated by decreased water channel expression whereas subsequent BBB permeability and the genesis of vasogenic oedema has been shown to correlate with increased expression of AQP4. The precise interval at which either cellular or vasogenic oedema arises is often not clearly understood. Typically during the first 5 h of ischaemia, BBB integrity is maintained. If CBF is not re-established during this period no net increase of brain water content is observed despite the existence of cellular oedema (Betz et al. 1989). Upon ‘reperfusion’ or return of CBF, there is a swift increase in ECF accumulation and
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corresponding rise in brain water content in conjunction with a sharp elevation in ICP. Two principal factors are seen to determine the timing of these phases, namely the severity and initial duration of reduced CBF, and the time at which CBF is restored (Avery et al. 1984). Critically however, altered expression of AQP4 has been shown to correlate with this biphasic nature of brain ischaemia (Manley et al. 2000; Zador et al. 2009).

1.4.1.6 Traumatic cerebral oedema

As discussed previously, TBI often comprises a mixture of cerebral insults such as hematoma, subarachnoid haemorrhage, DAI, focal lesions and contusions. Similarly, the genesis and type of traumatic cerebral oedema that results is highly dependent on the primary injury sustained. Classical views have long suggested that vasogenic oedema predominates soon after injury due to a distinct disruption of the BBB (Marmarou et al. 1994; Marmarou et al. 1994). However, more recent research has found that although barrier permeability is often breached in close proximity to the site of focal contusion (Bullock et al. 1999), widespread brain oedema is principally cellular by nature within hours after the diffuse impact (Dixon et al. 1991; Marmarou et al. 2006).

1.4.1.7 Focal, perifocal and diffuse traumatic cerebral oedema

Cerebral contusions are typified by a haemorrhagic core with necrotic tissue that is no longer adequately perfused. CBF within the surrounding perifocal regions is also characteristically diminished. Traditional studies of traumatic brain oedema have employed cryogenic injury models which produce a well-defined necrotic focus surrounded by a permeable BBB (Reulen et al. 1978), and whilst these experiments have accurately reproduced vasogenic swelling, they fail to adequately mimic clinical scenarios. Research involving controlled cortical impact models (Dixon et al. 1991) have demonstrated that focal and perifocal
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traumatic injuries are predominately cytotoxic. The controlled cortical impact model produces a focal necrotic core surrounded by an expanding oedematous zone, which when subsequently imaged by magnetic resonance (MRI), including diffusion weighted imaging and a map of the apparent diffusion coefficient (ADC), clearly demonstrates a principally cellular swelling (Stroop et al. 1998; Unterberg et al. 2004). As such current dogma is that a predominately cellular oedema occurs following TBI (Marmarou et al. 2006). MRI of patients shortly after injury clearly revealed increased ADC values (increased extracellular water) solely within the core of the contusion, with this region being notably surrounded by a widespread decrease in ADC levels (Kawamata et al. 2000; Maeda et al. 2003). Findings such as these do provide substance to the theory of a primarily cytotoxic oedema soon after TBI. However, positron emission tomography (PET) studies have not shown support for a critical reduction in CBF during the subacute stages post TBI (Diringer et al. 2002) and hence the contribution of ischaemia to cellular swelling remains controversial. What role the type of injury plays in the temporal profile of oedema is unclear, with focal versus diffuse models potentially giving different results (Marmarou et al. 2000). Yet despite the variability in these studies, it is clear that the expression of brain AQP channels are in response to the permutations typically observed in the continuum of oedema and as such play a key role in the regulation of cerebral fluid homeostasis.

1.4.2 Mixed models of traumatic cytotoxic oedema and the role of AQP4

As mentioned previously, cytotoxic brain oedema has been shown to be a prevailing form of cerebral swelling in many forms of TBI (Kimelberg 1995; Barzo et al. 1997; Stroop et al. 1998; Marmarou et al. 2000; Marmarou et al. 2006). While this form of oedema is commonly associated with ischaemia, it can also occur independently irrespective of CBF (Marmarou et al. 2000). Mechanical injury associated with TBI often impairs the synthesis of essential
brain n-acetyl-aspartate (NAA) by mitochondria, typically triggering numerous energy reliant pathways to fail (Signoretti et al. 2001). There is a resultant increase in intracellular Ca\(^{2+}\), as well as an opening of mitochondrial permeability transition pores. Transmembrane electrogentic gradients are lost, oxidative phosphorylation is uncoupled and the swelling of mitochondria ensues. Energy dependent ion pumps fail, membrane stability breaks down and excess water begins to enter cells.

The movement of water into and out of cells is thought to be mediated via the Kir4.1 channel in association with the AQP4 channels, which are colocalised in astrocytic end feet (Nagelhus et al. 1999). However, the joint relationship between these channels has been brought into question given that in pathophysiological situations, dissociation in the expression of the Kir4.1 and AQP4 can occur (Saadoun et al. 2005). The most significant evidence to support this view has been shown in α-syntrophin knockout mice who demonstrated a delayed onset of oedema (Vajda et al. 2002), suggesting that AQP4 mislocalisation on astrocytic end feet plays a far greater part in the movement of water across cell membranes alone, than with its proposed coordinated effect with the Kir4.1. Indeed, multiple studies involving principally cytotoxic events, in which the BBB remained intact, show that decreases in perivascular AQP4 expression attenuate the progression of injury induced cellular swelling (Nakahama et al. 1999; Manley et al. 2000). Further insight into how perivascular AQP4 serves in the inhibition of cellular oedema can be seen in the channels’ altered expression during the biphasic nature of ischaemia. In experimental models of cerebral ischaemia, expression of AQP4 was observed to reduce within the initial 48 h following injury (Sato et al. 2000; Yamamoto et al. 2001) however, was significantly upregulated at time points thereafter (Taniguchi et al. 2000). Upregulation of AQP4 is seen to occur in pathologies in which BBB integrity is breached, namely brain tumour metastasis and focal cold lesions (Saadoun et al. 2002). Taken together these results suggest that perivascular AQP4 serves as a route for
intracellular fluid transport in events where the BBB remains intact whilst in injuries that have increased BBB permeability, upregulation of the channel facilitates extracellular fluid clearance. However, the temporal profile of AQP4s 4 & 1 expression after diffuse TBI is poorly understood, and a far more complete study is required in order to understand the precise role that these channels serve in brain fluid dynamics post head injury.

1.4.3 AQP4 and mediators of brain oedema

It is widely accepted that a number of secondary mediators are involved in the formation of oedema post TBI, however the identification and characterisation of the role of AQP4s in the genesis and resolution of cerebral swelling would seem critical. As such, mention will be made of agents previously described to both interact with AQP4 and play a role in the development of brain oedema.

1.4.3.1 Hypoxia-inducible factor 1-alpha

Hypoxia-inducible factor 1-alpha (HIF-1α), a heterodimeric transcription factor, is an oxygen sensor known to play an integral role in the maintenance of body tissues (Semenza 2000). Under oxygen replete conditions, HIF-1α is expressed at negligible levels due to swift proteasomal breakdown (Jaakkola et al. 2001). However, during pathological events that result in oxygen depletion, chemical degradation of the protein cannot occur and thus it rapidly accumulates (Wang et al. 1995; Li et al. 2008). In some circumstances the build-up of HIF-1α in ischaemic or hypoxic tissue may well promote adaptive cell mechanisms for survival (Bergeron et al. 1999). Indeed research has shown that following an upregulation of HIF-1α during periods of hypoxia, as typically seen to occur in cytotoxicity, expression of AQP4 is increased (Kaur et al. 2006). At 5 h post diffuse TBI levels of the transcription factor are reported to be at their lowest compared to all other injury time points however, they
significantly increase thereafter, peaking at 24 – 48 h following injury (Ding et al. 2009; Higashida et al. 2011). Inhibition of HIF-1α has also been shown to correlate with a reduction in AQP4 and the attenuation of traumatic cerebral swelling (Shenaq et al. 2012), confirming that (Shenaq et al. 2012) the transcription factor does contribute to the genesis of brain oedema.

Research in brain tumours has also drawn a link between increased HIF-1α expression and the upregulation of vascular endothelial cell growth factor (VEGF) (Carmeliet and Storkebaum 2002), which increases the localisation of AQP4 in astrocytic end feet facing the glia limitans externa (Rite et al. 2008). Such findings suggest that in the event of BBB disruption, AQP4 adjacent to the glia limitans is physiologically upregulated in concert with other mediators such as TNF-α and VEGF so as to facilitate extracellular fluid clearance.

1.4.3.2 Tumour necrosis factor-alpha

Proliferation of glial cells and their progenitors is a key part of the neuroinflammatory process, often resulting in the inhibited repair of neurons. In conjunction with the hypertrophy of astrocytes, excessive production of the cytokine tumour necrosis factor alpha (TNF-α) has been reported to occur in microglia (Zou et al. 2012), along with a paralleled increase in astrocytic AQP4 (Alexander et al. 2008). An association has also been shown between TNF-α and the endopeptidase MMP-9 (Hosomi et al. 2005; Jia et al. 2010), a known mediator of BBB dysfunction. Other studies have suggested a direct role of TNF-α in brain oedema (Shohami et al. 1997; Hosomi et al. 2005) given its participation in opening the BBB (Yang et al. 1999). Indeed inhibition of the cytokine was observed to correlate with a decreased expression of MMP-9 production and thus attenuated injury induced BBB permeability. Such findings are of significant relevance to the role AQP4 plays in the pathophysiology of brain swelling, given that elevations in TNF-α which parallel BBB
dysfunction and ergot vasogenic oedema, are seen to correlate with increased expression of the water channel.

1.4.3.3 *Matrix metalloproteinases*

Matrix metalloproteinases (MMPs) are a group of extracellular zinc- and calcium-dependent endopeptidases well known to degrade essential extracellular matrix (ECM) proteins, most notably the neurovascular basal lamina and TJ proteins of the BBB (Wang and Tsirka 2005). Numerous studies have demonstrated an elevation in MMPs post TBI (Wang *et al.* 2000; Noble *et al.* 2002) as well as following cerebral ischaemia (Burk *et al.* 2008). Thus MMPs, and in particular MMP-9, have been suggested to play a major role in the pathogenesis of neurological disorders (Yong *et al.* 2001). Indeed it is widely accepted that elevations in the endopeptidase following TBI increase capillary permeability and subsequently result in the formation of vasogenic oedema (Rosenberg and Navratil 1997). Consistent with this view, inhibition of MMP-9 post TBI has been shown to ameliorate barrier permeability (Higashida *et al.* 2011), reversing the loss of essential vascular basal lamina protein.

1.5 *Aquaporins*

Eighty percent of the brain is water and although BBB permeability has been studied extensively, far less is known about cerebral fluid physiology. Classically it was suggested that water transport occurred singularly and slowly through phospholipid bilayers (Ishige *et al.* 1987) or additionally via small pores on endothelial cells (Hawkins and Egleton 2006). However, given that water passes through cells at a speed far beyond the capabilities of simple diffusion, another route for fluid transport was suspected to exist. Indeed early studies into red blood cells clearly demonstrated that their barrier membranes are more permeable to water than is possible through lipid bilayer diffusion alone (Herve *et al.* 2008). In 1988,
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Nobel Prize laureate Peter Agre described the first water channel, termed Aquaporin 1 (Moon et al. 1993; Lang et al. 1998). Subsequently, at least 13 AQPs have been found in mammals, where it is suggested that these ‘water channels’ evolved so as to actively regulate swift osmotic diffusion across the plasma membrane (Bilz et al. 1999). To date three primary subgroupings of the AQP gene family exist, namely aquaporins, aquaglyceroporins and so termed unorthodox aquaporins (Finn et al. 1996). The aquaporin (AQP) group is singularly responsible for the movement of water alone, whereas the aquaglyceroporins are able to serve as a route for water, glycerol and other nonpolar solutes. The unorthodox aquaporins are seen to transfer ions as well as water. Structurally the AQP channel assembles within cell membranes as tetramers (Kumagai et al. 1987) with each monomer possessing approximately 300 amino acids and six transmembrane domains, with cytoplasmic C- and N- termini, capable of transporting water in both directions (Badaut et al. 2002; Zelenina 2010).

Typically AQPs increase water permeability across epithelia, permitting swift flow of water in accompaniment to active salt transport (Agre 2006). Under normal physiological conditions AQPs are typically involved in the maintenance of cellular water homoeostasis, enabling the movement of water and other small solutes across biological membranes. However, the more recent discovery of these channels also serving as essential players in the formation and resolution of cerebral oedema (Zador et al. 2007; Saadoun et al. 2008; Zador et al. 2009; Kimura et al. 2010; Saadoun and Papadopoulos 2010; Verkman 2012) has illustrated their clear potential as targets of pharmaceutical development in the modulation of brain swelling (Migliati et al. 2009; Devuyst and Yool 2010). Of the 6 AQPs expressed in the rodent brain (AQP1, AQP3, AQP4, AQP5, AQP8, AQP9), AQP4 & 1 are clearly the most prominent and as such they will be the channels of focus within this thesis.
1.5.1 Cerebral aquaporins 4 & 1

1.5.1.1 Aquaporin 4

Aquaporin 4 (AQP4), a subtype of the AQP family, is the most abundant channel in the brain, largely due its high concentrations on the end foot process of astrocytes (Badaut et al. 2000; Jablonski and Hughes 2006). In order to retain such polarity it is believed that AQP4 interacts with an adaptor protein (α-syntropin), forming a complex with the cytoplasmic protein dystrophin and anchoring it to the cell membrane (Jablonski et al. 2004). Anatomically the channel is strongly expressed at brain tissue-fluid interfaces between the parenchyma and major fluid compartments, most notably in perivascular (brain–blood-barrier), glia limitans (brain–subarachnoid cerebrospinal fluid) and ependymal (brain–ventricular CSF) astrocytes (Araque et al. 1999; Zador et al. 2009). Being localised at such junctions between the brain tissues and fluid would therefore suggest that AQP4 serves as an integral part of cerebral hydrodynamics. Indeed previous research has demonstrated that shortly after TBI perivascular AQP4 expression rapidly decreases, however, is simultaneously upregulated at the basolateral surface of ventricular ependymal cells (Ghabriel et al. 2006), supporting the notion that the water channel serves as a facilitator in the clearance of extracellular fluid. Yet such notable redistribution of the protein following brain injury would appear to be highly dependent upon the type of trauma sustained. As described in section 1.4.1, AQP4 knockout attenuates brain swelling following acute water intoxication (Manley et al. 2000), a form of cellular oedema without the loss of BBB integrity. A similar effect is observed after permanent middle cerebral artery occlusion. In experimental models of hydrocephalus, AQP4 knockout animals showed significantly higher levels of brain swelling as compared to wild types (Papadopoulos et al. 2004), promoting the idea that in such injuries the channels play an essential part in the resolution of excess extracellular fluid. In predominately vasogenic...
models of brain injury, such as cortical freezing, AQP4 knockout animals demonstrated an increase in cerebral oedema formation when compared to wild types.

Beyond pathology, AQP4 is also believed to play an essential part in typical brain hydrodynamics. Indeed further to the classic cerebral fluid circulation proposals (see Aquaporins and brain fluid movement), AQP4 is now thought to serve in the essential role of water reabsorption from the extracellular space along with the traditional view of bulk flow clearance into the CSF, as well as transport back into the cerebral vasculature (Reulen et al. 1978; Marmarou et al. 1994).

### 1.5.2 The role of AQPs in the brain

AQP4 and α-syntropin knockout studies in mice have revealed three notable roles for the water channels within the brain, namely fluid movement, facilitation of neuronal propagation and astrocyte migration as well as metabolism. AQP1 on the other hand is principally believed to be involved in the secretion of CSF.

#### 1.5.2.1 Aquaporins and brain fluid movement in pathology

As previously discussed, the human intracranial cavity (approximately 1400ml), comprising the brain parenchyma (1200ml), cerebrospinal fluid (CSF) (100ml) and intravascular (100ml) (Tait et al. 2008) compartments, is composed of a highly organised architecture of neurons, supportive glia and complex neuronal-vascular relationships. Fluid moves between these compartments in response to alterations in hydrostatic or osmotic pressure. In many pathologies the brain itself may swell due to an excess accumulation of water within the parenchyma, with AQPs suggested to play differing roles according to the type of oedema. Mounting evidence strongly suggests that inhibition of AQP4 in principally cytotoxic models of injury, including water intoxication, focal cerebral ischaemia (Manley et al. 2000) and
bacterial meningitis (Yang et al. 2006) may attenuate brain oedema formation. In contrast, in models of injury that have an open BBB with the development of vasogenic oedema, AQP4 activity is thought to promote movement of ECF water into the vasculature (Manley et al. 2000; Amiry-Moghaddam et al. 2003; Amiry-Moghaddam et al. 2004; Huber et al. 2007; Moeller et al. 2009). This view is somewhat controversial given that it promotes the idea of interstitial water being eliminated via an AQP4 dependent route, as opposed to classically proposed bulk flow clearance into the CSF and arachnoid granulations (Reulen et al. 1978).

In terms of pharmacological manipulation of AQPs, it seems that AQP4 inhibition may be the suitable option in principally cytotoxic brain swelling, whilst upregulation of the channel may be beneficial in vasogenic oedema and hydrocephalus.

1.5.2.2 Aquaporins and astrocyte migration

It has well been established that glial cell scarring (see Astrogliosis) is a significant obstacle to neuronal regeneration (Brooks et al. 2000) and as such, the involvement of AQPs in cell migration may provide a potential target for improving outcomes following TBI. Indeed inhibition of AQP4 may enhance synaptogenesis and axonal sprouting post injury by delaying the onset of glial scarring (Nedergaard et al. 2003). Previous studies have shown that glial cell migration was significantly impeded in AQP4 knockout mice (Niemietz and Tyerman 2002), whilst disruption of AQP1 reduced angiogenesis and cell migration. The precise mechanism associated with this action remains unclear, although it has been suggested that intracellular ion influx at the forward edge of migrating cells increases localised cytoplasm osmolality, thereby inducing water influx (Saadoun et al. 2005). Such water entry results in a local rise in hydrostatic pressure, which in expanding the cell membrane, thus facilitates cell migration towards regions of hypo osmolality. Inhibition of AQP4 impairs the ability of astrocytes to migrate towards the site of injury (Auguste et al. 2009).
2007), thus preventing further glial scarring. In predominantly cytotoxic events, downregulation of AQP4 may well serve a dual role by inhibiting the uptake of excess extracellular fluid and attenuating glial cell migration.

1.5.2.3 Aquaporins, neuronal activity and Kir4.1

As described above, AQP4 is colocalised with the inward rectifying potassium channel, Kir4.1 on astrocytic end feet (Badaut et al. 2000; Badaut et al. 2000; Jablonski and Hughes 2006), where it is suggested to play a key role in glial cell K\(^+\) homoeostasis. Indeed delayed glial cell K\(^+\) reuptake has been demonstrated in mouse AQP4 knockout studies (Binder et al. 2006), supporting the idea of the channel playing a part in the facilitation of water diffusion along K\(^+\) concentration gradients. Similarly, other AQP4 knockout research has shown altered glutamate release following high K\(^+\) depolarisation (O'Connor et al. 2006). Typically both glutamate and K\(^+\) are released during neuronal activity and are cleared from the extracellular space via astrocytes. The colocalisation of AQP4 with the Kir 4.1 is likely to work alongside the glial cell EAAT in providing the fundamental basis for water, ionic and synaptic glutamate homeostasis. However, in a setting of post-traumatic bioenergetic depletion, cellular swelling and excitotoxicity, the role of AQP4 becomes even more important. Studies using AQP4 or α-syntropin null mice have demonstrated higher seizure thresholds in response to consultants or electrode stimulation (Reulen et al. 1977; Binder et al. 2006), further implicating an electrochemical relationship between K\(^+\) transport through the Kir4.1 and water via AQP4.

Research in oocytes has demonstrated that AQP4 does not possess a voltage or K\(^+\) sensor (Marmarou et al. 2006), suggesting that the influence of the ion on the water channel may be mediated by changes in extracellular K\(^+\) content or cell membrane potential. Other studies have proposed that AQP4, the Na\(^+\)/K\(^+\)-ATPase and mGluR5 work in concert with one another
in astrocytes so as to regulate cellular water volume and K\(^+\) homeostasis (Illarionova et al. 2010). It may also be that K\(^+\) activation of AQP4 is partially mediated via stimulation of the voltage-sensitive Na\(^+\), HCO\(_3^-\) cotransporter (NBC), also found abundantly in astrocytes (Nimmo et al. 2004). Elevations in extracellular K\(^+\) lead to depolarization of astrocytic membranes and the subsequent uptake of HCO\(_3^-\). Increased intracellular HCO\(_3^-\) then activates soluble adenylyl cyclase, increasing cAMP production (Hamm et al. 1994) and activating protein kinase A (PKA), and potentially the subsequent phosphorylation of AQP4. Activation of the protein kinase family results in a rapid decrease in AQP4 water permeability (Elliott and Jasper 1949) with prolonged activity resulting in internalisation of the channel (Haj-Yasein et al. 2011).

Taken together these findings would suggest that astrocyte spatial buffering of extracellular K\(^+\) levels, integral to typical neuronal activity, relies on a functional AQP4 channel and that alteration in the regulation of the AQP4 channel during brain oedema is likely to influence functional outcome post TBI.

1.5.2.4 Aquaporin localisation and alpha-syntrophin

Alpha-1-syntrophin (α-syntrophin), a member of the dystrophin protein complex, is colocalised with AQP4 and is believed to serve a key role in the channel’s adhesion to the membrane of astrocytes. The dystrophin protein complex is comprised of a large membrane assembly connecting the cytoskeleton to the extracellular matrix (Neely et al. 2001). On the cytoplasmic side, dystrophin binds to dystrobrevin and α-syntrophin to it. Recent studies have demonstrated that AQP4 anchors itself to a PSD95-Drosophila disc large-ZO-1 protein (PDZ) structural domain, located on α-syntrophin via a C-terminal (Jablonski et al. 2004). The presence of AQP4 appears to be contingent on its adhesion to α-syntrophin, given that α-syntrophin knockout mice demonstrate significant loss of the water channel (Engelborghs et
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al. 1998; Amiry-Moghaddam et al. 2004). Similarly, α-syntrophin knockout mice demonstrate a delayed onset of oedema in principally cellular models of brain swelling (Vajda et al. 2002). The key polar localisation of AQP4 on perivascular, ependymal and subpial astrocytic process, therefore appears dependent upon the activity of α-syntrophin, enabling the protein to serve as the fundamental cornerstone of brain tissue-fluid interfaces.

1.5.2.5 Aquaporin 1

Aquaporin 1 (AQP1) is principally expressed in the apical membrane of the choroid plexus, where is serves to play an integral role in the secretion of CSF (Kimelberg 2004). AQP1 is not expressed within the endothelia of brain capillaries (Dolman et al. 2005), as it is in the peripheral circulation. As such it has been long suggested that brain astrocytic end feet, in close proximity to cerebral capillaries, signal adjacent endothelia to switch off expression of AQP1. However, the precise mechanism involved in this process remains unclear.

The rate at which the choroid plexus transports water and salt into the brain via the production of CSF is significant and depends heavily on the active transport of Na⁺ across its AQP1 rich apical membrane. It is therefore not surprising that the altered expression of AQP1 correlates with the onset of hydrocephalus. Indeed, so critical is the role of AQP1 in CSF dynamics that inhibition of the channel swiftly results in decreased ICP and reduced CSF formation along with hydrocephalic oedema (Castaneyra-Ruiz et al. 2013).

1.5.3 Treatment approaches in cerebral oedema

The primary aim of neurosurgical techniques and critical care management in cerebral oedema is to ameliorate elevated ICP, maintain global and regional CBF, address bioenergetic and metabolic deficits and inhibit secondary neuronal injury (Siesjo and Wielcoh 1985). However, at their most rudimentary level, current treatment approaches for
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Brain swelling have failed to take into account the mechanisms underlying cerebral oedema formation (Tominaga and Ohnishi 1989). Present clinical management of brain oedema follows a distinct set of guidelines involving optimal patient head and neck positioning to facilitate intracranial venous outflow, establishing sufficient airway clearance, avoiding systemic hypotension and dehydration and the maintenance of normothermia (Tominaga and Ohnishi 1989; Boake et al. 2000; Ding et al. 2001). Therapeutic interventions include controlling hyperventilation, administering osmotherapies and diuretics, and targeting cerebral metabolism (Yamamoto et al. 1999; Yool 2007; Devuyst and Yool 2010). In the most severe cases, surgical decompression remain a mainstay of treatment even today (Kallakuri et al. 2003). The efficacy of such interventions is significantly limited as they fail to address the mechanisms associated with brain swelling. In this respect, development of pharmaceutical agents directed at AQPs offer promise of a non-invasive, mechanistic approach that could revolutionise critical care. Indeed given that contemporary approaches for this life-threatening condition remain inadequate (Bullock et al. 1999), an ideal agent in treating cerebral oedema would be one that mobilises and or inhibits its formation, provides swift onset, has a sustained duration of action and carries minimal adverse effects.

1.6 Synopsis

This thesis will examine the potential role that modulation of AQP water channels plays in the development of cerebral oedema following diffuse traumatic brain injury. Recent research has implicated AQP channels in oedema formation following acute CNS injury. The focus of this thesis will be to establish whether there is a significant role of AQP channels following experimental TBI in rats, and specifically whether modulation of this AQP activity is associated with the attenuation of oedema and functional deficits. Emphasis will firstly be focussed on characterising AQP 4 and 1 immunoreactivity within the brain after TBI.
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Thereafter, this thesis will investigate the effects of AQP channel receptor agonist and antagonist administration following diffuse TBI on both oedema formation and functional outcome. Finally, the timing of administration of the AQP modulators will be investigated to ascertain the therapeutic window of each following TBI, and whether combined, yet temporally distinct, administration of both compounds offers a therapeutic advantage.

A brief introduction will precede each experimental investigation, along with a summary of the methodological protocol, which will be fully outlined in Chapter Two. Although each chapter will report results specific to that chapter, it is expected that many of the results will have implications not only for the present investigation but also for other aspects raised in the thesis. This will result in some overlap across the chapters in interpretation and discussion. Finally, a concluding general discussion will integrate the major conclusions drawn from each chapter.
2 MATERIALS & METHODS
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2.1 Animals

2.1.1 Ethics

All experimental procedures presented in this thesis were conducted in accordance with the guidelines established by National Health and Medical Research Council and approved by the University of Adelaide Animal Ethics Committee (M-2012-157 R).

2.1.2 Housing

A total of 204 adult, male Sprague-Dawley rats weighing between 380-420g were randomly assigned to sham, vehicle or treatment groups. Animals were group housed in a conventional rodent room on a 12 h day-night cycle and given ad libitum access to standard rodent pellets and water. A standard 3-day acclimation time was observed post transportation and prior to use in any experiments.

2.1.3 Anaesthesia

2.1.3.1 Isoflurane

Isoflurane obtained as an aqueous solution (Veterinary companies of Australia, Kings Park) was stored at room temperature within a drug safe away from direct sunlight and heat. General anaesthesia was induced by placing the animal in a translucent induction chamber and delivering 5% Isoflurane in 0.3L/min O\textsubscript{2} and 1L/min air for 5 min via a calibrated vaporiser. Rats were subsequently intubated and mechanically ventilated using a rodent ventilator (Harvard) and anaesthetised to a surgical level using 1.5% Isoflurane in 0.6L/min O\textsubscript{2} and 2L/min N\textsubscript{2} at a stroke rate 95bpm and volume of 2mL/min.
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2.1.3.2 **Lignocaine**

Lignocaine in aqueous solution (Lignocaine hydrochloride, 2% Mavlab, Australia) was kept at room temperature within a drug safe away from direct sunlight and heat. It was applied to all animals to provide local anaesthesia prior to surgical incision. Lignocaine was administered subcutaneously using a 25gauge, 12.5mm needle at a dose of 0.5mL of 4% solution per site of injection.

2.1.3.3 **Pentobarbital (Lethobarb)**

Pentobarbital in aqueous solution (pentobarbitone sodium, 60mg/mL; Rhone Merieux) was stored at room temperature within a drug safe away from direct sunlight and heat. Administered ip with a 25gauge, 12.5mm needle at a dose of 60 mg/kg, pentobarbital was used as a means of humanely euthanising animals. Needle insertion was maintained in the left caudal region of the abdominal cavity so as to avoid puncture of vital organs.

2.1.4 **Impact-acceleration diffuse TBI**

2.1.4.1 **The impact acceleration model of diffuse TBI**

Marmarou's well-established impact acceleration model of diffuse TBI is one of the most widely used rodent models of diffuse head injury today (Marmarou *et al.* 1994). The trauma apparatus consists of a plexiglas cylindrical tube through which a 450 g brass weight falls freely by gravity from a designated height. The animal’s exposed skull is protected from skull fracture by a stainless steel disc (3mm in depth by 10mm in diameter) which is adhered centrally to the skull between the bregma and lambda sutures using a cyanoacrylate based adhesive. The probability of skull fracture is minimal as the mass-height combination from impact is evenly distributed across the cranial vault, generating a brain acceleration of 900G.
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and a brain compression gradient of 0.28mm. The accelerating weight is decelerated after impact using a 30x12x12cm foam bedding whereupon the animal is placed in the prone position with the steel disc positioned precisely under the plexiglas cylinder. The 450g brass weight is released from a height of two meters directly onto the metallic disc. The possibility of a second impact is avoided by quickly removing the foam platform with the animal still attached once impact has been delivered. Possible death primarily arises as a consequence of respiratory depression of the brain stem and as such mechanical ventilation via intubation significant lowers the mortality rate (Marmarou et al. 1994). This model of injury has been shown to produce widespread axonal injury, reduced cerebral blood flow, elevated intracranial pressure, alterations in tissue energy metabolism, loss of cerebral autoregulation, oedema and persistent functional deficits (Marmarou et al. 1994; Vink et al. 1994; Engelborghs et al. 1998; Prat et al. 1998; Kallakuri et al. 2003; Marmarou 2003). (Marmarou et al. 1994)

2.1.4.2 Surgery and injury induction

Diffuse TBI was induced using the well-established impact acceleration model as previously described (Marmarou et al. 1994) (Fig 2.1). Anaesthetised animals were placed on a thermostatically controlled heat pad in a prone position to ensure that body temperature remained constant throughout all surgical procedures. Isoflurane anaesthetic was maintained at 1.5% during surgery and then reduced to 1% following head trauma. After shaving the dorsal surface of the head and prior to incision, 0.5mL of the local anaesthetic lignocaine was subcutaneously injected at the surgical site. A subcutaneous scalp incision of approximately 1-2cm was then made along the midline, exposing the underlying skull. The skin and muscle were then retracted and the bone cleaned and dried with a cotton bud so as to visualise the bregma and lambda sutures. A stainless steel disc (3mm in depth by 10mm in diameter) was
then adhered to the skull with a quick dry cyanoacrylate based adhesive, placed centrally
between the exposed sutures along the midline. Adhesion of this disc served jointly as a
protective mechanism so to reduce the incidence of skull fracture whilst simultaneously
distributing the weight of impact equally across the cranial surface. Rats were then
temporarily disconnected from mechanical ventilation, placed in the prone position on a
30x12x12cm foam bed and the injury induced by dropping a 450g brass weight from a height
of two metres through a cylindrical plexiglas tube directly onto the fastened disc. The foam
bedding was swiftly removed once initial impact had been made so as to avoid any rebound
contact. Immediately after injury all animals were returned to mechanical ventilation and
placed on to the thermostatically controlled heat pad. At this time, the disc was removed from
the skull, the cutaneous incision sutured, treatments administered (at the designed time
points) and anaesthesia gradually terminated to permit recovery.

2.1.4.3 Animal exclusions

In all instances of animal skull fracture due to weight drop, the animal was immediately
euthanised with a lethal dose of sodium pentobarbital (60 mg/kg) and eliminated from the
investigation. A total mortality of n = 12 rodents were recorded throughout these studies as a
result of either surgical complications or skull break and were consequently excluded from
the data presented.

2.1.4.4 Sham controls

Sham controls throughout these studies were surgically prepared as identical to protocols
applied to injury and treatment groups, although were not subject to diffuse TBI. At the
conclusion of the sham surgery normoxia was restored and the animals immediately weaned
from the ventilator (Yamamoto et al. 1999).
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2.1.4.5 Hypoxic period

Respiratory depression is often observed in both the clinical and experimental setting of head injury (Yamamoto et al. 1999) and carries with it multiple secondary complications such as hypoxia and increased ICP. As such a set period of controlled post traumatic brain oxygen deprivation is applied in the current experiments to minimise experimental variability, whilst also closely replicating known secondary injury factors contributing to worsened outcome. Following induction of injury, animals were immediately re-connected to the ventilator and a 30 min period of hypoxia induced via inhalation of 1.5% Isoflurane in 240mL/min O2 and 2L/min N2. At the conclusion of the hypoxic period, normoxia was restored and the animals immediately weaned from the ventilator (Yamamoto et al. 1999).
Figure 2.1 Impact acceleration model of diffuse TBI in rats as illustrated by the author.
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2.1.4.6 Post-surgery recovery

During the post-surgical recovery period, animals were weaned off the ventilator and allowed to recover from anaesthesia on the heating pad. Once conscious and ambulatory, the rats were subsequently returned to their home cage with ad libitum access to food and water. Although not requiring any special post-surgical care, all animals were closely monitored during the post-operative period to ensure that there are no unexpected adverse effects.

2.1.5 Drug preparation and administration

All animals were randomly allocated to either treatment, sham or vehicle treatment groups and administered with the appropriate intervention at predetermined time points. Specific details of drug administration are provided in each chapter. For iv administration while still anaesthetised, an elastic rubber band was placed around the proximal end of the rat's tail and pulled together tightly with artery clamps so as to form a tourniquet, enabling constriction of the vein and easy localisation for the iv administration. For drug administration at later time points, the ip injection was administered in the left caudal region of the abdominal cavity so as to avoid puncture of vital organs.

2.1.5.1 AQP modulators AqB013 and AqF026

Specific AQP antagonists and agonists have recently been developed making it possible to investigate the ability of these drugs to treat post-traumatic cerebral oedema. The arylsulphonamide AqB013 (G. Flynn, Spacefill Enterprises, LLC, Tucson, AZ) has been shown to have a defined inhibitory effect on AQP4 & 1 water channels in vitro (Yool 2007), functioning as an antagonist when administered at a dose of 0.8mg/kg (Migliati et al. 2009). Alternatively the compound derivative AqF026 has been shown to bind at a separate site to specifically enhance AQP4 channel opening when given at dose of 0.2mg/kg (Devuyst and
Yool 2010), thereby serving as an AQP4 agonist. Previous research has also shown that neither compound is systemically toxic when administered as detailed. Both compounds were originally made up as 100 mM stock solutions in dimethyl sulfoxide (DMSO) and stored at 4°C. Experimental solutions of \( \leq 100\mu\text{M} \) final concentration in 0.1% DMSO were prepared by slow addition of drug stock into a final volume of isotonic saline during rapid mixing. For concentrations > 100\( \mu\text{M} \), drugs were sonicated in saline with 0.1% DMSO final. Vehicle solutions for control groups was composed of equal volume 0.1% DMSO in saline.

**Pharmaceutical mechanism of action**

Pharmacologically, the novel compounds used within this study are structurally based around the loop diuretic bumetanide. The physiological mechanism of loop diuretics is such that they act on the \( \text{Na}^+\text{-K}^+\text{-2Cl}^- \) co-transporter, inhibiting sodium and chloride reabsorption via competition for the \( \text{Cl}^- \) binding site. Calcium reabsorption is also partially dependent upon concentrations of intracellular \( \text{Na}^+ \) and \( \text{Cl}^- \) and therefore loop diuretics may also play a role in inhibiting its reabsorption. Prior studies have shown that systemic levels of bumetanide are tolerated without overt toxicity at doses as high as 100 mg/kg, when administered over both a long and short duration (McClain and Dammers 1981).

Previous in silico docking of AqB013 supported the notion of an intracellular candidate for its binding site in rat AQP4, suggesting that the block involves occlusion of the water pore at the cytoplasmic side (Yool 2007). AqF026 is thought to upregulate the activity of the channel via alterations in the OAPs subunit structure, induced by the binding of a ligand that increases the rate of water permeation through the pore. Such allosteric modulation of the pore is seen to enhance the probability of the channel opening, without physically interfering with water flux (Devuyst and Yool 2010). Given the physiological scaffold upon which these
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compounds are based, it makes them attractive agents in the pharmaceutical attenuation of secondary injuries, yielding mechanistic efficacy with minimal side effects.

2.2 Post traumatic assessments

2.2.1 Analyses of oedema

Cerebral oedema was calculated by the wet weight-dry weight methodology measured using a Mettler Toledo – HG63 halogen moisture analyser. At preselected time points, animals were reanaesthetised with 5% Isoflurane in 0.3L/min O₂ and 1L/min air and once a surgical level of anaesthesia was reached (as assessed by pinch reflex) they were decapitated and their brains swiftly removed from the skull. The olfactory bulb was then removed and each hemisphere separated using a 22-blade scalpel. The left hemisphere was placed on a 4.8g glass superfrost slide, previously weight tared in the moisture analyser, before being sliced evenly into three smaller segments. A second superfrost slide was then positioned on top of the first and clamped together until brain tissue was evenly distributed across each surface. Both slides were then placed into the moisture analyser, wherein it was weighed and then heated dry over a 10 min period. The same protocol was also performed on the right hemisphere in a second moisture analyser immediately after the first hemisphere had begun to heat. Brain moisture content was determined as a percentage of tissue water content as calculated by the wet weight-dry weight formula (Elliott and Jasper 1949).

\[
\% \text{ water} = \frac{(\text{wet weight} - \text{dry weight}) \times 100}{\text{wet weight}}
\]

The HG63 halogen moisture analyser performs this calculation automatically, however individual hemisphere wet and dry weights were also recorded into an Excel spreadsheet.
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2.2.2 **BBB permeability**

BBB permeability was assessed and quantified using the established spectrophotometric method for Evans Blue (EB) albumin complex extravasation, as well as using semiquantified ex vivo autofluorescent imaging (O'Connor *et al.* 2005). Subsequent to injury and 30 min prior to the predetermined time of transcardical perfusion animals were administered EB dye (MW 69,000; Sigma, E-2129) intravenously via the tail vein under general anaesthesia (0.4mL/animal; 0.10g EB in 10mL phosphate buffered saline, pH 7.4). 10 min prior to perfusion, animals were ip injected with 0.1mL of heparin (Pfizer, Australia; 1000 IU/mL) so as to facilitate the rinsing of EB from the circulation and subsequently transcardically perfused with 100mL of 0.9% NaCl. Animals were then decapitated, their brains extracted, the olfactory bulb removed and each hemisphere separated before being weighed. Each hemisphere was then placed in a vial and homogenised in 7.5mL of phosphate buffered saline. 2.5mL of trichloroacetic acid (Sigma, T-0699) was subsequently added to the samples which were then stored overnight at 4°C. Samples were then centrifuged at 1000 r.p.m for 30 min. An Ultraviolet–visible spectrophotometer was then employed to quantify the EB absorbency at 620nm in the resulting supernatant. The quantity of EB, expressed as μg/g of brain tissue, was subsequently calculated against a previously calibrated standard curve.

A further subset of animal brains were imaged prior to homogenisation using a Xenogen IVIS Imaging System 100 equipped with a Cy5.5 filter set so as to qualitatively measure EB autofluorescence intensity at a wavelength between 615-665nm. Identical illumination settings (lamp voltage, filters, lens focussing) were used for all images, and fluorescence emissions were normalised to fluorescent efficiency where the RGB value of each pixel in an image represented the fractional ratio of fluorescence emitted per incident of photons excited. High counts of fractional fluorescent ratios were representative as either red or green colour.
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channel pixels, and low to none as blue channel pixels, ranging between the values of 0-255. In quantifying the EB in an image, all scans were post processed on an identical sized pixel canvas, relevant background noise, blue colour channels removed and all red and green colour channel pixel counts tallied.

2.2.3 Evaluation of motor deficits

Post traumatic motor deficits were assessed in all groups via an unblinded assessor using the rotarod device as described in detail by Hamm and colleagues (Tominaga and Ohnishi 1989; Hamm et al. 1994; Boake et al. 2000; Ding et al. 2001). Consisting of a motorised rotating assembly of 18 rods (1mm in diameter), the rotarod device requires animals to grip in order to maintain their ability to traverse the rods. These rods thereby incorporate a grip strength component to the task making the rotarod task one of the most sensitive tests for assessing motor deficits following TBI (Hamm et al. 1994; Nimmo et al. 2004).

The rotational speed of the rotarod ranges from 0 - 30 revolutions per minute (rpm) and is determined by a control box that is operated by the assessor. Animals were placed on the rotarod, which at first remained stationary for the first 10 seconds, after which the rotational speed was increased by 3 rpm every 10 seconds for a maximum total time of 120 seconds. The rotarod score recorded for each individual animal was the length of time that the animal could remain walking on the rotarod or until the task ended. The task ended if the animal gripped onto the rods without walking for 2 consecutive revolutions, or completely fell off the rotarod. Animals were trained once daily for a five-day period prior to injury to establish a normal, pre-injury baseline. Motor outcome testing commenced at 24 hours post injury and was conducted at the same time each day for a 7-day period.
2.3 **Histological assessment**

2.3.1 **Transcardial Perfusion**

At the preselected time points of 5 h, 48 h and 7 d following TBI, animals were removed from their home cage and reanaesthetised using 5% Isoflurane in 0.3L/min O₂ and 1L/min air. Once the animal was unconscious, 0.4mL of Pentobarbital was administered ip to the left caudal region of the abdominal cavity, inducing a deep general anaesthesia. 0.1mL of Heparin was then administered ip so as to facilitate blood flow. When pain reflexes were absent by pinch, rats were placed in the supine position on a steel perforated grid plate and a bilateral thoracotomy performed so as to expose the heart. A blunt 19 gauge, 37mm needle was inserted into the apex of the left ventricle and guided into position within the ascending aorta before introducing 100mL of 10% formalin (pH 7.4). After commencing transcardial perfusion the right atrium was incised so as to permit vascular flushing. Perfusion was complete once the fluid leaving the right atrium became clear, the body rigid, the liver pale in colour and the lungs inflated. Following perfusion, animals were left intact for 1 h, and then decapitated, their brains removed and post-fixed in formalin for 1 week.

2.3.2 **Paraffin embedding and sectioning**

After 1 week of post fixation, brains were placed in a Kopf rodent tissue blocker (Kopf; USA) and 2 mm coronal sections cut. The resultant sections were subsequently processed over a 24 h period sequentially with 20 min of graded ethanol (50%, 70%, 80%, 95%, 100%, 100%), followed by 2 xylene baths over 1.5 h each and finally paraffin baths of increasing time allocations at (30, 60, 60, 90 min). The 2 mm sections were then embedded in paraffin wax and stored. When required, paraffin blocks were cut into 5μm serial sections using a
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microtome (Microm; HM330) before being floated onto a water bath and mounted on silane-coated glass slides where they were permitted to dry overnight.

2.3.3 **Haemotoxylin and Eosin Staining**

Haemotoxylin and eosin (H&E) staining was performed on brain sections to enable observation of morphological changes to neurones. Given their significance to the functional motor pathway, the regions of interest were the primary and secondary motor cortex, the caudate putamen (striatum) and the globus pallidus (Hall and Lindholm 1974). The cortex directly beneath the site of impact was also examined. Sections of interest were dewaxed via gentle heating so as to molten the residue and then placed into 2 changes of xylene and 100% alcohol for a period of 2 min each to clear any remaining excess wax. Sections were then rinsed in water, submerged in Mayer's haemotoxylin for 5 min and subsequently washed once again under running water. Following this, they were placed into lithium carbonate for 10-15 seconds until appearing blue, so as to fix the haemotoxylin stain. They were then rinsed again under water and then submerged in eosin for 1 minute. Finally, sections were submerged in 2 changes of ethanol and cleared with 2 changes of histolene for 2 min each so as to dehydrate. Sections were then mounted and permitted to dry before being imaged under an Olympus light microscope and then digitally scanned with a Hamamatsu nanozoomer.

2.3.4 **Immunohistochemistry procedures**

For immunohistochemical assessment of AQP4 and 1, sections were first dewaxed via 2 baths of xylene and dehydrated in 2 baths of 100% alcohol for a period of 2 min each. Nonspecific peroxidase activity was then blocked by submerging sections in methanol solution/0.5% hydrogen peroxide for 30 min so as to reduce background staining. The sections were then rinsed twice in PBS (pH 7.4) and then heated by microwave in retrieval buffer (pH 6.0) 5L dH2O / 10.5g citric acid / 25ml 5N NaOH, for 3.5 min on medium to high,
and then a further 10 min on medium (Power level 2; for two racks of section slides). The sections were then permitted to cool at room temperature to below 40°C and then washed once more in PBS. They were then incubated at room temperature in 30% normal horse serum (NHS) for a period of 30 min in a humidified chamber. Sections were then incubated overnight with the appropriate primary antibody for AQP4 (mouse monoclonal anti-aquaporin 4 antibody; ab9512; 1:200; Abcam) or for AQP1 (rabbit polyclonal anti-aquaporin 1 antibody; ab2219; 1:4000; Millipore). The following day sections were washed twice in PBS (pH 7.4) and treated with the secondary antibody in NHS for 30 min (biotinylated goat anti-rabbit IgG antibody; BA-1000; 1:250; Vector). They were subsequently incubated with a streptavidin horseradish peroxidase conjugate (SPC; 1:1000; Pierce), diluted in 1:1000 NHS for 60 min and thereafter reactively visualised with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma), containing 0.01% H₂O₂ for 7 min. Sections were then thoroughly washed in water and counterstained with haematoxylin before being dehydrated in 2 baths of alcohol and cleared with 2 changes of histolene. After mounting, sections were allowed to air prior to being imaged under an Olympus light microscope and then digitally scanned with a Hamamatsu nanozoomer. All antibodies were optimised by dilution and pre-screened for efficacy via the staining of negative controls. Positive protein controls for the protein being investigated were also visualised prior to tissue staining to make sure that any staining was due to binding of the primary antibody rather than nonspecific, background binding.

For immunohistochemical assessment of brain albumin content, protocols mirrored visualisation of AQP4 and 1 with the exception of prior retrieval. Following overnight incubation with the appropriate primary antibody (rabbit polyclonal anti-human albumin; 1:1000; DakoCytomation), sections were blocked with NHS and washed in PBS (pH 7.4) before being treated with the secondary antibody in NHS for 30 min (biotinylated goat anti-rabbit IgG Antibody; BA-1000; 1:250; Vector). Following this, sections were similarly
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incubated with SPC (Pierce, 1:1000) and thereafter visualised with DAB, counterstained with haematoxylin, dehydrated, cleared, mounted and imaged.

2.3.5 Antibodies

2.3.5.1 Aquaporin 4

A mouse monoclonal anti-aquaporin 4 antibody (ab9512; Abcam) with a stock dilution of 1:200 in NHS was employed for visualisation of AQP4 in perivascular and periventricular astrocytic end feet, as well as subpial process adjacent to the glia limitans externa.

2.3.5.2 Aquaporin 1

A rabbit polyclonal anti-aquaporin 1 antibody (ab2219; Millipore) with a stock dilution of 1:4000 in NHS was utilized for visualization of AQP1 within ependymal cells of the choroid plexus and any other observable areas within the cortex.

2.3.5.3 Albumin

A rabbit polyclonal anti-human albumin (DakoCytomation) with a stock dilution of 1:1000 in NHS was used to determine the magnitude of brain albumin content. Cellular proteolysis of albumin is also an indicator of cellular health and hence is of relevance when considering the effect oedema formation has on brain metabolism.

2.3.6 Colour Deconvolution

Automated colour deconvolution analysis was conducted on all immunohistochemistry slides imaged via the nanozoomer. As previously described in detail elsewhere (Helps et al. 2012), the protocol entails digital imaging of whole coronal 5μm slide sections which are then exported and subject to colour deconvolution. DAB staining is separated from the
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haemotoxylin and eosin colour vector images and the amount of DAB precipitate (antigen content) estimated using the histogram analysis of the DAB image using a mathematical formula, determining the percentage of weighted DAB (DABwt%) per image. The histogram numerical export is then imported into an Excel spreadsheet where the quantity of DAB is calculated by summing pixel frequency as a product of pixel intensity and then expressing this as a percentage of the total (Helps et al. 2012). Given the key widespread nature of the diffuse brain injury employed in these studies, whole immunohistochemistry slices were analysed for quantification of DAB. Whereas brain tissue fluid interfaces appropriate to the focus of this thesis, namely perivascular (brain–blood-barrier), glia limitans (brain–subarachnoid cerebrospinal fluid) and ependymal (brain–ventricular CSF) astrocytes, were chosen for qualitative investigation of AQP4 & 1 water channel activity, as well as albumin leakage.

2.4 Statistical Analyses

Results are presented as mean ± standard error (SEM). A repeated measures two-way analysis of variance (ANOVA) followed by a Bonferroni’s multiple comparisons posthoc test was performed on the cerebral oedema measurements and motor outcome where a p value of < 0.05 was considered to be statistically significant. A one-way ANOVA with a Bonferroni’s multiple comparisons posthoc test was employed to assess EB autofluorescence by spectrophotometry, ex vivo imaging and semiquantification of DAB visualisation on sectioned slides. Prism Graphpad software was used to conduct all statistical analyses.
3 A HISTOLOGICAL ANALYSIS OF AQP4 & 1 IN TRAUMATIC BRAIN INJURY
3.1 Introduction

Cerebral water homoeostasis is of pivotal physiological and clinical importance. Under physiological conditions cerebral water transport is tightly regulated so as to maintain a homoeostatic balance between the parenchyma, vasculature and CSF (Kimelberg 2004). However, traumatic disruption to such equilibrium inevitably results in an increase in cerebral water content, significantly contributing to the pathophysiology of brain oedema (Fishman 1975). The unyielding nature of the skull provides little capacity to compensate for intracranial volume changes and once surpassing a narrow threshold results in a rapid increase in ICP (Saadoun and Papadopoulos 2010). Elevations in ICP typically lead to a decrease in cerebral perfusion pressure (CPP), which by impairing essential blood flow result in hypoxia, ischaemia, tissue necrosis and ultimately death. Despite the clinical importance of brain water balance, little is known about the molecular mechanisms responsible for alterations in cerebral fluid homoeostasis. Thus present therapeutic interventions are limited to intravenous administration of hyperosmolar agents, which often demonstrate limited efficacy, and as a last resort, neurosurgical decompression (Boake et al. 2000). More recently, a family of molecular water channels termed aquaporins (AQP) have been identified within the brain (Badaut et al. 2002) and may play a crucial role in the mechanisms of cerebral oedema.

AQP4s are integral transmembrane proteins which are known to participate in the reabsorption and production of cerebral fluid and provide the principle route for cellular water transport (Agre et al. 1998) (Tait et al. 2008; Amiry-Moghaddam et al. 2010). In the brain AQP4 is the most widely expressed member of this protein family, particularly prominent at the blood-brain and brain-CSF tissue fluid interfaces (Badaut et al. 2000; Badaut et al. 2002). Highly concentrated at the end foot processes of astrocytes, AQP4 is found to line the cells of the
glia limitans and ventricular ependyma, surfaces in direct contact with the CSF and subarachnoid fluid filled space. AQP4 is also expressed in high levels within the end foot processes of astrocytes encircling the cerebral vascular wall, providing it with immediate access to cerebral blood flow (CBF) (Badaut et al. 2002; Amiry-Moghaddam et al. 2010). Another subtype of the AQP group, AQP1, is selectively expressed within the cells of the choroid plexus, where it serves an integral role in the secretion of CSF (Brown et al. 2004; Brodbelt and Stoodley 2007). Irrespective of the immediate importance these channels may play in maintaining brain fluid homoeostasis, few studies have characterised the role AQPs may serve in cerebral oedema following diffuse TBI.

Within the rodent brain, several members of the AQP family are present, the most significant of these being AQPs 4 & 1 (Badaut et al. 2002). Their role in cerebral oedema is unclear, with expression either decreasing or increasing depending on the type of oedema that is present. In cellular models of brain oedema, such as water intoxication and focal cerebral ischaemia, AQP4 knockout mice demonstrate significantly lower levels of brain water content (Manley et al. 2000; Friedman et al. 2009). In experimental hydrocephalic rats, there is an upregulation of subpial AQP4 accompanied by decreases in the expression of AQP1 within the choroid plexus (Kalani et al. 2012; Castaneyra-Ruiz et al. 2013). Conversely in vasogenic injury models in which the blood-brain-barrier (BBB) becomes permeable, upregulation of AQP4 would appear to significantly attenuate levels of cerebral oedema (Papadopoulos et al. 2004). Diffuse TBI has been previously shown to cause an initial vasogenic oedema with a subsequent predominant cellular phase (Marmarou et al. 2006; O’Connor et al. 2006), although it is likely that the observed oedema is in reality a mixture of the two. The role of the AQP channels in this situation is unclear. Accordingly (Walz 1992; Illarionova et al. 2010), this study will characterise the histological expression of AQP4 & 1 following diffuse TBI in rats at both 5 h and 48 h after TBI, which represent the maximal
points of vasogenic and cellular oedema in this model, respectively (Marmarou et al. 1994; O'Connor et al. 2006; Helps et al. 2012).
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3.2 Methods

3.2.1 Surgery and injury induction

Moderate to severe diffuse injury was induced using the well-established impact acceleration model (Marmarou et al. 1994) as detailed (Marmarou et al. 1994) in Chapter 2. Briefly, impact acceleration injury was induced in intubated, Isoflurane anaesthetised Sprague-Dawley rats (n=10; 380 ± 420g) by dropping a 450g brass weight from a height of two meters onto the animal’s skull. The skull was protected from fracture by a 3mm in x 10mm diameter stainless steel disc, fixed centrally to the animals exposed skull with a cyanoacrylate based adhesive. At the time of impact, the head of the animal was supported by a 30x12x12cm foam bedding which acted to decelerate the brass weight following impact. Immediately after injury, all animals were exposed to a 30 min period of hypoxia induced via intubated inhalation of Isoflurane 1.5%, 240mL/min, O₂, 2L/min N₂, before being returned to a normoxic mixture and immediately weaned from the ventilator. Throughout surgery and during the immediate recovery phase rats were placed on a thermostatic heatpad. After regaining ambulation, animals were returned to their home cages with restored access to food and water ad libitum.

3.2.2 Drug preparation and administration

All animals were randomly allocated to either sham surgery or injury and vehicle treatment groups. Vehicle animals were administered an appropriate dose mg/kg of DMSO as described previously in Chapter 2. For iv administration while still anaesthetised, an elastic rubber band was placed around the proximal end of the rat's tail and pulled together tightly with artery clamps so as to form a tourniquet, enabling constriction of the vein and easy localisation for the iv administration. For drug administration at later time points i.e. pre-perfusion, an ip
injection was administered in the left caudal region of the abdominal cavity so as to avoid puncture of vital organs.

3.2.3 **Immunohistochemistry**

For AQP4 & 1 immunohistochemistry (n=5/group) animals were injured and perfusion fixed with 20% formalin (pH 7.4) at the preselected time points of 5 h and 48 h after TBI. Another subset of animals (n=5) were surgically prepared although not injured (shams). Once extracted, all brains were fixed for a further week in 10% formalin, blocked, sectioned and subsequently stained using Anti-Aquaporin 4 antibody [4/18] (ab9512) (Abcam) and Anti-Aquaporin 1 Antibody (aB2219) (Millipore) as described in detail in Chapter 2. Digitally acquired nanozoomed images of slides were then evaluated for protein content via the automated colour deconvolution analyses. For repeated immunohistological analyses across all groups coronal sections labelled incrementally A-M were obtained from a slice matrices, with a 2mm interval, enabling precise and consistent blocking prior to microtome slicing. Repeated sections of E were chosen for slicing and subsequent analyses as anatomic ally these regions most appropriately included the relevant brain tissue fluid interfaces namely, ventricular and choroid plexus whilst also perivascular at the site of injury and glia limitans externa.

3.2.4 **Statistical Analysis**

Colour deconvolution data was analysed for statistical significance using a one-way ANOVA with a Bonferroni’s multiple comparisons posthoc test and presented as mean ± standard error (SEM).
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3.3 Results

3.3.1 AQP4 immunoreactivity post diffuse TBI

Brain tissue fluid interfaces, namely perivascular (brain–blood-barrier), glia limitans (brain–subarachnoid cerebrospinal fluid) and ependymal (brain–ventricular CSF) astrocytes were chosen for investigation of AQP4 water channel activity (Fig 3.1).

The cortex of sham animals directly beneath the potential impact site demonstrated moderate perivascular AQP4 immunoreactivity, reflecting normal baseline expression of AQP4 in astrocyte end foot process (Fig 3.2 A). At 5 h after injury (Fig 3.3 A) there is a decrease of AQP4 immunoreactivity surrounding the cerebral vasculature which by 48 h following TBI (Fig 3.4 A) had increased to above expression levels seen without injury (sham animals).

Periventricularly, moderate AQP4 immunoreactivity was observed in sham animals (Fig 3.2 B) and at 5 h following TBI (Fig 3.3 B). However, at 48 h (Fig 3.4 B) following injury there was a clear increase in periventricular water channel expression.

The glia limitans bordering the subarachnoid space showed moderate AQP4 immunoreactivity in sham animals (Fig 3.2 C). However, at 5 h post TBI (Fig 3.3 C) there was a notable increase in water channel protein activity within this region. This increase was still apparent increased along the glia limitans and in some peripheral regions of the surrounding parenchyma at 48 h following injury (Fig 3.4 C).
Figure 3.1 AQP4 immunoreactivity anatomical regions of interest. 
(A) Perivascular AQP4 directly beneath the site of impact. (B) Periventricular region. 
(C) Glia limitans externa.
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Figure 3.2 AQP4 stained section from uninjured sham Sprague-Dawley rats. (A) Moderate perivascular AQP4 immunoreactivity directly beneath the potential site of impact. (B) Moderate periventricular AQP4 immunoreactivity. (C) Moderate AQP4 immunoreactivity of the glia limitans bordering the subarachnoid space.

Figure 3.3 AQP4 stained section from injured Sprague-Dawley rats at 5 h post diffuse TBI. (A) Faint perivascular AQP4 immunoreactivity directly beneath the site of impact. (B) Moderate periventricular AQP4 immunoreactivity. (C) Moderate-intense AQP4 immunoreactivity of the glia limitans bordering the subarachnoid space.

Figure 3.4 AQP4 stained section from injured Sprague-Dawley rats at 48 h post diffuse TBI. (A) Intense perivascular AQP4 immunoreactivity directly beneath the site of impact. (B) Intense periventricular AQP4 immunoreactivity. (C) Intense AQP4 immunoreactivity of the glia limitans bordering the subarachnoid space.
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Colour deconvolution analyses of whole coronal sections stained for AQP4, showed a significant decrease \( p < 0.001 \) \( (7.660) \) in water channel immunoreactivity at 5 h following diffuse TBI \( (12.280 \pm 0.849) \) as compared to sham (uninjured) animals \( (19.940 \pm 1.155) \) (Fig 3.4). This decrease was transient with AQP4 expression returning to sham levels by 48 h after injury. While the AQP4 expression at 48 h post injury \( (21.260 \pm 0.583) \) was significantly \( p < 0.001 \) \( (9.430) \) greater than the 5 h timepoint, there was no statistical difference between AQP4 immunoreactivity in sham animals and those assessed at 48 h following injury, even though there was slightly greater immunoreactivity as reflected in the qualitative evaluation (Figs 3.2 and 3.4).
**Figure 3.5** Colour deconvolution of AQP4 immunoreactivity in sham and injured rats at 5 and 48 h post diffuse TBI.

Vehicle treated animals at 5 h post diffuse TBI demonstrated significantly less AQP4 immunoreactivity as compared to sham controls *** ($p < 0.001$). Comparably at 48 h following injury, vehicle administered groups showed a significant increase in AQP4 as compared to animals assessed at 5 h +++ ($p < 0.001$).
3.3.2 AQP1 immunoreactivity post diffuse TBI

Since expression of AQP1 occurs primarily within the apical membrane of the choroid plexus and to a lesser extent in ventricular ependyma, these regions were chosen for qualitative evaluation of protein activity (Fig 3.6). AQP1 is not expressed within the endothelia of brain capillaries (Dolman et al. 2005) and an examination of whole brain coronal sections did not yield evidence of the water channel elsewhere in any experimental groups (results not shown).

The periventricular region of sham animals (Fig 3.7 A) showed a faint-moderate expression of AQP1, with a small decrease in AQP1 activity within this region noted at 5 and 48 h post TBI (Fig 3.8 A and 3.9 A). Conversely the choroid plexus of sham animals (Fig 3.7 B) demonstrated intense staining of AQP1, which markedly decreased at 5 and 48 h following injury (Fig 3.8 B and 3.9 B).
Figure 3.6 AQP1 immunoreactivity anatomical regions of interest. 
(A) Periventricular and choroid plexus AQP1 (3rd ventricle). (B) Choroid plexus AQP1 (lateral ventricle).
Figure 3.7 AQP1 stained section from uninjured sham Sprague-Dawley rats.
(A) Faint-moderate periventricular and choroid plexus AQP1 immunoreactivity (3rd ventricle).
(B) Intense choroid plexus AQP1 immunoreactivity (lateral ventricle).

Figure 3.8 AQP1 stained section from injured Sprague-Dawley rats, at 5 h post diffuse TBI.
(A) Faint periventricular and choroid plexus AQP1 immunoreactivity (3rd ventricle). (B) Faint choroid plexus AQP1 immunoreactivity (lateral ventricle).

Figure 3.9 AQP1 stained section from injured Sprague-Dawley rats, at 48 h post diffuse TBI.
(A) Faint periventricular and choroid plexus AQP1 immunoreactivity (3rd ventricle). (B) Faint choroid plexus AQP1 immunoreactivity (lateral ventricle).
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Colour deconvolution analyses of whole coronal sections stained for AQP1 showed a significant decrease $p < 0.05$ (1.180) in water channel immunoreactivity at 5 h following TBI (3.960 ± 0.169) as compared to uninjured sham (5.140 ± 0.384) animals (Fig 3.8). No statistical difference was seen between AQP1 expression in sham controls and groups injured at 48 h (4.300 ± 0.228), although a qualitative evaluation would imply a slight decrease at this time. There was no significant difference in AQP1 immunoreactivity between animals injured at either time point.

**Figure 3.10** Colour deconvolution analyses of AQP1 immunoreactivity in sham and injured rats at 5 and 48 h post TBI.

Results demonstrate that at 5 h post diffuse TBI vehicle treated animals show significantly less AQP1 immunoreactivity as compared to sham controls * ($p < 0.05$).
3.4 Discussion

The current study has demonstrated that the regional expression of cerebral AQPs 4 & 1 is significantly altered following diffuse TBI, supporting suggestions that AQPs may play an important role in the pathophysiology of brain oedema (Papadopoulos et al. 2002).

Classically four forms of brain oedema have been identified, namely cellular, osmotic, vasogenic and hydrocephalic (Klatzo 1967; Klatzo 1987; Klatzo 1994), although it is the cytotoxic and vascular derived variants which predominate in diffuse TBI.

Cytotoxic oedema forms when cerebral cells are unable to maintain regulation of fluid and ion volume, in spite of an intact BBB. Such events then lead to an intracellular increase in water and a reduction in the capacity of the extracellular space (Klatzo 1987). Trauma induced hypoxia and ischaemia, well known to prelude the formation of cellular oedema, are also observed to be accompanied by impaired astrocytic regulatory volume decrease (RVD) and severe cytotoxic swelling (Strange 1992). Brain energy depletion and the ensuing failure of Na⁺/K⁺-ATPase pump activity, increases the cellular accumulation of sodium (Na⁺) which consequently leads to the passive transport of water into cells, causing them to swell.

However, given the localisation of AQP water channels at glial end foot process, the protein has been implicated as a major player in the pathophysiology of cellular oedema. Indeed, a number of studies have shown that AQP4 is important in water influx into the brain under conditions that promote cytotoxic oedema (Manley et al. 2000). Consistent with these previously published studies, the present results have demonstrated that at 5 h post diffuse TBI, perivascular AQP4 was downregulated at a time when the cells are vulnerable to cellular oedema (Maeda et al. 2003), supporting the notion of the water channel internalising so as to inhibit further fluid influx. Indeed in the setting of early onset post traumatic cellular swelling, channel phosphorylation by activation of various secondary mediators such as
protein kinase C (PKC) drives the internalisation of the pore (Carmosino et al. 2001; Gunnarson et al. 2004). Concomitant to shifts in astrocyte osmotic gradients, injury induced glutamate release results in extracellular levels of the excitatory neurotransmitter reaching neurotoxic proportions. The deleterious effect of excess glutamate on neuronal survival is well understood however, high levels of the EAA can also increase the water permeability of astrocytes via interactions with the G protein-coupled receptor mGluR5. Prior research has proposed that AQP4 may well assemble with mGluR5 and the Na\(^+\)/K\(^+\)-ATPase, so as to establish an electrochemical gradient across plasma membranes in the transport of K\(^+\), thus facilitating neuronal activity (Illarionova et al. 2010). Given the importance of cellular K\(^+\) homoeostasis in both the maintenance of cell volume and in neuron propagation, when coupled with the function of AQP4 in fluid transport, it is possible that in the setting of excitotoxicity such a microdomain would play a part in mediating the activity of the water channel, inhibiting further cellular swelling and attempting to restore neuronal health.

The internalisation of AQP4 has previously been demonstrated to be an effective regulatory mechanism for cellular water permeability in tissues other than brain (Carmosino et al. 2001; Madrid et al. 2001). In these tissues, it is likely that internalisation of AQP4 at tissue fluid interfaces serves as an inhibitory mechanism against cytotoxic swelling. Yet despite its significant role in atypical fluid transport, the precise pathways associated with the altered expression of AQPs in brain oedema remains to be elucidated. PKC may serve such a role given that reduced water permeability has previously been shown in the xenopus oocyte expression system following a prolonged incubation with a PKC activator (Moeller et al. 2009), where a decrease in the AQP4 water channel activity associated with the inhibition of cellular swelling. The effect of a PKC activator in downregulating AQP4 activity may well operate via direct phosphorylation of the channel at the structural site Ser180, leading to an allosteric alteration of the molecule that decreases its water permeability (Zelenina et al.}
CHAPTER 3: HISTOLOGY OF AQP4 & 1 CHANGES IN TBI

2002). These findings directly correlate with the mechanism of action proposed to occur in novel pharmaceutical compounds which antagonise the activity of AQP4 (Yool 2007). Similarly in the setting of post traumatic intracellular Ca\(^{2+}\) cascades, pathophysiological activation of PKC might well play a fundamental part in reducing the water permeability of AQP4, thereby inhibiting intracellular fluid influx.

In contrast, vasogenic oedema is known to arise with an increase in BBB permeability caused by, for example, disruption of tight junctions (TJ) situated between endothelial cells (Klatzo 1994). Protein and fluid from the cerebral vasculature subsequently enter the interstitial space and leads to an expansion of the extracellular compartment, typically resulting in an elevation of ICP. However, in contrast to its cytotoxic counterpart, experimental models of principally vasogenic oedema have shown that deletion of AQP4 exacerbates brain swelling, most probably due to an inhibition of cerebral water outflow. Indeed, the findings of this study showed that at 48 h post diffuse TBI, an intense upregulation of perivascular, subpial and periventricular AQP4 was observed, at a time at which mechanisms that would resolve oedema would be expected to become activated. Recent studies have also demonstrated that during the later stages following diffuse head injury, CBF may potentially become impaired (Marmarou et al. 2006), facilitating hypoxia and ischaemia. In such a setting, large quantities of damaging ROSs are generated, activating mediators of BBB permeability such as MMP-9 and the cytokine TNF-\(\alpha\). The synthesis of oxygen sensors, namely HIF-1\(\alpha\), are also triggered. Increases in TNF-\(\alpha\) and HIF-1\(\alpha\) are not only correlated with a loss of BBB integrity, but also parallel an upregulation of perivascular AQP4 (Hosomi et al. 2005; Kaur et al. 2006; Jia et al. 2010). Increases in AQP4 appear to ameliorate cerebral swelling (Papadopoulos et al. 2004), and it is possible that physiological upregulation of the channel during the late stages of oedema serves to resolve the accumulation of excess extracellular fluid.
The formation of hydrocephalus is not a typical occurrence with diffuse TBI, however, it is the known activity of water channels in hydrocephalus that is of direct relevance to the findings of this study. Hydrocephalic oedema is characterised by an inhibition the typical CSF flow within the ventricular space, or in its communicating variant where by an obstruction arises distal to the ventricles, producing an impaired absorption of CSF into the subarachnoid compartment (Gjerris and Børgesen 2000). During such events, elevations in intraventricular pressure lead to a migration of CSF through the AQP4 prominent ependyma into the periventricular white matter, raising extracellular fluid volume (Milhorat 1992). Rich in Na\(^+\) content, the ensuing oedematous fluid subsequently damages the structural integrity of the periventricular regions such that there is diffuse separation of glia and axons. Remaining astrocytes consequently swell and ultimately undergo a progressive atrophy (Milhorat et al. 1970). Our results clearly show a sharp decrease in choroid plexus AQP1 activity at 5 and 48 h following diffuse TBI. Such a finding is relevant given that other studies have demonstrated a similar result in experimental models of hydrocephalus(Kalani et al. 2012), namely a decrease in choroidal AQP1 and importantly an elevation in subpial AQP4 (Castaneyra-Ruiz et al. 2013). It is therefore reasonable to suggest that physiological reduction of choroid plexus AQP1 post diffuse TBI serves to decrease CSF production and potentially slow the onset of further oedema formation. The increase in perivascular and subpial AQP4 works in tandem with this downregulation so as to facilitate excess fluid clearance during the resolution phase of brain swelling.

Taken together, the findings of this study suggest that the biphasic expression of AQP channels 4 & 1 serves in a complementary fashion to regulate brain swelling following diffuse TBI. Given that brain swelling following acute head injury is typically a result of a modification to water transport between the cerebral fluid filled compartments, it is not surprising that proteins with the principal role of facilitating intracellular water movement are
seen to play an integral role in this process. Presumably, appropriate modulation of these channels at time points of pathophysiological and clinical significance are likely to attenuate the formation and resolution of post traumatic oedema.
4 THE EFFECT OF AQP MODULATION ON CEREBRAL OEDEMA:

A TIME COURSE STUDY
CHAPTER 4: AQP MODULATION IN CEREBRAL OEDEMA

4.1 Introduction

Cerebral water is continuously transported between differing brain compartments, principally via transmembrane protein channels known as aquaporins (AQP). Disturbances in such finely tuned water homoeostasis inevitably lead to deleterious effects on brain function and may result in death. The characteristic genesis of cerebral oedema following traumatic brain injury (TBI) typically leads to an expansion of brain volume, which as it increases, elevates intracranial pressure (ICP), decreases cerebral perfusion and contributes to the development of widespread hypoxia and potentially ischaemic injury (Klatzo 1987; Klatzo 1994; Engelborghs et al. 1998). Classically, two primary forms of oedema have been associated with TBI namely cytotoxic, as a consequence of sustained intracellular water influx, and vasogenic which is an accumulation of extracellular fluid due to a disruption in the blood-brain-barrier (BBB) (Klatzo 1967; Klatzo 1994). A third form of oedema termed osmotic oedema may also arise due to an osmotic imbalance between cerebral circulation and its tissues, whereas a hydrocephalic variant of brain swelling occurs as a direct result of obstruction to cerebrospinal fluid (CSF) outflow (Brodbelt and Stoodley 2007). Given the complexity of cerebral water dynamics it is common for hybrid variations of brain swelling to arise post injury, such as is the case in transependymal oedema whereby a failure of the ependymal lined ventricular wall subsequently results in an increase in periventricular interstitial fluid (Ho et al. 2012). Thus it is unlikely that in TBI a singular type of oedema solely exists.

Two members of the cerebral AQP family are localised in high concentrations at regions of juncture between brain tissue and its fluid filled interfaces. Aquaporin 4 (AQP4) is found in the end foot processes of astrocytes encircling the cerebral vascular wall, as well as adjacent to the cells of the glia limitans and ventricular ependyma, enabling the channel to be in direct
CHAPTER 4: AQP MODULATION IN CEREBRAL OEDEMA

contact with blood, CSF and the subarachnoid fluid filled space (Amiry-Moghaddam et al. 2010). The aquaporin 1 (AQP1) channel is selectively expressed within the cells of the choroid plexus, where it serves an integral role in the secretion of CSF (Brown et al. 2004; Brodbelt and Stoodley 2007). When considering atypical shifts in water balance following acute head injury it is crucial to consider the route by which fluid may flow.

Widespread cytotoxic swelling typically arises because of energy failure, resulting in the impairment of the ATP dependent Na\(^+\)/K\(^+\) pumps, increased levels of cellular ionic content, an elevation in overall cell osmolality and subsequent influx of water into cells (Klatzo 1987). Such transport of fluid from the extracellular to intracellular compartment does not in itself lead to an increase in brain water content, and is therefore unlikely to generate a rise in ICP. However, the genesis of cytotoxicity does directly impair cell function by altering well-tuned intracellular metabolite concentrations. Numerous inflammatory mediators are also released and contribute to the characteristic 'secondary injury' cascade. Such fluctuations in brain aerobic metabolism in conjunction with the release of various vasoactive agents, subsequently impair vascular barrier integrity and therein produce the hallmark of vascular oedema formation. As such it is clear that a chronologically biphasic profile of brain swelling exists following TBI.

Some controversy has persisted over the past several decades regarding the precise temporal profile of oedema form following TBI. Early research suggested that vascular oedema due to BBB permeability was the primary contributor to brain swelling after diffuse brain injury (Marmarou et al. 1994). Comparably other studies provided evidence that vascular barrier loss only predominated in the earlier stages of brain trauma (Reulen et al. 1977; Marmarou et al. 1980). However, these experiments were criticised as being fundamentally simplistic, largely employing cryogenic models of injury and failing to reproduce many of the clinical features associated with diffuse TBI. Some magnetic resonance imaging (MRI) studies have
CHAPTER 4: AQP MODULATION IN CEREBRAL OEDEMA

indicated that vasogenic oedema prevails within the surrounding region of focal contusion injuries (Marmarou et al. 2006), supporting previous findings of a transient opening of the BBB early post TBI (Barzo et al. 1996; O'Connor et al. 2006). Yet far more contemporary research incorporating both measures of cerebral blood flow (CBF) and MRI have suggested that traumatic brain oedema is principally a cellular pathology in both diffuse and focal injuries following an initial opening of the BBB, a hallmark of vasogenic leakage (Maeda et al. 2003; Marmarou et al. 2006). The surrounding area in focal injuries have demonstrated high ADC values, indicative of extracellular water and by inference, increased BBB permeability. However, in diffuse models of trauma a linear correlation is observed between lower ADC measures, implying a principally cellular swelling (Marmarou et al. 2006).

Indeed O’Connor and colleagues demonstrated via use of a diffuse model of TBI in rats that following weight drop impact male animals show significant, albeit incrementally decreasing, oedema formation over a 5 day period, with a peak at 5 h post injury (O'Connor et al. 2005). Taken together these results would imply a transient disruption in BBB permeability within the first 30 min to 5 h following TBI which then swiftly evolves into a principally cytotoxic swelling. Vascular oedema may re-emerge after 24 h.

Having characterised the activity of AQP4 & 1 expression following diffuse TBI in the previous chapter, the current study will examine the effects of pharmaceutical AQP modulation on brain water content throughout the time course of cerebral oedema after TBI. Either an AQP4 & 1 antagonist or AQP4 agonist was administered at 30 min, 5, 12, 24 or 48 h following head injury induced using the well-established rodent weight drop model of diffuse TBI (Marmarou et al. 1994), and quantification of cerebral oedema evaluated at 5 h post drug administration via the wet weight-dry weight methodology (Elliott and Jasper 1949) as measured by a Mettler Toledo – HG63 halogen moisture analyser.
CHAPTER 4: AQP MODULATION IN CEREBRAL OEDEMA

4.2 Methods

4.2.1 Surgery and injury induction

Animals were injured using the well-established weight drop model of diffuse brain injury (Marmarou et al. 1994) as detailed in Chapter 2. Briefly, impact acceleration injury was induced in intubated, Isoflurane anaesthetised Sprague-Dawley rats (n=120; 380 ± 420g) by dropping a 450g brass weight from a height of two meters onto a 3mm in depth by 10mm diameter stainless still disc, fixed centrally to the animals exposed skull with a cyanoacrylate based adhesive. The head of the animal was supported by a 30x12x12cm foam bedding which acted to decelerate the brass weight following impact. Immediately after injury all animals were exposed to a 30 min period of hypoxia induced via intubated inhalation of Isoflurane 1.5%, 240mL/min, O₂, 2L/min N₂. Throughout surgery and during the immediate recovery phase rats were placed on a thermostatic heatpad. After injury all wounds were sutured and following completion of the hypoxic episode, animals withdrawn from anaesthesia and returned to their home cages after recovery.

4.2.2 AQP modulators AqB013 and AqF026

Novel AQP modulators have been recently developed making it possible to investigate the ability of these drugs to treat post-traumatic cerebral oedema. The arylsulphonamide AqB013 (G. Flynn, Spacefill Enterprises, LLC, Tucson, AZ) has been shown to have an inhibitory effect on AQP4 and 1 water channels (Yool 2007; Migliati et al. 2009). Whereas the compound derivative AqF026 has been shown to bind at a separate site to specifically enhance AQP4 channel opening (Devuyst and Yool 2010; Yool et al. 2013). Both compounds were originally made as stock 100mM solutions in dimethyl sulfoxide (DMSO) and stored at 4°C. Experimental solutions of ≤ 100µM final concentration were prepared by
slow addition of the DMSO stock into a final volume of isotonic saline during rapid mixing. For concentrations > 100µM, drugs were sonicated in saline with 0.1% DMSO final. Vehicle solutions for control groups was composed of equal volume 0.1% DMSO in saline.

Once prepared single doses of either the AQP4 & 1 antagonist (AqB013; 0.8 mg/kg) or the AQP4 agonist (AqF026; 0.2 mg/kg) were administered iv via the tail vein at the pre-determined time points of 30 min, 5, 12, 24 and 48 h following TBI (n=6/group). At 5 h post drug administration brains removed from euthanised rodents were then assessed for moisture content.

4.2.3 Analyses of oedema

4.2.3.1 Brain moisture content

Cerebral oedema was calculated using the previously described wet weight-dry weight methodology (Elliott and Jasper 1949) as detailed in Chapter 2. In brief, brain water content was measured using a Mettler Toledo – HG63 halogen moisture analyser. Prior to evaluation, animals were reanaesthetised via chamber inhalation at 5 h post drug administration (5, 10, 17, 29 and 53 h). Once a surgical level of anaesthesia was reached rats were decapitated and their brains swiftly removed from the skull. The olfactory bulb was then removed and each hemisphere separated using a scalpel blade. The left hemisphere was placed on a 4.8g glass superfrost slide, previously weight tared in the moisture analyser, before being sliced evenly into three smaller segments. A second superfrost slide was then positioned on top and the first and the two slides clamped together until brain tissue was evenly distributed across each surface. Both slides were then placed into the moisture analyser, wherein it was swiftly weighed and then heated to dryness over 10 min. The same protocol was also performed on the right hemisphere in another identical moisture analyser immediately after the first
hemisphere had begun to heat. Brain moisture content was expressed as the percentage of tissue water content as calculated by the established wet weight-dry weight formula (Elliott and Jasper 1949).

4.2.4 Statistical Analysis

Levels of brain moisture content between and within experimental groups were analysed for statistical significance using a repeated measures two-way ANOVA with a Bonferroni’s multiple comparisons posthoc test and presented as mean ± standard error (SEM).
CHAPTER 4: AQP MODULATION IN CEREBRAL OEDEMA

4.3 Results

Brain moisture content in injured, vehicle treated animals peaked at 5 h (81.300 ± 0.102) post diffuse TBI and then progressively decreased thereafter, reaching its lowest point at 48 h following injury (78.746 ± 0.008) (Fig 4.1). These findings are consistent with the time course of oedema formation previously demonstrated in this model of injury (O'Connor et al. 2006). Single iv administration of the AQP4 & 1 antagonist at 30 min (77.051 ± 0.009) and 5 h (77.537 ± 0.024) following diffuse injury significantly reduced cerebral oedema +++ p < 0.001 (4.248) when compared to vehicle groups (Fig 4.1). However, when the same compound was given at 48 h (80.245 ± 0.014) after diffuse TBI, it not only failed to reduce levels of atypical brain moisture content but significantly increased cerebral oedema **** p < 0.001 (1.498) compared to vehicle treated groups.

Single dose iv administration of the AQP4 agonist at 30 min (80.080 ± 0.020) and 5 h (79.504 ± 0.018) also significantly ameliorated brain moisture content when compared to vehicle treated animals *** p < 0.001 (1.375) albeit that the levels were always significantly ♠♣♣ p < 0.001 (2.431) greater than sham levels of water content from 5 – 48 h (77.073 ± 0.020) after injury (Fig 4.1). When administered at either 24 h (78.070 ± 0.012) or 48 h (77.642 ± 0.012) post TBI, the AQP4 agonist also significantly □□□ p < 0.001 (1.105) attenuated brain moisture content although at these time points, values were similar to sham levels and significantly p < 0.001 (2.603) less than vehicle groups and AQP4 & 1 antagonist treated animals. ANOVA analyses also showed that there was a significant main effect for treatment groups F (3, 20) = 9269; p < 0.001) and a significant temporal interaction F (4, 80) = 178.4; p < 0.001) post TBI.
4.3.1 Cerebral oedema in treated animals post diffuse TBI

The AQP4 & 1 antagonist significantly ameliorated cerebral oedema at early time points +++ (p < 0.001) when compared to vehicle groups, albeit that the levels were always significantly greater than sham levels of water content from 5 – 48 h after injury mmm (p < 0.001). At the later time point however of 48 h the antagonist significantly increased brain moisture when compared to vehicle group’s www (p < 0.001). The AQP4 agonist reduced brain moisture content compared to the vehicle group at 5 h *** (p < 0.001) and 48 h ppp (p < 0.001), yet at the earlier time point failed to reduced cerebral oedema bellow pathological levels.
4.4 Discussion

The present study demonstrates that administration of an AQP4 & 1 antagonist early after diffuse TBI attenuates oedema formation, yet exacerbates oedema formation when administered at later time points. In contrast, administration of an AQP4 agonist attenuates oedema formation at all time points, although only reduces oedema to sham levels if administered at later time points. Such a finding is highly significant given that current treatment approaches for brain swelling have failed to keep up with the advances in the mechanisms underlying cerebral oedema (Rosenberg 2000), and have simply focussed on reducing symptoms (Bullock et al. 1999). The current compounds target the water channels themselves which are integral in oedema formation.

Previous research has shown that administration of sulfhydryl reactive based mercurials can inhibit the activity of AQP4 (Preston et al. 1993; Niemietz and Tyerman 2002). However, these molecules are nonspecific in their action and highly toxic to living cells; in essence, they are not suitable for therapeutic use. In contrast, the novel compounds employed in this study use the clinically used bumentanide as the structural scaffold, therefore enabling clinical translation (Devuyst and Yool 2010). To date, these are the only known agents worldwide to pharmacologically modulate brain AQP channel activity without associated toxicity. Other candidates for blocking AQP1 activity have been proposed, including acetazolamide (Ma et al. 2004), tetraethylammonium (Brooks et al. 2000) and DMSO (Yang et al. 2006), however these have proven ineffective in significantly inhibiting the water channels. More recent studies have suggested downregulation of AQP4 activity with multiple antiepileptics (Huber et al. 2007), although this has yet to be verified with the use of in vivo assays. Given that in the previous chapter we have shown that both AQP4 & 1
respond in a complementary manner to injury, the targeting of both channels would appear to be desirable in the optimal inhibition of cellular oedema.

Previously some debate arose over the time course of oedema form post diffuse TBI. However, a substantial body of evidence would now strongly support the notion of an early vasogenic leakage followed by a principally cellular oedema, with a potential re-emergence of vascular derived brain swelling at a later time point. Similarly, some disparity exists within the literature regarding the expression of brain AQP activity following head trauma (Manley et al. 2000; Badaut et al. 2002; Papadopoulos et al. 2004; Amiry-Moghaddam et al. 2010), although is slowly beginning to resolve (Zelenina 2010). It is now becoming evident that AQP localisation is highly dependent upon the injury form. The findings from the present chapter and the one before it also suggests that not only is AQP4 & 1 expression altered following diffuse TBI, but that pharmaceutical intervention which complements the temporal nature of such changes significantly ameliorates injury induced brain swelling. The results from Chapter 3 clearly demonstrated a physiological downregulation of AQP4 & 1 early after diffuse TBI. Similarly, the current study demonstrated that inhibition of the water channels immediately after diffuse head injury significantly reduced cerebral swelling. It is thus probable that administration of an AQP4 & 1 antagonist at this time significantly inhibits the onset of water influx. This observation is consistent with research demonstrating that induced experimental hydrocephalus in rats results in an early downregulation of choroidal AQP1 and increase in subpial AQP4, that was associated with the amelioration of brain swelling (Castaneyra-Ruiz et al. 2013). Such results are directly related to the observations of this study demonstrating a comparable downregulation in choroid plexus AQP1, decrease in perivascular AQP4 and moderate increase in the expression of the channel along the glia limitans externa during the acute stages following diffuse TBI. Hence internalisation of the channels shortly after diffuse brain injury would appear to minimise the deleterious effects of
perivascular and choroid plexus fluid influx, whilst simultaneously providing a route for extracellular water clearance at the subpial level. It is also worth noting that results from this present chapter show administration of an AQP4 agonist, when administered at 5 h post TBI, also reduces brain moisture content albeit to a far lesser extent than the antagonist at this time. Findings of this nature may be explained by the results of Chapter 3, in which subpial AQP4 expression appears to moderately increase at the earlier time of 5 h following injury in conjunction with an intense downregulation of perivascular water channel activity. Indeed arguably increases in AQP4 expression solely at the perivascular level at 5 h post injury, in the setting of prevailing cytotoxicity, would worsen neurological outcome. However, if in concert with severe increases to ICP at 5 h following TBI, cerebral AQP4 expression was universally increased, it is plausible that enhanced expression of water channel activity at the subpial level would assist somewhat in the clearance of oedematous fluid towards the subarachnoid space.

Previous research has shown that numerous secondary mediators contribute to the initial opening of the BBB following head injury. During this period in which the BBB is permeable, an upregulation in the availability of AQP channels would draw water into the surrounding tissues worsening oedema. However, when protein transport begins to slow and the barrier is closed at a later time point, an increase in the expression of the channel may well allow water to leave. Indeed as was observed in the previous study, a significant increase in perivascular, periventricular and subpial AQP4 arose at 48 h following diffuse TBI. This time course also correlates with the amelioration of cerebral swelling when a pharmaceutical agonist of the channel was administered.

Collectively, the results of this study and the findings shown in the chapter prior demonstrate that appropriate temporal modulation of AQP channels following brain injury can significantly attenuate cerebral oedema. Early downregulation of AQP4 & 1 channels
CHAPTER 4: AQP MODULATION IN CEREBRAL OEDEMA

ameliorates water influx and superfluous CSF outflow, whereas increases in AQP4 expression at a later stage correlate with an upregulation of oedema clearance. As such pharmaceutical agents that are set to reduce atypical brain water content should be administered in a complementary fashion to the innate biphasic nature of channel expression post TBI. In the next chapter, we will examine the effects of the AQP modulators on AQP expression, BBB permeability and motor outcome when the modulators are administered at their optimal time points for reducing oedema.
5  THE EFFECT OF AQP MODULATION AT OPTIMAL TIME POINTS ON CEREBRAL OEDEMA, BRAIN ALBUMIN CONTENT & MOTOR FUNCTIONAL OUTCOME
5.1 Introduction

It is widely accepted that brain swelling (cerebral oedema) is a characteristic pathology in acute head injury. Yet despite its significance within intensive care units worldwide, cerebral oedema continues to be a principle cause of morbidity and mortality following traumatic brain injury (TBI) (Fleminger and Ponsford 2005; Le´on-Carri´on et al. 2005). Typically, the brain establishes a fluid equilibrium throughout its primary compartments via precise regulation of water transport across cellular barriers, namely, endothelia of the blood-brain-barrier, ependyma of the ventricular cerebrospinal fluid interface and epithelia of the choroid plexus (Kimelberg 2004). Any disruption to such well controlled cerebral water homoeostasis frequently leads to an increase in brain water volume, which results in an elevation in intracranial pressure, a decrease in perfusion pressure, and widespread hypoxia and ischaemia (Klatzo 1987). Equally, however, subtle alterations in the composition of the extracellular fluid that bathes the cells of the brain parenchyma, significantly affect the function of neurons and cell metabolism.

Under physiological conditions levels of intracellular potassium are higher, whilst sodium and calcium are significantly lower than that of the extracellular fluid. These concentration gradients are primarily maintained by energy dependent ion pumps, specifically the Na⁺/K⁺ and Ca²⁺-ATPases. However, loss of brain energy as is sometimes seen to accompany TBI, swiftly results in the disturbance of ionic pump homoeostasis, leading to an intracellular accumulation of Na⁺, impairment to cytoskeletal integrity and disturbance in neuronal propagation. The resultant cell swelling is associated with an increase in intracellular Ca²⁺ concentration, which is proposed as being one of the central secondary events leading to the functional and structural breakdown of the cell membrane (Siesjo and Wielcoh 1985). As such, cytotoxic events are characteristically correlated with the disruption in neuronal action.
potential, In tandem with these early ionic and metabolic events following TBI, there is an indiscriminate release of excitatory amino acids (EAA), which through activating persistent intracellular Ca\(^{2+}\) influx, result in mitochondrial impairment, decreases in oxidative and glucose metabolism and axonal injury (Patro and Mohanty 2009). Therefore cerebral oedema is frequently associated with impairments to motor function as a consequence of the underlying ionic and metabolic deficits (Ishige \textit{et al.} 1987; Tominaga and Ohnishi 1989; Yan \textit{et al.} 2011). Sustained excitotoxicity following TBI can also in of itself contribute to further cellular swelling. In the context of bioenergetic depletion, glial and neuronal efforts to repair altered electrolyte equilibrium exacerbates an already established energy crisis and the associated failure of cell regulatory volume, which is further comprised by Ca\(^{2+}\) mediated increases in the production of damaging reactive oxygen species (ROS). Such mechanisms are of particular relevance when considering the role of AQPs in cellular swelling and motor functional impairment, as it is a primary responsibility of AQP4 rich astrocytes to clear the extracellular space of excess glutamate.

Serum albumin, the most abundant of the blood plasma proteins, is known to regulate colloidal osmotic (oncotic) pressure. Water will typically follow albumin and therefore, its role in brain water homeostasis makes it worthy of consideration when investigating the role of water channels during cerebral oedema. As a large molecule, albumin is typically unable to cross capillary walls and has therefore traditionally been used as a marker for BBB permeability (Hawkins and Egleton 2006; Hawkins and Egleton 2008). While the loss of endothelial cell tight junction (TJ) integrity was thought to facilitate albumin extravasation, more recent research has clearly demonstrated that albumin can be transported across cellular barriers by means other than TJ breakdown (Herve \textit{et al.} 2008). Once into the brain parenchyma, water follows down its osmotic gradient resulting in vasogenic oedema. Increased vascular permeability occurs early after diffuse TBI, resulting in the leakage of
CHAPTER 5: MODULATING AQP AT OPTIMAL TIME POINTS

large molecules such as albumin into the cerebral interstitial space (Klatzo 1967; Marmarou et al. 1994). Therefore this study will also consider the role of albumin in parallel to the part AQPs play in the resolution and genesis of cerebral oedema.

Having previously established the optimal time points at which to effectively administer the AQP modulators so as to attenuate the formation of cerebral oedema, the present study will give an AQP4 & 1 antagonist at 5 h or an AQP4 agonist at 48 h post diffuse TBI and assess effects on AQP4 & 1 expression, levels of brain albumin content as well as motor outcome.
CHAPTER 5: MODULATING AQP AT OPTIMAL TIME POINTS

5.2 Methods

5.2.1 Surgery and injury induction

Animals were injured using the weight drop model of diffuse brain injury (Marmarou et al. 1994) as described in detail in Chapter 2. Male Sprague-Dawley rats (380 ± 420g) were anaesthetised in Isoflurane, intubated and then maintained at a surgical level throughout injury induction and recovery. Immediately after injury (450g weight from 2 metres) all animals were exposed to a 30 min period of hypoxia, induced via intubated inhalation of Isoflurane 1.5%, 240mL/min, O2, 2L/min N2. All wounds were sutured during this period, and thereafter animals weaned from ventilated anaesthesia and returned to their home cages following recovery.

5.2.2 AQP modulators AqB013 and AqF026

At 5 h post injury the AQP4 & 1 antagonist (AqB013; 0.8 mg/kg) was administered iv, or at 48 h the AQP4 agonist (AqF026; 0.2 mg/kg) was administered iv, both via the tail vein. At 5 h following drug administration rodents were then assessed for either AQP4 & 1 immunohistochemical changes following brain fixation, or for brain albumin extravasation. Another subset of animals was assessed over a 7-day period following injury for motor functional performance.

5.2.3 Immunohistochemistry

AQP4 & 1 immunohistochemistry (n=5/group) was performed as described in detail in Chapter 2. Briefly, at 5 h following drug administration, rats were transcardially perfused with 10% formalin (pH 7.4). Once removed, all brains were stored in 10% formalin and subsequently stained using Anti-Aquaporin 4 antibody [4/18] (ab9512; Abcam) or Anti-
Aquaporin 1 Antibody (aB2219; Millipore). Digitally acquired nanzoomed images of stained slides were then evaluated for antigen content via the automated colour deconvolution analysis.

Albumin immunohistochemistry was initiated by overnight incubation with the appropriate primary antibody (rabbit polyclonal Anti-Human Albumin (optimal dilution 1:1000 (DakoCytomation). Sections were then blocked with NHS and washed in PBS (pH 7.4) before being treated with the secondary antibody in NHS for 30 min (biotinylated goat Anti-Rabbit IgG Antibody; 1:250; BA-1000; Vector). Sections were then incubated with SPC (Pierce, 1:1000) and thereafter visualised with DAB, counterstained with haematoxylin, dehydrated, cleared, mounted and imaged before applying colour deconvolution for quantitation.

Standard H&E were performed as previously described in detail in Chapter 2.

5.2.4 Brain albumin content

5.2.4.1 EvansBlue

Brain albumin extravasation was also evaluated using the spectrophotometry protocol for the EvansBlue (EB) albumin dye (Haj-Yasein et al. 2011) and by ex vivo autofluorescent imaging as detailed in Chapter 2. In brief, 30 min prior to perfusion animals (n=5/group) were administered EB dye (0.4mL/animal iv at 10 mg/kg) via the tail vein under general Isoflurane anaesthesia. Animals were administered Heparin 10 min prior to EB administration to facilitate transcardial perfused. Animals were then decapitated, their brains removed, the olfactory bulb removed and each hemisphere separated before being weighed. Each hemisphere was then placed in a vial and homogenised in 7.5mL of phosphate buffered saline. 2.5mL of trichloroacetic acid (Sigma, T-0699) was subsequently added to the samples
which were then stored overnight at 4°C. Samples were then centrifuged at 1000 r.p.m over a 30 min period. A UV/V spectrophotometer was then employed to quantify the EB absorbance in the resulting supernatant at 620nm, and converted μg/g of brain tissue using a standard curve.

Prior to homogenisation a subset of animal brains (n=5/group) were imaged for EB autofluorescence (615-665nm) prior to homogenisation, using a Xenogen IVIS Imaging System 100 equipped with a Cy5.5 filter. High counts of fractional fluorescent ratios were represented as either red or green channel pixels, while low to none as blue channel pixels, with a total range between 0-255 arbitrary units. In quantifying EB, all scans were post processed on an identical sized pixel canvas, relevant background noise and blue colour channels removed, and all red and green colour channel pixels tallied.

5.2.5 *Timed motor function performance*

Animals were assessed for functional motor deficits (n=5/group) over a week post injury using the rotarod device (Hamm et al. 1994) as described in detail in Chapter 2. In brief, animals were placed on a rotarod device consisting of a motorised rotating assembly of 18 rods (1 mm in diameter). Rotarod rotational speed ranged from 0 - 30 revolutions per minute (rpm), as determined by a control box operated by the assessor. Animals were placed on the rotarod, which at first remained stationary for the first 10 seconds, after which the rotational speed was increased by 3 rpm every 10 seconds for a maximum duration of 2 minutes. The point at which the animal fell from the rods or gripped the rods and spun for two consecutive revolutions, as opposed to actively walking, was then recorded in seconds. Animals were trained once daily over a 5-day period prior to injury so as to establish their normal pre-injury baseline. Thereafter, assessments commenced at 24 h post injury and were conducted at approximately the same time each day over a 7-day period.
5.2.6 Statistical Analysis

Results are presented as mean ± standard error (SEM). A repeated measures two-way analysis of variance (ANOVA) followed by a Bonferroni’s multiple comparisons posthoc test was performed on motor functional outcome. A one-way ANOVA with a Bonferroni’s multiple comparisons posthoc test was employed to assess EB autofluorescence by spectrophotometry, ex vivo imaging and semiquantification of DAB visualisation on sectioned slides. A p value of < 0.05 was considered to be statistically significant.
5.3 Results

5.3.1 AQP4 immunoreactivity in treated animals post diffuse TBI

Chapter 3 demonstrated noticeable expression of AQP4 at brain tissue fluid interfaces, namely perivascular (brain–blood-barrier), glia limitans (brain–subarachnoid cerebrospinal fluid) and ependymal (brain–ventricular CSF) regions. Accordingly these regions were again investigated in the current experiment.

At 5 h after treatment with the antagonist there is a low level of perivascular AQP4 immunoreactivity within the cortex directly beneath the site of impact (Fig 5.1 A). Moderate periventricular AQP4 immunoreactivity was observed in antagonist treated animals (Fig 5.1 B), while in the glia limitans, antagonist treated animals show moderate-intense AQP4 immunoreactivity (Fig 5.1 C).

In TBI animals treated with the agonist, at 5 h after drug intervention there was a marked perivascular AQP4 immunoreactivity within the cortex beneath the impact site (Fig 5.2 A). Similarly, there were high levels of immunoreactivity in the periventricular region (Fig 5.2 B) as well as in the glia limitans (Fig 5.2 C).
Figure 5.1 AQP4 stained section of Sprague-Dawley rats treated with an AQP4 & 1 antagonist at 5 h post diffuse TBI. (A) Faint-moderate perivascular AQP4 immunoreactivity directly beneath the site of impact. (B) Moderate periventricular AQP4 immunoreactivity. (C) Moderate-intense AQP4 immunoreactivity of the glia limitans bordering the subarachnoid space.

Figure 5.2 AQP4 stained section of Sprague-Dawley rats treated with an AQP4 agonist at 48 h post diffuse TBI. (A) Very intense perivascular AQP4 immunoreactivity directly beneath the site of impact. (B) Very intense periventricular AQP4 immunoreactivity. (C) Intense AQP4 immunoreactivity of the glia limitans bordering the subarachnoid space.
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Colour deconvolution analyses of the whole coronal sections stained for AQP4, as visualised with DAB, showed no significant difference in animals treated at 5 h post TBI with an AQP4 & 1 antagonist (11.020 ± 0.381) when compared to those treated with DMSO vehicle (12.280 ± 0.849) (Fig 5.3). There was however, a significant difference * p < 0.05 (3.900) observed in animals treated with an AQP4 agonist at 48 h (17.720 ± 0.447) following injury as contrasted with vehicles (21.620 ± 0.583) treated at this time.

**AQP4 Colour Deconvolution**

![AQP4 Colour Deconvolution](image)

**Figure 5.3** Colour deconvolution analyses of AQP4 immunoreactivity in injured and AQP modulator treated rats at 5 and 48 h post diffuse TBI.

Immunohistochemistry was performed at 5 h after drug treatment. No statistical difference was seen between vehicle and AQP4 & 1 antagonist treated groups at 5 h however, at 48 h there was a small change between AQP4 agonist and vehicle treated groups (p < 0.05).
5.3.2 AQP1 immunoreactivity in treated animals post diffuse TBI

Chapter 3 demonstrated changes in the expression of AQP1 after TBI within the apical membrane of the choroid plexus and to a lesser extent in ventricular ependyma. These regions were therefore selected for evaluation in the current study.

The periventricular region of injured animals treated with the antagonist demonstrated faint AQP1 immunoreactivity at 5 h after drug treatment (Fig 5.4 A). There was little expression in the choroid plexus at this time point in these animals treated with the antagonists.

Similarly, in animals treated with the AQP4 agonist, there was little periventricular (Fig 5.5 A) or choroid plexus (Fig 5.5 B) AQP1 immunoreactivity at 5 h after drug administration. Examination of whole brain coronal sections did not yield evidence of the water channel elsewhere in the experimental groups.
Figure 5.4 AQP1 stained sections from Sprague-Dawley rats administered an AQP4 & 1 antagonist at 5 h post diffuse TBI.

(A) Faint periventricular and choroid plexus AQP1 immunoreactivity (3rd ventricle). (B) Faint choroid plexus AQP1 immunoreactivity (lateral ventricle).

Figure 5.5 AQP1 stained sections from Sprague-Dawley rats administered an AQP4 agonist at 48 h following TBI.

(A) Faint periventricular and choroid plexus AQP1 immunoreactivity (3rd ventricle). (B) Faint choroid plexus AQP1 immunoreactivity (lateral ventricle).
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Colour deconvolution analyses of whole coronal sections stained for AQP1 as visualised with DAB, showed no significant difference in animals treated at 5 h post TBI with an AQP4 & 1 antagonist (4.440 ± 0.129) when compared to vehicle controls (3.960 ± 0.169) (Fig 5.6). Similarly there was no statistical difference between rodents treated with an AQP4 agonist (4.300 ± 0.138) at 48 h and vehicles (4.300 ± 0.228) at this time point.

**AQP1 Colour Deconvolution**

![AQP1 Colour Deconvolution](image)

**Figure 5.6** Colour deconvolution of AQP1 immunoreactivity in injured and treated rats at 5 h after drug administration. No statistical difference was seen between either of the AQP modulator groups and vehicle controls at any time point.
5.3.3 Albumin immunoreactivity in treated and untreated animals post diffuse TBI

Brain tissue fluid interfaces including perivascular (brain–blood-barrier), ependymal (brain–ventricular CSF) and choroid plexus were chosen for regions of investigation for albumin immunoreactivity, as previous research has indicated the notable localisation of water channels in these areas. In sham animals, faint-moderate perivascular levels of albumin were observed surrounding vessels directly beneath the site of injury (Fig 5.7 A). In contrast, very intense perivascular albumin immunoreactivity was seen at 5 h post TBI in vehicle groups (Fig 5.8 A), whilst at 48 h these levels appeared to slightly subside (Fig 5.9 A). In animals treated with either the AQP4 & 1 antagonist at 5 h (Fig 5.10 A) or with the AQP4 agonist at 48 h (Fig 5.11 A), there was reduced perivascular albumin staining at 5 h after drug administration.

In the periventricular region and choroid plexus of sham animals, moderate levels of albumin immunoreactivity were observed (Fig 5.7 B). However, at 5 h (Fig 5.8 B) and 48 h (Fig 5.9 B) post TBI, there is an unequivocal increase in periventricular and choroidal albumin immunoreactivity, as well as a noticeable appearance of the protein within the surrounding parenchyma. In contrast, animals treated at 5 h with the AQP4 & 1 antagonist (Fig 5.10 B) or at 48 h with the AQP4 agonist (Fig 5.11 B) show significantly less periventricular and surrounding parenchymal albumin staining. The presence of the protein in the choroid plexus in these treated animals appears comparable to sham controls.

In the glia limitans and surrounding parenchyma, albumin immunoreactivity in sham animals is moderate in intensity (Fig 5.7 C). However, at both 5 h (Fig 5.8 C) and 48 h (Fig 5.9 C) after diffuse TBI there is an unequivocal increase in glia limitans albumin immunoreactivity as well as throughout the adjacent parenchyma. In animals treated with the antagonist at 5 h
(Fig 5.10 C) or the AQP4 agonist at 48 h (Fig 5.11 C), there is a noticeable increase in glia limitans albumin staining when compared to sham controls. When compared with vehicle treated groups, there is an observable decrease in the protein throughout the surrounding parenchyma.
**Figure 5.7** Albumin stained section from uninjured sham Sprague-Dawley rats.
(A) Faint-moderate perivascular albumin immunoreactivity directly beneath the site of impact. (B) Moderate periventricular and choroid plexus albumin immunoreactivity. (C) Moderate albumin immunoreactivity of the glia limitans bordering the subarachnoid space.

**Figure 5.8** Albumin stained section from vehicle treated Sprague-Dawley rats, at 5 h post diffuse TBI.
(A) Very intense perivascular albumin immunoreactivity directly beneath the site of impact. (B) Moderate-intense periventricular and choroid plexus albumin immunoreactivity. (C) Very-intense albumin immunoreactivity of the glia limitans bordering the subarachnoid space.

**Figure 5.9** Albumin stained section from vehicle treated Sprague-Dawley rats, at 48 h post diffuse TBI.
(A) Intense perivascular albumin immunoreactivity directly beneath the site of impact. (B) Moderate-intense periventricular and choroid plexus albumin immunoreactivity. (C) Intense albumin immunoreactivity of the glia limitans bordering the subarachnoid space.
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Figure 5.10 Albumin stained section of Sprague-Dawley rats treated with an AQP4 & 1 antagonist at 5 h post diffuse TBI.
(A) Faint perivascular albumin immunoreactivity directly beneath the site of impact. (B) Faint-moderate periventricular and choroid plexus albumin immunoreactivity. (C) Very-intense albumin immunoreactivity of the glia limitans bordering the subarachnoid space.

Figure 5.11 Albumin stained section of Sprague-Dawley rats treated with an AQP4 agonist at 48 h post diffuse TBI.
(A) Faint perivascular albumin immunoreactivity directly beneath the site of impact. (B) Faint-moderate periventricular and choroid plexus albumin immunoreactivity. (C) Moderate-intense albumin immunoreactivity of the glia limitans bordering the subarachnoid space.
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Colour deconvolution analyses of whole coronal sections stained for albumin demonstrated that administration of an AQP4 & 1 antagonist at 5 h (12.525 ± 0.804) following diffuse TBI significantly *** p < 0.001 (10.30) reduced levels of brain albumin content at 5 h after drug administration as compared to vehicle (22.820 ± 0.806) groups (Fig 5.12). Administration of an AQP4 agonist (14.275 ± 0.765) at 48 h post injury reduced brain albumin levels when compared to vehicle (18.840 ± 1.923) controls, although the difference was not statistically significant. When compared to shams (13.325 ± 1.141) however, both the vehicle treated groups demonstrated significantly higher levels of cerebral albumin content at 5 h +++ p < 0.001 (9.495) and 48 h •••• p < 0.001 (5.515) respectively, indicative of atypical levels of the protein within the brain.
Figure 5.12 Colour deconvolution analyses of albumin immunoreactivity in injured and treated rats.

Assessed at 5 and 48 h post diffuse TBI with an AQP 4 &1 antagonist, and an AQP4 agonist, respectively. An AQP4 & 1 antagonist when administered early at 5 h and following injury significantly ameliorated atypical levels of cerebral albumin when compared to vehicle controls (p < 0.001). An AQP4 agonist given later at 48 h also notably reduced atypical levels of brain albumin following injury however, this difference was not statistically significant. Vehicle groups also demonstrate a significant peak in atypical levels of cerebral albumin at 5 h 5 h +++ (p < 0.001) which then significantly decreases at 48 h post injury *** (p < 0.001), when compared to sham controls.
5.3.4 **Evans Blue extravasation**

Using Evans blue extravasation to determine BBB permeability, when administered at 5 h after TBI, the AQP4 & 1 antagonist (4.439 ± 0.011) significantly ++ p < 0.001 (0.963) reduced levels of brain albumin content at 5 h after administration when compared to vehicle controls (5.402 ± 0.027) (Fig 5.13). The AQP4 agonist given at 5 h following injury (5.382 ± 0.040) demonstrated no such effect. When administered at 48 h after TBI, the AQP4 agonist (4.642 ± 0.029) significantly *** p < 0.001 (0.516) reduced levels of EB extravasation when compared to vehicle controls (5.159 ± 0.020). In contrast, the AQP4 & 1 antagonist (5.139 ± 0.030) administered at the same time point had no effect. Using autofluorescence to assess EB extravasation after injury and treatment gave qualitatively similar results (Fig. 5.14). Measures of EB extravasation also demonstrated that in vehicle treated animals at 48 h following diffuse TBI there was significantly **** p < 0.01 (0.225) less cerebral albumin as compared to those injured at 5 h, although when compared to shams this level of brain protein is still physiologically atypical.
Figure 5.13 Brain albumin post diffuse TBI as assessed by EB extravasation. An AQP4 & 1 antagonist when given at 5 h post TBI significantly reduced levels of brain albumin +++ (p < 0.001), when compared to vehicle controls. Alternatively administration of an AQP4 agonist at 48 h following injury attenuated atypical levels of cerebral albumin as compared with vehicles at the same time *** (p < 0.001). Levels of EB extravasation also indicate a peak at 5 h post TBI and a subsequent significant decrease at 48 h +++ (p < 0.01) follow trauma, although at the later time point not below that of pathological levels.
Evans Blue concentration by autofluorescence

Figure 5.14 Brain Evans blue content post diffuse TBI as assessed by autofluorescence.

When administered at 5 h following injury, the AQP4 & 1 antagonist notably reduced levels of brain EB autofluorescence at 5 h after drug administration when compared to vehicle controls. The AQP4 agonist demonstrated no such effect at this time point. When administered at 48 h after injury, the AQP4 agonist clearly reduces EB autofluorescence at 5 h after drug administration when compared to vehicle controls. The AQP4 & 1 antagonist administered at the same time point shows no effect.
5.3.5 Motor functional performance in treated animals post diffuse TBI

Treatment with the AQP4 & 1 antagonist at 5 h (61.2 ± 3.7) following impact significantly improved *** $p < 0.001$ (19.00) motor functional performance when compared to vehicle controls (42.2 ± 4.7) at the same time point. Administration of the AQP4 agonist (64.0 ± 3.2) also significantly improved +++ $p < 0.001$ (17.20) motor functional performance after administration at 48 h (but 5 h prior to testing) when compared to vehicle controls (46.8 ± 4.7). By 7 days after TBI, both treatment groups (antagonist 110.8 ± 1.9, agonist 105.2 ± 4.0) demonstrated significantly ••• $p < 0.001$ (14.20) improved motor functional performance when compared to vehicle controls (96.6 ± 5.1). Throughout the motor assessment period, the AQP4 & 1 antagonist was the most effective of the two compounds, although this difference was never statistically significant. ANOVA analyses also showed that there was a significant main effect for treatment groups $F(3, 128) = 1078; p < 0.001$ and a significant temporal interaction $F(7, 128) = 428.3; p < 0.001$ over the 7 d period following injury.
Figure 5.15 Effects of aquaporin modulators on motor outcome as assessed by the rotarod test.

Administration of an AQP4 & 1 antagonist at 5 h post TBI significantly attenuated injury induced impairment to motor function *** (p < 0.001) as compared with the vehicle group at this time. Alternatively an AQP4 agonist when given at 48 h following injury improved motor functional performance +++ (p < 0.001), although still not beyond that of an AQP4&1 antagonist given at 5 h. Assessed at 7 days after injury both the AQP4 & 1 antagonist when given at 5 h and AQP4 agonist when administered at 48 h improved TBI induced motor functional impairment ••• (p < 0.001) however, in comparing the two results would suggest a non-significant trend towards the antagonist being more effective at this time.
5.3.6 Neuronal cell morphology in treated animals post TBI

For the histological examination, the primary motor cortex, caudate putamen (striatum) and the globus pallidus were chosen as regions of interest (Fig 5.16) given their significance to the functional motor pathway.

In sham animals, healthy neuronal cell bodies were observed within the primary motor cortex (Fig 5.17 A). However, within the same region of injured rodents at 5 h (Fig 5.18 A) and 48 h (Fig 5.19 A) post TBI, there were some dark and contracted neuronal cell bodies with minor vacuolisation. In animals treated with the AQP4 & 1 antagonist at 5 h (Fig 5.20 A) or with the AQP4 agonist at 48 h (Fig 5.21 A), there were only limited numbers of abnormal shaped dark cells along with minimal vacuolisation.

The caudate putamen (striatum) region of sham animals demonstrated healthy neuronal cell bodies (Fig 5.17 B), whereas in injured rodents at 5 h (Fig 5.18 B) and 48 h (Fig 5.19 B) following trauma, there were a number of dark and contracted neuronal cells. In rodents treated with the AQP4 & 1 antagonist at 5 h (Fig 5.20 B) there were still some dark and abnormal shaped neuronal bodies interspersed with healthy cells. In contrast, in animals administered the AQP4 agonist at 48 h (Fig 5.21) there appears to be predominantly healthy neuronal bodies.

In the globus pallidus of sham animals, healthy cells were clearly apparent throughout (Fig 5.17 C). In injured rodents at 5 h (Fig 5.18 C) and 48 h (Fig 5.19 C) post TBI, there appears to be no notable dark or contracted neuronal bodies within the same region. Not surprisingly, the AQP4 & 1 antagonist at 5 h (Fig 5.20 C) and the AQP4 agonist at 48 h (Fig 5.21 C) had little effect in comparison to what was observed in vehicle treated animals.
Figure 5.16 Anatomical regions of interest as assessed by H&E.
(A) Primary motor cortex. (B) Caudate putamen (striatum). (C) Globus pallidus.
Figure 5.17 H&E stained section from uninjured sham Sprague-Dawley rats. 
(A) Healthy neuronal cell bodies of the primary motor cortex. (B) Healthy neuronal cell bodies of the caudate putamen (striatum). (C) Healthy neuronal cell bodies of the globus pallidus.

Figure 5.18 H&E stained section from vehicle treated Sprague-Dawley rats, at 5 h post diffuse TBI. 
(A) Some dark & contracted neuronal cell bodies of the primary motor cortex with notable vacuolisation. (B) Some healthy neuronal cell bodies of the caudate putamen (striatum). (C) Healthy neuronal cell bodies of the globus pallidus.

Figure 5.19 H&E stained section from vehicle treated Sprague-Dawley rats, at 48 h post diffuse TBI. 
(A) Some dark & contracted neuronal cell bodies of the primary motor cortex with healthy cell bodies interspersed. (B) Healthy neuronal cell bodies of the caudate putamen (striatum). (C) Healthy neuronal cell bodies of the globus pallidus.
Figure 5.20 H&E stained section of Sprague-Dawley rats treated with an AQP4 & 1 antagonist at 5 h post diffuse TBI.

(A) Some healthy neuronal cell bodies of the primary motor cortex, with few abnormal shaped dark cells with minor vacuolisation. (B) Some healthy neuronal cell bodies of the caudate putamen (striatum), with minor abnormal shaped dark cells. (C) Primarily healthy neuronal cell bodies of the globus pallidus.

Figure 5.21 H&E stained section of Sprague-Dawley rats treated with an AQP4 agonist at 48 h post diffuse TBI.

(A) Notable dark neuronal bodies of the primary motor cortex with some healthy cells and minor vacuolisation. (B) Predominantly healthy neuronal cell bodies of the caudate putamen (striatum). (C) Predominantly healthy neuronal bodies of the globus pallidus, with some dark cell change.
5.4 Discussion

The current study has demonstrated that administration of an AQP4 & 1 antagonist at 5 h or an AQP4 agonist at 48 h following diffuse TBI, significantly reduces levels of brain albumin accumulation and improves motor functional outcome. Administration of the AQP4 & 1 antagonist at 5 h also did not alter the relative protein expression of water channels however, at 48 h the administration of AQP4 agonist did, albeit to a small extent. Results from the previous chapter also clearly showed that intervention with either of these compounds at these optimal time points also significantly attenuated injury induced brain swelling.

It is evident from previous studies that cellular metabolism is integrally bound to the homoeostasis of cell volume (Lang et al. 1998), with AQPs playing a key role in this process. Injury induced alterations to brain bioenergetic balance disrupt the equilibrium of net solute entry and exit and thus result in either unmediated cellular swelling (hypotonicity) or cell shrinkage (hypertonicity). In addition to the deleterious effects disruption to cell volume has on intracellular metabolism, pathological changes in transmembrane ion fluxes across neuronal cell membranes equally impair propagation and generation of action potentials (Iwasa et al. 1980) and thus may be one possible route for the observed deficits in motor function. Prior studies have also demonstrated that cell volume, largely dependent upon the activity of water channels, directly mediates the facility of protein metabolism, such as proteolysis and activation of apoptotic enzymes (Finn et al. 1996; Bilz et al. 1999). The present study has demonstrated that appropriate pharmaceutical modulation of AQPs post TBI significantly reduces posttraumatic levels of cerebral albumin, at first suggesting that these modulators may regulate BBB permeability. However, no such role has been previously described for aquaporin channels, and given the selectivity of the modulators used in the current study, a direct effect on BBB permeability is unlikely. Alternative, given that cell
volume homoeostasis is inextricably linked with functioning metabolism, pharmacologic regulation of AQP mediated changes in water flux may well serve to attenuate oedema related damage to cellular proteolytic machinery, thus enabling the more rapid degradation of the deleterious intracellular accumulation of albumin.

Nonetheless, there is a potential association between albumin transport across the BBB and AQP4 channels. For example, albumin is known to cross the blood brain barrier after TBI via transcytosis (Simionescu 1983; Predescu et al. 2004; Nag et al. 2007; Nag et al. 2011). The movement of albumin across the cell layer occurs via flask shaped invaginations of the plasma membrane known as caveolae (Alonso and Millan 2001). These caveolae are also thought to regulate signal transduction, including that mediated through protein kinase C (PKC). Given that the cellular localisation of AQP4 channels is thought to be regulated by PKC (as well as PKA), where internalisation of the channel will reduce water permeability (Haj-Yasein et al. 2011), the adsorptive mediated transcytosis of albumin via caveolae in diffuse TBI may potentially signal for the early internalisation of perivascular AQP4, thereby slowing the onset of cellular swelling. Any inhibition of caveolae lined transcytotic pits after TBI may therefore regulate AQP expression, and potentially the reverse may also be possible. This interrelationship requires further investigation that is beyond the scope of the present study.

Results from this chapter also indicate that the AQP4 & 1 antagonist when administered at 5 h following injury did not alter water channel expression when compared to vehicle groups. However, when given at 48 h following trauma the AQP4 agonist did reduce water channel expression at this time. The finding that the antagonist did not alter water channel activity is not at first surprising given that the novel AQP compounds used in this thesis are based around the loop diuretic bumetanide. Yet the small decrease as was observed in water channel expression for animals treated with the agonist at 48 h post injury, when compared to
vehicles, may support the hypothesis of each of these compounds serving very different
effects. The AQP4 agonist, having already increased water channel activity at a time when
oedema efflux is beneficial, may pave way for less of a physiological need to clear fluid
further via the upregulation of the protein. The AQP4 & 1 antagonist however, when
administered at 5 h following injury in the setting of greater brain moisture content and
atypical albumin transport, is less likely to gain from further reductions to water channel
expression when there is already a stark downregulation of the protein at the perivascular and
to a lesser extent ventricular level.

An interrelationship between TBI and the onset of motor functional impairment has widely
been shown, with axonal injury playing a significant part in this process (Englander et al.
1996; Swaine and Sullivan 1996; Ding et al. 2001; Walker and Pickett 2007). However, the
precise mechanism by which cerebral oedema contributes to this pathological progression
towards motor impairment is unclear. Certainly changes in the concentration of solutes within
intra and extracellular fluid can typically affect action potentials and thus neuronal activity.
AQP4, Na+/K+-ATPase and mGluR5 work in concert with one another on astrocytes to
regulate cell water volume and K+ homoeostasis (Illarionova et al. 2010), and thus facilitate
neuronal activity. It is also well known that elevations in extracellular K+ leads to
depolarization of astrocytic membranes and the subsequent uptake of HCO$_3^-$.
Increased intracellular HCO$_3^-$ then activates soluble adenylyl cyclase, increasing cAMP production
(Hamm et al. 1994), activating protein kinase A (PKA) and thus likely contributing to the
internalisation of AQP4. Indeed mouse AQP4 knockout studies have shown a delayed
reuptake of K+ (Binder et al. 2006), supporting the notion of the channel serving a role in ion
homeostasis. As such brain astrocytes, which are rich in AQP4, are not surprisingly more
than simply structural support to the ‘neurovascular unit’. Numerous studies have shown that
the cerebral glia play an integral role not only in the maintenance of the BBB, but are
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providers of neuronal metabolic support, mediate synapse activity via transmitter
uptake/release, can modulate CBF and even promote neuroregeneration (Araque et al. 1999;
Therefore the health of astrocytes and their function, which may be strongly linked to the
activity of the AQP water channels, may directly impact upon neuronal activity and thus
serves as a mediator of motor functional impairment. Accordingly, pharmaceutical
modulation of AQP activity may well indirectly affect normal neuronal activity and thus have
an impact on functional outcomes.

While the individual administration of the antagonist or agonist has clear beneficial effects on
oedema, as well as intraparenchymal albumin accumulation, tissue histomorphology and
functional motor outcome, it is unclear whether the sequential administration of the
antagonist followed by the agonist will be superior to the agents administered on their own.
The next chapter will investigate this possibility.
6 SEQUENTIAL ANTAGONIST/AGONIST TREATMENTS: THE EFFECTS ON CEREBRAL ODEMA, BRAIN ALBUMIN CONTENT & MOTOR FUNCTIONAL OUTCOME
CHAPTER 6: SEQUENTIAL AQP MODULATOR TREATMENT IN TBI

6.1 Introduction

In the previous chapters, we have established that early single administration with an AQP4 & 1 antagonist or later administration with an AQP4 agonist after TBI significantly attenuate cerebral oedema, reduced brain albumin accumulation and improved motor functional outcome. Equally we have demonstrated that neither pharmaceutical agent alters the expression of brain water channels after diffuse TBI, and thus appear to specifically modulate AQP 4 and 1 activity after acute head trauma.

It is well established that the genesis of cerebral oedema, increased ICP, impaired CBF, hypoxia, ischaemia, and further development of brain swelling are significant prognostic factors in determining patient clinical outcome following TBI (Marmarou et al. 1980; Vink et al. 1994; Shima 2003; Nimmo et al. 2004; Yang et al. 2005). It is also well understood that not only is such an injury cascade complex, but it is also played out along a chronological continuum, making the efficacy of clinical intervention highly dependent upon a temporal window of opportunity. Indeed no more so is this chronological profile evident than in the transition of oedema from one form to the other following TBI, with AQPs seen to play an integral part in this process (Bramlett and Dietrich 2004; Kimelberg 2004; Pasantes-Morales and Cruz-Rangel 2010). Development of such a progressive secondary injury cascade is known to clearly involve multiple molecular pathways, which presumably work in conjunction with the temporally altered mediators of brain oedema. Hence therapeutic approaches targeting single factors are unlikely to be effective, and given our results from Chapter 4, may potentially be deleterious when administered at the wrong time points.

Accordingly, the aim of the current study is to examine the efficacy of combined, sequential treatment with both of the AQP modulators at their previously determined optimal time
CHAPTER 6: SEQUENTIAL AQP MODULATOR TREATMENT IN TBI

points in ameliorating cerebral oedema, atypical brain albumin content and motor functional impairment following diffuse TBI.
6.2 Methods

6.2.1 Surgery and injury induction

Animals were injured using the weight drop model of diffuse brain injury (Marmarou et al. 1994) as described in detail in Chapter 2. Briefly, male Sprague-Dawley rats (380 ± 420g) were anaesthetised in Isoflurane, intubated and then maintained at a surgical level with the same anaesthetic. Animals were then injured (450 g from 2 m) and exposed to 30 min of hypoxia, induced via intubated inhalation of Isoflurane 1.5%, 240mL/min, O₂, 2L/min N₂. During the hypoxic period, all wounds were sutured, and thereafter, animals were weaned from intubated anaesthesia and returned to their home cages following recovery of normal ambulation.

6.2.2 AQP modulators AqB013 and AqF026

For sequential therapy, the AQP4 & 1 antagonist (AqB013; 0.8 mg/kg) was administered at 5 h post-TBI followed by the AQP4 agonist (AqF026; 0.2 mg/kg) at 48 h after TBI, both iv via the tail vein. At 5 h following final drug administration (that is 53 h) rodents were assessed for either AQP4 & 1 immunohistochemical changes, brain oedema or brain albumin accumulation. Another subset of animals was assessed for motor functional performance over a 7 day period following injury.

6.2.3 Analyses of oedema

6.2.3.1 Brain moisture content

Cerebral oedema was calculated using the previously described wet weight-dry weight methodology (Elliott and Jasper 1949) as detailed in Chapter 2. Briefly, brain moisture content was measured using a Mettler Toledo – HG63 halogen moisture analyser. Animals
were reanaesthetised at 5 h post drug administration (53 h) and decapitated to facilitate rapid brain removal from the skull. After removal, the olfactory bulb was removed and each hemisphere separated using a scalpel blade. The left hemisphere was placed on a 4.8g glass superfrost slide, previously weight tared in the moisture analyser, before being sliced evenly into three smaller segments. A second superfrost slide was then positioned on top of the first and both slides clamped together until the brain tissue was evenly distributed across the surface. Both slides were then placed into the moisture analyser, wherein it was swiftly weighed and then heated to dryness over a period of 10 min. The right hemisphere was subject to the same protocol in another identical moisture analyser immediately after the first hemisphere had begun to heat. Brain moisture content was determined as a percentage of tissue water using the established wet weight-dry weight formula (Elliott and Jasper 1949).

6.2.4 Immunohistochemistry

At 5 h following final pharmaceutical intervention, injured and treated rodents (n=5/group) were transcardially perfused using 100mL of 10% formalin (pH 7.4). Removed brains were then stored in 10% formalin and subsequently stained using Anti-Aquaporin 4 antibody [4/18] (ab9512; Abcam) or Anti-Aquaporin 1 Antibody (aB2219; Millipore) before antigen retrieval and visualisation of antigen as described in detail in Chapter 2. Digitally acquired nanozoomed images of slides were then evaluated for antigen content via the automated colour deconvolution analyses as outlined previously (Helps et al. 2012).

Brain albumin immunohistochemistry was initiated by overnight incubation with the appropriate primary antibody (rabbit polyclonal Anti-Human Albumin; 1:1000; DakoCytomation). Sections were then blocked with NHS and washed in PBS (pH 7.4) before being treated with the secondary antibody in NHS for 30 min (biotinylated Goat Anti-Rabbit IgG Antibody; 1:250; BA-1000; Vector). Subsequently, sections were incubated with SPC
(Pierce, 1:1000) and thereafter visualised with DAB, counterstained with haematoxylin, dehydrated, cleared, mounted and imaged for analysis by colour deconvolution.

Haemotoxylin and eosin (H&E) staining was also performed post TBI so as to observe any morphological changes. Standard H&E protocols were followed as previously detailed in Chapter 2, with sections finally being digitally imaged using a Hamamatsu nanozoomer.

6.2.5 Brain albumin extravasation

6.2.5.1 Evans Blue

Barrier permeability was estimated using the spectrophotometry protocol for the Evans Blue (EB) dye and semi quantified via ex vivo autofluorescent imaging as detailed in Chapter 2. In brief, 30 min prior to perfusion, animals (n=5/group) were administered EB dye (0.4mL/animal iv at 10 mg/kg) via the tail vein under general anaesthesia. All animals were ip injected with 0.1mL of Heparin (Pfizer, Australia; 1000 IU/mL) 10 min prior to perfusion so as to facilitate transcardial perfusion. Animals were then decapitated, their brains removed, the olfactory bulb removed and each hemisphere separated before being weighed. Each hemisphere was then placed in a vial and homogenised in 7.5mL of phosphate buffered saline before adding 2.5mL of trichloroacetic acid and storing the samples overnight at 4°C. Samples were then centrifuged at 1000 r.p.m over a 30 min period. An UV/V spectrophotometer was then employed to quantify the EB absorbency in the resulting supernatant at 620nm. The quantity of EB was expressed as μg/g of brain tissue using a standard curve for conversion.

A subset of animal brains were imaged for EB autofluorescence (615-665 nm) prior to homogenisation using a Xenogen IVIS Imaging System 100 equipped with a Cy5.5 filter. High counts of fractional fluorescent ratios were representative as either red or green colour...
channel pixels, while low to none as blue channel pixels, with a total range 0-255 arbitrary units. In quantifying EB, all scans were post processed on an identical sized pixel canvas, relevant background noise and blue colour channels removed, and all red and green colour channel pixel counts tallied.

6.2.6 Motor functional performance

Animals were assessed for motor functional deficits (n=5/group) over a week period using the rotarod device (Hamm et al. 1994) as described in Chapter 2. Briefly, animals were placed on a rotarod device consisting of a motorised rotating assembly of 18 rods (1 mm in diameter). The presence of these rods incorporates a grip strength component to the task making the rotarod the most sensitive and efficient test for assessing motor deficits following diffuse TBI (Hamm et al. 1994; Nimmo et al. 2004). Rotarod rotational speed ranges from 0 - 30 revolutions per minute (rpm) as determined by a control box operated by the assessor. Animals were placed on the rotarod, which at first remained stationary for the first 10 seconds, after which the rotational speed was increased by 3 rpm every 10 seconds for a maximum duration of 2 minutes. The point at which the animal fell from the rods or gripped the rods and spun for two consecutive revolutions, as opposed to actively walking, was then recorded in seconds. Animals were trained once daily over a 5 day period prior to injury so as to establish a normal pre-injury baseline and after injury were commenced at 24 h post injury and conducted at approximately the same time each day over the 7-day period.

6.2.7 Statistical Analysis

Levels of cerebral oedema and motor functional outcome were analysed by a repeated measures two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons posthoc tests. A one-way ANOVA with Bonferroni's multiple comparisons posthoc tests was employed to assess EB autofluorescence by spectrophotometry, ex vivo.
imaging and DAB visualisation on sectioned slides. All results are presented as mean ± standard error (SEM).
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6.3 Results

Intravenous administration of either the AQP4 & 1 antagonist at 5 h (77.53 ± 0.024) or the AQP4 agonist at 48 h (77.64 ± 0.012) significantly *** p < 0.001 (1.209) attenuated injury induced brain swelling when compared to vehicle controls at 48 h obtained from Chapter 5 (78.74 ± 0008) (Fig 6.1). Notably, sequential treatment (77.34 ± 0.015) with the antagonist followed by the agonist at these same time points resulted in an even greater attenuation of oedema than either the AQP4 & 1 antagonist alone • p < 0.05 (0.191) or the AQP4 agonist alone +++ p < 0.001 (0.295). ANOVA analyses also showed that there was a significant main effect for treatment groups F (4, 160) = 935.4; p < 0.001) and a significant temporal interaction F (7, 160) = 597.4; p < 0.001) over the 7 d period following injury.
6.3.1 Cerebral oedema in sequentially treated animals post diffuse TBI

**Figure 6.1** Measures of cerebral oedema formation post diffuse TBI in response to AQP modulation.

(As assessed via levels of brain moisture content). Sequential treatment with both of the AQP modulators at the optimal times of 5 h for the AQP4 & 1 antagonist • (p < 0.05 ) and then followed by the AQP agonist at 48 h +++ (p < 0.001) resulted in a significant attenuation of oedema as compared to either of the modulators on their own. There was no statistical difference between either of the single treatments when compared with one another. However, as was shown in Chapter 4, single treatments with each compound did ameliorate cerebral swelling *** (p < 0.001) when compared to vehicle controls.
6.3.2 **AQP4 immunoreactivity in sequentially treated animals**

Brain tissue fluid interfaces, namely perivascular (brain–blood-barrier), glia limitans (brain–subarachnoid cerebrospinal fluid) and ependymal (brain–ventricular CSF) regions were chosen for analysis of AQP4 expression given the changes in immunoreactivity noted in these areas in the previous chapters.

At 5 h after administration of the agonist there was faint-moderate perivascular AQP4 immunoreactivity within the cortex region directly beneath the site of impact (Fig 6.2 A) in animals treated sequentially with each of the AQP modulators. Similarly, within the periventricular area in the same treatment group, faint-moderate AQP4 expression was observed (Fig 6.2 B). However, within the glia limitans, there appears to be intense AQP4 immunoreactivity in sequentially treated animals (Fig 6.2 C).
Figure 6.2 AQP4 immunoreactivity in Sprague-Dawley rats sequentially treated with the AQP 4 & 1 antagonist and the AQP4 agonist at 5 and 48 h post diffuse TBI, respectively.

(A) Faint-moderate perivascular AQP4 immunoreactivity directly beneath the site of impact. (B) Faint-moderate periventricular AQP4 immunoreactivity. (C) Intense AQP4 immunoreactivity of the glia limitans bordering the subarachnoid space.
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Colour deconvolution analysis of whole coronal sections stained for AQP4 in sequentially treated animals showed a significant *** p < 0.001 (10.26) decrease in water channel immunoreactivity at 53 h following diffuse TBI (9.68 ± 0.741) as compared to uninjured, sham animals (19.94 ± 1.155) obtained from Chapter 5 (Fig 6.3). No significant difference was observed in AQP4 expression in sequentially treated rodents compared with animals administered a single AQP4 & 1 antagonist at 5 h (11.02 ± 0.381) post injury.
Figure 6.3 Colour deconvolution analyses of AQP4 immunoreactivity in sham, injured and treated rats.

Results show that when administered at 5 h post TBI the AQP4 & 1 antagonist did not alter expression of the protein within the brain. However, at 48 h of administration the AQP4 agonist did significantly change levels of water channel expression + (p < 0.05), although such a small difference may not be physiologically relevant. Comparably in sequentially treated groups, at 53 h post diffuse TBI there was significantly less AQP4 immunoreactivity as compared to uninjured sham groups *** (p < 0.001).
6.3.3 **AQP1 immunoreactivity in sequentially treated animals**

Prior research has demonstrated principle expression of AQP1 within the apical membrane of the choroid plexus and to a lesser extent in ventricular ependyma. These regions were therefore selected again for qualitative evaluation of water channel activity in the present study.

At 5 h following administration of the agonist, the periventricular region of sequentially treated animals demonstrated faint AQP1 immunoreactivity (Fig 6.4 A). In the choroid plexus, AQP1 immunoreactivity of sequentially treated groups showed moderate channel immunoreactivity (Fig 6.4 B). Summary examination of whole brain coronal sections did not yield evidence of the water channel elsewhere in any experimental groups.
Figure 6.4 AQP1 stained sections from sequentially treated Sprague-Dawley rats at 53 h post diffuse TBI.

(A) Faint periventricular and choroid plexus AQP1 immunoreactivity (3rd ventricle). (B) Moderate choroid plexus AQP1 immunoreactivity (lateral ventricle).
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Colour deconvolution analyses of whole coronal sections stained for AQP1 showed no statistical difference in water channel immunoreactivity of sequentially treated rodents at 5 h following agonist administration (4.5 ± 0.176) as compared to either of the single treatment groups (Fig 6.5). This is despite qualitative examination suggesting a slight increase of AQP1 immunoreactivity in sequentially treated animals back to almost preinjury levels. There was however, significantly * p < 0.05 (1.18) less AQP1 immunoreactivity in 5 h vehicle groups (3.96 ± 0.169) compared to sham animals (5.14 ± 0.384).
Figure 6.5 Colour deconvolution analyses of AQP1 immunoreactivity in sham, injured and treated rats at 5 and 48 h post TBI.

Quantitative results of whole coronal sections demonstrate no significant different in AQP1 immunoreactivity between any treated or vehicle groups. However, a statistical change was seen between sham and 5 h vehicles * (p < 0.05).
6.3.4 Albumin immunoreactivity in sequentially treated animals after TBI

Given that the previous chapters have shown changes in albumin at the brain tissue fluid interfaces including perivascular (brain–blood-barrier), ependymal (brain–ventricular CSF) and choroid plexus, these regions were again assessed for the presence of albumin following acute brain injury in sequentially treated groups.

Faint-moderate perivascular levels of albumin immunoreactivity was observed surrounding vessels directly beneath the site of impact in sequentially treated rodents (Fig 6.6 A).

Similarly, the periventricular region of sequentially treated groups demonstrated moderate-faint levels of albumin (Fig 6.6 B). Albumin immunoreactivity of the glia limitans, but not surrounding parenchyma, in sequentially treated animals showed intense levels of the protein (Fig 6.6 C).
Figure 6.6 Albumin stained section of sequentially treated Sprague-Dawley at 5 h after agonist administration.

(A) Faint perivascular albumin immunoreactivity directly beneath the site of impact. (B) Moderate-faint periventricular and choroid plexus albumin immunoreactivity. (C) Intense albumin immunoreactivity of the glia limitans bordering the subarachnoid space.
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Colour deconvolution analysis of whole coronal brain sections stained for albumin, demonstrated that sequentially treated animals (12.32 ± 0.528) have significantly less levels of the protein when compared to vehicle treated animals at 5 h (22.82 ± 0.806) *** p < 0.001 (10.50) and 48 h (18.84 ± 1.923) + p < 0.05 (6.515) (Fig 6.7). No significant difference however, was shown in levels of brain albumin content between either single or sequentially treated groups, although qualitative.
**Figure 6.7** Colour deconvolution analyses of Albumin immunoreactivity in sham, injured and treated rats.

Quantitative results demonstrate that no significant difference was observed between levels of cerebral albumin in either single or sequentially treated groups. However, a statistical difference was seen between sequential groups and vehicle treated controls at 5 h *** (p < 0.001) and 48 h + (p < 0.05) respectively.
6.3.5 Evans Blue extravasation in sequentially treated animals

Figure 6.8 illustrates that sequentially treated animals demonstrated significantly less brain EB extravasation at 53 h after injury (4.277 ± 0.015) when compared to either the single AQP4 & 1 antagonist at 5 h (4.439 ± 0.011) * p < 0.05 (0.161) or the AQP4 agonist at 48 h (4.642 ± 0.029) ++ p < 0.01 (0.365). Similarly, EB autofluorescence (Fig. 6.9) qualitatively confirmed that sequentially treated animals had less EB extravasation than either of the AQP modulators administered on their own.

**Figure 6.8** Brain EB extravasation after diffuse TBI.
Quantitative results show that sequentially treated animals as assessed at 53 h following injury have significantly less EB within the brain as compared to either the single antagonist at 5 h * (p < 0.05) or agonist at 48 h ++ (p<0.01).
EvansBlue autofluorescence concentration

Figure 6.9 Measures of brain EB extravasation after diffuse TBI as assessed via EB autofluorescence.
6.3.6 Motor functional performance in sequentially treated animals

**Figure 6.10** Rotarod performance in animals treated with AQP modulators after TBI. Results show that sequentially treated animals perform significantly better in motor functional assessment at 48 h when compared to either the AQP4 & 1 antagonist * (p < 0.05) or the AQP4 agonist +++ (p < 0.001) administrations alone. Similarly at 7 d post injury, sequentially treated groups perform significantly better than either the AQP4 & 1 antagonist +++ (p < 0.01) or AQP4 agonist ppp (p < 0.001).

With respect to motor outcomes, sequentially treated animal’s demonstrated significantly improved motor functional outcome when compared to either the AQP4 & 1 antagonist 61.2 ± 3.7) * p < 0.05 (7.00) or the AQP4 agonist (64.0 ± 3.2) alone +++ p < 0.001 (17.80).

This difference was observed as early as 2 days after injury and was sustained over the remaining period of assessment. At 7 d, sequentially treated (119.0 ± 1.2) animals showed statistically greater motor function compared to either the AQP4 & 1 antagonist (110.8 ± 1.9) +++ p < 0.01 (8.20) or AQP4 agonist (105.2 ± 4.0) p < 0.001 (13.80).
6.3.7 Neuronal cell morphology in sequentially treated animals post TBI

In sequentially treated animals, healthy neuronal bodies were mainly present within the primary motor cortex (Fig 6.11 A) and caudate putamen (Fig 6.11 B). Similarly, the neuronal bodies in the globus pallidus were largely healthy although there was some minor dark cell change (Fig 6.11 C).
Figure 6.11 H&E stained sections of sequentially treated Sprague-Dawley rats at 53 h post diffuse TBI.

(A) Predominantly healthy neuronal bodies of the primary motor cortex with minor dark cells interspersed. (B) Healthy neuronal cell bodies of the caudate putamen (striatum). (C) Predominantly healthy neuronal bodies of the globus pallidus, with some dark cell change.
6.4 Discussion

Within this study, we have demonstrated that sequential treatment with both of the AQP modulators at their optimal time points is significantly more efficacious in ameliorating injury induced cerebral oedema, albumin extravasation, levels of brain albumin content and impaired motor functional deficit as compared to either of the single treatments alone. Such a result is highly promising given that in certain clinical scenarios the time points at hand may provide a relevant temporal window of opportunity for improved pharmaceutical intervention in attending to victims of TBI.

Immunohistological analyses of whole brain coronal sections stained for AQP4 demonstrated a significant downregulation in the water channel in sequentially treated groups as compared to either single administration alone. However, a qualitative increase in AQP4 was observed along the glia limitans in sequentially treated groups at 48 h post TBI. No significant difference was seen in the expression of AQP1 from analyses of coronal sections between treatment groups, although a qualitative upregulation of the protein was observed within the choroid plexus of sequentially treated rodents. Findings of this nature may well provide some clue as to how activity of the channels improves neurological outcome following TBI.

As was previously shown in Chapter 5, decreases in the expression of perivascular and periventricular AQP4 early on after diffuse TBI would appear to serve in inhibiting the entry of water into the brain parenchyma. Equally downregulation of choroid plexus AQP1 at 5 h following TBI is likely to play a role in reducing fluid entry into the brain. Alternatively at the later stages of injury, increases in perivascular, periventricular and glia limitans expression of AQP4 would serve in the resolution of oedema. It would therefore be logical that in sequentially treated animals there is less of a physiological need to increase the expression of AQP4 at a later time point following diffuse TBI, given that the first part of the
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therapy has already significantly attenuating oedema onset. Nonetheless, in sequentially treated rodents at 5 h after agonist administration, there did appear to be a qualitative upregulation in AQP4 along the glia limitans, as was indeed the same case in vehicle treated animals at the same time point. Water channel expression of this form both in sequentially treated groups and vehicle controls would therefore imply that at the later stages of diffuse TBI, increases in AQP4 along the glia limitans serve as a potential route for extracellular fluid clearance during the resolution phase of oedema. As compared with the single AQP4 agonist treatment and vehicle groups at 48 h, which showed an increase in water channel activity in perivascular, periventricular and the glia limitans regions, sequentially administered animals need only upregulate AQP4 at a later time point along a tissue fluid interface which would directly enable water exit.

A similar hypothesis of a cumulative pharmaceutical effect would also hold true for the present results showing that sequentially treated animals exhibited significantly reduced levels of brain albumin content and improved motor functional outcome when compared to single administrations alone. As was suggested in Chapter 5, the effects on albumin may represent both an indirect effect on protein extravasation from the vasculature, and also a reflection of improved protein catabolism within cells. Appropriate cell volume, largely dependent upon the activity of water channels, directly mediates the process of protein metabolism, such as proteolysis of intracellular albumin (Bilz et al. 1999). Similarly, in view to the integral role AQP4 rich astrocytes play in neuronal metabolic support, appropriate K+ buffering, modulation of excitotoxicity and promotion of neuroregeneration, sequential treatment approaches are likely to yield a cumulative efficacy in attenuating injury, as reflected in both the improved cellular morphology in the current study, as well as the improved motor outcome.
As has been described previously, the expression of brain AQPs is not only highly dependent upon injury form, but their expression also varies with time. Chapter 5 demonstrated that choroidal plexus expression of AQP1 was downregulated early after diffuse TBI, most likely in a protective role to inhibit further entry of cerebral fluid during a time of profound water influx. During the same time, an increase in the expression of AQP4 was observed along the glia limitans and as well as a decrease at the perivascular and periventricular level. In contrast, in the later stages after TBI, upregulation of AQP4 would appear to serve in the promotion of water clearance. Sequential treatment with each of the AQP modulators at their optimal time points is therefore supplementing the physiological changes in AQP channel expression that occur after TBI in an effort to prevent and resolve cerebral oedema.
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This thesis describes the effects of pharmacological modulation of AQPs 4 & 1 on cerebral oedema, BBB permeability, motor functional performance and cell morphology following experimental diffuse TBI. We have also established (a) the temporal profile of AQP4 & 1 expression following diffuse TBI and (b) the chronology of brain moisture content following trauma.

Characterisation of the temporal profile of AQPs 4 & 1 after diffuse TBI in Chapter 3 showed that brain AQP4 expression is generally downregulated at the perivascular and periventricular tissue fluid interfaces at 5 h after TBI, although appears to be moderately increased along the glia limitans externa. At the same time, cerebral AQP1 expression is decreased, most notably within the cells of the choroid plexus. By 48 h following diffuse TBI, brain AQP4 is generally upregulated at all described tissue fluid interfaces. Choroid plexus AQP1 expression also appears to marginally increase at this time. Such findings are difficult to directly compare to other research as no previously published work has characterised the expression of AQP4 & 1 in a specifically diffuse brain injury model. A number of studies have described the expression of brain AQPs 4 & 1 in uninjured rodents (Badaut et al. 2002) and those findings were consistent with the sham findings in the current study. In primarily cellular models of brain oedema in mice, including water intoxication and focal cerebral ischaemia, AQP4 expression is downregulated at perivascular and ventricular surfaces thus limiting water entry (Manley et al. 2000; Friedman et al. 2009). Taya and colleagues demonstrated in a similarly cytotoxic model of controlled cortical impact in rats, that brain AQP4 protein expression significantly decreased post injury when combined with induced hypoxia however, increased as compared to uninjured controls without the presence of secondary hypoxic insult (Taya et al. 2010). In experimental models of hydrocephalus in rats, decreases in the expression of AQP1 within the choroid plexus are paralleled by an increase in subpial AQP4 (Kalani et al. 2012; Castaneyra-Ruiz et al. 2013). In contrast, in principally
vasogenic injury models, such as cold lesion, AQP4 deletion has resulted in higher ICP and brain moisture content (Papadopoulos et al. 2004), suggesting that removal of the channel in these circumstances may impair resolution of oedema via the glial limitans and ependymal barriers. My results in the diffuse TBI model clearly demonstrate that AQP4 was largely downregulated during the earlier time points following diffuse TBI and then conversely upregulated at the later stages of oedema. Taken in concert, such data strongly implies an early influx of water followed by a later resolution of oedema. Not only was the altered expression of brain water channels linked with injury form, but also that AQPs 4 & 1 served to limit excess fluid influx at a time when the BBB is more permeable to proteins, while during the later stages in which vascular leakage has begun to resolve, upregulation of the channels aids in the facilitation of cerebral fluid clearance.

The potential mechanisms underlying such dynamic mobilisation of brain AQPs in response to disturbances of cerebral fluid homeostasis may involve a number of alternative pathways. Protein kinase C (PKC) activators have been shown to downregulate expression of AQP4, suggesting that internalisation of the channel at its intracellular structural site Ser180, is promoted via secondary injury cascades, thereby decreasing water influx (Zelenina et al. 2002; Yool et al. 2009). Indeed, it well known that injury-induced Ca\(^{2+}\) cascades ensue following diffuse TBI resulting in the activation of numerous PKC pathways (Yang et al. 1993). As such the temporal nature of secondary excitatory mechanisms may well correlate with the early downregulation of AQP4 activity by 5 h post diffuse TBI. During the later stages follow diffuse injury, at which point BBB permeability has partially resolved and excitotoxicity has largely subsided, a physiological attempt to increase cerebral water channel activity would enable the resolution of excess oedematous fluid. Consideration may also be given to the essential inclusion of a hypoxic period in this model of diffuse TBI, as cerebral AQP expression has been shown to alter in the presence of secondary injury oxygen
deprivation. Indeed as has been described prior, cerebral hypoxia is a well characterised pathological hallmark of the secondary injury cascade following severe brain trauma, often exacerbating the damages caused by oedema (Ishige et al. 1987; Hellewell et al. 2010). Hence the demonstration by Taya et. al. that the inclusion of secondary hypoxic events post TBI significantly downregulate cerebral AQP4 expression when compared to injuries alone (Taya et al. 2010), would provide support to the hypothesis of brain water channels internalising in response to deprived oxygen supply. Previously in astrocyte cultures hypoxia has been shown to downregulate AQP4 (Fu et al. 2007; Zhu et al. 2009) and also critically observed to increase beyond its initial control levels once reoxygenation occurs (Yamamoto et al. 2001; Zhu et al. 2009). As such this early decrease in the water channels expression and subsequent increase at a later time point in response to oxygen supply, would coincide with the temporal profile of AQP4 activity as demonstrated in this Chapter.

Having established the temporal profile of AQP4 & 1 expression in diffuse TBI, the next step was to confirm the cerebral oedema profile after diffuse TBI and then administer either AQP antagonists or agonists at relevant time points along this continuum so as to determine their efficacy in ameliorating injury induced brain swelling. My results in Chapter 4 demonstrated that oedema peaked at 5 h after diffuse TBI and then progressively decreased thereafter to reach normal levels by 48 h after injury. These results were consistent with the time course of oedema formation as demonstrated in previously published research in this model of rat TBI (O'Connor et al. 2006) and confirmed that maximum water influx was occurring early after TBI and that the oedema was resolving by 48 h after injury. Administration of an AQP4 & 1 antagonist at 5 h post diffuse TBI was significantly attenuated cerebral oedema formation, whereas administration at the later timepoint of 48 h increased brain water content. In contrast, an AQP4 agonist given at 48 h significantly ameliorated cerebral oedema as compared to the antagonist treated group at this time. When given at 5 h after TBI the AQP4
agonist also reduced brain swelling, albeit not substantially below that of pathological levels. Intriguingly the nature of effective AQP modulation changed at around 24 h post diffuse TBI, providing an insight into the driving forces associated with water influx and efflux following diffuse TBI. Soon after diffuse brain injury, BBB permeability to large protein entry increases (Maeda et al. 2003; Marmarou et al. 2006). At the same time, there is net influx of Na\(^+\) and Ca\(^{2+}\) into the cells, in part because of excitotoxicity during the early phase after TBI. Water then flows down its osmotic gradient via AQP channels, resulting in oedema formation. The net driving force for water at this early phase after TBI is therefore from the vasculature to the brain parenchyma. Internalisation of AQP4 channels or inhibition using the AQP antagonists would attenuate oedema formation under these circumstances. At later time points, when BBB permeability to proteins has abated, the AQP4 channels increase their presence on the astrocyte end foot processes and facilitate resolution of oedema. The net driving force for water is from the brain parenchyma to the vasculature and inhibition of this flow exacerbates oedema. As such, my findings from Chapter 4 would not only imply a seamless continuum from one form of oedema to another following TBI, but also support the hypothesis suggested in Chapter 3 that brain AQPs 4 & 1 mobilise so as to at first inhibit and then help to resolve brain swelling following diffuse injury.

Immunohistological results from Chapter 5 subsequently demonstrated that the AQP4 & 1 antagonist when administered at 5 h after TBI did not significantly alter the physiological expression of brain water channels. The AQP4 agonist however, when given at 48 h did change water channel expression, albeit to a small extent. The findings obtained from administration of the antagonist at 5 h are not surprising given that the structure of the novel AQP compounds used in this thesis are based around the loop diuretic bumetanide (Migliati et al. 2009; Yool et al. 2009; Devuyst and Yool 2010) and accordingly were not expected to alter protein expression. However, the result showing that the AQP4 agonist when given at 48
h post injury did reduce water channel expression, as compared to vehicle groups, is possibly suggestive of the compound having already increased water channel activity at a time when oedema efflux is beneficial and therein reducing a physiological need to upregulate expression of the protein at this time.

Despite the variable roles AQP channels are seen to play within the brain, there are currently no other suitable pharmacological candidates for clinical development and deployment. Previous studies have shown that administration of sulfhydryl reactive based mercurials does inhibit the activity of AQP4 channels (Niemietz and Tyerman 2002), however, these ions are highly toxic to living cells and nonspecific in their action and thus prove to be entirely inappropriate for therapeutic use. Other candidates for blocking AQP1 activity have been reported such as acetazolamide (Ma et al. 2004), tetraethylammonium (Brooks et al. 2000) and DMSO (Yang et al. 2006), however, subsequent testing has demonstrated that none of these compounds display significant water channel inhibition. More recent studies have demonstrated the ability of multiple antiepileptics to downregulate AQP4 activity (Huber et al. 2007), although the use of specific assays has failed to verify such findings. The demonstration that the novel agents used in the current thesis are able to attenuate cerebral oedema without impeding the physiological expression of brain water channels is highly significant, and supports that the compounds directly modulate water channel function and not channel protein expression.

In addition to effects on water channel expression, Chapter 5 also demonstrated that the AQP4 & 1 antagonist and the AQP4 agonist administered at their optimal timepoints of 5 h and 48 h, respectively, both significantly reduced levels of brain albumin content and improved motor functional outcome following diffuse TBI. Brain albumin content has long been established as a marker for BBB integrity (Hawkins and Egleton 2006) given that as a large molecule it is generally unable to pass through the cerebral capillary wall without prior
disruption to barrier permeability. While able to pass between endothelial cells following
disruption of the tight junction, other studies have shown that albumin can traverse through
the barrier via caveolin mediated transcytosis (Anderson et al. 1992; Parton et al. 1994;
Indeed, it is widely held that the TJ between endothelial cells remains intact in the early
period after TBI and that increased BBB permeability to proteins after TBI is primarily
through upregulated transcytosis. Given that previous work has also shown that caveolin-1
can downregulate abluminal AQP channel activity (Jablonski and Hughes 2006), potentially
via PKC activity, it is at least plausible that in the event of increased brain albumin transport
after TBI, water channels internalise when the protein is being actively transported and then
partially returning to their apical state once BBB integrity is restored. However, as yet no
such role has been previously described for the water channels, and a direct or indirect effect
on BBB permeability remains speculative. A clue however, might be found in considering the
work of Taya and colleagues who demonstrated, in a rodent model of controlled cortical
impact that in the presence of secondary hypoxia post injury there is a downregulation of
cerebral AQP4 in parallel to increased levels of EB (Taya et al. 2010). Such a finding when
coupled with the results obtained from this present Chapter may therefore provide some
evidence to the hypothesis that cerebral AQPs serve to limit additional fluid influx at a time
when brain albumin transport is increased following TBI via their internalisation, whereas
upregulate their expression at a later time when vascular permeability has begun to resolve. It
may also be the case that levels of brain albumin content are more indicative of cellular
metabolism rather than simply a reflection of BBB permeability. Cell health is integrally
bound to the homoeostasis of cellular volume (Lang et al. 1998), which has been linked to the
cell’s ability to undertake protein metabolism (Finn et al. 1996; Bilz et al. 1999). AQPs play
a key role in regulating cellular volume and any pharmaceutical intervention that regulates
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AQP activity may potentially influence protein metabolism. This may include the degradation of any albumin that has accumulated with injury. My results on BBB permeability also demonstrated a partial albeit lesser increase in BBB permeability during the later stages of diffuse brain trauma (48 h). Such a notion has already been contemplated in earlier published work (Maeda et al. 2003; Marmarou et al. 2006), and although further exploration of this phenomena is beyond the scope of this present thesis, it is relevant to note the significant efficacy of the AQP agonist at these later time points in resolving excess fluid accumulation.

We subsequently showed that administration with either of the AQP modulators at their most efficacious time point with respect to attenuating oedema also significantly attenuated posttraumatic motor deficits, although the AQP4 & 1 antagonist was the most effective of the two compounds. In part, this may be due to the early administration (5 h) of the antagonist as opposed to the late administration (48 h) of the agonist. However, given that the water channels assist in maintaining basic ionic cell homeostasis, it is also possible that appropriate modulation of AQP activity post diffuse TBI may facilitate recovery of normal neuronal function. At their most rudimentary level, AQP4 rich astrocytes contribute to extracellular K$^+$ reuptake/release, the appropriate buffering of which is a basic component of neuronal synaptic function. As such, astrocyte health can integrally influence neuronal survival during the acute stages of brain insult, as well as mediate neurite outgrowth during the recovery period post TBI. Glutamate toxicity is also a critical mechanism associated with neuronal cell death in brain trauma. Glial glutamate reuptake requires transport against a steep concentration gradient and this transport hinges heavily upon sufficient reserves of ATP. Hence the appropriate modulation of astrocytic AQP4, assisting in the key maintenance of glial energy metabolism, is likely to facilitate improved neuronal activity post TBI. Excess glutamate is also known to increase glial cell water permeability via mGluR5, as well as activate AQP4 (Illarionova et al. 2010). Thus the functionality of the proposed AQP4,
Na+/K+-ATPase, mGluR5 microdomain would also be facilitated via the appropriate pharmaceutical support of water channel activity post TBI.

My results from Chapter 6 showed that sequential administration of both the antagonist and the agonist reduced brain moisture content, levels of cerebral albumin and improved impaired motor functional outcome to a significantly greater extent than animals treated with the single interventions alone. Immunohistological results assessing whole brain coronal sections demonstrated that sequentially treated animals also had significantly lower levels of AQP4 at 48 h when compared to the individual treatments, although there was an increase in AQP4 along the glia limitans at this time. It would therefore seem that in sequentially treated animals there is less of a physiological need to overexpress AQP4 at later stages because the need for oedema clearance has been reduced. The results in this chapter also showed that in sequentially treated rodents, AQP1 expression within the choroid plexus appeared to return to pre-injury levels. This observation is consistent with other studies in hydrocephalic rats that demonstrated a downregulation of choroid plexus AQP1 in parallel with an increase in glia limitans AQP4 (Paul et al. 2009; Kalani et al. 2012; Castaneyra-Ruiz et al. 2013). Water channel expression changes of this nature would support the notion of brain AQPs 4 & 1 working in concert with one another to attenuate brain oedema formation, with their expression being dependent upon both time and injury form.
7.1 Conclusion.

In the present thesis, I have shown that single administration of an AQP4 & 1 antagonist at 5 h, an AQP4 agonist at 48 h and the sequential treatment with both of the compounds at their optimal time points is beneficial to physiological and functional outcome following diffuse TBI. At a physiological level there was an attenuation of post traumatic cerebral oedema and of brain albumin content, with these beneficial effects occurring in the absence of any changes in water channel expression. Functionally, each compound improved functional motor outcome after TBI when they were administered at their optimal time point. Sequential treatment with both compounds proved even more efficacious than single interventions. The sequential treatment with the antagonist and then the agonist augmented what seemed to be a protective response of the brain against posttraumatic oedema, namely an initial downregulation of AQP channels followed by a later upregulation. This alteration in expression was mimicked by initial inhibition with the antagonist followed by facilitation of water transport during the resolution phase of oedema. Taken together these results provide evidence in favour of a pharmaceutical treatment for the attenuation of injury-induced brain swelling, which when administered at the optimal time points may deliver a much needed novel, therapeutic intervention for this life threatening condition.
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