Combinatorial ion channel inhibitor therapy for the treatment of CNS injury: a therapeutic optimisation approach

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B.Sc. Neuroscience (Hons)

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&
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This work is dedicated to anyone that has ever doubted me, to my family for supporting me, and to Rhianna, who believed in me every step of the way.
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**Abbreviations**

AAD  acutaxonal degeneration
ADP  adenosine diphosphate
AM   acetoxymethyl
AMPA $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AnkG ankyrin-G
AQP4 aquaporin 4
ATP  adenosine triphosphate
Bbb  blood brain barrier
BBB  Basso, Beattie and Bresnahan
Ca   calcium
CaM  calmodulin
cADP cyclic adenosine diphosphate
cGMP cyclic guanosine monophosphate
Cl   chloride
CMH$_2$DCF chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate
CNP  2′,3′-Cyclic nucleotide 3′-phosphodiesterase
CNS  central nervous system
CPP  3-(2-carboxypiperazin-4-yl) propyl-1-phosphoric acid
CSF  cerebrospinal fluid
Cu   copper
Cu,Zn,SOD copper, zinc, super oxide dismutase (super oxide dismutase catalysed dismutation of superoxide)

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DAP7 2-amino-7-phosphonoheptanoate
DCF 2’,7’-dichlorofluorescein
DHE dihydroethidium
DNA deoxyribose nucleic acid
EAE experimental autoimmune encephalomyelitis
ED1 antibody against cellular marker CD68
EGTA ethylene glycol-bis (β-aminoethyl ether)-N, N, N’, N’-tetra acetic acid
eNOS endothelial nitric oxide
ER endoplasmic reticulum
FADH flavin adenine dinucleotide hydroquinone
Fe iron
GAP43 growth associated protein 43
GFAP glial fibrillary acidic protein
GluR glutamate receptor
GPx glutathione peroxidase
H2O2 hydrogen peroxide
HIPPR high protein and peptide recovery
HNE 4-hydroxynonenal
HO heme oxygenase-1
HPLC high performance liquid chromatography
HVA high voltage activated
InsP3 inositol triphosphate receptor
IL-1 interleukin-1
INQ  see YM872
K  potassium
LC/MS/MS  liquid chromatography tandem mass spectrometry
Lom  Lomerizine
LVA  low voltage activated
MAG  myelin-associated glycoprotein
MBP  myelin basic protein
MCT1  monocarboxylate transport 1
MCT2  monocarboxylate transport 2
MDA  malondialdehyde
Mem  memantine
MK801  Dizocilpine
MnSOD  manganese superoxide dismutase
MPT  mitochondrial permeability transition
MRM  multiple reaction monitoring
Na  sodium
NAADP  nicotinic acid adenine dinucleotide phosphate
NADH  nicotinamide adenine dinucleotide hydrate
NADPH  nicotinamide adenine dinucleotide phosphate
NBQX  2,3-dihydroxy-6-nitro-7-sulfamoylbenzo (F) quinoxaline
NF155  neurofascin155
NF186  neurofascin186
NG2  neural/glial antigen-2
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NrCAM</td>
<td>neuronal cell adhesion molecule</td>
</tr>
<tr>
<td>O₂</td>
<td>oxygen</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>superoxide anion</td>
</tr>
<tr>
<td>O₂⁻²</td>
<td>peroxide</td>
</tr>
<tr>
<td>OH⁻</td>
<td>hydroxyl ion</td>
</tr>
<tr>
<td>·OH</td>
<td>hydroxy radical</td>
</tr>
<tr>
<td>8OHDG</td>
<td>8-hydroxy-2'-deoxyguanosine</td>
</tr>
<tr>
<td>OLIG1</td>
<td>oligodendrocyte transcription factor-1</td>
</tr>
<tr>
<td>OLIG2</td>
<td>oligodendrocyte transcription factor-2</td>
</tr>
<tr>
<td>ON</td>
<td>optic nerve</td>
</tr>
<tr>
<td>OPC</td>
<td>oligodendrocyte progenitor cell</td>
</tr>
<tr>
<td>oxATP</td>
<td>oxidised adenosine triphosphate</td>
</tr>
<tr>
<td>P2X</td>
<td>adenosine triphosphate-gated cation channel family</td>
</tr>
<tr>
<td>P2Y</td>
<td>purinergic g protein-coupled receptor family</td>
</tr>
<tr>
<td>PDGFα</td>
<td>platelet-derived growth factor subunit alpha</td>
</tr>
<tr>
<td>PLP</td>
<td>proteolipid protein</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>SCI</td>
<td>spinal cord injury</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SMI32</td>
<td>anti-neurofilament H non-phosphorylated</td>
</tr>
<tr>
<td>TBI</td>
<td>traumatic brain injury</td>
</tr>
<tr>
<td>TEAB</td>
<td>triethylammonium bicarbonate</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor alpha</td>
</tr>
<tr>
<td>VGCC</td>
<td>voltage gated calcium channel</td>
</tr>
<tr>
<td>WD</td>
<td>Wallerian degeneration</td>
</tr>
<tr>
<td>YM872</td>
<td>[2,3-dioxo-7-(1H-imidazol-1-yl)6-nitro-1,2,3,4-tetrahydro-1-quinoxalinyl] acetic acid monohydrate</td>
</tr>
<tr>
<td>Zn</td>
<td>zinc</td>
</tr>
</tbody>
</table>
Abstract

Spinal cord injury (SCI) is a devastating event that can result in paralysis or even death. Following SCI, cells beyond the primary injury site undergo secondary degeneration, contributing to chronic deterioration of functional outcomes. Reducing the extent of secondary degeneration following SCI is likely to result in greater preservation and/or recovery of function, but treatment options have thus far been limited. Calcium (Ca\(^{2+}\)) overload and oxidative stress is understood to exacerbate myelin abnormalities and the loss of neurons, glia and function due to secondary degeneration following neurotrauma. Given that Ca\(^{2+}\) can enter cells of the central nervous system (CNS) through multiple ion channels, therapeutic strategies to reduce Ca\(^{2+}\) influx are likely to require inhibition of several ion channels. We have previously assessed efficacy of various combinations of three ion channel inhibitors for treatment of chronic secondary degeneration: using lomerizine (Lom), YM872 and/or oxATP to block voltage gated L- and T-type channels, Ca\(^{2+}\) permeable AMPA receptors and purinergic P2X\(_7\) receptors (P2X\(_7\)-R) respectively. We demonstrated that the combination of three ion channel inhibitors preserved structure and function following neurotrauma in vivo. However, at this time we had not established if efficacy was due to direct effects on intracellular Ca\(^{2+}\) concentration, nor had we assessed the effects of the inhibitor combinations during the early time phase of secondary degeneration. Finally, we were yet to test the optimal combination of ion channel inhibitors in a clinically relevant model of neurotrauma. Here we used a variety of models to: (1) define the oxidative events during early time phases of secondary degeneration; (2) define the effects of various combinations of ion channel inhibitors in vitro and in vivo, and (3) assess the efficacy of the most optimal combination of ion channel inhibitors in a clinically relevant contusion model of thoracic SCI.

The first study was designed to identify contributors to, and consequences of oxidative stress in white matter tracts, vulnerable to secondary degeneration. Partial dorsal transection of the optic nerve (ON) was used to model secondary degeneration in ventral nerve unaffected by the primary injury. Reactive species were assessed using fluorescent labelling and liquid chromatography/tandem mass spectroscopy. Fluorescent indicators of reactive oxygen species and/or nitrogen species increased at 1, 3 and 7 days after partial injury to the CNS. Similarly, immunoreactivity for glutathione peroxidase and haem oxygansase-1 increased in vulnerable tissue at 3 and 7 days after injury, respectively.
Despite increased antioxidant activity, DNA oxidation was evident from 1 day, lipid peroxidation at 3 days, and protein nitration at 7 days after injury. Nitrosative and oxidative damage was particularly evident in mature oligodendrocytes, at times after injury at which structural abnormalities of the node of Ranvier/paranode complex have been reported.

To help determine the effects of multiple combinations of ion channel inhibitors on intracellular Ca\(^{2+}\) concentration, we directly measured changes in intracellular Ca\(^{2+}\) concentration in an in vitro CNS injury model. When assessing Ca\(^{2+}\) concentration and viability of primary mixed cortical cultures in vitro, most combinations of inhibitors that included oxATP significantly decreased Ca\(^{2+}\) concentration and increased cell viability following 30 minute \(\text{H}_2\text{O}_2\) stress. However, reductions in intracellular Ca\(^{2+}\) concentration were not always linked to viability of neurons and glial cells. Combinations of inhibitors including oxATP preserved NG2-positive non-oligodendroglia, but preservation of astrocytes and neurons required additional inhibitors. Interestingly, NG2-positive/Olig2-positive oligodendrocyte precursor cells (OPCs) were not preserved by any inhibitor combinations in vitro.

To determine the optimal combination of ion channel inhibitors, we assessed the effects of the inhibitors on axonal degeneration, node of Ranvier structure, oxidative stress and OPCs at 3 days following injury in vivo, in tissue vulnerable to secondary degeneration. Following partial ON injury, most combinations of ion channel inhibitors were effective in restoring the lengths of the paranode and the paranodal gap, indicative of the length of the node of Ranvier, following injury. However, only all three inhibitors in combination restored to normal Ankyrin G (AnkG) length at the node of Ranvier. Similarly, lipid peroxidation and loss of OPCs were only prevented by treatment with all three ion channel inhibitors in combination. Other indicators of oxidative stress were unchanged or reduced by Lom and/or the three ion channel inhibitors in combination; visual function was intermediate between injured and normal levels with all inhibitor combinations at this acute time point. Data indicate that inhibiting any of a range of ion channel combinations including the VGCC inhibitor Lom preserves specific elements of node structure and limits some oxidative damage following injury, whereas Ca\(^{2+}\) flux through a number of additional specific channels must be inhibited to prevent acute lipid peroxidation and
preserve AnkG distribution and OPCs. From these data, it was determined that Lom, YM872 and oxATP in combination was the optimal combination of inhibitors.

The efficacy of the optimised combination of ion channel inhibitors in a clinically relevant *in vivo* model of spinal cord injury was then assessed, to provide preclinical data necessary if translation of this therapeutic strategy to the clinic is to be achieved. Fischer (F344) rats were subjected to a moderate (150kD) contusive SCI at thoracic level T10 using an Infinite Horizons impactor. Lom in butter was delivered orally twice daily, and YM872 and oxATP were delivered *via* osmotic mini-pump immediately after SCI thereafter until 2 weeks following SCI. Behavioural analyses were conducted from day 0 to 70 days, post injury. Open field locomotion analysis revealed that treatment with the three inhibitors in combination improved the rate of functional recovery of the hind limb (compared to controls) as early as 1 day post injury, with beneficial effects persisting to 14 days post injury, while all three inhibitors were present. Functional improvement observed with acute combinatorial treatment was associated with significantly decreased cyst size, tissue sparing, increased immunoreactivity of β-III tubulin+ve axons and myelin basic protein, as well as preservation of CC1+ve oligodendrocytes and NG2+ve/PDGFα+ve oligodendrocyte progenitor cell densities, and reduced lipid peroxidation by-products, compared to control animals. The combination of Lom, oxATP and YM872 shows preclinical promise for control of secondary degeneration following SCI and further investigation of long term continued treatment is warranted.

We have shown that combinatorial treatment for CNS injury using specific combinations of ion channel inhibitors reduces intracellular Ca\(^{2+}\) influx following insult to CNS cells, resulting in reduced cell death, axonal and node of Ranvier/paranode complex structure changes, oxidative damage, cyst formation, astrogliosis, and preserved oligodendroglia. Treatment with Lom, oxATP and YM872, was observed to improve acute hindlimb function in a clinically relevant model of spinal cord injury. The effects of the optimised combination of Lom, oxATP and YM872 provides mechanistic information and a key step-forward in identifying further therapeutic targets following CNS injury.
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Chapter One

Introduction and Review of Literature

Neurotrauma is a serious debilitating injury that can quickly lead to brain damage, paralysis and even death. It can be caused by a wide range of insults to the central nervous system (CNS), from blunt physical trauma to the spinal cord, to ischemic stroke in the brain. Despite decades of research, no cure or effective treatment exists. Secondary degeneration leads to the spread of injury to vulnerable undamaged tissue, resulting in loss of function beyond the lesion site of the initial injury. Secondary degeneration is associated with loss of calcium (Ca\(^{2+}\)) homeostasis in cells of the nervous system, resulting in the spread of oxidative stress and consequent damage to nervous tissue. Limiting excessive Ca\(^{2+}\) influx and/or oxidative stress in neurons and glia has been investigated as a therapeutic strategy to reduce secondary degeneration, particularly in white matter. Pre-clinical trials utilising single Ca\(^{2+}\) channel inhibitors have shown a number of beneficial effects of limiting excessive Ca\(^{2+}\) influx into neurons and glia, however these inhibitors seldom progress further than early phase clinical trials.

This chapter reviews the literature covering the causes and mechanisms of secondary degeneration following neurotrauma, with particular focus on white matter, Ca\(^{2+}\), reactive oxygen species and oxidative stress, and the development of effect treatment strategies. The review will first provide an overview of the CNS with focus on myelinating glia and the structure of myelin to provide necessary background information to understand the changes that occur as a consequence of neurotrauma. The final aspect will address the idea of combinatorial treatment strategies; in particular, the rationale and use of combining multiple ion channel inhibitors for the treatment of CNS injury.

1.1 The Central Nervous System (CNS)

The nervous system is subdivided into the peripheral nervous system (PNS) and the CNS. The CNS, composed of the brain and spinal cord, is surrounded by the bony boundaries of the skull and vertebral column, and protected by all three layers of the meninges (dura, arachnoid and pia) and cerebrospinal fluid (CSF). The brain itself has a number of subdivisions, composed of the cerebrum (divided into left and right cerebral
hemispheres), the cerebellum and the brainstem (Figure 1.1). The spinal cord lies in the vertebral canal, continuous with the medulla oblongata of the brainstem. The spinal cord can be divided into cervical, thoracic, lumbar, sacral and coccygeal regions, according to the parts of the body its nerves supply. Regions can be further subdivided into segments, each giving rise to a pair of spinal nerves; eight cervical (C1-C8), twelve thoracic (T1-T12), five lumbar (L1-L5), five sacral (S1-S5) and one coccygeal.

Despite the generous size, widespread distribution and complexity of the CNS, it contains only two principal categories of cells – nerve cells, or neurons, and glial cells. Although neurons and glial cells are not limited to the CNS, and can be found throughout the PNS; this review will primarily focus on cells in the CNS. Neurons are electrically excitable cells; whose primary role is the chemical and electrical transmission of signals and information processing. Neurons consist of a cell body (soma) which contains the nucleus, and other organelles that support the metabolic needs of the neuron. Most neurons have many branching dendrites, and at least one long axon extending away from the soma that receives information from other neurons via synaptic contacts. Cytoplasmic components and neurotransmitters are synthesized in the soma and then transported along the axon and dendrites. Historically, glia is Greek for “glue”, a term given to cells of the CNS that ‘filled the spaces’ between neurons and appear to hold them in place. Although

Figure 1. Key anatomical features of the spinal cord. A sagittal section through human CNS (a) and a transverse section through human spinal cord (b). (Thuret et al., 2006).
glial cells provide structural support, it is now well established that CNS glial cells serve additional, more important roles. CNS glial cells include: ependymal cells, microglial cells, astrocytes and oligodendrocytes (Nolte, 2009)(Figure 1.2). Overall, the complexity of the CNS is governed by the wide range of glial cells present, maintaining the optimum conditions for neuronal function. In the following sections, the key glial subtypes, oligodendrocytes and their progenitors, will be described together with the myelin they generate.

![Figure 2. Cells of the CNS. The CNS is composed of different cell types. These include neurons as well as the glial population; astrocytes, microglia, and oligodendrocytes (Allen and Barres, 2009).](image)

### 1.1.1 Oligodendrocytes

In mammalian white matter of the CNS, oligodendrocytes are considered to be the most common of glial cells (Hildebrand et al., 1993), with the myelin sheath constituting the most abundant membrane structure in all vertebrates. Oligodendrocytes generate myelin as an extension of their plasma membrane, sending out extensions, each of which simultaneously wrap around several axonal segments multiple times. The resultant
structure forms a tightly compacted and insulating myelin sheath consisting of layers of lamellae held together with proteins (Hildebrand et al., 1993; Nave, 2010a). Lipids constitute 70-80% of myelin, with the remaining 20-30% made up of four major proteins: myelin basic protein (MBP); proteolipid protein (PLP); myelin associated glycoprotein (MAG); and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) (Hildebrand et al., 1993) (Figure 1.3).

Figure 3. Oligodendrocytes and myelin composition. Myelin contains a complex arrangement of complex lipids and proteins (MBP, PLP, MAG and CNP). (Podbielska et al., 2013).

The development of oligodendrocytes and myelination of axons is a highly regulated mechanistic process, including: extracellular ligands, secreted molecules and axonal activity (Emery, 2010). From early embryonic development through to early post-natal life, oligodendrocyte progenitor cells (OPCs) are generated in the ventral neuroepithelium of the neural tube (Pringle et al., 1998), and dorsoventral regions of spinal cord and hindbrain (Vallstedt et al., 2005; Kessaris et al., 2006). Several antigen markers allow specific identification of OPCs using immunohistochemistry, such as the proteoglycan.
NG2 (Nishiyama et al., 1999a) and platelet derived growth factor receptor α (PDGFRα) (Pringle et al., 1992). Other markers may be used to identify transcription factors in cells further along the oligodendroglial lineage; Olig1, Olig2, Nkx2.2 (Qi et al., 2001; Lu et al., 2002; Fancy et al., 2004), and O4 (Gard and Pfeiffer, 1990). However, some non-oligodendroglia also express these markers; for example, astrocytes can typically express Olig2 (Marshall et al., 2005). Thus, combinations of various markers are required to correctly identify OPCs and myelinating oligodendrocytes appropriately.

OPCs express most neurotransmitter receptors including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Yoshioka et al., 1995; Matute et al., 1997) and N-methyl-D-aspartate (NMDA) receptors (Bakiri et al., 2009a; Bakiri et al., 2009b) (Figure 1.4). Neuronal activity and AMPA receptor activation are among the positive signals necessary for regulating OPC proliferation and differentiation (Barres and Raff, 1993; Fannon et al., 2015). As such, axon signals are important for the proper myelination of the CNS. In rat cerebellum, CNP is synthesized by developing oligodendrocytes, followed 2-3 days later by the appearance of MBP along with PLP trafficking under neuronal control, immediately before the formation of myelin (Trajkovic et al., 2006).

![Figure 1.4](image)

*Figure 4. Glutamate receptor expression on oligodendrocyte lineage cells. The development of oligodendrocytes and myelination of axons is a highly regulated mechanistic process, including: extracellular ligands, secreted molecules and axonal activity. During development, OPCs, immature and mature oligodendrocytes express glutamate receptors (Kolodziejczyk et al., 2010).*

The final stage of oligodendrocyte development is myelination. Here, these proliferative cells migrate to developing white matter, leave the cell cycle, and differentiate into mature
oligodendrocytes, extending several cytoplasmic extensions (filipodia) to find suitable axons for myelination. Microtubules then begin to invade the filipodia, enlarging the processes and converting them to lamellipodia (Lee et al., 2005). During development, myelination onset depends on both positive and negative axonal signals (Coman et al., 2005). Extrinsic signalling mechanisms control both oligodendrocyte differentiation and myelination via axonal surface ligands, secreted molecules and axonal activity (see review Emery, 2010).

1.1.2 Myelin

Oligodendrocytes not only form myelin, but have a physiological role in supporting the functional integrity of axons, with oligodendrocyte defects known to cause axonal degeneration (Lappe-Siefke et al., 2003; Kassmann et al., 2007). Given that compacted myelin acts as an insulator, it inevitably limits the axonal compartment from free access to glucose from the extracellular environment (Nave, 2010b). Therefore, it is likely that oligodendrocytes transfer metabolites, feeding the axon. Recently it was suggested that the most likely route of metabolite transfer is ‘myelinic channels’ of non-compacted myelin, providing a physical connection between glial cytoplasm and the periaxonal space of myelinated axons. Funfschilling (2012) demonstrated this with the uptake of blood-derived glucose by glycolytic oligodendrocytes and delivery of glycolysis products (pyruvate/lactate) via monocarboxylate transports (MCT1 and MCT2) to myelinated axons.

In addition to provision of metabolic support, the myelination of axons is thought to benefit neurons in two further ways. Firstly, myelination strongly reduces the energy consumption needed for normal maintenance of ion gradients by sodium (Na⁺), potassium (K⁺)-ATPase (Boulton et al., 1986). Secondly, myelination of axons allows for a 100-fold increase in conduction velocity, resulting in the efficient transmission of neuronal signals and information within the central and peripheral nervous systems (Stys, 2004b; Nave, 2010a; Stirling and Stys, 2010). In addition to the highly specialised insulation of the myelin sheath, there is a coordinated organisation of axonal ion channels, supplying the necessary voltage-dependent ionic permeabilities to longitudinally move current along the axon (Stys, 2004a). A fundamental characteristic of myelinated axons is their distinct molecular domains, organised to allow for saltatory action potential propagation.
These domains include nodes of Ranvier, paranodes and juxtaparanodes (Buttermore et al., 2011).

### 1.1.3 Nodes of Ranvier

Separating the myelin sheaths of oligodendrocytes are distinctive, highly specialised junctions: the nodes of Ranvier (Hildebrand et al., 1993). At the nodes, high densities of voltage-gated Na\(^+\) channels are clustered in the axonal membrane, and are responsible for conducting inward depolarising currents (Kaplan et al., 1997; Stirling and Stys, 2010), paramount for the propagation of current along myelinated axons (Waxman and Ritchie, 1993). In addition to voltage-gated Na\(^+\) channels, K\(^+\) channels and several other transmembrane and cytoskeletal proteins exist in the axolemma at the node, including: Neurofascin-186 (NF186), a 186-kDa splice variant (Davis and Bennett, 1993; Davis et al., 1993; Tait et al., 2000); neuronal cell adhesion molecules (NrCAM) (Davis et al., 1996); actin-binding protein βIV-spectrin (Berghs et al., 2000), and the cytoskeletal adaptor ankyrin-G (AnkG) (Kordeli et al., 1990; Kordeli et al., 1995). AnkG is responsible for anchoring Na\(^+\) channels to the cytoskeleton of the nodal axolemma; NrCAM and NF186 bind via βIV-spectrin attachment (Figure 1.5), the resulting complexes restricting Na\(^+\) and K\(^+\) channels (see below) to specialised regions (Nave, 2010a). Rodent models with mutations in these cytoskeletal complexes have reductions in Na\(^+\) channel density and AnkG expression at nodes of Ranvier, demonstrating the importance of proper organisation at these regions (Lacas-Gervais et al., 2004; Yang et al., 2004).

### 1.1.4 Paranodal junctions

Nodes of Ranvier are flanked by paranodal axoglial junctions (paranodes) (Nave, 2010a). Paranodes are enormous and very unique structures which anchor the myelin sheath, seal the terminal loops of each myelin segment to the axon, on both sides of the node of Ranvier (Figure 1.5 (Rosenbluth, 2009) and thus allow for bidirectional signalling between axons and oligodendrocytes throughout life (Nave and Trapp, 2008).
Figure 5. Paranodal junctions. Schematic diagram depicting each sheath of compacted myelin (purple) anchoring to the axon at paranodal axoglial junctions. These paranodal junctions flank each node of Ranvier (Nave, 2010b; Devaux and Faivre-Sarrailh, 2013).

Like the node of Ranvier, the paranodal junction (PNJ) is maintained via neuron-glia interactions. The major components of paranodes are lipid raft-associated proteins: contactin-associated protein (Caspr-1) (Rios et al., 2000), a transmembrane neurexin superfamily molecule (Einheber et al., 1997); as well as the complex glycoproteins Contactin-1 and Neurofascin-15 (Nf155). Axonal Caspr and Contactin-1 form cis-heteromers localised at the axon in paranodal domains where myelin loops establish
axoglial junctions (Buttermore et al., 2011), and interact in trans with glial expressed Nf155. Similar to axonal Nf186, Nf155 is a 155-kDa splice variant of the Neurofascin gene, however, it is only expressed by oligodendrocytes. Caspr-1 contains a super-secondary structure for binding to the scaffolding 4.1B protein, co-localizing with ankyrin-B, αII and βII-spectrin (Faivre-Sarrailh and Devaux, 2013)(Figure 1.5). Axoglial junctions play a critical role in the organisation and maintenance of molecular domains in myelinated axons and deficiency in either Contactin-1, Caspr-1 or Nf155 induces disruption of paranodal junctions (Maier et al., 2007) and a reduction of nerve conduction velocity (Sherman and Brophy, 2005; Sherman et al., 2005), potentially leading to severe neurological and physiological defects, such as ataxia (Garcia-Fresco et al., 2006).

1.1.5 Juxtaparanodes

The juxtaparanode is located just beyond the innermost PNJ (Poliak and Peles, 2003) and is a region of enriched voltage-gated, delayed-rectifier K⁺ channels; Kv1.1, Kv1.2 and their Kvβ2 subunit (Figure 1.6) (Peles and Salzer, 2000; Buttermore et al., 2011). The delayed-rectifier K⁺ channels stabilize conduction by promoting repolarisation and maintaining the internodal resting potential (Rasband et al., 1998). At the juxtaparanodes, a heteromeric complex of Contactin-2 (or TAG-1) and Caspr-2 are co-expressed with a number of similar scaffolding proteins (4.1B protein, ankyrin-B, αII and βII-spectrin), in both axonal and glial membranes (Faivre-Sarrailh and Devaux, 2013).

Figure 6. Axons, myelin and the juxtaparanode. Oligodendrocytes make myelin as an extension of their cell membrane which wraps around and covers a long segment of axon. The myelin is segmented, with each segment flanked by nodes of Ranvier. Beyond the innermost PNJ is the juxtaparanodal region containing a cluster of K⁺ channels (Nave, 2010a).
1.2 Neurotrauma

Traumatic injury to the brain or spinal cord is a seriously debilitating medical emergency that can quickly lead to brain damage, paralysis, or death (Ditunno et al., 1994). In Australia, the predominant causes of traumatic brain injury (TBI) and spinal cord injury (SCI) include transport accidents, falls, collisions and water related accidents. Despite high incidence, morbidity, extensive research and clinical advancements in SCI and TBI research, these injuries are a continual cause not only of disability and mortality, but of profound physical, emotional and social impact on today’s society (Sekhon and Fehlings, 2001). Given the population size of over 300 million people in the United States, it is estimated that the annual incidence of SCI is approximately 40 cases per million population, or 17,000 new SCI cases each year alone (Center, 2015).

The pathophysiology of SCI and TBI involves both primary injury; occurring during initial insult, and secondary injury; often gradual and involving an array of mechanisms. Primary and secondary injury following neurotrauma can affect both grey and white matter (Blakemore, 1975; Liu et al., 1997; McIntosh et al., 1998; Lee et al., 1999; Pitt et al., 2000; McDonald and Sadowsky, 2002).

1.2.1 Primary injury

The primary injury following neurotrauma encompasses the direct physical and mechanical assault that occurs at the time of injury (Norenberg et al., 2004; Park et al., 2004). Spinal cord injury can be classified into four categories depending upon the extent of injury: 1) maceration, whereby the morphology of the cord is severely distorted; 2) laceration, as a result of clear-cut disruption(s) of the surface anatomy of the cord; 3) contusion, which can lead to spinal hematoma (hematomyelia) and the formation of cysts in the cord (syringomyelia); and 4) solid cord injury; which compared to contusion has no central focus of necrosis (see review Bunge et al., 1993) (Figure 1.7). Complete transection of the spinal cord, whereby all axonal fibres are severed, is extremely uncommon in a clinical setting. Hemi-sections, partial tearing or contusion injuries are far more common (Sekhon and Fehlings, 2001). Typically, mechanical and vascular insults result in a rapid loss of cells (necrosis) at the injury site. However, the extent of these events is dependent on the type and severity of injury (i.e. laceration injury vs. contusion). Outside the primary injury area, neurons and glia, although not directly
damaged, remain vulnerable to secondary degeneration and may undergo a variety of secondary phenomena (Schwab and Bartholdi, 1996; Sekhon and Fehlings, 2001; McDonald and Sadowsky, 2002; Bradbury and McMahon, 2006).

Figure 7. Spinal cord injury gross anatomy. Cross sections of normal spinal cord (A). Solid cord injury showing loss of architecture and myelin (B). Cyst development following contusion injury (C). Laceration injury resulting in the breaching of pial surface (D). Compression injury showing fragmentation, distortion and haemorrhage (E). Note: images are of human material (Norenberg et al., 2004).

1.2.2 Secondary injury mechanisms

Following the primary insult, cells vulnerable to secondary degeneration may undergo delayed death via downstream mechanisms, involving multiple cellular and molecular cascades. These mechanisms contribute to secondary phenomena such as haemorrhage, inflammation, neuronal changes, Wallerian degeneration, glial scarring and demyelination. Overall, this leads to a progressive degenerating injury to the brain or spinal cord termed “secondary injury” (Tator and Fehlings, 1991; Lu et al., 2000a; Park et al., 2004), which is temporally and spatially separated from cell death at the primary injury site (Figure 1.8). Given the importance of secondary degeneration, it is important for CNS injury models to separate primary from secondary injury. Partial transection of the optic nerve (ON) has emerged as a useful model to study secondary degeneration in the CNS. Dorsal axons of the ON are axotomised, leaving ventral axons intact, but
vulnerable to secondary degeneration. This leaves neurons and glia undamaged by the primary injury spatially separated from the primary injury, allowing for a reproducible and comprehensive assessment of events (Levkovitch-Verbin et al., 2003). Given the progressive nature of secondary injury, the multiple secondary pathological and physiological mechanisms involved provide important targets in developing therapeutic strategies for treatment of neurotrauma (Park et al., 2004). These therapeutic targets will be discussed in greater detail later in this chapter.

Figure 8. Primary versus secondary injury. Schematic diagram of CNS injury showing spatial separation between primary (epicentre) and secondary injury. Secondary injury mechanisms involve haemorrhage and oedema, inflammation, axonal changes and Wallerian degeneration, glial scarring and myelin changes (adapted from Franklin and Ffrench-Constant, 2008).
1.3 Consequences of secondary degeneration

The CNS has a high demand for energy and disruptions in energy supply result in the loss of ionic homeostasis, depolarisation, and rapid conduction failure of central fibres (Stys, 2004a). In the absence of a constant energy supply, axoplasmic K⁺ concentrations fall and Na⁺ and Ca²⁺ levels rise (Stirling and Stys, 2010), associated with oxidative stress. A complex inflammatory response (Hausmann, 2003) is initiated and begins almost immediately after neurotrauma (initial 1-2 hours), involving vascular changes, cellular responses and chemical mediators (Norenberg et al., 2004), which can spread the damage to tissue initially spared (Fleming et al., 2006; Weishaupt et al., 2010). Later phases of injury are characterised by haemorrhage and oedema, progressing/ongoing inflammation, axonal changes and Wallerian degeneration, glial scarring and various changes to myelin (Norenberg et al., 2004). Selected facets of secondary injury will now be discussed in greater detail.

1.3.1 Haemorrhage and oedema

When compromised by vascular events, the CNS becomes highly susceptible to damage (Acker and Acker, 2004; Peña and Ramirez, 2005; Martirosyan et al., 2011). A common consequence following primary injury is the disruption of surrounding microvasculature. Upon injury, the blood brain barrier (Bbb) is compromised, propagating plasma fluid leakage into the extracellular space, spreading oedema, and ionic disturbances. Disturbances in intracellular Ca²⁺ levels are typically associated with swelling of blood vessels, astrocytes and neurons (Sykova et al., 1999; Unterberg et al., 2004). Cytoplasmic free Ca²⁺ is sequestered by mitochondria, in turn depleting ATP (discussed in further detail below). Depletion of ATP is accompanied by further intracellular influx of Cl⁻ via chloride channels, which increases intracellular osmolarity, driving inflow of water through a number of water channels including Aquaporin (AQP) channels. Studies from our research group have demonstrated a significant upregulation in AQP4 immunoreactivity in optic nerve vulnerable to secondary degeneration (Wells et al., 2012). Similarly, previous studies have demonstrated this secondary phenomenon in reactive astrocytes (Rao et al., 2010; Rao et al., 2011), with elevated AQP4 for several months following SCI (Nesic et al., 2006).
Severe haemorrhages in the grey matter (Sekhon and Fehlings, 2001) and major changes to microcirculation of capillaries and venules at the injury site may also occur (Senter and Venes, 1978), with the presence of haemorrhage in the spinal cord following injury indicative of poor functional outcomes (Flanders et al., 1996; Tewari et al., 2005). Similarly, reductions in spinal cord blood flow (hypoperfusion) has been shown to persist in secondary regions, below the level of SCI (Li et al., 2017). Destruction of the vasculature tissue surrounding the injury site also leads to problems in cardiac output, such as: hypotension, bradycardia, and peripheral resistance (Tator and Fehlings, 1991; Tator and Koyanagi, 1997).

1.3.2 Inflammation

Breakdown of the Bbb following CNS injury results in invasion of neutrophils and microglia/macrophages, which can contribute further to secondary injury mechanisms (Fitch and Silver, 1997; DiNapoli et al., 2008). Inflammation is a ubiquitous consequence of CNS trauma, however investigation of lesion models of injury have demonstrated differences between brain and spinal cord inflammatory responses, following injury (Schnell et al., 1999). Inflammation following CNS injury and neurodegenerative diseases has both beneficial and detrimental effects depending on when it occurs in the SCI timeline. In more recent years, this paradigm has been explored, in particular, the diverse functional phenotypes of microglia, ranging from pro-inflammatory M1 phenotypes, to anti-inflammatory M2 phenotypes (see reviews Cherry et al., 2014; Tang and Le, 2016). Indeed, it is increasingly understood that individual cells can display characteristics of both M1 and M2 phenotypes, illustrating that the complexities of macrophage characterisation are not yet well understood (see review Martinez and Gordon, 2014). Studies investigating the efficacy of depleting immune cells (immunosuppression) in models of experimental SCI have demonstrated improved locomotor function and neuroanatomical repair (Taoka et al., 1997; Popovich et al., 1999). As part of normal intercellular communication, neurons and glia synthesize pro-inflammatory cytokines such as tumour necrosis factor-α (TNFα) and Interleukin-1 (IL-1) (see review Hopkins and Rothwell, 1995). Following injury, inflammatory cells typically become over activated, causing sustained elevation and/or dysregulation of pro-inflammatory factors, leading to neuronal and glial death (Mander and Brown, 2004; Gibbons and Dragunow, 2006; Ling et al., 2006; Block and Hong, 2007). For example,
IL-1 may contribute to neuronal injury by increasing Ca^{2+} influx through NMDA receptors (Viviani et al., 2003). These overactivated inflammatory cells can overproduce superoxide and nitric oxide, which combine to form the highly toxic compound, peroxynitrite (Chatzipanteli et al., 2002; Xiong et al., 2007). Overproduction of reactive species coupled with sustained TNFα can impair glutamate metabolism by astrocytes which serves as a feed-forward mechanism of glutamate excitotoxicity (discussed in greater detail below). Despite a plethora of studies demonstrating the pathogenic consequences of inflammation in CNS injury, some have shown the beneficial effects of inflammation after CNS injury, in vitro and in vivo (see review Donnelly and Popovich, 2008). For example, selective depletion of macrophages in SCI rats has been shown to promote partial hindlimb recovery and neuroanatomical repair (Popovich et al., 1999). Similarly, immunomodulation via injections of mesenchymal cells following SCI promotes functional recovery and increased white matter tissue sparing (Hodgetts et al., 2013). Therefore, immunoregulatory strategies may prove effective for the treatment of CNS injury.

### 1.3.3 Axonal changes and Wallerian degeneration

When the normal functions of neurons are compromised by trauma or disease, a pattern of morphological and molecular changes known as Wallerian degeneration (WD) can occur. WD is a secondary event following primary injury, characteristic of axotomised axons, where cytoskeletal disassembly occurs with granular degeneration of axons distal to the injury site. This is typically followed by a breakdown of the Bbb, distortion and fragmentation of myelin sheaths, altered axoplasm, and infiltration of reactive glial cells (Tsao et al., 1994; Norenberg et al., 2004; Kerschensteiner et al., 2005; Wang et al., 2012; Weber, 2012).

The initial phase of WD is termed acute axonal degeneration (AAD). Following injury to the nerves, the axonal segments proximal and distal to the primary injury site degenerate, mediated at least in part by extracellular Ca^{2+} influx and the Ca^{2+} -dependent protease, calpain. A number of studies have shown the key role calpain plays in axonal injury following TBI (Saatman et al., 1996a; Saatman et al., 1996b; McCracken et al., 1999) and SCI (Li et al., 1995; Schumacher et al., 1999). Inhibition of calpain can enhance axonal survival and improve neurological function following SCI (Schumacher et al., 2000). Phase two of WD involves an initial latency period of the distal axons whereby
they remain capable of physiologically normal functionality (Tsao et al., 1994), followed by axonal retraction and the formation of axonal bulbs at the injury site (Kerschensteiner et al., 2005; Erturk et al., 2007). Axonal swelling is a key characteristic following acute injury to white matter and can persist from as early as 1 day (Norenberg et al., 2004), until 6 months following injury (Payne et al., 2012). Axonal swellings may be a consequence of pinched axons at the proximal end of the transection (injury) with continued axoplasmic flow, or it may represent dysfunction of the axon, resulting in focal blockage of axonal transport (see review Povlishock and Christman, 1995). From approximately 72 hours after injury, there is a rapid fragmentation and cytoskeletal breakdown along the distal axon with increased glial infiltration, primarily macrophages, (Zhang and Guth, 1997; Zhang et al., 1997) and astrocytes, producing the glial scar (Figure 1.9).

![Diagram of Wallerian degeneration]

**Figure 9. Wallerian degeneration.** Mediated by extracellular Ca\(^{2+}\) influx and intracellular Ca\(^{2+}\)-dependent protease calpain, axonal segments proximal and distal to the injury site exhibit short distance acute axon degeneration. A slower axonal retraction follows, and the formation of axonal bulbs at the injury site occur. This is then followed by a period of latency in which distal axons remain morphologically stable. At more than 72 hours following injury, rapid fragmentation and cytoskeletal breakdown occur along the full length of the axon, followed by increased glial influx for clearing of debris. (Wang et al., 2012).
1.3.4 Glial scarring

Following primary injury, astrocytes containing abundant mitochondria and endoplasmic reticula (ER) become reactive and undergo hypertrophy (Fawcett and Asher, 1999; McGraw et al., 2001; Norenberg et al., 2004). Similar to the M1/M2 paradigm, reactive astrocytes can be categorised into A1 and A2 types. A1 astrocytes are typically induced via Il-1α, TNFα, and C1q secretions from activated microglia, (Liddelow et al., 2017), and express an ‘injury-type specific’ detrimental gene profile known to exacerbate neurodegeneration, driving synaptic destruction (Zamanian et al., 2012). In contrast, A2 astrocytes can up-regulate a number of neurotrophic factors, strongly promoting neuronal survival and tissue repair. In particular regards to SCI, reactive astrocytes have been shown to be functionally important in wound and Bbb repair, restricting inflammation, protecting neurons and glia, and preserving function (Faulkner et al., 2004). However, the end stage of this reactive astrogliosis can be the formation of a glial ‘scar’ (Norenberg et al., 2004). The scar is surrounded by extracellular matrix, and comprises interwoven astrocyte processes attached to one another by tight junctions (Berry et al., 1983). The network of processes mix with invading connective tissue, and impedes axonal/neurite growth. However, the glial scar may serve the role of excluding the injury site from healthy tissue, preventing overwhelming inflammation and limited cellular degeneration (Silver and Miller, 2004) with the removal chronic scars following SCI shown to impede spontaneous axon regrowth, and exacerbate tissue degeneration (Anderson et al., 2016). Thus, the concluding benefit of glial scarring is the overall survival of the animal, at the sacrifice of long-term functional regeneration.

1.3.5 Myelin abnormalities

A growing body of evidence suggest that myelin is essential for neuronal survival. The myelin diseases such as multiple sclerosis and leukodystrophies are associated with axonal loss in regions of chronic demyelination (Ferguson et al., 1997; Trapp et al., 1997; Grigoriadis et al., 2004). Demyelination of intact axons leads to a decrease in conduction velocity (Lang and Rosenbluth, 2003), and loss of motor function and coordination (Rosenbluth et al., 1994; Jeffery and Blakemore, 1997). Changes in myelin have been shown to be a pathological feature and key contributor to functional impairment that occurs following white matter insult, such as SCI (Blight, 1983; Jeffery and Blakemore, 1997; Totoiu and Keirstead, 2005; Payne et al., 2011). Gutierrez et al. (1995) suggested
that the degree of myelination and the packing density (decreases indicated by decompaction) of the myelin sheath affects conduction velocity by ON fibres and may lead to increased functional impairments in central fibres.

Following neurotrauma, the long-term stability of oligodendrocytes, myelin and myelin repair remains a matter of speculation. Some studies report abnormal or loss of myelination during CNS injury and secondary degeneration (Waxman, 1989; Guest et al., 2005b; Payne et al., 2011; Payne et al., 2012; Savigni et al., 2013), whilst others report a thin layer of myelin following injury, likely due to remyelination (McDonald and Belegu, 2006; Franklin and Ffrench-Constant, 2008; Powers et al., 2012). We have shown in recent studies that myelin surrounding ON axons vulnerable to secondary degeneration displayed increasing abnormalities, with significant increases in decompaction of myelin (dysmyelination) (Figure 1.10) as secondary degeneration progressed (Fitzgerald et al., 2009a; Payne et al., 2011). Myelin disruptions may be linked to secondary events such as energy depletion; excitotoxicity; over production of ROS (e.g. NO), and lipid peroxidation, (Fitzgerald et al., 2010; Szymanski et al., 2013). Furthermore, PLP deficient mice, express a number of ultrastructural abnormalities of myelin and axon degeneration, reminiscent of the changes in secondary degeneration (Klugmann et al., 1997; Rosenbluth et al., 2006).

![Figure 10. Myelin decompaction following partial CNS injury. Representative transmission electron microscopy images of axons with decompacted myelin in normal optic nerve (ON; A-B), and at 3 months (C) and 6 months (D) following partial ON transection are indicated with arrows (>>) (Payne et al., 2012).](image)
It is plausible that the changes seen in myelin structure reflect oligodendrocyte disturbances resulting in perturbed axoglial support. However, some studies have shown that mature oligodendrocytes are relatively resistant to aspects of secondary degeneration, whereas oligodendrocyte progenitor cells (OPCs) appear more vulnerable (Back et al., 1998; Juurlink et al., 1998a; Back et al., 2001; Back et al., 2005). Indeed, chronic depletion of OPCs is observed following partial CNS injury, at 9 days, 1, 3 and 6 months after injury (Fitzgerald et al., 2010; Payne et al., 2011; Payne et al., 2012; Payne et al., 2013). Nevertheless, recent evidence from our laboratory suggests that mature oligodendrocytes vulnerable to secondary degeneration exhibit increased indicators of oxidative stress. Szymanski et al. (2013) observed significant increases in carboxymethyl lysine, an oxidative stress marker detecting glycation of proteins and lipids, in mature oligodendrocytes vulnerable to secondary degeneration. This infers that both OPCs and mature oligodendrocytes are vulnerable, and may succumb to secondary injury mechanisms, as a result of CNS trauma.

### 1.3.6 Node of Ranvier and paranode abnormalities

The nodal domains of the axolemma contain specific sets of ion channels, cell adhesion molecules and cytoplasmic adaptor proteins. Therefore, disruptions of these complexes are likely to impair nerve signalling and function. It is understood that changes in paranodal proteins results in the redistribution/disorganisation of the nodal and paranodal complexes. Early studies modelling head acceleration injury in non-human primates demonstrated fragmentation and enlargement of the nodal axoplasm (Gennarelli et al., 1993). Similarly, stretch injury in white matter tracts may cause loss of microtubules at the node of Ranvier and internodes (Maxwell and Graham, 1997). We have reported significantly increased lengths of paranodes and the nodes of Ranvier in the days and months following injury in ventral ON vulnerable to secondary degeneration (Figure 1.11).
Figure 11. Node and paranode length changes following partial CNS injury. Typical nodes and paranodes are characterised by a β-III tubulin immunopositive area (node of Ranvier) flanked by two Caspr immunopositive clusters (paranode; A). Following partial injury to the optic nerve, at day 1 there is an increase in both paranode (B) and node length (C). At three months, node length was significantly increased, whereas paranode length was not significantly different from controls. Difference from normals is indicated by *(p<0.05; scale bar = 1µm) (Szymanski et al., 2013).

Alterations including diffusion of Nav channels along the demyelinated axon in the node of Ranvier have been documented in white matter lesion models, such as for multiple sclerosis (MS) (Moll et al., 1991; Coman et al., 2006) and experimental autoimmune encephalomyelitis (EAE) (Zoupi et al., 2013). Paranode length increases are also seen within demyelinating lesions, with Nf155 immunoreactivity spreading along the internodes in MS (Howell et al., 2006). Similarly, increases in the length of node of Ranvier has been observed in EAE, (Fu et al., 2011), ageing (Hinman et al., 2006), and SCI (Ouyang et al., 2010; Sun et al., 2012).

A number of pathological conditions including SCI result in paranodal disruption and retraction of the myelin sheath (Blight and Young, 1989; Shi and Blight, 1997; Nashmi
and Fehlings, 2001; Jensen and Shi, 2003). In ex vivo spinal cord white matter, glutamate induces myelin splitting and retraction at paranodal domains of axons (Fu et al., 2009). Similarly, the detachment of the myelin sheath has also been observed following exposure to the lipid peroxidation bi-product, acrolein (Shi et al., 2011a; Shi et al., 2011b). Consequentially, myelin retraction causes the exposure of K+ channels in the juxtaparanodes, with redistribution of the channels (Kv1.2) causing a further breakdown of the axo-glial junctions (Fu et al., 2009). Further to this, it has been demonstrated that following SCI, aberrant Caspr localisation occurs, with the overlapping of Caspr, Kv1.1 and Kv1.2 in the paranodal regions (Karimi-Abdolrezaee et al., 2004). These alterations of the paranodal axo-glial junctions, and the exposure of Kv1 channels, might contribute to conduction defects and consequent functional deficits.

The remainder of this chapter will focus primarily on the cellular and molecular mechanisms resulting in damage and progressive loss of neurons and myelin that occur during secondary degeneration, following CNS injury.

1.4 Mechanisms of secondary degeneration

1.4.1 Calcium in the CNS

In the CNS, Ca2+ is known to perform important physiological roles, involving information processing, cell growth, survival and differentiation (Komuro and Rakic, 1996; Gomez and Spitzer, 2000; Spitzer et al., 2000). Ca2+ also plays a salient role both in functional recovery and in cell death, with Ca2+ over-activation, caused by excessive Ca2+ influx, comprising the final common pathway for neuronal death, preceded by massive soma swelling (Prilloff et al., 2007). In neurons, the normal resting cytoplasmic [Ca2+] is ~50-100nM, with physiological Ca2+ signals resulting in 100-1000nM concentrations. Resting cytoplasmic [Ca2+] in glial cells varies from 30-40 to 200-400nM, with free cytoplasmic Ca2+ contributing to <0.001% of total Ca2+ in glial cells. This variation occurs among subtypes of glial cells, but also within the same population of cells (see reviews Verkhratsky et al., 1998; Verkhratsky and Petersen, 1998).

Intracellular Ca2+ concentration is determined by the combined mechanisms of Ca2+ influx (from extracellular space), Ca2+ release (from internal stores), Ca2+ sequestration, Ca2+ efflux (to extracellular space) and Ca2+ buffering (see review Gilabert, 2012). Ca2+
transporters are members of several superfamilies of transmembrane Ca\(^{2+}\)-permeable channels, ATP-driven pumps, and electrochemically driven exchangers. The resulting Ca\(^{2+}\) fluxes determine whether Ca\(^{2+}\) is delivered or removed from the cytoplasm of the cell. Ca\(^{2+}\) is normally trapped upon entering the cell by Ca\(^{2+}\)-binding proteins, dependent upon the Ca\(^{2+}\) -buffering capacity of the cell. Ca\(^{2+}\) transporters are localised in the cell membrane and in the intracellular organelles (e.g. mitochondria, endoplasmic reticulum, golgi complex and nucleus), forming the intracellular Ca\(^{2+}\) storage system, which accumulates Ca\(^{2+}\), bound to intraluminal proteins, and rapidly releases it via intracellular Ca\(^{2+}\) channels (Pozzan et al., 1994).

Intracellular Ca\(^{2+}\) plays an important role as a messenger for intracellular and intercellular signalling (see review Rottingen and Iversen, 2000). Intracellular Ca\(^{2+}\) signals begin with transient changes in cytoplasmic Ca\(^{2+}\) characterised by an initiating trigger (e.g. action potential of neurons), followed by mechanisms that propagate Ca\(^{2+}\) signals to neighbouring cells (Sanderson et al., 1994). Although the main form of communication by neurons is electrical signals, the exchange of Ca\(^{2+}\) also occurs, with rises in intracellular Ca\(^{2+}\) closely related to synaptic functioning (Zucker, 1999; Takasu et al., 2002). Changes in intracellular Ca\(^{2+}\) concentrations are the predominant signal by which glial cells regulate their own, and influence neuronal, behaviour (Scemes, 2000; Scemes et al., 2000). Ca\(^{2+}\) signals can be communicated amongst astrocytes, and between astrocytes and other cell types, including; vascular endothelial cells (Leybaert et al., 1998; Paemeleire and Leybaert, 2000), microglia (see review Moller, 2002), NG2- expressing glia (Hamilton et al., 2009), oligodendrocytes (Kirischuk et al., 1995a; Kirischuk et al., 1995b; Chen et al., 2007) and neurons (Nedergaard, 1994; Parpura et al., 1994; Parri et al., 2001).

Ca\(^{2+}\) is known to enter neurons and glia, including oligodendrocytes, through a range of glutamate receptors and Ca\(^{2+}\) channels including, but not limited to: 1) N-methyl-D-aspartate (NMDA) receptors (see review Wong and Kemp, 1991); 2) voltage-gated Ca\(^{2+}\) channels (VGCCs) (see review Catterall et al., 2005) 3) reversal of the Na\(^{+}\)-Ca\(^{2+}\) exchanger (Siesjo and Bengtsson, 1989); 4) ionotropic P2X receptors (see reviews Ralevic and Burnstock, 1998; North, 2002); and 5) \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors lacking the GluR2 subunit (Hollmann et al., 1991; Verdoorn et al., 1991; Takahashi et al., 1998; Takahashi et al., 2002). Although intracellular Ca\(^{2+}\) levels are typically very low (~10\(^{-7}\) M), cells that are excessively
depolarised, allow the influx of intracellular free Ca\textsuperscript{2+} via VGCCs. Furthermore, in the presence of extracellular glutamate, NMDA and AMPA receptor activation promotes further intracellular Ca\textsuperscript{2+} influx and subsequent depolarisation (Doble, 1999). In summary, Ca\textsuperscript{2+} signalling pathways are known to play a vital role in neuronal and glial survival (Wildburger et al., 2009). Due to the feed-forward nature of secondary degeneration in relation to Ca\textsuperscript{2+}, neurons and glia, such as oligodendrocytes, are continuously vulnerable long after injury. The various pathways by which Ca\textsuperscript{2+} can enter the cells of white matter will now be discussed in greater detail.

1.4.2 Internal stores

The majority of Ca\textsuperscript{2+} is bound to Ca\textsuperscript{2+} binding proteins and sequestered in organelles; primarily endoplasmic reticulum (ER) and mitochondria (see review Clapham, 1995). The major contributors to intracellular Ca\textsuperscript{2+} concentration from internal stores are likely to be: (1) from the mitochondrial matrix (Thayer and Miller, 1990; Nicholls et al., 2003); (2) ER release via distinct receptors including ryanodine receptors, Inositol(1,4,5)P\textsubscript{3} (InsP3) receptors, nicotinic acid adenine dinucleotide phosphate [NAADP]- sensitive receptors, and sphingolipid Ca\textsuperscript{2+} release-mediating protein of ER (Pozzan et al., 1994; Berridge et al., 2000; Fill and Copello, 2002; Rutter, 2003); and (3) release from cytosolic Ca\textsuperscript{2+} -binding proteins including parvalbumin, calbindin-D28K, and calmodulin (CaM)(Heizmann and Hunziker, 1991; Kostyuk and Verkhratsky, 1994; Schwaller et al., 2002). Several studies support the idea that the above sources of intracellular Ca\textsuperscript{2+} play an important role in traumatic CNS injuries. Specifically, in conjunction with rapid influx of extracellular Ca\textsuperscript{2+}, intra-axonal Ca\textsuperscript{2+} overload occurs via: ryanodine receptor-mediated release from ER following L-Type Ca\textsuperscript{2+} channel activation (Ouardouz et al., 2003); release via InsP3 receptors (Nikolaeva et al., 2005); and release from the mitochondrial stores (Ouardouz et al., 2005)(Figure 1.12).

Mitochondria store and release intracellular Ca\textsuperscript{2+} (Parekh, 2008), regulated under normal physiological conditions primarily via the mitochondrial Na/Ca exchanger (Castaldo et al., 2009). However, under abnormal conditions, both the Ca\textsuperscript{2+} uniporter and permeability transition pore (MPT) can also release Ca\textsuperscript{2+} (see reviews Bernardi and Rasola, 2007; Rasola and Bernardi, 2007). Ca\textsuperscript{2+} influx is further mediated by the reversal of the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger. Because the exchanger is an electrogenic ion transporter, collapse of the Na\textsuperscript{+} gradient due to impaired activity of the membrane will attenuate the electrochemical
driving force and promote $\text{Ca}^{2+}$ influx through it (Doble, 1999; Stys, 2004b). This process is thought to be irreversible; thus it is plausible that the rises in intracellular $\text{Ca}^{2+}$ are the secondary trigger of excitotoxic cascades through feed-forward mechanisms of $\text{Ca}^{2+}$-mediated $\text{Ca}^{2+}$ influx (Frandsen and Schousboe, 1992; Lei et al., 1992; Doble, 1999; Sattler and Tymianski, 2000; Park et al., 2004)(Figure 1.12). Accordingly, the degree of injury is thought to be related to the amount of $\text{Ca}^{2+}$ accumulated within the axoplasm (Stys, 2004b). In 2005, it was suggested that the majority of mitochondrial $\text{Ca}^{2+}$ release during CNS injury (in this case, ischemia) is mediated via the Na/Ca exchanger (Nikolaeva et al., 2005). However, they found that three inhibitors of mitochondrial $\text{Ca}^{2+}$ release: CGP37157, a blocker of mitochondrial Na/Ca exchanger; Ru360, an inhibitor of the mitochondrial Ca uniporter; and cyclosporine A, a MPT blocker, reduced intracellular $\text{Ca}^{2+}$ rises much more effectively when in combination compared to when administered alone. This study demonstrates the importance of controlling multiple sources of $\text{Ca}^{2+}$ following CNS injury.

**Figure 12. Molecular cascades and intracellular calcium ($\text{Ca}^{2+}$) pathways.** Intracellular $\text{Ca}^{2+}$ concentration is determined by $\text{Ca}^{2+}$ influx, $\text{Ca}^{2+}$ release, $\text{Ca}^{2+}$ sequestration, $\text{Ca}^{2+}$ efflux and $\text{Ca}^{2+}$ buffering (Verkhratsky et al., 1998).
1.4.3 L-type Ca\(^{2+}\) channels and ryanodine receptors

VGCCs comprise a broad class of channels with a high selectivity for Ca\(^{2+}\) and wide variety of voltage-dependent activation and inactivation features (Peterson et al., 1999; Catterall, 2011). VGCCs play a crucial role in the CNS, and form an important pathway for Ca\(^{2+}\) entry (Agrawal et al., 2000; Calin-Jageman and Lee, 2008). VGCCs are categorised into low- (LVA) and high-voltage activated (HVA) (Catterall, 2000). HVA channels can be further subdivided based on biophysical, pharmacological, and molecular features. Traditionally, they are classified as L-, P/Q, N- and R-type Ca\(^{2+}\) channels, which have been found in neurons (Mintz et al., 1992; Randall and Tsien, 1995; Tottene et al., 1996; Lin et al., 1997; Bonci et al., 1998) astrocytes (Latour et al., 2003), microglia (Silei et al., 1999), pericytes (Zhang et al., 2002), and both progenitor and mature oligodendrocytes in vitro (Verkhratsky et al., 1990; Blankenfeld et al., 1992; Kirischuk et al., 1995b).

In particular, high-voltage activated (L-type) Ca\(^{2+}\) channels, widely distributed in the CNS (Bertolino and Llinas, 1992), have been shown to regulate membrane excitability and intracellular signal transduction in neurons (Calin-Jageman and Lee, 2008). T-type Ca\(^{2+}\) channels are also known to contribute to intracellular Ca\(^{2+}\) release under ischemic conditions (Fern, 1998; Nikonenko et al., 2005). T-type Ca\(^{2+}\) channels, are a type of low-voltage activated Ca\(^{2+}\) channel, are expressed predominantly in neurons (Talley et al., 1999; Heady et al., 2001) but are also found in glial cells (Bringmann et al., 2000). Thus following injury, activation of L- and T-type Ca\(^{2+}\) channels can lead to a large elevation of Ca\(^{2+}\) within cells (Young, 1992), which then can activate a variety of mechanisms, setting in motion a cascade of Ca\(^{2+}\)-mediated events that eventually results in cell injury and death (Trump and Berezesky, 1995; Wojda et al., 2008).

1.4.4 P2X receptors

Extracellular ATP is a major excitatory neurotransmitter in the CNS and can contribute to neuronal excitotoxic activation of P2X (ionotropic) and P2Y (metabotropic) receptors (Ralevic and Burnstock, 1998; North, 2002). ATP-gated P2X channels are formed by P2X\(_{1-7}\) subunits and are highly permeable to Ca\(^{2+}\) (Matute et al., 2007a). P2Y receptors are G-protein linked receptors that increase intracellular InsP3 and mobilise Ca\(^{2+}\) from InsP3-sensitive stores, whilst P2X receptors are ligand-gated cation channels that mediate
Ca\(^{2+}\) entry into the cell. P\(_2\)Y receptors are activated by ATP concentrations in the nanomolar range, whilst micromolar concentrations are needed to activate P\(_2\)X receptors (James and Butt, 2001a, b). In neurons, the specific subtype of P\(_2\)X receptors, P\(_2\)X\(_7\) receptors, are an appreciable source of Ca\(^{2+}\) influx (North, 2002; Egan and Khakh, 2004). P\(_2\)X\(_7\) receptors are also expressed and serve a variety of functions in astrocytes (Ballerini et al., 1996; Duan et al., 2003b), Schwann cells (Graf et al., 1999) and a number of inflammatory and immunomodulatory cells (Alcaraz et al., 2003). There is evidence showing that oligodendrocytes and their progenitors express P\(_2\)X\(_7\) receptors \textit{in vitro and in situ} (James and Butt, 2001b). High concentrations of ATP activate P\(_2\)X receptors responses in optic nerve oligodendrocytes, causing rapid and prolonged increases in cytosolic Ca\(^{2+}\) concentrations (James and Butt, 2001b). Purinergic receptors, including P\(_2\)X\(_7\) receptors have also been shown to participate in oligodendrocyte development, with Ca\(^{2+}\) increases stimulating oligodendrocyte growth, migration and differentiation (Agresti et al., 2005). ATP concentrations typically increase in response to injury (Anderson and Nedergaard, 2006). Intracellular ATP concentration is within the millimolar range, therefore injured cells have the potential to release large amounts of ATP which can directly injure vulnerable cells (Matute et al., 2007b). Hence, with an abnormally large release of ATP following SCI, there is the potential for significant death of nearby neurons and oligodendrocytes (Wang et al., 2004).

1.4.5 Glutamate receptors

Ca\(^{2+}\) waves are also associated with increased spread of extracellular glutamate (Innocenti et al., 2000). Astrocytic glutamate release can be vesicular (Pasti et al., 2001; Bal-Price et al., 2002), anion transport related (Jeremic et al., 2001) or \textit{via} P2X\(_7\) receptor channels (Duan et al., 2003a). However, these mechanisms appear to have differing Ca\(^{2+}\) sensitivities: vesicular release and anion transporter related release is Ca\(^{2+}\)-dependent (Parpura and Haydon, 2000; Jeremic et al., 2001), whereas release through hemi-channels or P2X\(_7\) receptors cannot be blocked \textit{via} intracellular Ca\(^{2+}\) buffering, nor \textit{via} emptying Ca\(^{2+}\) stores with thapsigargin (Duan et al., 2003a; Ye et al., 2003). Glutamate acts on ionotropic and metabotropic glutamate receptors activating Ca\(^{2+}\) entry or its mobilisation from stores (Nakahara et al., 1997). Glutamate not only excites neurons, but also glial cells (Bowman and Kimelberg, 1984; Kettenmann et al., 1984a; Kettenmann et al., 1984b; Gallo and Ghiani, 2000).
1.4.5.1 AMPA receptors

AMPA receptors are a class of ionotopic, ligand-gated, cation channel, glutamate receptors in the CNS, typically allowing the entry of Na\(^+\) and the exit of K\(^+\) (see review Michaelis, 1998). They are composed of a combination of four sub-units (GluR1-4) and require the binding of glutamate for activation (Park et al., 2004; Kowaltowski et al., 2009). However, if an AMPA receptor has a relative absence of the GluR2 subunit, it becomes permeable to Ca\(^{2+}\) (Figure 1.13) (Hollmann et al., 1991; Hume et al., 1991; Geiger et al., 1995). In the spinal cord, GluR3 and GluR4 are the predominant AMPA subunits expressed in oligodendrocytes and astrocytes, respectively (Park et al., 2003). Ca\(^{2+}\)-permeable AMPA receptors expression is thought to be low compared to the expression of Ca\(^{2+}\)-impermeable receptors (McIntosh et al., 1996; Doble, 1999). It is now understood that the GluR2 subunit is poorly expressed in many types of neuroglia with decreases in GluR2 expression rendering AMPA receptors permeable to Ca\(^{2+}\) influx and leading to increased cytosolic Ca\(^{2+}\) in neurons, as well as astrocytes oligodendrocytes and NG2\(^+\) glial cells (see review Verkhratsky and Kirchhoff, 2007).

![Figure 13. AMPA receptor permeability to calcium (Ca\(^{2+}\)). AMPA receptors typically have four subunits (GluR1-4). However, when the receptors lack the GluR2 subunit, they become Ca\(^{2+}\)-permeable. Following neurotrauma, GluR2 expression is downregulated, thus rendering AMPA receptors Ca\(^{2+}\)-permeable and allowing for increased Ca\(^{2+}\) influx into cell. (https://www.drugabuse.gov/news-events/nida-notes/2009/12/rare-glutamate-receptor-proliferates-after-cocaine-withdrawal).](image)

1.4.5.2 NMDA receptors

N-methyl-D-aspartate or NMDA receptors, are ionotropic glutamate gated ion channels, which possess high Ca\(^{2+}\) permeability, and unique voltage-dependent sensitivity to
magnesium, governed by glycine (see review Monyer et al., 1992). Glutamate activation of NMDA receptors allows for Ca\(^{2+}\) influx, critical for activity dependent synaptic plasticity (Schiller et al., 1998). NMDA receptors are non-specific cation channels, permeable for Na\(^+\), K\(^+\) as well as Ca\(^{2+}\); the fraction of Ca\(^{2+}\) contributing to the total cation current through NMDA receptors is about 6-12% (Schneggenburger et al., 1993; Burnashev et al., 1995; Rogers and Dani, 1995; Garaschuk et al., 1996). It is important to note that the specific functional properties of NMDA receptors are determined by the subunit composition (Neyton and Paoletti, 2006; Paoletti and Neyton, 2007), the phosphorylation status of the receptor (Ultenius et al., 2006), and the membrane potential of the cell. For example, NMDA receptor action is perturbed in spinal cord neurons by removing physiological concentrations of magnesium (Mayer et al., 1984). NMDA receptors have further been shown to have a role in traumatic SCI, linked to increased intracellular Ca\(^{2+}\) concentration in neurons (MacDermott et al., 1986), and have also been implicated in Ca\(^{2+}\)-mediated cell death in various neurodegenerative disorders (Rothman and Olney, 1987; Regan and Choi, 1991; Agrawal and Fehlings, 1997).

### 1.5.1 Glutamate excitotoxicity

Excitotoxicity is the pathological processor response of neuronal death caused by excessive or prolonged activation of receptors via neurotransmitters following a CNS insult (Stys, 2004b; Lau and Tymianski, 2010; Nave, 2010a). Glutamate is the major excitatory transmitter within the mammalian CNS (Watkins and Evans, 1981; Doble, 1999), and is considered a key contributor to excitotoxicity (Lucas and Newhouse, 1957; Olney, 1969). Subsequent to insult or axotomy, glutamate is released from damaged neural tissue into the extracellular space (Globus et al., 1995; Xu et al., 2004). This can result in additional excitotoxic damage of nearby tissue, thereby propagating the cascade of secondary events (Vorwerk et al., 2004; Casson, 2006). Glutamate excitotoxicity is largely attributed to the over activation of glutamate receptors which are permeable to both Na\(^+\) and Ca\(^{2+}\), thus contributing to secondary injury via Ca\(^{2+}\) secondary messenger cascades (MacDermott et al., 1986; Dingledine et al., 1999). The full mechanisms underlying glutamate excitotoxicity are complex (Doble, 1999; Matute et al., 2002; Dong et al., 2009a) and involve a number of channels, in a variety of cell types (e.g. oligodendrocytes). Within white matter, the Ca\(^{2+}\)-dependent contribution to the
excitotoxic cascade (Choi, 1985; Doble, 1999; Lau and Tymianski, 2010) is particularly important, and will be discussed in further detail below.

1.5.2 Mitochondrial dysfunction

Mitochondria are complex organelles which function primarily to provide energy in the form of ATP, which is generated through oxidative phosphorylation. This process is driven via an electrochemical gradient (via the electron transport chain) across the inner membrane of the mitochondrion (Saraste, 1999). The electron transport chain (ETC; Figure 1.14) consists of four complexes: Nicotinamide adenine dinucleotide dehydrogenase (NADH), succinate dehydrogenase, cytochrome c reductase and cytochrome c oxidase. Ubiquinone and cytochrome c are responsible for the transfer of electrons between complexes. Electrons are sourced from NADH and succinate, and to a less extent, FADH (Guarente, 2008) all of which are produced by the citric acid cycle (Tretter and Adam-Vizi, 2000).

![Electron Transport Chain](image)

*Figure 14. Electron transport chain. ATP production is driven through oxidative phosphorylation via an electrochemical gradient of the electron transport chain; consisting of four complexes (OpenStax, Anatomy & Physiology. OpenStax CNX. Feb 26, 2016 http://cnx.org/contents/14fb4ad7-39a1-4eee-ab6e-3ef2482e3e22@8.24).*

Normal mitochondrial function involves the production of reactive oxygen species (ROS) (Cadenas and Davies, 2000; Turrens, 2003; Kowaltowski *et al.*, 2009; Murphy, 2009).
ROS are a group of oxygen containing molecules which form through the sequential reduction of oxygen through the addition of electrons, leading to the formation of superoxide, hydrogen peroxide, hydroxyl radical, hydroxyl ion and nitric oxide (Camello-Almaraz et al., 2006) (Figure 1.15). Under normal physiological conditions, several ROS are regulated at low levels by enzymatic antioxidant defence systems such as glutathione peroxidase (GPx), and manganese superoxide dismutase (MnSOD), together with non-enzymatic anti-oxidants including ascorbic acid and glutathione.

Figure 15. Reactive oxygen species. Electron structures of common reactive oxygen species formed through the sequential reduction of oxygen.

Mitochondrial dysfunction contributes to delayed death of neurons, oligodendrocytes and astrocytes following cerebral ischemia, hypoxia and trauma. One of the key events leading to mitochondrial dysfunction is an abnormal increase in intracellular Ca\(^{2+}\). Mitochondrial mediated cell death can be caused by conditions of excess cytoplasmic Ca\(^{2+}\) in the mitochondrial matrix, via opening of the mitochondrial membrane permeability transition (MPT) in response to elevated Ca\(^{2+}\) and ROS (Peng and Jou, 2010), leading to death and autophagy (Lemasters et al., 1998). Further, increased permeability of the mitochondrial membrane can lead to swelling and rupture of mitochondria, with activation of pro-apoptotic factors, as cytochrome \(c\) is released into the cytosol (Brustovetsky et al., 2002; Baines et al., 2005). Increased permeability also results in slowed electron transfer from complex III to complex IV, leading to increased ROS production and oxidative damage (Kowaltowski et al., 1996a; Kowaltowski et al., 1996b).
In conditions of excess Ca$^{2+}$ and altered mitochondrial membrane potential, increases in electron leakage at mitochondrial complexes I and III of the ETC can result in increased formation of superoxide (St-Pierre et al., 2002). Ca$^{2+}$ may increase mitochondrial ROS production via a number of mechanisms, including: activation of ROS-generating enzymes (Tretter et al., 2007); inhibition of mitochondrial respiration via increased NO generation (Ghafourifar et al., 1999); and the release of cytochrome $c$ due to excessive Ca$^{2+}$ accumulation (Kowaltowski et al., 2009). Furthermore, Ca$^{2+}$ may accelerate the electron influx into the ETC promoting greater ROS generation overall, although outcomes depend on the substrate and system studied (Tretter et al., 2007).

Nitric oxide (NO) is a free radical synthesized from L-arginine by nitric oxide synthase (NOS) (Palmer et al., 1988a; Palmer et al., 1988b) and is involved in a number of cellular functions such as neurotransmission (Bult et al., 1990) and the immune response (see review Bogdan, 2001). Studies have also demonstrated that NO is involved in cell-cell propagation of Ca$^{2+}$ signalling, and can also trigger intracellular Ca$^{2+}$ waves. NO is synthesised via oxidation of L-arginine by nitric oxide synthase (NOS)(Lowenstein and Snyder, 1992)). NOS exists in three isoforms in the CNS: neuronal NOS (nNOS); endothelial NOS (eNOS) which is also present in astrocytes and microglia (Murphy et al., 1993; Shafer et al., 1998); and inducible NOS (iNOS) in endothelial cells, microglia, astrocytes and neurons (see review Nathan and Xie, 1994). In response to increased Ca$^{2+}$ levels, nNOS and eNOS synthesise NO, activating guanylyl cyclase and increasing cytoplasmic cGMP, which in turn activates ADP-ribosylycyclase to produce cADP ribose (Galione, 1993). In astrocytes, cADP ribose triggers ryanodine receptor mediated Ca$^{2+}$ release (Willmott et al., 2000a). NO may also increase Ca$^{2+}$ through cGMP-independent mechanisms, by mobilising Ca$^{2+}$ stores (Bowman et al., 2001; Bal-Price et al., 2002) or by provoking Ca$^{2+}$ entry (Willmott et al., 2000b; Matyash et al., 2001).

Like most free radicals, NO can be either neuroprotective or destructive depending on its redox state (Lipton, 1993; Lipton et al., 1993) and/or source (see review Iadecola, 1997), with NO implicated in glutamate-mediated neuronal cell death by the formation of toxic peroxynitrite (Beckman et al., 1990; Dawson et al., 1991). Peroxynitrite is formed when NO rapidly reacts with superoxide anions (Beckman et al., 1990). Peroxynitrite can damage neural tissue by three distinct pathways (see review Simonian and Coyle, 1996): (1) decomposition to hydroxyl radicals (Beckman et al., 1990; Hogg et al., 1992); (2)
oxidation of sulfhydryl groups, lipids, DNA and proteins (Radi et al., 1991; Gatti et al., 1994), and; (3) tyrosine and phenylalanine nitrosylation in proteins (Ischiropoulos et al., 1992; Beckman, 1994; Beckman et al., 1994). High levels of NO, coupled with pro-inflammatory cytokines such as tumour-necrosis factor-α (TNF-α) are likely to be released from microglia/macrophages and astrocytes during secondary degeneration (Giovannoni, 1998; Munoz-Fernandez and Fresno, 1998; Fitzgerald et al., 2009b; Fitzgerald et al., 2010). High levels of NO may also lead to S-nitrosylation of one of the major myelin proteins, PLP, resulting in a disturbance of homophilic bonding of PLP molecules between adjacent myelin lamellae, and decompaction of myelin at the level of the intraperiodic line (Bizzozero et al., 2001a; Bizzozero et al., 2001b; Bizzozero et al., 2004).

1.5.3 Oxidative stress

Oxidative stress occurs when there is an imbalance between ROS production and the metabolism of these species (see reviews Uttara et al., 2009; Wang and Michaelis, 2010). Oxidative stress is a hallmark of neurodegenerative diseases such as glaucoma and CNS injury as well as other chronic neurodegenerative diseases (e.g. Alzheimer’s disease), with a highly interactive relationships between intracellular Ca$^{2+}$ accumulation, ROS overproduction and glutamate excitotoxicity (Panter et al., 1990; Yamamoto et al., 1998; Castilho et al., 1999). The body of research evidence of ROS-induced cellular damage in CNS injury is well characterised in models of TBI and SCI. It has been suggested that oxidative stress plays a key role in both primary damage following acute TBI (see reviews Kontos and Povlishock, 1986; Ikeda and Long, 1990) and in secondary processes associated with inflammatory mediators and neutrophil-mediated inflammation (Feuerstein et al., 1997; Juurlink and Paterson, 1998a). Increases in superoxide and hydroxyl radicals have been observed as early as 5 mins following spinal cord injury in rats (Liu et al., 2004). When overproduction of the ROS described above overcome endogenous antioxidant capacities, subsequent oxidation of lipids, proteins and DNA may occur.

1.5.3.1 Lipid peroxidation

The most validated and predominant form of oxidative damage that occurs in CNS injury is lipid peroxidation (see reviews Hall et al., 2010; Hall, 2011). Lipid peroxidation occurs
when oxygen free radicals react with polyunsaturated fatty acids. In the CNS, highly reactive oxygen species reacts with membrane polyunsaturated fatty acids such as arachidonic acid, to form lipid radicals, consequently disturbing membrane and cellular machinery (Alexander-North et al., 1994; Refsgaard et al., 2000). These lipid radicals can react with other radicals or radical scavengers to form both toxic and non-toxic end products. Lipid peroxidation is a manifestation of glutamate excitotoxicity and/or oxidative stress (see review Doble, 1999), and is a known pathophysiological event that occurs following CNS injury (Foxley et al., 1991; Anghileri, 1993; Roof et al., 1997; Kaptanoglu et al., 2004; Sanli et al., 2012a; Sanli et al., 2012b). Two highly toxic end-products of lipid peroxidation are 2-propenal (acrolein) and 4-hydroxynonenal (4-HNE)(Hamann and Shi, 2009; Hall et al., 2010; Bains and Hall, 2012) which can bind to amino acids such as lysine and sulfhydryl-containing cysteine residues in cellular proteins, altering their structure and functional properties (see review Hall, 2015). The elevated presence of these markers as an indicator of oxidative stress has been shown in animal models of TBI and SCI (Baldwin et al., 1998; Inci et al., 1998; Hall et al., 2004; Hamann et al., 2008). The measurement of malondialdehyde (MDA) has also been used as a marker for lipid peroxidation (Ohkawa et al., 1979), with increased levels following diffuse head injury (Vagnozzi et al., 1999), weight drop injury (Inci et al., 1998) and SCI (Barut et al., 1993; Qian and Liu, 1997). Consequences of increased lipid peroxidation and its by-products include impairment of glutamate transport in cortical astrocytes (Blanc et al., 1998), disrupted synaptosomal mitochondrial function (Keller et al., 1997) and glutamate uptake (Springer et al., 1997).

### 1.5.3.2 Protein & DNA oxidation

Oxidative damage to biological molecules involves the combined action of ROS and trace elements such as iron (Fe) and copper (Cu). The site and extent of oxidative damage is governed by site-specific iron-binding properties of Fe, and its relative amount (Floyd and Carney, 1992). A number of aromatic and sulfur-containing amino acids are sensitive to oxidative damage (Jesberger and Richardson, 1991; Stadtman and Oliver, 1991). Therefore, the extent of oxidative damage can be determined by assessing the relative amount of oxidised proteins (Levine et al., 1990). The oxidation of proteins is a key consequence of oxidative stress in cerebral ischemia (Carney et al., 1991; Dubey et al., 1995; Hall et al., 1995). Protein oxidation involves modifications of three-dimensional protein structures and physiochemical properties that can result in fragmentation,
aggregation, and increased susceptibility to proteolysis (see reviews Berlett and Stadtman, 1997; Butterfield and Lauderback, 2002; Stadtman and Levine, 2003). Additionally, research has demonstrated that increased levels of oxidized protein is often associated with loss of proteosomal activity (Keller et al., 2000; Petropoulos et al., 2000; Szweda et al., 2002a; Szweda et al., 2002b). Given the role of oxidative stress in CNS injury and disease, it is not surprising to also see increased levels of oxidative damage to proteins in models of neurodegenerative disorders (Smerjac and Bizzozero, 2008).

Overproduction of ROS/RNS can also cause several different DNA lesions including DNA strand breaks, abasic site production and nucleotide modifications. Of all nucleotide bases, guanine is the most susceptible to oxidative modifications due to its low reduction potential. Hydroxyl radicals can interact with the imidazole ring of guanine, resulting in the formation of 8-hydroxyguanosine (8-OHGD) (see review Cooke et al., 2003). 8-OHGD can potentially pair with adenine, promoting G to T transversion, resulting in mutagenic DNA lesions (Bruner et al., 2000). Both mitochondrial and nuclear DNA may undergo hydroxyl oxidation. However, mitochondrial DNA is more susceptible to ROS stress than nuclear DNA (Yakes and Van Houten, 1997). Using antibodies against 8-OHGD, oxidative modifications of DNA have been observed as early as 1 day to 21 days following SCI in rats (Kotipatruni et al., 2011). Similarly, DNA strand breaks have also been observed in acute and chronic time points following SCI (Dagci et al., 2009).

1.5.3.3 Abnormal anti-oxidant activity

CNS cells have multiple capacities to manage oxidative stress, with enzymes being particularly important, scavenging superoxide anions and peroxides. Superoxide dismutase (SOD) together with phagocytic (NADPH) oxidase contribute to increased formation of hydrogen peroxide produced from superoxide (see review Fridovich, 1995). Measurements of enzymatic activity may be used as an indirect marker of radical formation. The elimination of ROS is controlled by glutathione and Cu,ZnSOD, with elevated levels of glutathione following SCI demonstrating significant protection against lipid peroxidation (Lucas et al., 2002). Studies within our research group using the partial ON transection model, have demonstrated rapid (within 5 min) elevations in MnSOD immunoreactivity in hypertrophic astrocytes in areas of nerve vulnerable to secondary degeneration (Fitzgerald et al., 2010). Astrocytes are known to be reactive following SCI in rats (Popovich et al., 1997) with astrocytic networks contributing to the secondary
spread of oxidative stress by amplifying and propagating Ca\(^{2+}\) waves and glutamate excitotoxicity (Nicotera et al., 1997; Osborne et al., 2004; Hamilton et al., 2008; Hartwick et al., 2008). Reactive astrocytes are responsible for neuroprotection from oxidative stress via glutathione dependent mechanisms (Chen et al., 2001) and glutamate uptake (Anderson and Swanson, 2000). However, during excitotoxic conditions, astrocytic function and anti-oxidant activity may be compromised or reversed, thus further contributing to the cascade of nervous tissue damage (Takahashi et al., 1997; Parpura et al., 2004).

1.6 Calcium as a therapeutic target for CNS injury

Given effects of excessive Ca\(^{2+}\) influx into neurons and glia, there have been a considerable number of in vitro and in vivo pre-clinical trials evaluating the therapeutic benefits of attenuation of excessive Ca\(^{2+}\) flux via a variety of channels. Many inhibitory agents have been developed and tested in various models of ischemia, neurodegeneration and neurotrauma (see review O'Hare Doig and Fitzgerald, 2015). Promising results have been achieved in many of these pre-clinical studies of ion channel inhibitors, however, clinical trials have been limited and/or disappointing, indicating a clear need for a novel approach. It is increasingly recognised that combinatorial treatment strategies are likely to be required to limit the multifaceted and detrimental facets of neurotrauma (Lu and Tuszynski, 2008; Kelso et al., 2011). A description of promising ion channel inhibitors we propose for a combinatorial treatment strategy follows, whereby excessive Ca\(^{2+}\) influx through multiple routes of entry is inhibited simultaneously, following neurotrauma.

1.6.1 VGCC inhibitors & Lomerizine

Intra-axonal rises in Ca\(^{2+}\) can occur through ER release, triggered by L-Type mediated Ca\(^{2+}\) influx, which in turn activates intracellular ryanodine receptors (Rios and Brum, 1987; Stirling and Stys, 2010). In spinal cord models of ischemia, the application of the L-Type Ca\(^{2+}\) blocker Nimodipine resulted in significant functional recovery (Ouardouz et al., 2003). Furthermore, blockade of Ca\(^{2+}\) influx through L-Type Ca\(^{2+}\) channels attenuated mitochondrial injury and apoptosis following hypoxia (Tanaka et al., 2004). However, specific effects on glia have not been comprehensively evaluated. A systematic review of randomized controlled trials in TBI evaluated nine studies with a total of 2,182 patients, seven of which assessed the efficacy of nimodipine (Xu et al., 2013). It was found that
Nimodipine only appears to be beneficial in patients with subarachnoid haemorrhage, where risk of death may be lower. Nimodipine has been found to cause hypotension and further ischemic injury to the spinal cord, and only in SCI animal studies where hypotension is avoided, are improvements in spinal cord function observed (Fehlings et al., 1989).

Lomerizine (Lom; KB-2796) is a dual L- and T-type Ca\(^{2+}\) channel blocker which is currently in clinical use for the treatment of migraine: minimal adverse cardiovascular effects are observed (Nakashima and Kanamaru, 1989; Gotoh et al., 1995; Hara et al., 1995; Tamaki et al., 2003). Lom does not cause hypertension, and has localised effects on the CNS (Hara et al., 1999; Toriu et al., 2000). An in vitro study has shown that Lom reduced glutamate induced neurotoxicity, however this required pre-treatment with Lom prior to the antagonist delivery. Delivery of Lom before and directly after in vivo ischemia/reperfusion damage, resulted in significant reduction of retinal damage (Torii et al., 2000). Lom has further been shown to have protective effects against retinal, dorsal lateral geniculate nucleus and superior colliculus damage, following injection of NMDA (Ito et al., 2010). Our research team has demonstrated reduced secondary retinal ganglion cell death, morphological disruption, macrophage infiltration and MnSOD aggregates as well as partial preservation of function (Fitzgerald et al., 2009a) and reduced myelin decompaction, following treatment of partial CNS injury with Lom (Savigni et al., 2013).

### 1.6.2 P2X receptor antagonists & oxATP

A limited number of novel P2X\(_7\) receptor antagonists have been reported (Baraldi et al., 2000; Baraldi et al., 2002; Baraldi et al., 2003), including oxidised ATP (oxATP) and Brilliant Blue G (Jiang et al., 2000; Carmo et al., 2014). Inhibition of P2X\(_7\) receptors via oxATP has been shown to protect against secondary injury in rats following acute compact SCI (Wang et al., 2004). OxATP is an irreversible, non-competitive antagonist of P2X\(_1\), P2X\(_2\) and P2X\(_7\) receptors (Kennedy, 2007). In the initial experiments of Wang et al. (2004) , pre-treatment of rats with oxATP resulted in a highly significant (>75%) reduction in the number of apoptotic (TUNEL-positive) cells in both grey and white matter, suggesting the P2X\(_7\) receptor antagonist limited secondary injury. Furthermore, when the antagonist was administered 1 hour after SCI, there was once again fewer TUNEL-positive cells and significantly improved functional recovery (Wang et al., 2004).
2004). These data support the notion that P2X$_7$ receptor antagonists, and more specifically oxATP, has potential as a therapeutic agent in neurotrauma.

### 1.6.3 AMPA receptor antagonists & YM872

Quinoxalinedione derivatives were among the first generation of antagonists of the AMPA receptor (Ouardouz et al., 2003). 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[f]quinoxaline-2,3-dione (NBQX) was shown to have neuroprotective effects following cerebral ischemia (Gill, 1994; Graham et al., 1996) and reduced excitotoxic insult in white matter following injury (Follett et al., 2000). However, NBQX has poor water solubility, thus renal toxicity is thought to limit the experimental and clinical efficacy of the drug (Xue et al., 1994). Therefore, improving the solubility of AMPA antagonists became an important goal to increase their suitability as effective neuroprotective agents (Takahashi et al., 2002). The novel competitive AMPA receptor antagonist zonampanel monohydrate (YM872) is highly soluble and selective and has undergone clinical neuroprotective drug trials for treatment of cerebrovascular disorders (Takahashi et al., 1998; Minematsu et al., 2008). However, YM872 was abandoned in phase-III trials for the treatment of acute ischemic stroke after failing an interim futility analysis (statistical evaluation of success in achieving primary outcome) (Klein and Engelhard, 2010).

It is thought that the antagonism of Ca$^{2+}$ permeable AMPA receptors by YM872 may prevent the activation of NMDA receptor channels and could also reduce activation of voltage-gated Ca$^{2+}$ channels (Miller, 1991; Wong and Kemp, 1991). Furthermore, YM872 may prevent Ca$^{2+}$ influx into neurons, oligodendrocytes and other glia by reversal of the Na$^+$-Ca$^{2+}$ exchanger. (Takahashi et al., 2002). Because of this, YM872 remains a candidate to be used in our model to target the molecular cascades involved in secondary degeneration following injury, and possibly prevent the associated oligodendrocyte and myelin abnormalities that occur.

### 1.6.4 NMDA antagonists & Memantine

Pharmacological antagonism of NMDA receptors is a potential strategy for reducing glutamate excitotoxicity following neurotrauma. There have been a number of inhibitory candidates trialled, with non-competitive NMDA receptor antagonists such as phencyclidine (PCP), ketamine, dextorphan, and dizocilpine (MK-801), showing an
advantage over competitive antagonists such as 2-amino-7-phosphonoheptanoate (D-AP7) and 3-(2-carboxypiperazin-4-yl)propyl-1-phosphoric acid (CPP) (Karschin et al., 1988). This is likely due to these non-competitive antagonists being lipophilic, which allows penetrance of the CNS (Kemp et al., 1987). From the early 1990’s to 2000’s, newer studies found little or no effect of NMDA antagonists in TBI models (Lanier et al., 1990; Buchan et al., 1991; Nellgard and Wieloch, 1992; Morris et al., 1999; Davis et al., 2000). On the contrary, there is a wealth of earlier literature that supports the therapeutic efficacy of NMDA antagonists, with some studies describing NMDA antagonism as beneficial in the treatment of TBI and SCI (Faden et al., 1988; Yum and Faden, 1990; Gaviria et al., 2000a; Gaviria et al., 2000b). In 1988, it was demonstrated that pre-treatment with PCP could limit long-term behavioural deficits following brain injury in a rat model (Hayes et al., 1988).

The challenge facing those trying to combat excitotoxicity is that the same processes, which in excess lead to excitotoxic cell death, are crucial for normal cellular function at lower levels. To be clinically acceptable, competitive antagonists of NMDA receptors must block excessive activation of the NMDA receptor, whilst leaving normal function unimpaired. However, competitive antagonists compete with the endogenous agonist (e.g. glutamate or glycine) (see review Paoletti and Neyton, 2007). Thus, inhibitor treatment is likely to lead to adverse events such as drowsiness, hallucinations or coma. Indeed, early clinical trials of NMDA inhibitors for stroke and traumatic injury to the brain and spinal cord have had severe and unacceptable side effects, rendering them clinically unviable (Davis et al., 2000; Sacco et al., 2001).

However, Seif el Nasr et al. (1990) discovered that the non-competitive NMDA receptor antagonist 3, 5-dimehtyl-1-adamantanamine (Memantine), was capable of blocking excitotoxic cell death, in a clinically tolerated matter (el Nasr et al., 1990; Pellegrini and Lipton, 1993; Chen and Lipton, 1997). In further studies, Memantine has been shown to provide neuroprotection without adverse side effects in animal models of ischemia (Block and Schwarz, 1996; Ehrlich et al., 1999). In 2003, the first randomized, placebo-controlled, multi-centre, phase III clinical trial showed the effectiveness of memantine for moderate-to-severe Alzheimer’s disease (Reisberg et al., 2003). Currently, memantine is the first and only novel class of medication approved by the U.S F.D.A and the European Medicines agency for the treatment of moderate to severe Alzheimer’s disease. More recently, memantine therapy has shown beneficial effects after brain injury.
(Abrahamson et al., 2014), including alleviation of neurobehavioral deficits (Huang et al., 2015).

1.7 Combinatorial strategies for the treatment of CNS injury

Nevertheless, after decades of research and clinical trials, there is still no effective pharmacological treatment for acute or chronic SCI. Neurotrauma as a whole, also lacks effective therapies and there is a great need for the development of effective treatments for neurodegeneration and neurotrauma to restore lost or damaged function (Tuszynski, 2005). The pathophysiology of neurotrauma is multi-faceted and rich with pharmacological targets. However, to date, most of the studies of neuroprotective agents are, or have been focused on a single component of the complex cascade of injury. Given the multiple potential routes of excess Ca\(^{2+}\) entry into neurons and glia, we have hypothesized that combinations of Ca\(^{2+}\) channel inhibitors that inhibit different routes of Ca\(^{2+}\) entry may limit myelin damage and reduce loss of function following neurotrauma (Savigni et al., 2013; O'Hare Doig and Fitzgerald, 2015). More recently, combinatorial therapy has arisen as a potential means of effective treatment (Lu et al., 2004; Tuszniski, 2005; Stack et al., 2006; Kelso et al., 2011). Jenkins et al. (1988) took a combinatorial approach by combining treatment of PCP with cholinergic antagonist scopolamine, and demonstrated reduced neuronal vulnerability to secondary ischemia. Furthermore, magnesium and ketamine in combination has been found to attenuate cognitive dysfunction following brain injury in rats (Smith et al., 1993). Combined application of nimodipine and the Na\(^+/\)Ca\(^{2+}\) exchange inhibitor KB-R7943 in spinal cord dorsal column ischemia did not result in additive effects, likely due to additional effects of KB-R7943 on VGCCs (Ouardouz et al., 2005). A novel study assessing the combined effects of Nogo-A antibody, neurotrophin-3, and elevation of NMDA receptor function on synaptic plasticity following SCI resulted in slightly better motor function in the absence of adverse effects (Schnell et al., 2011). A combinatorial treatment trial with Nimodipine was conducted in France in 1996, under the North American Spinal Cord Injury protocol (Bracken, 1990), where 100 patients with SCI were treated with nimodipine, with and without methylprednisolone sodium succinate. Although the combination of drugs was novel, benefit over placebo was not demonstrated in any treatment group (Petitjean et al., 1998).
However, studies assessing efficacy of multiple ion channel inhibitors delivered simultaneously are limited. We have recently assessed various combinations of three ion channel inhibitors for efficacy at reducing secondary degeneration following partial optic nerve transection in rat (Savigni et al., 2013). We used Lom to inhibit VGCCs, oxATP to inhibit purinergic P2X7 receptors, and/or YM872 (also referred to as INQ) to inhibit Ca2+ permeable AMPA receptors. The oxATP and YM872 were delivered by osmotic minipump directly to the site of injury, commencing at the time of injury and continuing for 2 weeks. Lom was administered orally twice daily, until outcomes were assessed at 3 months. Each of the treatments involving Lom significantly increased the proportion of axons with normal compact myelin, implying a role for excess Ca2+ entry via VGCC in myelin decompaction. Following traumatic injury to white matter the length of the Node of Ranvier increases, reflected in functional loss (Szymanski et al., 2013). Only treatment with the three ion channel inhibitors in combination resulted in preservation of the normal length of the Node of Ranvier and preservation of function, as measured using the optokinetic nystagmus visual reflex at 3 months after injury (Savigni et al., 2013). Other tested combinations of ion channel inhibitors were less effective. These results indicate that early inhibition of P2X7 and AMPA receptors, together with sustained inhibition of VGCCs, is helpful in maintaining structure of the node of Ranvier and function following neurotrauma. While our outcomes of treatment of secondary degeneration with three ion channel inhibitors in combination are encouraging, limitations and questions remain. Treatment with YM8732 and oxATP was confined to the first 2 weeks after injury, with outcomes assessed at 3 months. As such, it is not yet clear whether beneficial effects on node length and function could be achieved with fewer inhibitors if combined treatment was sustained. Memantine is yet to be included in the combinatorial treatment strategy: as such further beneficial effects from NMDA antagonism could be identified. The contribution of Ca2+ derived from intracellular stores is yet to be determined, as are the effects of the inhibitor combinations on intracellular Ca2+ concentration. Importantly, efficacy of the treatment strategy must be assessed in more clinically relevant models of neurotrauma such as contusion (incomplete) SCI.
1.8 Project overview & experimental design

1.8.1 Hypothesis

By using selected ion channel inhibitors (Lom, oxATP, YM872 and/or memantine) to limit Ca$^{2+}$ entry through the various Ca$^{2+}$ channels (L- and T-type Ca$^{2+}$ channels, P2X$_7$ receptors, AMPA receptors and NMDA receptors), I hypothesise that we can significantly reduce oxidative damage and excessive Ca$^{2+}$ flux in neurons and glia, and preserve functional outcomes following injury to the CNS.

1.8.2 Aim 1: Identifying the oxidative events following partial injury to the CNS during the early time phase of secondary degeneration

A clear time course of oxidative events following white matter injury is not yet established. The first aim will characterise the acute time course of reactive species and oxidative stress in vulnerable tissue following partial injury to the CNS.

1.8.3 Aim 2: Optimise and define the effects of various combinations of ion channel inhibitors *in vitro* and *in vivo*

Using an *in vitro* model of secondary degeneration, we will define the effects of various combinations of ion channel inhibitors, directly measuring changes in intracellular Ca$^{2+}$ concentration and cell viability (Aim 2a). Utilising our *in vivo* model of partial CNS injury, we will assess the effects of selected ion channel inhibitor combinations on various markers of axonal changes, oxidative stress, node/paranode complexes, and myelin, as well as assessing functional outcomes (Aim 2b). The combinations of ion channel inhibitors that demonstrate the most profound effects on the variety of measures taken, will be considered optimal.

1.8.4 Aim 3: Assess efficacy of the optimised combination of ion channel inhibitors in a clinically relevant model of spinal cord injury

Finally, I will assess efficacy of the optimised combination(s) of ion channel inhibitors in a clinically relevant *in vivo* rat model of neurotrauma, involving the partial laminectomy, and mild-moderate (150kD) contusive injury of thoracic spinal cord. This will provide the preclinical data necessary for the potential translation of the optimised combinatorial treatment to the clinic.
1.9 Introduction to a series of papers

The Introduction and Literature Review presented in this chapter overviews a wide body of literature. It highlights the multidisciplinary nature of the PhD project undertaken, which combines the fields of *in vitro* and *in vivo* pharmacology, with analysis techniques encompassing fluorescent labelling and liquid chromatography/tandem mass spectroscopy (LC/MS/MS), analysis of reactive species, live cell Ca$^{2+}$ imaging and immunohistochemistry, as well as analyses of animal behaviour to address the current gap in understanding of the time course of secondary degeneration and to test a potential therapeutical treatment for CNS injury.

Each of the following chapter addresses the results of the listed aims, and are presented in a series of published papers, submitted manuscripts, and prepared chapters, the citations of which are listed below. Chapters outline the evolution of experiments conducted in addressing these aims, highlighting experimental data generated in their completion.


Outlined below are peer-reviewed articles published during my PhD candidature that were not directly a part of the aims of this thesis.

**Original research articles**

using a combination of ion channel inhibitors: possible confounding effects of seromas. *Neural Regeneration Research* 12(2): 307-316


Reviews


Chapter Two

Identifying the oxidative events following partial injury to the CNS during the early time phase of secondary degeneration

2.1 Introducing ‘Reactive species and oxidative stress in optic nerve vulnerable to secondary degeneration’

Following trauma to the CNS, tissue vulnerable to secondary degeneration including cells in the ON, are exposed to significant changes in Ca$^{2+}$ dynamics following partial CNS injury (Wells et al., 2012; Lozic et al., 2014). Changes in Ca$^{2+}$ flux are associated with the overproduction of ROS, changes in antioxidant activity, and oxidative damage to proteins, lipids, and mitochondrial and nuclear DNA (Tavazzi et al., 2005; Mazzeo et al., 2009). Although oxidative stress is a well-known hallmark of CNS injury (Tator and Fehlings, 1991; Lu et al., 2000b; Park et al., 2004; Carrico et al., 2009), a comprehensive time course of oxidative events in the partial ON transection model had not yet been established. Therefore, the first aim of this thesis was to characterise the acute time course of changes in reactive species, antioxidant enzymes and oxidative stress in vulnerable tissue following partial injury to the CNS. This allowed us to gain a necessary indication of outcome measures with which to assess the efficacy of ion channel inhibitors for the treatment of CNS injury. Furthermore, having developed an oxidative stress time course using a suite of markers during my PhD candidature, we were able to identify a suitable time to assess the acute in vivo effects of the combinatorial treatment strategy using ion channel inhibitors, proposed in Aim 2b.

2.2 Detection of reactive species in vivo following partial CNS injury

Most ROS are highly reactive molecules, rendering them unstable and difficult to detect in perfusion fixed biological samples. Therefore, direct imaging of ROS in fresh biological samples is attractive, but extremely difficult. Therefore, this study detected ROS levels by detecting end products with chemiluminescence and immunofluorescence. To detect intracellular ROS, particularly hydrogen peroxide, we first used a chloromethyl
derivative of 2′,7′-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA), a cell permeable substrate which when cleaved by intracellular esterase’s and oxidised, produces a green fluorescent product, 2′,7′-dichlorofluorescein (DCF) (Bass et al., 1983; Sundaresan et al., 1995). Once cleaved by intracellular esterases, it becomes trapped intracellularly, facilitating post-fixation of sections and microscopy image analysis. However, DCF is also considered to be a general indicator of ROS, reacting with hydrogen peroxide, peroxynitrite and superoxide (Carter et al., 1994; Possel et al., 1997). Therefore, to more specifically detect superoxide production, dihydroethidium (DHE) was used, which freely permeates cell membranes, reacting with superoxide anions to form ethidium, a red fluorescent product (Rothe and Valet, 1990). However, ethidium has also been found to form due to other interactions, non-specific to superoxide production, but instead reflecting the redox status of the cell (Fink et al., 2004). Therefore, additional LC/MS/MS techniques were used to separate ethidium from its constituents (2-hydroxyethidium), to estimate intracellular superoxide levels in vivo, in non-fixed tissue. In addition, this study used immunohistochemical techniques to detect antioxidant enzymes and indicators of oxidative damage, in fixed tissue at various time points during the early time phase after injury, at 1 hour, and 1, 3 and 7 days, following injury.

Increases in fluorescent indicators of ROS and/or RNS were observed in ventral ON at 1, 3 and 7 days after injury. Increases in DCF intensity were observed 1 day post-injury, with similar increases in Amplex UltraRed intensity at 1 and 3 days post-injury. However, changes in dihydroethidium (DHE) intensity were not observed until 7 days-post injury in freshly frozen tissue. As noted above, H₂DCF reacts with a range of one-electron-oxidising species, therefore it cannot be used to quantify specific reactive species (Kalyanaraman et al., 2012). Therefore, the probe was used as a ‘general’ oxidative stress measure, suggesting the overall level of oxidative stress was greatest at day 1 post-injury.

2.3 Conclusions of ‘Reactive species and oxidative stress in optic nerve vulnerable to secondary degeneration’

Increases in glutathione peroxidase (GPx) and haem oxygenase immunoreactivity in a variety of cell types were observed 3, and 7 days post-injury, respectively, in ON vulnerable to secondary degeneration. Interestingly, immunoreactivity of GPx, an enzyme responsible for the reduction of free H₂O₂, was co-localised with mature, myelinating oligodendrocytes. Similarly, increases in protein nitrination markers were also
co-localised with mature oligodendrocytes. GPx is reported to reduce lipid peroxidation (Denicola and Radi, 2005; Schneider et al., 2008), with homozygous null mutations of GPx1 in mice, shown to have increased susceptibility to oxidative stress (de Haan et al., 1998). Interestingly however, elevations in lipid peroxidation at 3 days post-injury, despite increases in GPx were observed in this study.

From these data, we infer that increases in antioxidant responses seen at 3 days post-injury in mature oligodendrocytes may be inadequate to overcome oxidative stress, particularly lipid peroxidation. Thus, the combination of nitrosative damage and lipid peroxidation of oligodendrocytes observed in this study, may contribute to abnormal myelin (Payne et al., 2012) and node/paranode complex changes (Szymanski et al., 2013) observed during secondary degeneration in previous studies. Taken together the data indicate that acute oxidative stress, may be a therapeutic window and target for the treatment of secondary degeneration.

The study described in this chapter is presented below and is published as:

Regular Article

Reactive species and oxidative stress in optic nerve vulnerable to secondary degeneration

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Abstract
Secondary degeneration contributes substantially to structural and functional deficits following traumatic injury to the CNS. While it has been proposed that oxidative stress is a feature of secondary degeneration, contributing reactive species and resultant oxidized products have not been clearly identified in vivo. The study is designed to identify contributors to, and consequences of, oxidative stress in a white matter tract vulnerable to secondary degeneration. Partial dorsal transection of the optic nerve (ON) was used to model secondary degeneration in ventral nerve unaffected by the primary injury. Reactive species were assessed using fluorescent labelling and liquid chromatography/tandem mass spectroscopy (LC/MS/MS). Antioxidant enzymes and oxidized products were semi-quantified immunohistochemically. Mitophagy was assessed by electron microscopy. Fluorescent indicators of reactive oxygen and/or nitrogen species increased at 1, 3 and 7 days after injury, in ventral ON. LC/MS/MS confirmed increases in reactive species linked to infiltrating microglia/macrophages in dorsal ON. Similarly, immunoreactivity for glutathione peroxidase and haem oxygenase-1 increased in ventral ON at 3 and 7 days after injury, respectively. Despite increased antioxidant immunoreactivity, DNA oxidation was evident from 1 day, lipid oxidation at 3 days, and protein nitration at 7 days after injury. Nitrosative and oxidative damage was particularly evident in CC1-positive oligodendrocytes, at times after injury at which structural abnormalities of the Node of Ranvier/paranode complex have been reported. The incidence of mitochondrial autophagic profiles was also significantly increased from 3 days. Despite modest increases in antioxidant enzymes, increased reactive species are accompanied by oxidative and nitrosative damage to DNA, lipid and protein, associated with increasing abnormal mitochondria, which together may contribute to the deficits of secondary degeneration.

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Introduction
The pathophysiology of injury to white matter tracts in the central nervous system (CNS) involves both primary and secondary mechanisms. Outside the primary injury area, neurons and glia remain vulnerable to secondary degeneration, with structural changes and delayed cell death resulting in progressive loss of function (Lu et al., 2000; Park et al., 2004; Payne et al., 2012; Tator and Fehlings, 1991). Excess Ca2+ flux and oxidative stress have been implicated in vitro studies, as major contributors to damage following injury (Peng and Jou, 2010). However, the biochemical processes and contributors leading to damage and death in nerve tissue vulnerable to secondary degeneration in vivo have not been characterized. Partial transection of the optic nerve (ON), involving a precise primary lesion only to dorsal axons, leaves those in ventral ON intact but vulnerable to secondary degeneration (Fitzgerald et al., 2009a; Levkovitch-Verbin et al., 2003). This in vivo model allows spatial separation of primary from secondary injury, and the comprehensive assessment of biochemical pathways contributing specifically to secondary degeneration in a white matter tract of the CNS.

Oxidative stress is a hallmark of neurodegenerative diseases such as glaucoma (Izozzi et al., 2006; Tezel, 2006; Uttara et al., 2009) and CNS injury (Carrico et al., 2009; Fitzgerald et al., 2010; Lu et al., 2000; Park et al., 2004; Tator and Fehlings, 1991; Wells et al., 2012) and occurs as a consequence of excess reactive species. Reactive species play a dual role, with both beneficial and harmful effects well documented, as
reviewed by Valko et al. (2007). Enzymatic antioxidant defence systems such as glutathione peroxidase (GPx) and manganese superoxide dismutase (MnSOD), together with non-enzymatic antioxidants including ascorbic acid and glutathione, act to maintain redox balance (Valko et al., 2007). However, when there is an abnormal increase in production of reactive oxygen or nitrogen species (ROS/RNS) and/or a decrease in antioxidant activity, antioxidant defences are overwhelmed, resulting in oxidative stress (Kowaltowski et al., 2009). Excess ROS and RNS can oxidise DNA, lipids and proteins to toxic metabolites (Pamplona et al., 2005; Xiong and Hall, 2009). Moreover, an altered redox environment can activate signalling pathways leading to cell damage and death (Valko et al., 2007). Oxidative damage, particularly in the form of lipid oxidation, has been demonstrated following traumatic brain injury (Hall et al., 2010). Increases in a wide spectrum of indicators of oxidative stress provide circumstantial evidence that highly reactive oxidants mediate secondary degeneration following neurotrauma (Cornelius et al., 2013; Hallwell, 2006), and damage in a range of neurological conditions (Dagupta et al., 2013; Sultana et al., 2006). However, direct measures of the causes and consequences of oxidative stress in white matter exclusively vulnerable to secondary degeneration following neurotrauma are lacking.

ROS consist of radical and non-radical species that can be formed by the partial reduction of oxygen, and include superoxide anion radical (O$_2^\cdot$), hydrogen peroxide (H$_2$O$_2$), hypochlorous acid (HOCl) and the hydroxyl radical (OH) (Camelo-Almarraz et al., 2006). RNS also include radical and non-radical species, such as nitric oxide (NO) and peroxynitrite (ONOO$^-$). There is evidence that in ON vulnerable to secondary degeneration, mitochondrial dysfunction and oxidative stress occur early after injury (Cummins et al., 2013; Szymanski et al., 2013). However, the identities of the ROS and/or RNS that contribute, and the nature of oxidative damage that they cause, are not clearly understood. Most ROS are highly reactive molecules, rendering them unstable and difficult to detect in biological samples. Therefore, accurate detection of ROS/RNS requires labelling and/or imaging of live tissue (Pamplona et al., 2005; Xiong and Hall, 2009). Moreover, an altered redox environment can activate signalling pathways leading to cell damage and death (Valko et al., 2007). Oxidative damage, particularly in the form of lipid oxidation, has been demonstrated following traumatic brain injury (Hall et al., 2010). Increases in a wide spectrum of indicators of oxidative stress provide circumstantial evidence that highly reactive oxidants mediate secondary degeneration following neurotrauma (Cornelius et al., 2013; Hallwell, 2006), and damage in a range of neurological conditions (Dagupta et al., 2013; Sultana et al., 2006). However, direct measures of the causes and consequences of oxidative stress in white matter exclusively vulnerable to secondary degeneration following neurotrauma are lacking.

In vivo labelling with dihydroethidium (DHE) and detection of reactive oxygen or nitrogen species (ROS/RNS) and/or a decrease in antioxidant activity, antioxidant defences are overwhelmed, resulting in oxidative stress (Kowaltowski et al., 2009). Excess ROS and RNS can oxidise DNA, lipids and proteins to toxic metabolites (Pamplona et al., 2005; Xiong and Hall, 2009). Moreover, an altered redox environment can activate signalling pathways leading to cell damage and death (Valko et al., 2007). Oxidative damage, particularly in the form of lipid oxidation, has been demonstrated following traumatic brain injury (Hall et al., 2010). Increases in a wide spectrum of indicators of oxidative stress provide circumstantial evidence that highly reactive oxidants mediate secondary degeneration following neurotrauma (Cornelius et al., 2013; Hallwell, 2006), and damage in a range of neurological conditions (Dagupta et al., 2013; Sultana et al., 2006). However, direct measures of the causes and consequences of oxidative stress in white matter exclusively vulnerable to secondary degeneration following neurotrauma are lacking.

**Materials and methods**

**Animals**

Procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research, and were approved and monitored by the University of Western Australia Animal Ethics Committee. Adult, female Piebald-Virol-Glaxo (PVG) hooded rats were procured from the Animal Resources Centre (Murdoch, WA), and housed under temperature controlled conditions on a 12-h light/dark cycle, with access to standard rat chow and water *ad libitum*.

**Partial ON transection**

The partial ON transection procedure was conducted as described previously (Fitzgerald et al., 2010). Briefly, PVG rats were anaesthetized intraperitoneally (i.p.) (50 mg/kg ketamine hydrochloride and 10 mg/kg xylazil hydrochloride, Troy Laboratories, NSW, Australia). Right ONs were surgically exposed by incising the skin overlying the skull and deflecting lacrimal tissue. A diamond keratotomy knife (Geuder, Germany) was used to make a controlled dorsal incision in each ON, to a depth of 200 μm. Post-operative analgesia was administered subcutaneously (2.8 mg/kg carprofen, Norbrook Australia, Pty. Ltd., VIC, Australia). Controls were uninjured normal animals, as we have previously demonstrated no change following sham anaesthesia and surgery (Fitzgerald et al., 2009a). The contralateral ONs were not used as controls, due to demonstrated changes in the opposite eye following ON injury (Bodeutsch et al., 1999) and thus do not provide an appropriate or useful baseline for comparison of oxidative stress changes in our model. All animals were euthanased with Lethabarb® (800–1000 mg/kg i.p., Virbac Australia Pty. Ltd., NSW, Australia) at 1 h, 1, 3 or 7 days after injury (total n = 24/time point).

**Detection of reactive species in previously frozen sections**

With animals under ketamine-xylazil anaesthesia as described above, right ONs (n = 6/time point) were dissected from the ocular cavity, collected onto a microscope slide maintained at —20 °C over a bed of dry ice, mounted in optical cutting temperature compound then snap-frozen in Eppendorf tubes in liquid nitrogen, and stored in airtight zip-lock bags at —80 °C, to avoid desiccation. Tissue was cryosectioned longitudinally at —20 °C and free floating tissue sections (20 μm) from each experimental animal were collected into 30% sucrose in phosphate buffered saline (PBS) in one well of the top row of each of six 24-well trays, to facilitate subsequent labelling and avoid multiple freeze–thaw cycles. Trays containing sections were stored at —80 °C. Labelling and washing solutions were dispensed in rows of the trays and sections were transferred between solutions in droplets, using forceps that did not meet, to avoid damaging the tissue; incubations were timed to ensure equal durations. Sucrose was removed by washing sections in PBS followed by labelling at room temperature, in the dark, in either: 30 μM chloromethyl 2,7'-dichlorodihydrofluorescein diacetate (CM-H$_2$DCF) (Cell Biolabs, CA, USA) in PBS for 15 min; 100 μM Amplex UltraRed (Life Technologies, VIC, Australia) in 50 mM sodium citrate buffer (pH 6.5) containing 0.2 U/ml horse radish peroxidase (Life Technologies, VIC, Australia) for 30 min; or 10 μM dihydroethidium (DHE) (Life Technologies, VIC, Australia) in PBS for 10 min. Stock solutions of CM-H$_2$DCF and DHE were prepared in dimethyl sulphoxide (DMSO, Sigma-Aldrich, MO, USA) and stored at —20 °C under argon; Amplex UltraRed was used according to manufacturer's instructions. Some sections were pretreated with 666 μM polyethylene glycol superoxide dismutase (PEG–SOD) in PBS for 15 min or 2 mM phenyl–N-tert-butyl nitrite (TPBN) for 60 min as described (Ma et al., 2009). Tissue sections were washed for 10 min in PBS, and then fixed in 4% paraformaldehyde (PFA, ProSciTech, QLD, Australia) in PBS, for 10 min, washed in PBS, dried, and mounted on glass slides with Fluoromount-G (Southern Bio- tech, AL, USA). Preliminary studies indicated that exposure of the sections to light (required for imaging) increased fluorescence dramatically within seconds, and that fixation reduced this problem.

**In vivo labelling with dihydroethidium (DHE) and detection of reactive species by LC/MS/MS quantification of DHE and its oxidation products**

As DHE is only sparingly soluble in aqueous buffers, DHE was bound to bovine serum albumin (BSA, Fraction V essentially fatty acid free, Sigma-Aldrich, MO, USA) for the day of use as follows. Twenty-five millimolar DHE stock was prepared in argon purged DMSO with vigorous vortexing and minimal exposure to light. The DHE stock was added drop-wise to 7.5 mM BSA in sodium phosphate buffer (50 mM, pH 7.4) such that the final concentrations were DHE 2.5 mM, DMSO 10% and BSA 6.8 mM. Following incubation at 37 °C for 15 min, diethylenetriamine pentaacetic acid (DTPA, ProSciTech, QLD, Australia) in PBS, for 10 min, washed in PBS, dried, and mounted on glass slides with Fluoromount-G (Southern Bio- tech, AL, USA). Preliminary studies indicated that exposure of the sections to light (required for imaging) increased fluorescence dramatically within seconds, and that fixation reduced this problem.
incubated in situ in the presence of DHE-BSA for 1 h, with overlying tissue restored to its normal position to minimise light exposure; additional anaesthesia was administered if required. At the conclusion of DHE-BSA labelling, prior to euthanasia, right ONs were dissected from the ocular cavity, briefly rinsed in PBS to remove excess DHE-BSA, and collected onto a microscope slide maintained at −20 °C over a bed of dry ice. Frozen ONs were cut into dorsal and ventral segments using a scalpel, and then pooled dorsal or ventral ON segments from three animals were snap-frozen in liquid nitrogen as described above. Three sets of three ON segments from animals at each time point were collected \( (n = 3, \text{total animals} = 9/\text{time point}) \); similarly located and labelled ON segments from completely normal animals were used as controls.

The pooled ON segments were then subjected to quantification of DHE and its major oxidation products, ethidium \((E^+\)\), 2-hydroxyethidium \((2-OH-E^-)\), the superoxide-specific product of DHE, and 2-chloroethidium \((2-Cl-E^-)\), the HOCl-specific product of DHE) as described previously \( (\text{Maghzal et al., 2014}) \). For the detection of RNS, we used the putative nitrosated HE \((2-nitroethidium, 2-NOE^-)\) as the specific product of the reaction of ONOO\(^{-}\) with DHE. 2-NOE\(^{-}\) was detected using the general LC/MS/MS parameters described previously \( (\text{Maghzal et al., 2014}) \) and the following precursor to product ion transition, \( m/z 359 \rightarrow 312 \), with collision energy set at 35 V. Briefly, ON segments were cut into small pieces, 100 μl of 80% N₂-bubbled ethanol added and subjected to three freeze/thaw cycles in liquid N₂. After centrifugation at 14,000g \( (4 \degree C, 15 \text{ min}) \), DHE, \( E^+ \), 2-OH-E\(^-\), and 2-Cl-E\(^-\) were then detected and quantified in the supernate against authentic standards by LC/MS/MS analysis.

**Immunohistochemical assessments**

Following euthanasia, animals were transcardially perfused with 0.9% saline followed by 4% PFA \((0.1 \text{ M phosphate buffer; } pH 7.2)=\) ONs \( (n = 5/\text{time point}) \) were cryosectioned and immunohistochemical analyses conducted according to established procedures \( (\text{Fitzgerald et al., 2010}) \), using primary antibodies recognising: GPx1 \((1:250)=\) Abcam, Cambridge, UK); haem oxygenase-1 \((1:200)=\) Abcam, Cambridge, UK); 3-nitrotyrosine \((3NT)=\) Abcam, Cambridge, UK); 4-hydroxynonenal \((HNE)=\) J. Biomed. Sci.; 8-hydroxydeoxyguanosine \((80HDG)=\) Abcam, Cambridge, UK); colabelled with primary antibodies to identify specific cell types: activated microglia/macrophages, ED1 \((1:500)=\) Millipore, MA, USA) \((\text{Damoiseaux et al., 1994})\); resident microglia/macrophages, Iba1 \((1:400)=\) Novacem, VIC, AUS) \((\text{Ito et al., 1998})\); mature oligodendrocytes, CC1 \((1:500)=\) Millipore, MA, USA) \((\text{Fuss et al., 2000})\); astrocytes, GFAP \((1:1000)=\) Abcam, Cambridge, UK); and Hoechst nuclear stain \((1:1000)=\) Invitrogen, VIC, Australia). Secondary antibodies were species specific AlexaFluor® 555 and 488 \((1:500)=\) Invitrogen, VIC, Australia).

**Microscopy and image analysis**

Reactive species and immunohistochemical labelling were visualised in a single section of ventral ON directly below the primary injury site for each animal, and photographed using either a Leitz Dialplan fluorescence microscope \((\text{Leica, Germany})\) or, where co-localisation of markers with specific cell types was required, a Nikon Eclipse Ti inverted microscope \((\text{Nikon Corporation, Japan})\). A series of optical images at 0.5 μm increments along the z-axis were acquired from the middle 6 μm of each 14 μm section imaged using the Nikon microscope: images were collected and deconvoluted using autoquant blind deconvolution with Nikon Elements AR software. All images for each outcome measure were captured at constant exposures and in a single session. Image analysis was conducted on a single image using ImageJ/Fiji analysis software, setting constant arbitrary threshold intensities for all images in an analysis and semi-quantifying mean intensities of the whole ventral ON images and mean intensities and areas above the set threshold. Transmission electron microscopy \((\text{TEM})\) and quantification of mitochondrial autophagic profiles were conducted as described \( (\text{Cummins et al., 2013})\) \( (n = 4 \text{animals/time point}) \), using a using a Philips CM-10 TEM \((\text{Eindhoven, The Netherlands})\) attached to an Olympus Megaview III camera \((1376x1032 \text{pixels})\), at an accelerating voltage of 80 kV at 25,000 × magnification, corresponding to a field of view size of 10.4 μm².

**Statistical analyses**

All data were expressed as mean ± SEM; statistical analyses were conducted using SPSS statistical software \((\text{IBM})=\) Analyses of DHE in vivo labelling data employed two-way ANOVA comparing dorsal to ventral ON, and control to injured outcomes. All other data were analysed using one-way ANOVA \((F \text{ value, degrees of freedom (df)}=\) and Bonferroni Dunn or Dunnett’s post hoc tests as appropriate \((p \text{ value})\); or Student’s t-test where comparisons were between two values \((p < 0.05=\) significant).

**Results**

Increased reactive species in ON vulnerable to secondary degeneration

Dyes that fluoresce upon oxidation by a range of reactive species were used first to assess reactive species in fresh frozen tissue sections. In ventral ON vulnerable to secondary degeneration, there was a small but significant increase in mean CM-H₂DCF derived fluorescence intensity above an arbitrary but constant threshold, at 1 day post-injury \((F = 4.25, df = 4, p = 0.002; \text{Fig. 1A}, 127\% \text{of control})\), but no significant changes in mean area of fluorescence above that threshold \((F = 0.28, df = 4, p = 0.88; \text{Fig. 1B})=\). Similarly, there was a significant increase in the mean Amplex UltraRed derived fluorescence intensity above threshold at 1 and 3 days post-injury in ventral ON \((F = 32.23, df = 4, p < 0.0001; \text{Fig. 1D}, 165\% \text{and 143}\% \text{of control respectively})=\), but no significant changes in the mean fluorescence area above the threshold \((F = 1.91, df = 4, p = 0.14; \text{Fig. 1E})=\). There were no changes in the mean fluorescence intensities of the whole ventral ON image for CM-H₂DCF or Amplex UltraRed (not shown). In the primary injury, the particularly bright staining patterns obtained with CM-H₂DCF and Amplex UltraRed were reminiscent of infiltrating microglia/macrophages \((\text{Fig. 1C} \text{and F})=\) \((\text{Fitzgerald et al., 2010})\), although definitive immunohistochemical identification of these cells was precluded by the need to use non-fixed tissue. Additionally, although there were no significant changes in the mean DHE derived fluorescence intensity above threshold in the ventral ON at any day post-injury \((F = 1.14, p = 0.37; \text{Fig. 1G})=\), we detected a substantial and significant increase in the mean DHE derived fluorescence area above the threshold in ventral ON 7 days following injury \((F = 10.76, df = 4, p < 0.0001; \text{Fig. 1H})=\), representative images in J, 383\% of control)\); and the mean intensity of the whole ventral ON image \((F = 8.15, df = 4, p = 0.0002, \text{not shown})=\), indicating greater spread of reactive species recognised by this dye. Pre-treatment of sections with PEG-SOD resulted in a significant decrease in mean DHE derived fluorescence intensity in ventral ON at 3 days following injury \((p < 0.05; \text{Fig. 1I})=\); note that absolute values of fluorescence intensity are not comparable between experiments conducted on different days \( (\text{e.g. Fig. 1C} \text{and I})=\). Pre-treatment of sections with the reactive species scavenger TPN resulted in no difference in mean CM-H₂DCF derived fluorescence intensity \((p > 0.05=\), not shown).

In vivo labelling of ON with DHE-BSA, followed by LC/MS/MS analysis of tissue, allowed detection and quantification of: 2-hydroxyethidium \((2-OH-E^-)\), a O₂⁻-specific product \((\text{Zielonka et al., 2008a})\); ethidium \((E^+)\), a non-specific oxidation product arising from a number of species reacting with DHE \((\text{Zielonka and Kalyanaraman, 2010})\); and 2-chloroethidium \((2-Cl-E^-)\), a specific product formed from DHE by chlorinating species such as hypochlorous acid \((\text{HOCl})=\) \((\text{Maghzal et al., 2014})\) \((\text{Fig. 2})=\). However, we were not able to detect any significant
levels of putative 2-NO$_2$-E$^+$, a specific product of the reaction of HE with ONOO$^-$ (data not shown). Binding of DHE to BSA (used to prepare a form of DHE soluble in an aqueous buffer) did not apparently change its suitability as a redox dye, as there was no difference in the intensity or appearance of labelling of fresh frozen sections with DHE-BSA, compared to DHE (e.g. mean ± SEM fluorescence intensity above set threshold: DHE = 30.9 ± 3.1; DHE-BSA = 29.3 ± 1.4; n = 3/group). We did not detect any significant differences in the amounts of 2-OH-E$^+$ or E$^+$ in normal compared to injured ON segments ($F = 3.03$, dF = 2, $p = 0.09$; $F = 1.03$, dF = 2, $p = 0.39$ respectively), or between dorsal and ventral ON ($F = 3.32$, dF = 1, $p = 0.19$; $F = 4.09$, dF = 1, $p = 0.07$ respectively; Fig. 2A, B). Similarly, when the data were expressed as a ratio of oxidized to total probe detected (to account for any variation in DHE uptake), despite a trend towards an increased ratio of E$^+$/DHE following injury, there were no significant differences in the ratios of 2-OH-E$^+$ / DHE or E$^+$/DHE in normal compared to injured ON segments ($F = 1.47$, dF = 2, $p = 0.27$; $F = 3.30$, dF = 2, $p = 0.07$ respectively), or between dorsal and ventral ON ($F = 1.23$, dF = 1, $p = 0.29$; $F = 0.91$, dF = 1, $p = 0.36$ respectively; Fig. 2D, E). However, there was significantly more 2-Cl-E$^+$ in dorsal ON than ventral ON at days 1 and 7 following partial ON transection ($F = 6.62$, dF = 1, $p = 0.05$, $p = 0.03$ respectively; Fig. 2C, 344% and 764% of ventral 2-Cl-E$^+$ respectively). Furthermore, the ratio of 2-Cl-E$^+$ / DHE in dorsal ON was significantly increased at 1 and 7 days after injury compared to normal ($F = 17.18$, dF = 2, $p < 0.0001$, $p = 0.01$ respectively, 297% and 216% of control respectively), and was significantly higher than the ratio of 2-Cl-E$^+$ / DHE in ventral ON at these times ($F = 12.28$, dF = 1, $p = 0.01$, $p = 0.02$ respectively, 181% and 187% of ventral 2-Cl-E$^+$ / DHE respectively; Fig. 2F).

Increases in antioxidant enzyme immunoreactivity in ON vulnerable to secondary degeneration

There was a small but significant increase in the mean intensity of immunoreactivity of the antioxidant enzyme GPx1, when assessing the whole of ventral ON images at 3 days following injury ($F = 3.00$, dF = 4, $p = 0.05$; Fig. 3A, 119% of control) compared to normal ON. However there were no significant changes in the mean intensity above a set threshold ($F = 1.26$, dF = 4, $p = 0.32$, not shown) or the
area above that set threshold ($F = 2.78$, $df = 4$, $p = 0.06$; Fig. 3B), implying a diffuse increase, as observed in Fig. 3D. GPx1 immunoreactivity co-localised with cell somata of CC1+ cells, although some CC1+ cells were not GPx1 immunoreactive (arrowheads). Occasional co-localisation with ED1+ cells and MBP was much less pronounced (arrows, Fig. 4C–E). There was also a significant increase in intensity of HO-1 immunoreactivity when assessing the whole of ventral ON images at 7 days following injury ($F = 3.50$, $df = 4$, $p = 0.002$; Fig. 3F, 123% of control). While there were no significant changes in the mean intensity above a set threshold ($F = 2.58$, $df = 4$, $p = 0.07$, not shown), the area above that set threshold was substantially and significantly increased at 7 days ($F = 3.03$, $df = 4$, $p = 0.003$; Fig. 3G, 219% of control). HO-1 immunoreactivity was also present in a somewhat diffuse pattern across the ventral ON, that co-localised with Iba1+ cells at 3 days after injury (note that the particular image chosen to demonstrate co-localisation had higher than average numbers of Iba1+ cells, arrow, Fig. 3H).

### Increases in protein nitration, lipid oxidation and oxidative damage to DNA in ON vulnerable to secondary degeneration

We then investigated whether markers of oxidative damage were increased in ON vulnerable to secondary degeneration. We detected a significant increase in both the mean intensity of immunoreactivity of 3NT (a marker of protein nitration) in the whole ventral ON image ($F = 6.51$, $df = 4$, $p = 0.0003$; Fig. 4A, 160% of control), and a large increase in the area above a set threshold intensity ($F = 9.96$, $df = 4$, $p < 0.0001$; Fig. 4B, 430% of control) at 7 days following injury, compared to normal ON. While there appeared to be a significant change in the mean intensity above that threshold ($F = 2.98$, $df = 4$, $p = 0.04$, not shown), post hoc tests did not reveal a significant difference from normal, at any time point. 3NT immunoreactivity was not co-localised with MBP at 7 days (Fig. 4C), but was observed increasingly in CC1+ somata as time passed after injury (arrows, Fig. 4I).

There was no significant change in the mean intensity of HNE immunoreactivity in the whole ventral ON image after injury ($F = 1.51$, $df = 4$, $p = 0.25$; Fig. 4D), however the mean intensity above the set threshold was slightly increased at 3 days following injury ($F = 4.24$, $df = 4$, $p = 0.003$; not shown, 129% of control), and the area above that threshold increased substantially ($F = 3.94$, $df = 4$, $p = 0.002$; Fig. 4E, 231% of control). Representative images demonstrate that HNE immunoreactivity occasionally co-localised with MBP (Fig. 4F) and was co-localised to most CC1+ myelinating oligodendrocyte somata (green, arrows, Fig. 4G). HNE immunoreactivity also occasionally co-localised with GFAP (blue) immunoreactivity (purple, Fig. 4H).

There was also a significant increase in immunoreactivity of 8OHDG (a marker of DNA oxidation) in ventral ON from 1 day after injury, when assessing the mean intensity of the whole image ($F = 12.00$, $df = 4$, $p < 0.0001$; Fig. 4I, 135% of control at day 1 to 164% at day 7), the mean intensity of immunoreactivity above the set threshold ($F = 20.00$, $df = 4$, $p = 0.0001$, not shown, 110% of control at day 1 to 124% at day 7) and the mean area above that threshold ($F = 11.60$, $df = 4$, $p < 0.0001$; Fig. 4J, 198% of control at day 1 to 252% at day 7). Cellular distribution of 8OHDG immunoreactivity was widespread, with many cell somata demonstrating 8OHDG immunoreactivity by 1 day after injury (Fig. 4K).

### Increases in mitochondrial autophagic profiles in ON vulnerable to secondary degeneration

Ultrastructural analyses of mitochondria revealed a small subset which featured second double membranes, very high electro-density and/or vacuole-like features, clearly indicative of autophagic processes (Cummins et al., 2013). Given the complexities associated with definitive identification of mitophagy, these structures were classified as mitochondrial autophagic profiles and their incidence quantified (40 fields of view/animal, total area assessed = 416 μm²). We observed a large, significant increase in the incidence of mitochondrial autophagic profiles in ventral ON from 3 days after injury ($F = 73.01$, $df = 4$, $p < 0.0001$, Fig. 5A). Representative images illustrate mitochondrial autophagic profiles with a second double membrane (Fig. 5B, arrow), compared to normal mitochondria (Fig. 5C). Furthermore, the incidence of abnormal mitochondria was also increased during secondary degeneration: representative images at day 1 illustrate mitochondria with
swollen regions, that were highly electron dense or that featured a lysosomal sac (arrows, Fig. 5D–Fr e s t i v e l y). 

Discussion

Increased reactive species and resultant oxidative damage to DNA, lipid and protein have been implicated in secondary degeneration, largely based on in vitro studies (Peng and Jou, 2010), but in vivo characterisation of contributors to, together with consequences of, oxidative stress are lacking. We have used an in vivo ON partial transection model, enabling analysis of ON exclusively vulnerable to secondary degeneration, to characterise increases in reactive species in white matter vulnerable to secondary degeneration. Despite relatively modest increases in antioxidant enzyme immunoreactivity, increases in oxidative and nitrosative damage were detected in DNA, lipid and protein, particularly evident in oligodendrocytes. This oxidative damage may contribute to the structural and functional deficits of secondary degeneration following traumatic injury to the CNS.

Changes in reactive species during secondary degeneration

In conditions of excess Ca$^{2+}$ and altered mitochondrial membrane potential, electron leakage to oxygen at complexes I and III of the electron transport chain can increase, resulting in increased formation of O$_2^•$ (Kowaltowski et al., 2009; Pryde and Hirst, 2011). Superoxide dismutases reduce O$_2^•$ to H$_2$O$_2$ (Higgins et al., 2009), and their enzyme activity can increase in response to O$_2^•$ production (Li et al., 2010). We have previously shown increases in MnSOD immunoreactivity in hypertrophic astrocytes from 5 min to 3 days in ON vulnerable to secondary degeneration (Fitzgerald et al., 2010). This increase in MnSOD is associated with decreased catalase activity (Wells et al., 2012), consistent with the presence of oxidative stress and our observed increases in CM-H$_2$DCF and Amplex Ultrared fluorescence in ventral ON. However, it is important to bear in mind the limitations in conclusions one can draw from results derived from CM-H$_2$DCF and Amplex Ultrared (Kalyanaraman et al., 2012). H$_2$DCF reacts with a wide range of one-electron-oxidising species, including heme proteins such as cytochrome c and different ROS and RNS. As such, the probe cannot be used to specifically quantify H$_2$O$_2$ or other ROS (Kalyanaraman et al., 2012). Similarly, Amplex Ultrared-derived fluorescence requires the presence of H$_2$O$_2$ plus a peroxidase, so that any change in signal is not a direct measure of H$_2$O$_2$. These limitations may help explain why CM-H$_2$DCF staining was not more pronounced than Amplex Ultrared fluorescence in ventral ON. However, a range of sources of fluorescence on tissue sections may further preclude comparison of staining intensities or definitive identification of individual reactive species present. By comparison, in vivo labelling with DHE-BSA, followed by LC/MS/MS analysis of tissue, allows more definitive identification of some individual reactive species.
For example, the increased 2-Cl-E• that we observed in the primary injury following in vivo labelling is likely due to exposure of DHE to HOCl derived from myeloperoxidase, characteristic of infiltrating inflammatory cells (Maghzal et al., 2014), and coinciding with the marked fluorescence in dorsal ON, in tissue sections stained with CM-H2DCF and Amplex Ultrared.

The substantial increase in DHE derived fluorescence in ventral ON tissue sections could be interpreted as increased O2•− (Bindokas et al., 1996). However lack of increase in the O2•−specific product 2-OH-E• (Maghzal et al., 2014; Zielonka and Kalyanaraman, 2010) in ventral ON, labelled in vivo with DHE, indicates that increased DHE staining was more likely due to formation of ethidium, a non-specific oxidation product that can arise from a number of species reacting with DHE, as well as cytochrome c (Zielonka et al., 2008b). Our observed increases in Tunel• + oligodendrocyte precursor cells in ventral ON in the first week after injury are consistent with increased cytochrome c derived ethidium (Payne et al., 2013; Zielonka et al., 2008b). There was a trend towards increased ethidium detected using the LC/MS/MS approach, particularly in the primary injury site, but this did not reach significance, and caution should be exercised when interpreting these findings. Indeed, we have previously observed decreases in DHE staining in fixed tissue vulnerable to secondary degeneration (Szymanski et al., 2013), indicating significant variability depending upon the timing of fixation and analysis technique employed. Furthermore,
while PEG-SOD pre-treatment of tissue sections reduced DHE derived fluorescence, presumably due to reductions in primarily extracellular O$_2^-$ and possibly intracellular ROS in sectioned cells, we did not observe a decrease in TPBN scavenged reactive species detected using CM-H$_2$DCF. It has been demonstrated that effects of scavengers in cell free systems do not necessarily correspond to effects in dissociated retinae (Schlieve et al., 2006), and therefore perhaps in live tissue sections. Taken together, and bearing in mind the caveats already mentioned, our results indicate that infiltrating inflammatory cells at the primary injury are a significant source of reactive species, primarily HOCl. Additional reactive species including H$_2$O$_2$, 'OH and ONOO$^-$ may also contribute to the damage of secondary degeneration, but definitive identification of these species remains to be established in vivo.

Our results are consistent with a working model of changes in reactive species following partial ON injury, in which increased Ca$^{2+}$ (Knoerle et al., 2010) and SOD, together with phagocytic nicotinamide adenine dinucleotide phosphate (NADPH) oxidase lead to increased H$_2$O$_2$, produced from O$_2^-$, in mitochondria, the cytosol and extracellularly (Fig. 6). HOCl derived from myeloperoxidase-containing inflammmatory cells will also be present intra- and extracellularly at the primary injury site in the first day after injury (Fitzgerald et al., 2009a), but is not likely to diffuse to other parts of the tissue due to its high reactivity. However O$_2^-$ and particularly the more stable H$_2$O$_2$, together with excess Ca$^{2+}$, may spread to ventral ON vulnerable to secondary degeneration through membranes, in the case of H$_2$O$_2$, and via interconnecting gap junctions of the astrocytic syncytium, as has been demonstrated in endothelial cells (Tang and Vanhoutte, 2008). Increased MnSOD in ventral astrocytes (Fitzgerald et al., 2010) may exacerbate this phenomena by rapidly converting mitochondrial O$_2^-$ to H$_2$O$_2$. Astrocytes contact myelinating oligodendrocytes and axons at the Node of Ranvier and may also disseminate ROS through heme-channels into the extracellular space. Rapid spread of ROS from astrocytes to myelinating oligodendrocytes is consistent with our demonstrations of increased oxidative stress in oligodendrocytes and node/paranode abnormalities in secondary degeneration in the first day after injury (Szymanski et al., 2013). Inflammatory cells spread into ventral ON by 3 days after injury (Fitzgerald et al., 2009a), likely furthering the spread of ROS/RNS and associated oxidative stress. Direct reaction of DNA with reactive species such as HOCl, 'OH and ONOO$^-$ (Vaca et al., 1988; Yu et al., 2005) are likely to have led to our observed increases in oxidized DNA at this time, and may in part account for the early and sustained death of oligodendrocyte precursor cells in ON vulnerable to secondary degeneration (Payne et al., 2013)

and later death of neurons by necrosis and to a lesser extent, apoptosis (Fitzgerald et al., 2009b). It is likely that the proposed model is of relevance to neurotrauma and neurodegenerative conditions where glutamate excitotoxicity, associated with changes in Ca$^{2+}$ flux, initiate focal increases in reactive species. Such changes have been reported in models of optic nerve injury (Knoerle et al., 2010), Parkinson’s disease (Goldberg et al., 2012), spinal cord injury (Ferguson et al., 2008) and neurodegenerative diseases (Halliwell, 2006).

**Antioxidant responses and oxidative/nitrosative damage during secondary degeneration**

GPX1 is one of the enzymes that reduces free H$_2$O$_2$ to H$_2$O; increased immunoreactivity in ventral ON at 3 days may reflect increased synthesis of the enzyme as a compensatory response to high levels of H$_2$O$_2$ (Fan et al., 2003) present in a range of cell types in ventral ON. Specifically, the co-localisation of GPX1 immunoreactivity with CC1$^+$ oligodendrocytes and MBP within these cells, although modest, may indicate a response to limit H$_2$O$_2$ in oligodendrocytes. Oligodendrocytes form CNS myelin which is rich in lipid and iron and highly vulnerable to selective oxidative damage (Bongarzone et al., 1995; Griot et al., 1990). GPX1 has been reported to reduce some lipid hydroperoxides (Ursini and Bindoli, 1987) and consequent lipid oxidation leading to HNE (Denicola and Raci, 2005; Schneider et al., 2008). However, GPX1 increases were small, not sustained and were associated with increased HNE, followed by protein tyrosine nitration (Fig. 6), in oligodendrocytes as well as other cells. Although we have not directly measured antioxidant enzyme activity, from these data we can infer that antioxidant responses in oligodendrocytes vulnerable to secondary degeneration are inadequate to prevent lipid oxidation, perhaps contributing to the structural abnormalities in myelin and functional loss we observe at this time (Fitzgerald et al., 2009a; Szymanski et al., 2013), as well as myelin decompaction despite sustained oligodendrocyte numbers later after injury (Payne et al., 2012; Payne et al., 2013). Our findings are in accordance with other reports of oxidative damage in white matter and oligodendrocytes, associated with glutamate excitotoxicity, following injury to the CNS (Haider et al., 2011; Matute et al., 2007; Park et al., 2004), although vulnerability is thought to be maturation dependent in these cells (Back et al., 2005; French et al., 2009). It is interesting to note that the increased HNE immunoreactivity was due to an increased area of positive cells and a greater intensity above a set threshold, indicating more or swollen cells that were immunopositive for lipid.
oxidation, as well as increased lipid oxidation within these cells. Oxidized lipid is associated with structural changes to mitochondria, including progression to mitophagy in both axons and glia at this time. The substantial increase in incidence of abnormal mitochondria is likely to reflect profoundly altered oxidative metabolism and possibly increased reactive species, at least in this mitochondrial subset.

Protein tyrosine nitration is a major cytotoxic pathway in the CNS, potentially contributing to a range of CNS insults and neurodegenerative disorders (Crow et al., 1997; Giasson et al., 2000; Reynolds et al., 2005). The increased co-localisation of 3NT immunoreactivity with CC1+ myelinating oligodendrocytes indicates nitrosative damage in these cells, which may further contribute to abnormal myelin in secondary degeneration (Payne et al., 2012). A possible source of the implied reactive nitrogen species is myeloperoxidase (Gaut et al., 2002), consistent with our observed increase in 2-Cl-E". Nitration of tyrosine residues of MnSOD has been shown to inactivate the enzyme (Pacher et al., 2007). Our observed greater than fourfold increase in 3NT at 7 days after injury may explain the reduced immunoreactivity of MnSOD at this time (Fitzgerald et al., 2010), resulting in a feed forward loop of increased O2•− and other reactive species, including ONOO− (Fig. 6). Under oxidative stress conditions, free haem released from haem proteins also becomes highly cytotoxic (Jeney et al., 2002), accelerating the production of ROS. HO-1 catalyses the degradation of haem (Stocker, 1990), and the greater than twofold increase in immunoreactivity co-localised with resident activated microglia in ventral ON indicates a protective role for these cells, as has been previously reported (Min et al., 2006).

Conclusions

While ROS are generated as natural by-products of oxidative metabolism, important for cell signalling and homeostasis, when overproduced during secondary degeneration in vivo, they are associated with spread of reactive species and oxidative damage despite modest
inhibitors increase the antioxidant enzyme immunoreactivity. Myeloperoxidase-derived chlorinating species and increased lipid oxidation and protein damage likely contribute to the structural and functional abnormalities in myelin that are a feature of secondary degeneration in vivo. As such, oligodendrocytes and/or their precursors may present an attractive target for antioxidant therapeutic intervention to limit secondary degeneration following neurotrauma. This study provides an insight into therapeutic window of opportunity in the treatment of secondary degeneration in the brain.

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Chapter Three

Optimising and defining the effects of various combinations of \( \text{Ca}^{2+} \) channel inhibitors in vitro

3.1 Introducing ‘Specific combinations of ion channel inhibitors reduce excessive \( \text{Ca}^{2+} \) influx as a consequence of oxidative stress and increase neuronal and glial cell viability in vitro’

We have previously assessed various combinations of three ion channel inhibitors in vivo (Savigni et al., 2013), achieving very promising results improving behavioural function following partial ON transection. However, it is not clear whether beneficial outcomes following administration of ion channel inhibitors were due to the direct effects of inhibiting particular combinations of channels, or overall changes in intracellular \( \text{Ca}^{2+} \) concentrations. This study utilised a high throughput in vitro model of CNS injury to provide novel insight into the effects on \( \text{Ca}^{2+} \) concentration and cell viability of various combinations of Lom, oxATP, YM872 and NMDA antagonist Memantine, demonstrating protection of various cell types of the CNS from excess \( \text{Ca}^{2+} \).

3.2 \( \text{H}_{2}\text{O}_2 \) and CNS injury in vitro

This study employed the techniques of live cell \( \text{Ca}^{2+} \) imaging to measure changes in intracellular \( \text{Ca}^{2+} \) concentration and cell viability following oxidative insult to CNS neurons and glial cells, in mixed cortical cultures (Whittemore et al., 1994). Data in Chapter 2 demonstrated increases in DCF, Amplex UltraRed and DHE fluorescence, associated with increases in MnSOD and GPx enzymes responsible for catalysing conversion of superoxide and \( \text{H}_2\text{O}_2 \). \( \text{H}_2\text{O}_2 \) can be generated during inflammation via superoxide anion dismutation, and is known to cause and/or exacerbate cellular biochemical changes, such as DNA oxidation (Fahl et al., 1984), and lipid peroxidation (Rubin and Farber, 1984). \( \text{H}_2\text{O}_2 \) can also induce cellular injury and \( \text{Ca}^{2+} \) overload in ischemic tissue (Josephson et al., 1991), as well as mediate changes in channel function via alterations of thiol groups (Hool and Corry, 2007). Because \( \text{H}_2\text{O}_2 \) is a stressor with
relative stability, and a clear and substantial role in oxidative damage (Haskew-Layton et al., 2010), this reactive species was chosen as a stressor for this particular in vitro model.

3.3 Choosing an appropriate model of CNS injury in vitro

Initially, to address this aim, we used PC12 cell line cultures, originally derived from a pheochromocytoma of rodent adrenal medulla (Greene et al., 1987), to assess Ca\(^{2+}\) ion flux using the ratiometric Ca\(^{2+}\) indicator Fura-2 AM. However, as PC12 cell cultures are an immortalised cell line, these cultures do not provide clinically relevant information for our field of research due to the lack of co-cultured glial and neuronal phenotype cells. Therefore, a considerable amount of time was devoted to developing a more relevant culture model for our assessments. We conducted pilot experiments using a number of alternatives, including organotypic slice cultures from both brain and spinal cord of PVG rat pups (P0-P3). However, there was significant variability in Fura-2 AM signal and results were not sufficiently reproducible following H\(_2\)O\(_2\) insult. We then utilised dissociated primary cortical cell cultures, which involved the dissection of PVG rat pup cortices, and provided far more robust and reproducible in vitro outcomes of CNS injury. In short, this technique involves the dissection of PVG rat pup cortices, which are then chopped up and enzymatically broken down; cells are then plated onto coated coverslips, and allowed to grow for 10 days before experimentation. This culture period allows for the growth and/or proliferation of neurons and glial cells, including non-myelinating oligodendroglia, microglia/macrophages and astrocytes. The approximate ratio of neurons: astrocytes: oligodendrocytes: microglia was 4:4:2:1. It is difficult to determine whether these ratios are reflective of in vivo numbers due to widespread variation in reported values. Although likely not reflective of the adult rodent brain in vivo (Bandeira et al., 2009), the recorded in vitro glia/neuron ratio of approximately 1.75, still remains within the reported ranges during post-natal development. However, it is important to acknowledge the variability in ratios depending on the post-development age, and/or CNS region of interest, and/or size (see review Herculan-Houzel, 2014). Live cell Ca\(^{2+}\) imaging using Fura-2 AM dye was performed to visualise and quantify intracellular Ca\(^{2+}\) concentrations after H\(_2\)O\(_2\) exposure, with appropriate controls. We determined the most suitable H\(_2\)O\(_2\) concentration based upon previous studies and our own pilot experiments, in which 400\(\mu\)M provided a consistent, sustained increase in Ca\(^{2+}\) without biphasic effects or inconsistent oscillations (Herson et al., 1999). In initial experiments, individual
intracellular Ca\textsuperscript{2+} concentrations were determined, however as experiments were conducted on mixed cultures, it was not appropriate to report Ca\textsuperscript{2+} concentrations in µM, due to established variabilities between Ca\textsuperscript{2+} concentrations in neurons and glia (Suadicani et al., 2010). Varying Ca\textsuperscript{2+} may have led to confounding results, potentially masking the effects of the inhibitors, if any. Therefore, changes in Ca\textsuperscript{2+} concentrations were reported as ∆F-ratios.

3.4 Various changes in Ca\textsuperscript{2+} influx and cell viability observed following H\textsubscript{2}O\textsubscript{2} insult and ion channel inhibitor treatment

H\textsubscript{2}O\textsubscript{2} insult resulted in gradual increases in intracellular Ca\textsuperscript{2+} concentration in mixed cortical cells over a 30 min incubation, relative to control. However, as explained in Chapter 1, changes in intracellular Ca\textsuperscript{2+} can occur via influx of ions from the extracellular space, or release from intracellular Ca\textsuperscript{2+} stores. We therefore investigated the sources of intracellular Ca\textsuperscript{2+} following H\textsubscript{2}O\textsubscript{2} exposure, and determined additional sources of Ca\textsuperscript{2+} other than ryanodine receptor dependent release of Ca\textsuperscript{2+} from intracellular stores. Fura-2 AM has been reported to detect free Zinc (Zn) (Grynkiewicz et al., 1985), with oxidative signals triggering Zn liberation from intracellular sources, including metal-binding proteins, mitochondria, and via activation of ryanodine receptors (Woodier et al., 2015). Therefore, it was important to conduct additional experiments assessing the effects of H\textsubscript{2}O\textsubscript{2} in the presence of a Zn chelator, to ensure changes in ∆F-ratio were a true representation of changes in Ca\textsuperscript{2+} concentration, and not confounded by Zn dynamics. We found no significant decrease in ∆F-ratio in the presence of a Zn chelator, indicating that Zn was not contributing to the values reported in the study.

Live cell Ca\textsuperscript{2+} imaging revealed that a 30 min exposure to H\textsubscript{2}O\textsubscript{2} induced slow increases in intracellular Ca\textsuperscript{2+} concentration, associated with a loss of cell viability by 6 hours. The effects of all possible combinations of ion channel inhibitors Lom, oxATP, YM872 and Mem on intracellular Ca\textsuperscript{2+} concentration and cell viability were assessed. Interestingly, most combinations of inhibitors that included P2X\textsubscript{7} receptor antagonist, oxATP, significantly decreased Ca\textsuperscript{2+} and increased cell viability, following H\textsubscript{2}O\textsubscript{2} exposure. However, reductions in intracellular Ca\textsuperscript{2+} concentration were not always associated with cell viability. Therefore, the data presented in this study supports a ‘source specificity hypothesis’ whereby cell death is not simply a function of increased Ca\textsuperscript{2+} concentration, but instead is governed by excessive Ca\textsuperscript{2+} influx through specific Ca\textsuperscript{2+} channels. To
clearly demonstrate that the protective role of oxATP is Ca\(^{2+}\) dependent, additional experiments were conducted examining the effects of H\(_2\)O\(_2\) and oxATP on cell survival in low extracellular Ca\(^{2+}\) concentrations. EGTA, a Ca\(^{2+}\) chelator was added at various concentrations to reduce extracellular Ca\(^{2+}\) concentrations, while maintaining cell viability (Takadera et al., 2010; Takadera et al., 2011). In the presence of H\(_2\)O\(_2\), 1mM EGTA significantly increased cell viability compared to H\(_2\)O\(_2\) control, indicating the Ca\(^{2+}\) contribution of H\(_2\)O\(_2\) on cell viability. In the presence of H\(_2\)O\(_2\) and oxATP, all tested concentrations of EGTA significantly increased cell viability compared to H\(_2\)O\(_2\) control. Together with the demonstration of reduction of intracellular Ca\(^{2+}\) with oxATP, this additional experiment clarified that the role of oxATP is Ca\(^{2+}\) dependent.

We further investigated the efficacy of ion channel inhibitors by semi-quantifying the density of specific cell subpopulations; β-III tubulin\(^{\text{+ve}}\) neurons, GFAP\(^{\text{+ve}}\) astrocytes, NG2\(^{\text{+ve}}\)/Olig2\(^{\text{-ve}}\) non-oligodendroglial cells, Olig2\(^{\text{+ve}}\)/NG2\(^{\text{+ve}}\) oligodendroglia, and ED1\(^{\text{+ve}}\) microglia/macrophages within the mixed cortical culture, following H\(_2\)O\(_2\) and ion channel inhibitor treatment. Interestingly, no protection of olig2\(^{\text{+ve}}\) oligodendroglia was observed following treatment with any of the ion channel inhibitor combinations, reflecting the vulnerability of the oligodendroglial lineage to reactive species (Back et al., 1998) in this *in vitro* model.

**3.5 Conclusions of 'Specific combinations of ion channel inhibitors reduce excessive Ca\(^{2+}\) influx as a consequence of oxidative stress and increase neuronal and glial cell viability in vitro'**

This study demonstrated the contribution of inhibiting Ca\(^{2+}\) flux through specific channels to cell viability, and the effects of excessive Ca\(^{2+}\) influx in individual cell sub-populations, in an *in vitro* model of oxidative stress in the CNS. This is the first study to assess the effects (in a high throughput model) of all combinations of Lom, oxATP, YM872 and Mem, highlighting the effects of inhibition of individual and multiple ion channels on Ca\(^{2+}\) influx and cell viability of CNS cells.

The study described in this chapter is presented below and is published as:

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SPECIFIC COMBINATIONS OF ION CHANNEL INHIBITORS REDUCE EXCESSIVE Ca\(^{2+}\) INFLUX AS A CONSEQUENCE OF OXIDATIVE STRESS AND INCREASE NEURONAL AND GLIAL CELL VIABILITY IN VITRO

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Abstract—Combinations of Ca\(^{2+}\) channel inhibitors have been proposed as an effective means to prevent excess Ca\(^{2+}\) flux and death of neurons and glia following neurotrauma in vivo. However, it is not yet known if beneficial outcomes such as improved viability have been due to direct effects on intracellular Ca\(^{2+}\) concentrations. Here, the effects of combinations of Lomeronzine (Lom), 2,3-dioxo-7-(1H-imidazol-1-yl)-6-nitro-1,2,3,4-tetrahydro-1-quinoxalinyl]acetic acid monohydrate (YM872), 3,5-dimethyl-1-adamantanamine (memantine (Mem)) and/or adenosine 5′-triphosphate periodate oxidized sodium salt (oxATP) to block voltage-gated Ca\(^{2+}\) channels, Ca\(^{2+}\)-permeable \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, NMDA receptors and purinergic P2X\(_{\text{R}}\) receptors (P2X\(_{\text{R}}\)) respectively, on Ca\(^{2+}\) concentration and viability of rat primary mixed cortical (MC) cultures exposed to hydrogen peroxide (H\(_2\)O\(_2\)) insult, were assessed. The contribution of ryanodine-sensitive intracellular stores to intracellular Ca\(^{2+}\) concentration was also assessed. Live cell calcium imaging revealed that a 30 min H\(_2\)O\(_2\) insult induced a slow increase in intracellular Ca\(^{2+}\), in part from intracellular sources, associated with loss of cell viability by 6 h. Most combinations of inhibitors that included oxATP significantly decreased Ca\(^{2+}\) influx and increased cell viability when administered simultaneously with H\(_2\)O\(_2\). However, reductions in intracellular Ca\(^{2+}\) concentration were not always linked to improved cell viability. Examination of the density of specific cell subpopulations demonstrated that most combinations of inhibitors that included oxATP preserved NG2+ non-oligodendroglial cells, but preservation of astrocytes and neurons required additional inhibitors. Olig2+ oligodendroglia and ED-1+ activated microglia/macrophages were not preserved by any of the inhibitor combinations. These data indicate that following H\(_2\)O\(_2\) insult, limiting intracellular Ca\(^{2+}\) entry via P2X\(_{\text{R}}\) is generally associated with increased cell viability. Protection of NG2+ non-oligodendroglial cells by Ca\(^{2+}\) channel inhibitor combinations may contribute to observed beneficial outcomes in vivo. © 2016 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: Ca\(^{2+}\) channel inhibitors, intracellular Ca\(^{2+}\) concentration, oligodendroglia, NG2-glia, Cell viability.

INTRODUCTION

In the CNS, calcium (Ca\(^{2+}\)) plays a vital role in important physiological processes such as cell differentiation, growth and survival (Komuro and Rakic, 1996; Gomez and Spitzer, 2000; Spitzer et al., 2000). Changes in cytosolic Ca\(^{2+}\) stimulate multiple Ca\(^{2+}\)-dependent pathways, normally designed to maintain cell structure and function. These include, but are not limited to, calpain activation (Lipton, 1999), lipid peroxidation (Braugher and Hall, 1992), nitric oxide synthesis (Belanos et al., 1997) and mitochondrial free radical production (Camello-Almaraz et al., 2006). However, Ca\(^{2+}\) also plays a salient role in cell death, with excessive intracellular Ca\(^{2+}\) accumulation leading to over-activation of Ca\(^{2+}\)-dependent pathways that are the final common
Ca	extsuperscript{2+} is known to enter neurons and glia through a range of channels and receptors, including but not limited to: voltage-gated Ca	extsuperscript{2+} channels (VGCCs) (Agrawal et al., 2000); purinergic P2X-Rs (Matute et al., 2007); glutamate-gated, GluR2 subunit lacking, Ca	extsuperscript{2+} permeable ionotropic \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) (Hollmann et al., 1991); and \(N\)-methyl-D-aspartate receptors (NMDARs) (Matute, 2006). Increased glutamate is a common consequence following injury to the CNS (Doble, 1999) and can lead to over-activation of NMDA and AMPA receptors on neurons and glia, specifically oligodendrocytes, rendering these cells susceptible to increased Ca	extsuperscript{2+} influx and depolarization (Matute et al., 1997; Doble, 1999). ATP also typically increases in response to injury (Neary et al., 2005), which can directly injure vulnerable cells, as well as trigger ATP-gated Ca	extsuperscript{2+} influx (Matute et al., 2007). Reactive species such as cell permeant \(H_2O_2\) are generated as a consequence of injury both in vivo (Cornelius et al., 2013; O’Hare Doig et al., 2014a) and in vitro (Mandavilli et al., 2005; Ma et al., 2012), and also lead to increased influx through Ca	extsuperscript{2+} channels (Muralidharan et al., 2016). As such, over-activation of Ca	extsuperscript{2+} channels and receptors results in an appreciable influx of Ca	extsuperscript{2+} into cells. As a consequence of both Ca	extsuperscript{2+} and reactive species entry, mitochondria swell, oxidative metabolism is compromised, and cytochrome c is released into the cytoplasm, associated with oxidative damage to DNA, lipids and proteins, and neuronal and glial cell death (Gandhi et al., 2009; Huang et al., 2009; Kowaltowski et al., 2009).

Given the consequences of excessive Ca	extsuperscript{2+} entry into neurons and glia following injury, the administration of Ca	extsuperscript{2+} channel inhibitors has been assessed as a therapeutic strategy for treatment of CNS damage in vivo. In rodent models of ischemia, application of nimodipine, an \(L\)-Type VGCC inhibitor, resulted in significant functional recovery (Guarzdouz et al., 2003) and attenuated mitochondrial injury and apoptosis (Tanaka et al., 2004). However, nimodipine has also been found to cause hypotension and further ischemic injury to the spinal cord (Fehlings et al., 1989). Lomerizine (Lom), a dual \(L\)- and \(T\)-type VGCC inhibitor which is currently in clinical use for the treatment of migraines, has been shown to act more specifically on the CNS with adverse systemic side effects avoided (Hara et al., 1999). We have demonstrated reduced retinal ganglion cell death and microglia/macrophage infiltration, preservation of myelin compaction and limited functional improvements following treatment of partial CNS injury with Lom (Fitzgerald et al., 2009), but clinical trials of Lom for treatment of neurotrauma are lacking.

A relatively limited number of P2X	extsubscript{7}R antagonists have been developed (Baraldi et al., 2000, 2002, 2003). One of the more effective of these is adenosine 5'-triphosphate periodate oxidized sodium salt (oxATP), an irreversible, non-competitive antagonist of P2X	extsubscript{7}, P2X	extsubscript{2} and P2X	extsubscript{4} receptors, which has been shown to protect against secondary injury, and improve functional outcomes in rodent acute impact spinal cord injury (SCI) (Wang et al., 2004). NBOX, an AMPA antagonist, has been shown to have neuroprotective effects following cerebral ischemia (Gill, 1994; Graham et al., 1996) and reduce excitotoxic insult in white matter following injury (Follett et al., 2000). However, NBQX has poor water solubility, and renal toxicity has limited use of this drug in the clinic (Xue et al., 1994). More recently, a highly selective novel competitive AMPA receptor antagonist \(2,3\)-dioxo-7-(1H-imidazol-1-yl)-6-nitro-1,2,3,4-tetrahydro-1-quinoxalinyl]ace tic acid monohydrate (YM872 or “INQ”), with high solubility, was developed. YM872 has been assessed in a number of clinical trials of neuroprotection (Takahashi et al., 1998), however it was abandoned in phase-III human clinical trials for stroke in 2010, after failing an interim futility analysis (Klein and Engelhard, 2010). 3,5-Dimethyl-1-adamantanamine (memantine or “Mem”) is a non-competitive NMDAR antagonist, shown to provide neuroprotection with minimal side effects in animal models of ischemia (Block and Schwarz, 1996; Ehrlich et al., 1999). Memantine therapy has shown beneficial effects after brain injury, including alleviation of neurobehavioral deficits (Huang et al., 2015).

While promising results have been demonstrated in pre-clinical studies, the translation of these therapeutic agents to the clinic has been limited and outcomes have been disappointing, highlighting the clear need for a new approach. Given the multiple routes of Ca	extsuperscript{2+} entry associated with the detrimental aspects of neurotrauma, it is becoming increasingly recognized that a combinatorial treatment strategy may be required (Tuszynski, 2005; Stack et al., 2006). We recently tested this hypothesis in vivo, assessing the efficacy of various combinations of three ion channel inhibitors at reducing secondary degeneration assessed 3 months following partial optic nerve transection in Plebald Viral Glaxo (PVG) rats (Savigni et al., 2013). We used Lom to inhibit \(L\)- and \(T\)-type VGCCs, oxATP to inhibit P2X	extsubscript{2}, and/or YM872, to inhibit Ca	extsuperscript{2+} permeable AMPARs. Each of the treatment combinations involving Lom significantly increased the proportion of axons with normal compact myelin, implying a role for excess Ca	extsuperscript{2+} entry via VGCCs in myelin decomposition. In areas of nerve vulnerable to secondary degeneration, there is a significant increase in node of Ranvier length, associated with loss of visual function (Szymanski et al., 2013). Only administration of the three Ca	extsuperscript{2+} channel inhibitors in combination (Lom + oxATP + YM872) resulted in maintenance of normal node of Ranvier length (s), and preservation of function, with the remaining combinations proving less effective (Savigni et al., 2013).
However, an association between intracellular Ca\(^{2+}\) concentration and the viability of neurons and glia following treatment with combinations of Ca\(^{2+}\) channel inhibitors has not yet been established. Therefore here, the effects of combinations of the inhibitors (Lom, OxA TP, YM872 and/or Mem) on excess Ca\(^{2+}\) influx and associated cell death of multiple cell types were determined, using a high throughput, in vitro model of oxidative injury in primary mixed cortical (MC) cultures (Whittemore et al., 1994). Note that the phrase “Ca\(^{2+}\) channel inhibitors” is used at times, with the understanding that Ca\(^{2+}\) permeable AMPARs, P2X\(_7\)Rs and NMDARs are also permeable to other ions that may be playing a role. Hydrogen peroxide (H\(_2\)O\(_2\)) was chosen as the reactive species stressor given its relative stability and established role in inducing oxidative damage in neurons and glia (Haskew-Layton et al., 2010).

**EXPERIMENTAL PROCEDURES**

**Animals**
PVG rat pups (postnatal days 0–3) were obtained from the Animal Resource Centre (M urdoch, Western Australia). All procedures were approved by The University of Western Australia Animal Ethics Committee (Ethics Approval Number RA3/100/673) and conformed to the National Health and Medical Research Council (NHMRC) of Australia Code of Practice for use of Animals for Scientific Purposes. All efforts were made to minimize animal suffering and to reduce the number of animals used. Rat pups were euthanized with intraperitoneal injection of Lethabar® (P entobarbitral sodium 850 mg/kg, Virbac, Milperra, NSW, Australia). For each cell culture preparation, cortices from two to six animals were pooled together, depending upon cell numbers required. 85 animals were used in the experiments of the study.

**Tissue dissection**
Following euthanasia, the skin overlying the skull was removed, and the skull was peeled away. Deep cuts were made at the margin of the cortex and superior colliculi caudally (both sides) and again at the border of the olfactory lobes, and cortex rostrally. The middle section of the brain with cortices intact was removed from the skull, and transferred to a sterile petri dish containing cold (\(-4^\circ\)C) Neurobasal-A medium (NB-A, Life Technologies, Thermo Fisher Scientific Australia, Malaga, WA, Australia). The cortex was then peeled away from underlying midbrain structures and the meninges were teased off. The ‘naked’ cortex was then transferred to a second sterile petri dish containing cold NB-A. Once all tissue was collected, a scalpel blade was used to chop the cortices into small pieces, which were transferred using a transfer pipette to a 50 mL falcon tube containing 10 mL NB-A.

**Cell culture**
MC cells were prepared as described in (Whittemore et al., 1994). In brief: following an initial 3 min (min), 200 g centrifugation, tissue was enzymatically digested in 10 mL Phosphate Buffered Saline (PBS; Life Technologies) solution containing 165 U papain (Worthington, Lakewood, NJ, USA), 3000 U of DNase 1 from bovine pancreas (dissolved in Earls Balanced Salt Solution; both from Sigma–Aldrich, Sydney, NSW, Australia), 1.65 \(\mu\)M cysteine (Sigma–Aldrich) and 50 \(\mu\)M NaOH for 5 min (min), at 37 °C. Enzyme treated tissue pieces were centrifuged for 22 min at 200 g, enzyme solution was removed and tissue was dissociated in 10 mL of NB2 media, consisting of: Neurobasal-A medium (NB-A), containing 500 \(\mu\)M glutamine (Life Technologies), 2% (v/v) B27 (Life Technologies), 100 U/mL penicillin (Invitrogen) and 100 \(\mu\)g/mL streptomycin; (Life Technologies). All multi-well plates and coverslips were pre-coated with poly-L-lysine (10 \(\mu\)g/mL) in UltraPure™ distilled water, for one hour (h) at room temperature, followed by washing 3× with PBS prior to cell seeding. MC cells were seeded at 1.5 \(\times\) 10\(^5\) cells/cm\(^2\) in NB2: into 12-well plates containing 15-mm coverslips (for calcium imaging); or 24-well plates (for live/dead viability assay). Following seeding, cells were allowed to incubate for 24 h after which NB2 media were replaced with fresh media containing 57% NB2 and 43% NB1 media (NB2/NB1 growth media). NB1 media is NB2 media supplemented with 4.2% (v/v) fetal calf serum (Life Technologies), 1% (v/v) horse serum (Life Technologies), 26.7 \(\mu\)M l-glutamic acid monosodium salt hydrate (Sigma–Aldrich), and 22.2 \(\mu\)M 2-mercaptoethanol (Life Technologies). NB2/NB1 media were replaced every 48–72 h.

**Treatments**
Effects of combinations of ion channel inhibitors, Lom (LKT Labs, St Paul, MN, USA), OxA TP (Sigma), YM872 (LKT Labs), and/or Mem (Sigma-Aldrich) were assessed. Choices of treatment concentrations were based on previously published studies using these agents individually. Lom was dissolved in dimethyl sulfoxide (DMSO) before being added to medium at a final concentration of 1 \(\mu\)M (Tamaki et al., 2003): final concentration of DMSO was less than 1% v/v. OxA TP (1 mM; (Matute et al., 2007)), YM872 (240 \(\mu\)M; (Savigni et al., 2013)) and Mem (60 \(\mu\)M, (McAllister et al., 2008)) were dissolved in medium. Ion channel inhibitors were administered to cultures such that all possible combinations of single and multiple inhibitors were assessed.

**Analysis of inhibitor and H\(_2\)O\(_2\) stability**
Stability of the inhibitors in the presence and absence of H\(_2\)O\(_2\) were assessed by reverse phase High Performance Liquid Chromatography (HPLC), on a Waters 2695 HPLC with a Waters 2489 UV/vis detector with elution through a C18 analytical column (150 × 4.60 mm, 5 \(\mu\)m, 25 °C). For all inhibitors, the ratio of inhibitor:H\(_2\)O\(_2\) concentration equated to those used in cell analysis experiments, described below. For OxA TP, two samples were prepared by dissolving OxA TP in water in the absence and presence of H\(_2\)O\(_2\) (OxA TP/H\(_2\)O\(_2\); 1:0.4). A mobile phase of Acetonitrile (A):0.1 M Phosphate buffer pH 7 (B) was used with
gradient elution and varying flow rates as follows: 0 min, 100% B 0.85 ml/min; 4 min, 95% B at 0.8 ml/min; 8 min, 75% B at 1 ml/min; 12 min, 70% B at 1 ml/min. Injection volume was 10 μL. Detection wavelength was 254 nm with retention times of 5.4 min (OxATP) and 5.2 min (OxATP + H2O2). Total run time was 12 min.

For Lom and YM872 the mobile phase consisted of Water with 0.5% Trifluoroacetic acid:Acetonitrile (31:69 v/v) with isotropic elution at 0.5 ml/min. Total run time was 15 min, detection wavelength was 210 nm and sample injection volume was 1 μL. Lom was dissolved in methanol in the absence and presence of H2O2 (Lom: H2O2, 1:400) with retention times of 4.7 min. However, an additional peak at 3.3 min was evident in the presence of H2O2 indicating possible degradation of Lom. YM872 was dissolved in water in the absence and presence of H2O2 (YM872:H2O2, 1:1.65) with retention times of 2.9 min.

Stability of H2O2 in the presence and absence of the compounds was measured by fluorescence detection of H2O2 using the hemin-catalyzed oxidation of p-hydroxyphenylacetic acid to yield the fluorescent dimer. Briefly, p-hydroxyphenylacetic acid (80 μM) and hemin (8 μM) were dissolved in ammonia buffer, pH 10. This solution was then used to prepare samples of H2O2 (400 μM) in the absence and presence of: YM872, OxATP. Lom (see compound:H2O2 ratios detailed above) and Memantine (Memantine:H2O2, 1:6.7). Fluorescence was measured in triplicate on a Varian Cary Eclipse instrument with excitation at 320 nm and emission at 410 nm in a 1 mL cuvette with a 10-mm path length. Average fluorescence intensity was 40 ± 0.5 in the absence and presence of each of the compounds.

Cell analysis

The following experiments were undertaken following 10 days of culture in NB2/NB1 growth media at 37 °C (95% air/5% CO2 v/v). Control cultures were incubated in identical fashion but without H2O2, and/or without the addition of Ca2+ channel inhibitors or other modulators as detailed below.

Live Ca2+ imaging. Ca2+ imaging was performed on MC cells attached to glass coverslips at room temperature, using the ratiometric Ca2+-sensitive dye Fura-2AM (Invitrogen). All solutions were prepared immediately prior to imaging. Cells were loaded with 4 μM Fura-2AM in NB-A (+10 mM HEPES, without Phenol Red; Life Technologies) at room temperature, for 30 min, then gently washed with Hanks Balanced Salt Solution (HBSS; Life Technologies). Coverslips were then transferred to an RC-26G chamber system (Warner Instruments) containing 1 mL NB-A (+10 mM HEPES). Images were captured every 15 s for 2 min in order to measure basal Ca2+ levels. A pre-prepared solution containing a final H2O2 concentration of 400 μM ± ion channel inhibitors (at concentrations described above), and/or the nyanodine receptor agonist ryanodine (20 μM), 40 μM, or 60 μM: Sigma–Aldrich) or the Zn chelator N,N,N',N'-Tetrais(2-pyridylmethyl)ethylenediameine (TPEN, 1 or 10 μM; Sigma–Aldrich) was then applied to the chamber at the beginning of a 30-min acquisition. The choice of H2O2 concentration was based upon previous studies and our own pilot experiments that indicated 400 μM provides a sustained and consistent increase in intracellular Ca2+ without biphasic effects or oscillations (Herson et al., 1999). In initial experiments, individual intracellular Ca2+ concentrations were determined. MC cells were incubated with Fura-2 AM as above and imaged for 10 min following addition of 10 μM ionomycin (Molecular Probes, Life Technologies) in HBSS (+3 mM Ca2+, Mg2+) to determine Rmax values. MC cells were then washed gently with HBSS (Ca2+ free) and imaged for another 10 min following addition of 10 μM BAPTA AM (Molecular Probes). The mean intracellular Ca2+ concentration at baseline (no H2O2 present) was ~73 nM.

Cells were visualized using an Olympus BX51WI upright microscope equipped with an XM10 monochrome CCS camera (Olympus). Imaging was performed at 60× magnification, capturing a field of view (FOV) of 147.2 μm x 100.4 μm, with an exposure time of 20 ms and 2 x 2 pixel binning. Regions of interest (ROIs) per FOV were defined for each individual cell within the FOV, and were used to determine F340/F380 ratios using Olympus Xcellence RT software. One FOV per coverslip was assessed, consistent for all coverslips.

Cell viability. MC cells were incubated in H2O2 ± ion channel inhibitors and/or EGTA (10 μM, 0.1 mM or 1 mM; Sigma–Aldrich) for 30 min, 6 or 24 h. Immediately following the incubation, cells were gently rinsed with HBSS, and incubated with 1 μM Calcein-AM (Life Technologies) and 2 μM Ethidium homodimer (Ethd-1; Life Technologies) for 30 min. Cultures were then imaged at 40× magnification using an Olympus IX51 inverted fluorescence microscope. FOV were randomly assigned and consistent for all culture wells. Viability was quantified by counting all viable and dead cells in 2 FOV per well (450 μm x 350 μm), with three wells per condition. Data were expressed as the percentage of viable cells ± S.E.M.

Densities of individual cell populations. MC cells were incubated in H2O2 ± ion channel inhibitors for 30 min, 6 or 24 h. Following incubation, cell cultures were washed 3× with PBS, then fixed with 2% (v/v) paraformaldehyde (PFA; 0.1 M phosphate buffer; pH 7.2) for 10 min, followed by a further 20 min with 4% (v/v) PFA (0.1 M phosphate buffer; pH 7.2). Cells were then washed a further 3× with PBS and immunohistochemical analyses conducted according to established procedures (Fitzgerald et al., 2010), using primary antibodies to identify specific cell types: neurons, β III-tubulin (1:1000; Covance, Sydney, NSW, Australia); astrocytes, glial fibrillary acidic protein (GFAP; 1:500; Sigma–Aldrich); NG2+ non-oligodendroglial cells, NG2+ (1:500; Abcam, Melbourne, VIC, Australia)/Olig2 – (1:500, R and D Systems, Noble Park, VIC, Australia); oligodendroglia, Olig2; activated microglia/macrophages (ED1; 1:500; Abcam)
and Hoechst nuclear stain (1:2000; Life Technologies). Secondary antibodies were species specific AlexaFluor® 488 and 555 (1:400; Life Technologies). Cultures were imaged at 40× magnification using an Olympus IX51 inverted fluorescence microscope. Quantification of densities of individual cell populations was conducted by counting all immunopositive cells in 4 FOV per well (450 µm × 350 µm), with 2 wells/treatment group. FOV were randomly assigned and consistent for all culture wells. Mean number of immunopositive cells were expressed per mm².

Statistical analyses

All statistical analyses were performed using SPSS® Version 20 (IBM©) analysis software. For analysis of live cell calcium imaging, all data were derived from 3 to 5 independent experiments, each experiment using cells prepared from separate groups of rat pups. Variability between experiments was examined and we did not find outliers from specific experiments. Because of the large number of inhibitor combinations being compared it was not feasible to conduct all assessments within each single experiment. Therefore, in accordance with best practice in the published literature (Zhang et al., 2005), data from the experiments were combined for the final statistical analyses. A minimum of 14 and frequently as many as 40 cells in total were assessed for each treatment condition. The baseline F340/F380 ratios were averaged and at each time point, F340/F380 ratios were divided by the mean baseline (F340/F380 ratio before addition of H₂O₂ +/− inhibitors) to give a ΔF-Ratio. As experiments were conducted on mixed cultures it was not appropriate to convert ΔF-Ratios to Ca²⁺ concentrations, due to established variabilities between Ca²⁺ concentrations in neurons and glia (Suadicani et al., 2010) and altered responses to stressors (van den Pol et al., 1992). ΔF-Ratios were compared using two-way repeated measures analysis of variance (ANOVA), with Games-Howell post hoc tests (α = 0.05) to compare H₂O₂ ± Ca²⁺ channel inhibitors to H₂O₂ treated cultures, at each time point assessed during the 30-min incubation period. Analyses of cellular viability were conducted using a two-way ANOVA (α = 0.05), with Games-Howell post hoc tests to compare H₂O₂ ± Ca²⁺ channel inhibitors to H₂O₂ control at each time point. Analyses of densities of individual cell types were conducted using a two-way ANOVA (α = 0.05), with Dunnett’s post hoc tests to compare H₂O₂ ± Ca²⁺ channel inhibitors to H₂O₂ control at each time point. All data were expressed as means ± standard error of the mean (S.E.M.); F and degrees of freedom (df) are reported for each ANOVA conducted and subsequent p values refer to post hoc test outcomes.

RESULTS

Differential reduction in intracellular Ca²⁺ with various Ca²⁺ channel inhibitor combinations

H₂O₂ insult resulted in gradual increases in mean ΔF-Ratio (indicative of changes in intracellular Ca²⁺ concentration) in MC cells over a 30 min incubation period, relative to control (Fig. 1B), with a significant increase demonstrated at 30 min (df = 1, F = 15.67, p < 0.05; representative images Fig. 1A). The intracellular Ca²⁺ concentration of MC cells not exposed to H₂O₂ did not change (p > 0.05, Fig. 1A, B). Increases in intracellular Ca²⁺ concentration can occur via influx across the cell membrane (extracellular source) or release from intracellular Ca²⁺ stores. The contribution of ryanodine receptor-dependent release of Ca²⁺ from the endoplasmic reticulum was assessed by challenging the cultures with increasing concentrations of the antagonist ryanodine simultaneously with H₂O₂ insult. 40 µM ryanodine was required to reduce the ΔF-Ratio at 30 min relative to H₂O₂ control (df = 4, F = 32.27, p < 0.05, Fig. 1B). However, the ΔF-Ratio in the presence of 40 µM ryanodine remained higher than in controls without H₂O₂ at this time (p < 0.05, Fig. 1B), indicating additional sources of intracellular Ca²⁺.

Fig. 1. Hydrogen peroxide insult increases intracellular Ca²⁺ in MC cells via intracellular and extracellular sources. Representative images show increased Fura340 fluorescence (blue) at 30 min in MC cells stressed with H₂O₂ (A); scale bar = 20 µm. Quantification of ΔF-Ratio through analysis of Fura-2 AM emissions under 340/380 nm excitation, immediately preceding and following 400-µM H₂O₂ insult, traces of mean data over time are shown (B): 3–4 separate experiments were conducted for each data point, encompassing a total of 27–40 cells/treatment group. MC cells treated with H₂O₂ ± ryanodine (Ry, 10 µM, 20 µM or 40 µM) for 30 min, traces of mean data shown (B): 3–4 separate experiments encompassing a total of 10–20 cells/treatment group. ΔF-Ratio in MC cells treated with H₂O₂ ± TPEN (1 µM or 10 µM) for 30 min, traces of mean data shown (D): 3–4 separate experiments were conducted, encompassing a total of 10–20 cells/treatment group; note that the coloring of traces for control and H₂O₂ are represented using the same colours as in B.
In the absence of cells, HPLC stability analyses of Lom, oxATP and YM872 incubated for 30 min in the presence of H$_2$O$_2$, demonstrated that there was no effect of H$_2$O$_2$ on oxATP or YM872 stability, but Lom was degraded by approximately 15% (data not shown). Memantine could not be analyzed due to the lack of a chromophore that could be detected by HPLC. Additionally, fluorescence detection of H$_2$O$_2$ indicated that H$_2$O$_2$ stability did not alter in the presence of each of the inhibitors, confirming a lack of a direct effect of the inhibitors on H$_2$O$_2$ and indicating that outcomes to be measured were due to effects of the inhibitors on cells.

The effects of all possible combinations of the ion channel inhibitors Lom, oxATP, YM872, and/ or Mem on the intracellular Ca$^{2+}$ concentration ($\Delta$F-Ratio) of H$_2$O$_2$ stressed MC cells were assessed. The changes in Ca$^{2+}$ concentration are expressed relative to the baseline Ca$^{2+}$ concentration in the same culture before the addition of H$_2$O$_2$ and inhibitors. Ion channel inhibitors were administered to cultures singly, in pairs, in groups of three or all four inhibitors, and $\Delta$F-Ratios were compared to outcomes from H$_2$O$_2$ treated cultures without inhibitors. Of the single inhibitors, only oxATP significantly reduced $\Delta$F-Ratio at 30 min (df = 16, $F = 20.78$, $p < 0.05$; Fig 2A, B). Of the combinations of two inhibitors, only Lom + YM872, Lom + Mem, oxATP + YM872, and oxATP + Mem significantly reduced $\Delta$F-Ratio (df = 16, $F = 20.78$, $p < 0.05$; Fig 2A, C). The trend to an increased $\Delta$F-Ratio with Mem + YM872 was not significant at any time point ($p > 0.05$). The finding that Lom + YM872 resulted in a significant reduction in $\Delta$F-Ratio whereas YM872 alone did not, confirmed that the concentration of Lom used was sufficient to cause a biological effect despite some degradation in the presence of H$_2$O$_2$. Of the remaining combinations of three or four inhibitors, only Lom + oxATP + YM872, oxATP + YM872 + Mem, and Lom + oxATP + YM872 + Mem significantly reduced $\Delta$F-Ratio (df = 16, $F = 20.78$, $p < 0.05$; Fig 2A, D). It is important to note that the apparently higher $\Delta$F-Ratio following treatment with Lom + oxATP + Mem was not significantly different to the H$_2$O$_2$ control. This trend toward an anomalous finding was likely due to differences in ROI numbers between the two groups.
and the somewhat high variability observed with this inhibitor combination. Comparisons between ΔF-Ratios from MC cells treated with the various inhibitor combinations that reduced intracellular Ca\(^{2+}\) concentration relative to H\(_2\)O\(_2\) without inhibitors, did not reveal significant differences between these inhibitor combinations at 30 min (\(p > 0.05\), Fig 2A).

**Differential reduction in cell viability with various Ca\(^{2+}\) channel inhibitor combinations**

The association of acute changes in Ca\(^{2+}\) concentration with cell viability at 30 min as well as at later time points (i.e. 6, 24 h) was assessed in MC cells exposed to H\(_2\)O\(_2\) insult. Following a 30 min incubation with H\(_2\)O\(_2\), the time point at which Ca\(^{2+}\) imaging was performed, there was no significant decrease in the percentage of viable cells relative to control (Fig 3A, B). At 6 h, the percentage of live cells was significantly reduced, and remained significantly reduced at 24 h (\(p < 0.05\), Fig 3A, B) relative to control. The density of cells in control cultures was 1001.36 ± 70.18 cells/mm\(^2\), and was not significantly reduced at 30 min (1129.36 ± 84.92 cells/mm\(^2\)) but was significantly reduced at 6 (379.36 ± 54.51 cells/mm\(^2\)) and 24 h (405.08 ± 117.72 cells/mm\(^2\), \(p < 0.05\)) relative to control.

![Fig. 3](image_url)

**Fig. 3.** Time-dependent changes in MC cell viability with \(H_2O_2\) insult. Mean ± SEM percent viable MC cells following incubation with 400 \(\mu\)M \(H_2O_2\) for 30 min, 6 or 24 h compared to control at each time point (A): statistically significantly different from control at each time point, \(p < 0.05\). Representative images of MC cells stained with Calcein-AM and Ethidium homodimer following incubation with 400 \(\mu\)M \(H_2O_2\) are shown (B), scale bar = 75 \(\mu\)m.

The effects of ion channel inhibitor combinations on cell viability were assessed at 6 h; the choice of 6 h as opposed to 24 h was based upon the aim of assessing changes in viability associated with altered Ca\(^{2+}\) concentrations at 30 min, while minimizing secondary effects likely to contribute to cell death at later time points. Of the fifteen ion channel inhibitor combinations, only seven combinations significantly increased the percentage of live cells compared to \(H_2O_2\) control (\(df = 16, F = 29.36, p < 0.05\)). Specifically, of the single inhibitors, only oxATP significantly increased the percentage of viable cells (\(p < 0.05\), Fig. 4). Note that reductions in intracellular Ca\(^{2+}\) concentration with single inhibitors (Fig 2A, B) were directly associated with improvements in cell viability. Of the combinations of two inhibitors, only Lom + oxATP, oxATP + YM872, and oxATP + Mem significantly increased the percentage of live cells compared to \(H_2O_2\) control (\(p < 0.05\), Fig. 4). It is interesting to note that improvements in cell viability with treatment with Lom + oxATP were not associated with reduced intracellular Ca\(^{2+}\) concentration, whereas treatment with oxATP + YM872 and oxATP + Mem were associated both with reduced intracellular Ca\(^{2+}\) concentration (Fig 2A, B) and improved viability. In contrast, Lom + YM872 and Lom + Mem treatments, which were observed to decrease intracellular Ca\(^{2+}\) concentration (Fig 2A, B), had no effect on cell viability (\(p > 0.05\), Fig. 4). Of the remaining combinations of three or four inhibitors, Lom + oxATP + YM872, Lom + oxATP + Mem, oxATP + YM872 + Mem, as well as Lom + oxATP + YM872 + Mem all significantly increased the percentage of viable cells (\(p < 0.05\), Fig. 4). These improvements in cellular viability were directly associated with reductions in intracellular Ca\(^{2+}\) concentration for Lom + oxATP + YM872, oxATP + YM872 + Mem, and Lom + oxATP + YM872 + Mem treatments (Fig 2A, B). However Lom + oxATP + Mem improved viability without altering intracellular Ca\(^{2+}\) concentration (Figs. 2A, 4A, B). The effects of oxATP on viability in decreased extracellular Ca\(^{2+}\) concentrations were assessed using increasing concentrations of EGTA to chelate Ca\(^{2+}\). The chosen EGTA concentrations have been shown to decrease extracellular Ca\(^{2+}\) concentration while maintaining cell viability (Takadera et al., 2010). EGTA alone had no significant effect.
on cell viability, and viability was significantly higher than H$_2$O$_2$ control at each of the tested concentrations (df = 11, $F = 23.90$; $p < 0.05$, Fig. 5). In the presence of H$_2$O$_2$, 1 mM EGTA significantly increased cell viability compared to H$_2$O$_2$ control, indicating that increased extracellular Ca$^{2+}$ contributes to H$_2$O$_2$ insult; lower EGTA concentrations had no significant effect. In contrast, in the presence of oxATP, all tested concentrations of EGTA significantly increased cell viability compared to H$_2$O$_2$ control ($p < 0.05$, Fig. 5), which together with the demonstrated reduction of intracellular Ca$^{2+}$ with oxATP (Fig. 2A), implies that the protective role of oxATP is Ca$^{2+}$ dependent.

Increased viability of neurons, astrocytes, and NG2+ non-oligodendroglial cells with various Ca$^{2+}$ channel inhibitor combinations

Early reductions in intracellular Ca$^{2+}$ concentration were not always associated with improvements in cell viability at 6 h. It was therefore postulated that the ion channel inhibitor combinations may have differential effects on viabilities of specific cell sub-populations within the MC cultures. Accordingly, a selection of ion channel inhibitor combinations with a range of effects was used, and densities of individual cell sub-populations assessed. The ion channel inhibitor combinations tested were oxATP, Lom + oxATP, Lom + oxATP + Mem, Lom + oxATP + YM872, and Lom + oxATP + YM872 + Mem. Cell sub-populations were: β-III tubulin+ cells that are predominantly neurons (Katsetos et al., 1993); astrocytes (GFAP+); NG2+ non-oligodendroglial cells, likely to be predominantly pericytes and in some circumstances macrophages (Dimou and Gallo, 2015) (NG2+/Olig2−); oligodendroglia (Olig2+); and activated microglia/macrophages (ED1+). The Olig2+ sub-population comprised approximately 70% Olig2+/NG2+ oligodendrocyte precursor cells; the remainder were Olig2+/NG2− and were therefore likely to be more mature oligodendrocytes. Following H$_2$O$_2$ insult in the absence of inhibitors, there was a statistically significant reduction in the density of β-III tubulin+, GFAP+, Olig2+/NG2+ oligodendrocyte precursor cells; the remainder were Olig2+/NG2− and were therefore likely to be more mature oligodendrocytes. Following H$_2$O$_2$ insult in the absence of inhibitors, there was a statistically significant reduction in the density of β-III tubulin+, GFAP+, Olig2+/NG2−, Olig2+ and ED1+ cells, compared to control (df = 7, $F = 14.38$, 4.82, 9.06, 22.95, respectively, $p < 0.05$, Fig. 6). In the presence of the six ion channel inhibitor combinations and H$_2$O$_2$, treatment with the five combinations that included oxATP resulted in significantly increased density of at least one of the cell sub-populations compared to control.
the H$_2$O$_2$ only control (p < 0.05, Fig. 6). However, no one inhibitor combination was uniformly effective. Specifically, H$_2$O$_2$ stressed cultures treated with Lom + oxATP + YM872 or Lom + oxATP + YM872 + Mem significantly increased the density of β-III tubulin+ cells (p < 0.05, Fig. 6A, B). However, while the increases were significant, the degree of improvement was minor (Fig. 6A). Only treatment with Lom + oxATP + YM872 + Mem resulted in significantly increased density of GFAP+ cells (p < 0.05, Fig. 6C), with densities following all remaining treatments not significantly different from H$_2$O$_2$ only control (p > 0.05, Fig. 6C, D). Treatment with oxATP, Lom + oxATP, Lom + oxATP + YM872, and Lom + oxATP + Mem significantly increased the density of NG2+/Olig2– cells compared to H$_2$O$_2$ only control (p < 0.05, Fig. 6E). However, only treatment with Lom + oxATP + YM872 and Lom + oxATP + Mem resulted in preservation of these NG2+/Olig2– cells to densities not significantly different to control (p > 0.05, Fig. 6E, F). No combinations of ion channel inhibitors were observed to have a significant effect on density of Olig2+ oligodendroglia or ED1+ microglia/macrophages, compared to H$_2$O$_2$ only control (p > 0.05; Fig 6G–J).

**DISCUSSION**

Using a high throughput in vitro model of CNS injury and multiple combinations of four ion channel inhibitors, we demonstrate that intracellular Ca$^{2+}$ concentration is not always directly related to cell viability. Furthermore, while individual cell sub-populations were vulnerable to H$_2$O$_2$ insult, only some sub-populations could be rescued by treatment with ion channel inhibitors. Specifically, most inhibitor combinations including oxATP preserved NG2+ non-oligodendroglial cells, but preservation of astrocytes and neurons required additional inhibitors. Olig2+ oligodendroglia and ED-1+ activated microglia/macrophages were not preserved by any of the inhibitor combinations.

Intracellular Ca$^{2+}$ has been shown to be sequestered into mitochondria during excitotoxicity, triggering mitochondrial dysfunction and oxidative stress (Dykens, 1994; Lau and Tymianski, 2010). Reactive oxygen species (ROS) are generated as natural by-products of oxidative metabolism, and are vital for cell signaling and homeostasis (Kowaltowski et al., 2009). However, exposure of mitochondria to increasing Ca$^{2+}$ influx results in a secondary feed-forward mechanism whereby ROS production is enhanced (Dykens, 1994). This phenomenon has now been demonstrated for many cell types including neurons (Kahlert et al., 2005), cardiac myocytes (Viola et al., 2007) and vascular smooth muscle (Chaplin et al., 2015). Excess ROS can spread, leading to oxidative damage in vivo (Kowaltowski et al., 2009; Fitzgerald et al., 2010). Following injury to the central nervous system, infiltrating inflammatory cells that enter the injury site are a significant additional source of ROS, including H$_2$O$_2$ (O’Hare Doig et al., 2014b). Multiple feed forward mechanisms that result in ROS initiate further increases in intracellular Ca$^{2+}$ levels, contributing to
additional cell death (Kristian and Siesjo, 1998). Proposed mechanisms by which H2O2 causes increased intracellular Ca2+ levels include activation of VGCCs (Roveri et al., 1992), nonspecific changes in membrane permeability to Ca2+ (Rojanasakul et al., 1993), changes in the Na+–Ca2+ exchanger (Kaneko et al., 1989), and H2O2 induced Ca2+ release from intracellular stores (Nicotera and Rossi, 1994). Our data indicate that both release of Ca2+ from ryanodine-sensitive intracellular stores and influx of extracellular Ca2+ through P2X7Rs as well as other Ca2+ channels contribute to the rise in intracellular Ca2+ levels following H2O2 insult.

Given the consequences of excessive Ca2+ entry into neurons and glia following injury, it was hypothesized that reductions in intracellular Ca2+ levels following treatment with Ca2+ channel inhibitors would be associated with increased cell viability. Reductions in intracellular Ca2+ concentration with oxATP treatment were directly associated with substantial improvements in cell viability. However, treatment with the inhibitor combinations Lom + oxATP, Lom + YM872 and Lom + Mem revealed that dissociation between intracellular Ca2+ concentration and cell viability can occur in this model system. Thus, one cannot simply assume that intracellular Ca2+ overload results in cell death. As cell viability was increased following treatment with all combinations of inhibitors that included oxATP, the results indicate that controlling intracellular Ca2+ concentration by limiting influx via P2X7Rs may play an important role in maintaining cortical cell viability in vitro following insult. As such, the data support the ‘source specificity hypothesis’ whereby Ca2+ cytotoxicity is not merely a function of increased Ca2+ concentration, but instead is linked to specific second messenger pathways activated by excessive Ca2+ entry through specific channels (Tymianski et al., 1993). Interestingly however, treatment with Lom + YM872 or Lom + Mem were not protective, and nor were YM872 or Mem alone. Furthermore, almost all combinations of Ca2+ channel inhibitors that included both YM872 + Mem were not protective: exceptions were treatments that included oxATP. While somewhat speculative at this stage, it is possible that Ca2+ influx through both Ca2+ permeable AMPAR and/or NMDAR is necessary for MC cell health following H2O2 insult, and that the cytotoxic consequences of excessive Ca2+ through these channels can be overcome by the inhibitory action of oxATP on P2X7Rs and subsequent downstream events. Inhibition of influx of other ions through Ca2+ permeable AMPAR, P2X7Rs and NMDAR may also influence viability.

The apparent dissociation between intracellular Ca2+ concentration and MC cell viability may have been due to masking of differential effects on individual cell sub-populations by the effects on the mixed cell population as a whole. Ca2+ imaging studies on pure neuronal and/or astrocyte cultures exposed to H2O2 stress and the multiple combinations of Ca2+ channel inhibitors were considered, however such studies would ignore the complexities of inter-cellular interactions, cytokine release in response to stress and inter-cellular transfer of reactive species via gap junctions. Increase in intracellular Ca2+ concentration in neurons via P2X7Rs ion channels plays a major role in mitochondrial dysfunction leading to apoptotic neuronal death (Nishida et al., 2012). However, treatment with oxATP was insufficient to protect neuronal density. Reports have indicated that neurons are particularly sensitive to H2O2 (Behl et al., 1994; Whittemore et al., 1994). Thus, it is likely that the 6-h H2O2 insult overwhelmed the protective effect of oxATP and additional inhibitors were required for even minimal neuroprotection. While there was a trend to protection of astrocytes by most of the tested combinations of Ca2+ channel inhibitors, only the combination of all four inhibitors resulted in significant protection. Astrocytes release glutamate and ATP in the injured scenario via a number of mechanisms (Bal-Price et al., 2002; Ye et al., 2003) including P2X7Rs (Duan et al., 2003) and this can have detrimental effects on adjacent cells. The loss of astrocytes despite treatment with most inhibitor combinations may have limited their contribution to damage to surrounding cell sub-populations. NG2-glia are an abundant population of cells in the adult CNS that can generate multiple cell types including oligodendrocytes, type 2-astrocytes and pericytes (Richardson et al., 2011). NG2-glia have been shown to evolve increased intracellular Ca2+ concentrations in optic nerves in situ following P2X7-R and AMPAR activation, with ATP alone evoking robust changes in intracellular Ca2+ (Hamilton et al., 2010). In the current study, inhibition of P2X7-R with oxATP resulted in increased NG2-glia viability. However, protection was only observed for NG2+ cells that were not of an oligodendroglial lineage. Olig2+ oligoden-droglia, were approximately 70% oligodendrocyte precursor cells, and not protected by any of the Ca2+ inhibitor combinations, likely reflecting the known selective vulnerability of OPCs to oxidative stress (Back et al., 1998). Despite a lack of protection of oligodendroglia by the tested inhibitor combinations in vitro, Lom treatment has been shown to preserve myelin compaction in vulnerable white matter following partial optic nerve transaction in vivo, and short term delivery of oxATP + YM872 together with sustained delivery of Lom preserved node/-paranode structure as well as visual function in this in vivo model (Savigni et al., 2013). These data indicate that the three ion channel inhibitors in combination have a beneficial effect on oligodendroglia in vivo. While the MC cells utilized in the current study contain many of the cell types found in vivo, three dimensional architecture and cellular interactions are lacking. It is increasingly understood that NG2-glia are required at the Node of Ranvier for axonal and myelin integrity (Butt et al., 1999). Our demonstration of protection of NG2+ olig2− cells by combinations of ion channels containing oxATP indicate that protection of these particular cells may be a critical element preserving structure and function of intact but vulnerable myelinated axons of the CNS in vivo.

CONCLUSIONS

The contribution of specific Ca2+ channels to excess Ca2+ influx in individual cell sub-populations in this in vitro model may not reflect the complexities of the
injured CNS. Nevertheless, the data provide insight into effects of inhibition of individual and multiple ion channels on cell sub-populations, with a breadth and scope not feasible in \textit{in vivo} studies. The demonstration of protection of MC viability with oxATP alone and in combination with other ion channel inhibitors provides support for further \textit{in vivo} investigation where the presence of oxATP is maintained long term after CNS injury.

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Chapter Four

Optimising and defining the effects of various combinations of ion channel inhibitors in vivo

4.1 Redox proteomics pilot study to detect oxidised proteins following partial CNS injury and treatment with ion channel inhibitors

As part of the original experimental design of Aim 2, we looked to use mass spectrometry instrumentation to identify any or all proteins within optic nerve tissue that were differentially oxidised following injury (redox proteomics). Based on the results of Chapters 2 and 3, we hypothesised that several proteins are likely to undergo oxidative changes, associated with overproduction of reactive species. Redox proteomics (Castegna et al., 2002; Dalle et al., 2006) serves as a powerful tool for the identification of oxidatively modified proteins and we hypothesised that this could be applied to enhance understanding of the pathogenesis of CNS injury and neurodegenerative disorders, including apoptosis, oxidative stress, metabolism and cytoskeletal organisation.

4.1.1 Redox proteomics materials and methods

Tissue from normal optic nerve and at 1 and 7 days after injury was collected and sent to Proteomics International Ltd, who conducted the tissue processing and mass spectrometry analyses. In brief, rat optic nerve samples were removed from -80°C storage and 20μL of Lysis buffer (50mM Tris pH7, 0.5mM EDTA, 20% (v/v) glycerol and protease inhibitors) added to each sample. Samples were sonicated seven times for 5 seconds each time and twice for 10 seconds. After sonication, samples were centrifuged at 13,000g for 5 minutes and the supernatant collected. Another 20μL of Lysis buffer was added to each nerve sample, sonicated twice for 10 seconds and centrifuged at 13,000g for 5 minutes. Again, the supernatant was collected and pooled with the previous supernatant and 240μL of acetone was added to each sample. Samples were incubated at -20°C for 1.5 hours, then centrifuged for 12 seconds and acetone was removed. Protein pellets were resuspended in 20μL of 0.5M triethylammonium bicarbonate + 0.1% sodium dodecyl sulfate buffer,
digested twice with trypsin for 3 hours and overnight respectively, at 37°C. Digested samples were applied to a ‘High Protein and Peptide Recovery’ detergent removal column (HiPPR; Thermo Scientific) and desalted on a Strata-X 33μm polymeric reversed phase column (Phenomenex). Peptides were analysed by LC/MS using an UltiMate 3000 nanoflow High-performance liquid chromatography (HPLC) system (Dionex) coupled to a 4000 Q-TRAP mass spectrometer (Sciex). A 1μL volume of optic nerve peptides was loaded onto a Zorbax 300SB-C18, 3.5μm column (Agilent Technologies) and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v) over 90 minutes. Multiple reaction monitoring (MRM) transitions for oxidized and non-oxidized peptides were searched for in the mass spectrometer. Peptide oxidation analysis of MRM data was performed with the Skyline software program.

4.1.2 Results and conclusions from redox proteomics pilot study

Pilot studies addressing the feasibility of the described approach were completed and oxidative changes to specific proteins were identified (Appendix Table 1). Originally, analyses were primarily focussed on the redox state of axonal and glial internode and node of Ranvier associated proteins. We then aimed to identify any redox changes that occurred following partial CNS injury, and to determine whether these changes were reversed following ion channel inhibitor treatment. However, many of these structural proteins were not detected, therefore we made the decision to investigate a number of other structural and enzyme associated proteins. Data showed that in ON vulnerable to secondary degeneration, there were 35 peptides showing increased oxidation levels compared to normal, with 9 peptides showing a >3-fold increase in oxidation levels across all three comparisons; Normal, 1 Day, 7 Day (Appendix Table 2). We identified a number of proteins that underwent oxidation as a result of injury, including but not limited to tubulins, antioxidant enzymes and haemoglobin subunits (Appendix Figure 1). We then looked to assess the effects of the ion channel inhibitor combination Lom + oxATP + YM872 on the redox changes stated above. Once tissue was processed and analysed, large amounts of variability in the outcome of results was observed. Specifically, the observed increases with injury observed in the data presented in Appendix Tables 1 and 2 and Figure 1 were not seen. Therefore, all experiments were repeated, only to once again experience high variability in the outcomes produced. We attempted to investigate the source of variability, however this was unsuccessful and it was agreed by all parties that
the redox proteomics data may not prove to be sufficiently reliable for publication. The time taken attempting to confirm the redox proteomics outcomes resulted in significant delays in the PhD program, therefore the final decision was made not to further pursue redox proteomics in the current body of work.

### 4.2 Introducing ‘Specific ion channels contribute to key elements of pathology during secondary degeneration following neurotrauma’

The data presented in Chapter 3 demonstrated the therapeutic effects of specific combinations of ion channel inhibitors on a variety of CNS cell types in vitro. However, we were yet to determine how controlling flux of Ca$^{2+}$ through specific ion channels contributes to the various pathologies and oxidative events observed during secondary degeneration. It was not feasible to test every combination of all ion channel inhibitors in vivo, as the number of combinations was prohibitively large. Therefore, we assessed the effects of selected ion channel inhibitor combinations deemed most promising based on the results of Chapter 3, on axonal degeneration, node of Ranvier/paranode structure, oxidative damage, and OPC numbers. Most outcomes were assessed at 3 days following injury, as results obtained in Chapter 2 identified a potential therapeutic window at this time point, with significant increases in a suite of fluorescent indicators of reactive species and immunohistochemically detected indicators of oxidative stress.

Furthermore, this study allowed us to speculate whether acute elements of oxidative and structural damage were associated with long term physiological and functional outcomes. For example, in earlier published work arising from my Honours thesis, we determined that treatment with Lom, oxATP and YM872 in combination, significantly increased visual function and the preservation of node of Ranvier and paranode complexes 3 months following partial CNS injury (Savigni et al., 2013). However, what remained uncertain at the conclusion of that study, was whether treatment of a specific acute event of secondary degeneration with the ion channel inhibitors, was associated with these long term positive outcomes.
4.3 Experimental design, materials and methods

The remainder of the study was a collaborative effort with Ph.D. Candidate, Ms. Wissam Chiha, and assessed the effects of selected ion channel inhibitor combinations on various outcomes of axonal integrity, oxidative stress, node/paranode complexes, and myelin changes following treatment with selected combinations of Lom, oxATP and YM872 *in vivo*. The paper provides a time course of changes in axonal proteins with injury; however, this work was predominantly the work of Ms Chiha, and will not be discussed in further detail here. The delivery route and duration of ion channel inhibitor treatment was determined by the pharmacological properties of Lom, YM872 and oxATP, based on previous studies. Lom is a lipid soluble reagent, able to cross the blood brain barrier, allowing for long term, daily oral delivery *via* food grade household butter. Although oxATP is water soluble, it cannot pass through the blood brain barrier. Therefore, oxATP and YM872 were delivered *via* an osmotic mini-pump, directly micro-perfusing to the injury site.

A range of normalisation techniques are employed in the literature to account for variation in intensity of immunohistochemical staining of tissue sections. Therefore, choices must be carefully considered for each outcome measure being assessed, and the model and analyses employed. For immunohistochemical intensity and area data, normalisation to background staining within the same section was employed to account for potential variation in section thickness and antibody application, as per reviewer recommendations. Normalisation to background is not possible for oxidative stress immunointensity data, as the oxidised proteins and DNA are diffusely distributed throughout individual cells and/or tissue and there is nowhere in the tissue that can be conclusively described as background (refer to Chapter 2, Figure 2.3 and 2.4). We did not normalise data relative to other proteins as these can change with the pathological state. For example, we have published that β-III tubulin immunointensity increases at 3 days after injury (Fitzgerald *et al.*, 2010). As such, normalising to tubulin would result in inappropriate interpretation of our data. While such a technique is very relevant when assessing growth of axons (Cho and Cavalli, 2012), it was not appropriate for the current chapter.
4.3 Conclusions of ‘Specific ion channels contribute to key elements of pathology during secondary degeneration following neurotrauma’

Various combinations of ion channel inhibitors reduced hyper-phosphorylation of Tau and acetylated tubulin, and increased Nogo-A immunoreactivity at day 3 after injury. However, only all three inhibitors in combination restored normal AnkG lengths at the node of Ranvier. Furthermore, HNE immunoreactivity and loss of oligodendrocyte precursor cells were only limited by treatment with all three ion channel inhibitors in combination. Data indicate that inhibiting any of a range of ion channels preserves specific axonal and nodal structures, associated with limited elements of oxidative damage following injury. However, all three ion channel inhibitors must be utilised to prevent lipid peroxidation and preserve AnkG distribution and OPC numbers. Moving forward from the conclusion of this study, one can speculate about the effects of a longer time course of inhibitor administration, beyond 3 and 7 days, or alternative drug regimen. The former concept is developed further in the additional chapters remaining in this thesis. For the latter, we acknowledge the potential for the addition of anti-inflammatories to the combinatorial regimen as persistent neuroinflammation and M-1 like activation is a prominent feature in a variety of neurodegenerative diseases, as well as TBI and SCI (see review Faden et al., 2016).

The study described in this chapter is presented below and is published as:

Specific ion channels contribute to key elements of pathology during secondary degeneration following neurotrauma

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Abstract

Background: Following partial injury to the central nervous system, cells beyond the initial injury site undergo secondary degeneration, exacerbating loss of neurons, compact myelin and function. Changes in Ca\(^{2+}\) flux are associated with metabolic and structural changes, but it is not yet clear how flux through specific ion channels contributes to the various pathologies. Here, partial optic nerve transection in adult female rats was used to model secondary degeneration. Treatment with combinations of three ion channel inhibitors was used as a tool to investigate which elements of oxidative and structural damage related to long term functional outcomes. The inhibitors employed were the voltage gated Ca\(^{2+}\) channel inhibitor Lomerizine (Lom), the Ca\(^{2+}\) permeable AMPA receptor inhibitor YM872 and the P2X7 receptor inhibitor oxATP.

Results: Following partial optic nerve transection, hyper-phosphorylation of Tau and acetylated tubulin immunoreactivity were increased, and Nogo-A immunoreactivity was decreased, indicating that axonal changes occurred acutely. All combinations of ion channel inhibitors reduced hyper-phosphorylation of Tau and increased Nogo-A immunoreactivity at day 3 after injury. However, only Lom/oxATP or all three inhibitors in combination significantly reduced acetylated tubulin immunoreactivity. Most combinations of ion channel inhibitors were effective in restoring the lengths of the paranode and the paranodal gap, indicative of the length of the node of Ranvier, following injury. However, only all three inhibitors in combination restored to normal Ankyrin G length at the node of Ranvier. Similarly, HNE immunoreactivity and loss of oligodendrocyte precursor cells were only limited by treatment with all three ion channel inhibitors in combination.

Conclusions: Data indicate that inhibiting any of a range of ion channels preserves certain elements of axon and node structure and limits some oxidative damage following injury, whereas ionic flux through all three channels must be inhibited to prevent lipid peroxidation and preserve Ankyrin G distribution and OPCs.

Keywords: Secondary degeneration, Neurotrauma, Traumatic injury, Ion channel inhibitor, Axonal degeneration, Node of Ranvier, Lipid peroxidation, Oligodendrocyte precursor cells, Oxidative stress

Background

Following trauma to the central nervous system (CNS), cells beyond the initial injury site succumb to degenerative events in a series of sequelae referred to as secondary degeneration. Secondary degeneration of white matter adjacent to a primary injury and vulnerable to damage can be modelled by partial transection of the optic nerve, where axons in the dorsal optic nerve are axotomised by the primary injury and ventral optic nerve is initially intact but vulnerable to secondary degeneration [1, 2]. Tracing of optic nerve axons using fluorescent dyes applied to ventral retina illustrates that...
ventral axons remain intact in the early stages following the partial transection [2]. Changes in Ca\(^{2+}\) flux occur following neurotrauma [3], and in optic nerve vulnerable to secondary degeneration following partial transection [4, 5]. Altered Ca\(^{2+}\) flux is associated with increased reactive oxygen species (ROS), enhanced anti-oxidant activity, and oxidative damage to DNA, protein and lipid in the first minutes and days following injury [6–8]. Cytosolic Ca\(^{2+}\) concentrations increase via influx from extracellular pools, and also via Ca\(^{2+}\)-mediated release from intracellular stores [9, 10]. Ca\(^{2+}\) enters cells of the CNS through a range of channels and receptors, including but not limited to: voltage-gated Ca\(^{2+}\) channels (VGCCs) [11]; purinergic P2X receptors (P2X,Rs) [12]; and Ca\(^{2+}\) permeable ionotrophic a-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid receptors (AMPARs) [13]. Intra-cellular levels of Ca\(^{2+}\) are typically low in neurons and glia, however excessive depolarization of neurons via glutamate excitotoxicity [14, 15] promotes intracellular Ca\(^{2+}\) influx through VGCCs [16]. Increasing extracellular ATP concentrations are also a typical consequence of injury [17], leading to increased Ca\(^{2+}\) flux through P2X,Rs [18].

Ca\(^{2+}\) influx following axonal injury activates deleterious cascades which induce the breakdown of cytoskeletal proteins and disruption of axonal transport, often leading to degeneration and neuronal death. Tau is a cytoskeletal phosphoprotein and although dynamic, site-specific phosphorylation of Tau is essential for its proper functioning, inappropriate and or hyper-phosphorylation at tyrosine 205 (Tau [pT205]), serine 262 (Tau [pS262]) and serine 396 (Tau [pS396]) [19, 20] renders it toxic and results in impaired microtubule assembly, disruption of anterograde and retrograde axonal transport and calpain mediated cell death [20–23]. The microtubule protein tubulin is acetylated in an activity-dependent manner via Ca\(^{2+}\) influx [24]. Reductions in acetylated tubulin are associated with Alzheimer’s disease and Parkinson’s disease [25, 26], and it is argued that tubulin acetylation may be a consequence of, rather than contributor to, microtubule stability. Limiting Ca\(^{2+}\) influx is neuroprotective via maintenance of the integrity of cytoskeletal proteins, however it is not known which cytoskeletal elements are essential for maintenance of axonal integrity, myelin structure and long term function following injury [27], and the timing of disruptions to axonal proteins during secondary degeneration is not yet clear.

Following injury, distribution of node of Ranvier and paranode proteins is also disrupted, contributing to functional impairment [28]. Caspr localization changes with spinal cord injury, with aberrant overlapping of Caspr, Kv1.1 and Kv1.2 in the paranodal regions [29]. Paranodal unfurling and lengthening of the paranode and paranodal gap are observed at 1 and 3 days as well as chronic time points in white matter vulnerable to secondary degeneration [30]. Myelin disruption is linked to its vulnerability to secondary events such as energy depletion, excitotoxicity [31], over-production of ROS and lipid peroxidation [32], which are elevated in optic nerve vulnerable to secondary degeneration [6]. Myelinating oligodendrocytes and oligodendrocyte precursor cells (OPCs) are particularly sensitive to excitotoxic insult and oxidative stress, with greater sensitivity of progenitor cells [33]. Early OPC proliferative responses and OPC depletion from 7 days are features of secondary degeneration [34], associated with myelin decompaction detected by electron microscopy from 3 months [35, 36]. However, mature oligodendrocyte numbers are maintained at relatively constant levels throughout [34]. The oligodendrocyte membrane protein NogoA is an inhibitor of axonal regeneration, located predominantly in the innermost and outer myelin membranes [37]. Studies demonstrate variable changes in the expression of NogoA following injury, with increases and decreases depending upon injury model and timing [38–40]. NogoA dynamics in secondary degeneration are unknown, yet the dynamic variation in NogoA expression could potentially explain different regenerative phenotypes and lack of a consistent therapeutic effect of NogoA inhibition [41].

Given the consequences of excessive Ca\(^{2+}\) during secondary degeneration, limiting Ca\(^{2+}\) entry into cells is hypothesized to reduce oxidative stress, and disruptions to axonal and myelin structure. The administration of single Ca\(^{2+}\) channel inhibitors have been used as a therapeutic strategy for CNS injury in vivo, reviewed in [42]. Whilst promising results have been demonstrated in pre-clinical studies, clinical trials have been disappointing [43, 44]. Given the multiple routes of entry of Ca\(^{2+}\) in the CNS, it is increasingly understood that a combinatorial approach to therapy, involving inhibition of multiple channels through which Ca\(^{2+}\) can pass, is likely to be required. We have previously assessed the efficacy of various combinations of three ion channel inhibitors for treatment of secondary degeneration: using lomerizine hydrochloride (Lom) [45], zonapanol monohydrate YM872 [46, 47] (also referred to as INQ) and/or oxidized ATP (oxATP) [48] to inhibit VGCCs, Ca\(^{2+}\) permeable AMPARs and P2X,Rs, respectively. We have demonstrated that the three ion channel inhibitors in combination were required to preserve myelin compaction, node of Ranvier length and behavioural function, in optic nerve vulnerable to secondary degeneration in the chronic phase, 3 months following injury [49]. However, in the acute phase after injury, it is not yet known whether ionic flux through particular channels is associated specifically with oxidative stress, axon degeneration, altered nodes and/or OPC loss. Here we used the
partial optic nerve transection model and assessed early cytoskeletal protein and Nogo-A changes in the first week after injury. In vivo administration of various combinations of the three ion channel inhibitors was used as a tool to determine the relative importance of influx of extracellular ions through specific channels, for acute disruptions to visual behaviour, axon and node of Ranvier structure, oxidative stress and OPC numbers. Outcomes of inhibitor treatment were assessed at three days following injury as this is a time point known to feature oxidative damage, nodal disruptions and OPC responses in nerve vulnerable to secondary degeneration [30, 34], and were related to chronic rescue of myelin structure and function by the inhibitors in this model [49]. Outcome measures were confined to those known to change in the early phase of injury rather than those that appear chronically; an immunohistochemistry based approach was employed to enable assessment of cell numbers, structural components and cell-specific responses to injury.

Methods

Animals, anaesthesia and surgery
Female Piebald Virol Glaxo (PVG) adult rats (160–200 g), obtained from the Animal Resource Centre (Murdoch, Western Australia), were housed in groups of three under standard conditions including 12 h (h) light/dark cycles and ad libitum access to chow and water. All procedures were carried out in accordance with National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and approved by The University of Western Australia Animal Ethics Committee, Approval No. RA3/100/673. Female rats were used in order to complement our body of work on secondary degeneration following partial optic nerve transection in female PVG rats [6, 35, 49, 50]. Using a single sex of animals minimises variability due to sex specific differences and female animals can readily be group housed, making them convenient to work with. Anaesthesia was administered intraperitoneally (i.p.) as a combination of Xylazine (Ilum Xylazil 20, 10 mg/kg, Troy Laboratories) and Ketamine (Ketamil, 50 mg/kg, Troy Laboratories). Partial transection of the optic nerve, in which retinal ganglion cell (RGC) axons in the dorsal aspect of the optic nerve are lesioned leaving those on the ventral side intact but susceptible to secondary degeneration, was conducted as described previously [50]. Briefly, the dorsal side of the right optic nerve was partially transected approximately 1 mm behind the eye, to a controlled depth of 200 μm using a diamond radial keratotomy knife (Geuder). Post-operative analgesia was administered subcutaneously (2.8 mg/kg carprofen, Norbrook). Control animals were uninjured normal animals, as sham injured animals have been shown to be no different to normal in terms of visual function, RGC numbers and other cellular parameters [50]. Animals were euthanized with Euthal (active constituents Pentobarbitone Sodium 170 mg/mL, Phenytoin Sodium 25 mg/mL) at days 1, 3 or 7 post surgery.

Treatments

Animals were randomly allocated into groups within three separate cohorts (described below) for testing of combinations of the three Ca²⁺ channel inhibitors, lomerizine (Lom; LKT Laboratories), OxATP (Sigma-Aldrich) and/or YM872 (LKT Laboratories). Treatment began on the day of surgery after full recovery from anaesthesia. Choices of treatment concentrations, routes and durations were based on previously published studies showing efficacy using these agents individually. Lom was administered orally twice daily in butter (30 mg/kg) [45]; all animals not receiving Lom received butter vehicle. OxATP (1 mM; Matute et al. [49]) and/or YM872 (240 μM) were dissolved in sterile phosphate buffered saline (PBS), and delivered at a rate of 0.5μL/h via a subcutaneously implanted, pre-loaded mini-osmotic pump (Model 2002; Alzet), attached to a cannula targeting the injured dorsal aspect of the optic nerve. Prior to the study, the stability of YM872 in the presence of PBS was assessed by reverse phase High Performance Liquid Chromatography (HPLC), on a Waters BEH C18 column with 275 nm UV detection. No degradation of YM872 dissolved in PBS was observed for 4 weeks at 37 °C. Similar analyses of oxATP showed some degradation after one day at 37 °C and almost complete degradation at 3 days, indicating that observed effects are due to the first day of oxATP treatment. Rats were housed individually to minimise disturbance of mini-pumps by cage-mates. Controls included injured animals treated with vehicle only (PBS in pumps and/or oral butter vehicle) and completely normal animals.

Optokinetic nystagmus

At 2 days post-surgery, a cohort of rats (cohort 2: 8/group, n = 48 animals) were anaesthetised as above and their uninjured left eye lids sutured shut. Following complete recovery from anaesthesia (Day 3), behavioural testing was conducted using the optokinetic nystagmus test for visual function as described previously [50, 51]. Briefly, after acclimatisation, responses of rats to rotation of black and white stripes in the anti-clockwise direction were recorded. Analysis was performed by counting the number of purposeful movements in the direction of the stripes within the period each rat was engaged in the task.
Tissue preparation
Tissue was collected from three separate cohorts of animals. Cohort 1 had 10 animals/group, total n = 30 animals, used for immunohistochemistry assessments of axonal changes at days 1 and 7 following injury, relative to normal control animals. Cohort 2 had 8 animals/group, total n = 48 animals, used for behavioural analyses and immunohistochemistry assessments of axonal and myelin structure following injury and treatment with ion channel inhibitor combinations or vehicle control, assessed at day 3. Cohort 3 had 5 animals/group, total n = 30, used for analyses of oxidative damage following injury and treatment with ion channel inhibitor combinations or vehicle control, assessed at day 3. For cohort 1 and 3, animals were euthanized with Euthal (Pentobarbitone sodium 850 mg/kg Phenytion sodium 125 mg/kg; Delvet i.p.) and transcardially perfused with 0.9% NaCl (MnSOD, 1:500; Abcam rabbit, SOD-110); DNA oxidation indicators manganese superoxide dismutase (MnSOD, 1:500; Abcam rabbit, SOD-110); and stored in airtight zip-lock bags at −80 °C, to avoid desiccation.

Immunohistochemical assessments
Tissue was cryosectioned longitudinally at −20 °C (20 μm). Tissue from cohort 2 was fixed in 50:50 methanol:acetone for 15 min (min), washed with PBS, post-fixed for 1 min with Bouins solution and again washed with PBS prior to primary antibody application overnight at 4 °C. Immunohistochemical analyses were conducted according to established procedures [52], using primary antibodies recognising: manganese superoxide dismutase (MnSOD, 1:500; Abcam rabbit, SOD-110); DNA oxidation indicator 8-hydroxyguanosine (8OHGD, 1:500; Abcam mouse Ab62623); advanced glycation end-product carboxy-methyl lysine (CML, 1:500; CosmoBio, KAL-KH024); lipid peroxidation products 4-hydroxynonenal (HNE, 1:200; Alpha Diagnostics, rabbit HNE11-S), and acrolein (1:1000; Abcam, rabbit Ab37110); protein nitration indicator 3-nitrotyrosine (3NT, 1:500; Abcam mouse Ab61392); myelin basic protein (MBP; 1:500; Abcam, rabbit Ab40390 or Santa Cruz, goat SC13914); β-III tubulin (1:500; Covance, mouse MMS-435P); ED1 for activated microglia/macrophages (CD68, 1:1000 Merk Millipore, mouse MAB1435); Iba1 for resident microglia/macrophages (1:1000 Abcam, goat Ab5076); Caspr (1:500, Abcam, rabbit Ab34151 or NeuroMab mouse 75-001), and Ankyrin G (AnkG, 1:200; Invitrogen, mouse 33-8800) for paranode and node of Ranvier structures; axonal components Tau (1:400, Invitrogen, mouse ABH0042), Tau p[S396] (1:200, Invitrogen rabbit 44752G), Tau p[S202] (1:400, Invitrogen, rabbit 44750G), Tau p[T205] (1:200, Invitrogen, rabbit 44738G), acetylated α-tubulin (1:500, Sapphire, mouse Ab24610), and NogoA (1:400, Millipore, rabbit Ab5888); olig2 (1:500, R and D Systems, goat AF2418) and NG2 (1:400, Merck Millipore, rabbit Ab5320 or mouse MAB5384) to identify OPCs. Antibody binding was visualized following 2 h incubation at room temperature with appropriate Alexa Flour 488 or 555 secondary antibodies (1:400; Molecular Probes, Life Technologies). Slides were cover-slipped using Fluoromount-G (Southern Biotechnology) and viewed using fluorescence microscopy.

Immunohistochemistry image analysis and quantification
Assessment of immunointensity in optic nerve sections was semi-quantitative, using established procedures [6, 30], and in line with best practice [53], in order to assess cell-type specific changes with injury and ion channel inhibitor treatment. In brief: a single image of the area directly ventral to the primary injury site was visualized and photographed using either a Leitz Dialplan fluorescence microscope (Leica, Germany) where cellular colocalisation was not required e.g. for oxidative stress measures, or a Nikon Eclipse Ti inverted microscope (Nikon Corporation) with a 20× objective or a 40×/1.3 N.A. oil immersion objective. For each outcome measure, all images were collected in a single session, with constant exposure and microscope settings to ensure uniformity of imaging parameters and consistency between measures. When using the Nikon microscope, a series of optical images at 0.5 μm increments along the z-axis were acquired from the middle 6 μm of each 20 μm section, sampling a field of view of 217.5 × 162.5 μm (for 40× objective) of the ventral area, vulnerable to secondary degeneration. All images were collected using Nikon Elements AT software and deconvulated using autoquant blind deconvolution. Deconvolution was performed using a custom written macro (Nathanael Yates) using the AQI_DeconvolutionND function and batch processing feature in NIS Elements. The images were then saved as new files for analysis; investigators were blinded to image identity. Image analysis was performed using a custom written macro in ImageJ. Briefly, each image was opened, and the visual slice along the z axis that was most in focus was selected for analysis. The image was then cropped to standard dimensions (500 × 500 pixel) in order to only include
the region of interest. The intensity above an arbitrary set threshold, and area above that threshold were measured with constant parameters for all files. Choice of threshold was based on a visual determination of the level that captured clearly immunopositive areas in a selection of images from all groups. Note that the degree of changes to outcomes did not change with the deconvolution process. A range of normalisation techniques are employed in the literature to account for variation in tissue sections, and choice of these must be carefully considered in light of the outcome measure being assessed and the model and analyses employed. For immunohistochemical intensity and area data, such as in the current study, normalisation to background staining within the same section can be employed to account for variation in section thickness and antibody application [54]. Here, all immunointensity data assessing cellular structures was normalised to background. Note that normalisation to background is not possible for oxidative stress immunointensity data, as the oxidised proteins and DNA are diffusely distributed throughout the cells and/or tissue and there is nowhere in the tissue that can be conclusively described as background. Following injury, it is not appropriate to normalise data relative to other proteins as these can change with the pathological state. For example, β-III tubulin immunointensity increases at 3 days after injury [52]. As such, normalising to tubulin would result in inappropriate interpretation of data. The choice of data to display in histograms displaying immunointensity was based upon the pattern of immunoreactivity and whether changes occurred due to an increase in the area of immunofluorescence or an increase in the intensity of that immunofluorescence, as previously described [6]. For analyses of phosphorylated Tau (Tau p) [S396], Tau p[T205] or Tau p[S202], the same area was used to analyse immunointensities, and data were expressed as a ratio of total Tau immunointensity.

For node/paranode analyses, 30 node/paranode complexes were assessed from a single defined and consistent field of view from a single z-series of images from each animal, collected as described above, measuring: the length of the paranodal gap, defined as the distance between two Caspr−paranodes [55]; the average length of the flanking paranodes (Caspr+); and AnkG length/distribution, defined as the length of AnkG+staining between flanking paranodes. A representative orthogonal z-projection is shown to illustrate that the majority of node/paranode complexes did not span more than three visual slices; each z-stack was assessed to ensure inclusion of all elements of the node/paranode complex within the visual sampling before analysis. Axons of the optic nerve are predominantly medium caliber (mean ± SD = 0.64 ± 0.29) [56]; as such, the imaging strategy employed enabled detection of the majority of axons. NG2+/olig2+ OPCs were identified by colocalisation of immunoreactivity and the number of OPCs in a single visual slice of a single image of ventral optic nerve for each animal, collected as described above, was counted. A single visual slice was utilised to ensure co-localisation of NG2 and olig2. Numbers of ED1+ activated microglia/macrophages and Iba1+ resident microglia were counted from a single visual slice of single images from both dorsal and ventral optic nerve for each animal, collected as described above.

Statistical analyses

Results were analysed using the statistical package StataView for Windows (SAS Institute Inc.) or IBM SPSS Statistics. Equality of variance F-tests were conducted to test for homogeneity of variance in groups within experiments. Data were natural log transformed, where necessary to achieve a normal distribution. All data are expressed as means of each treatment group ±SEM, unless otherwise stated. ANOVAs followed by Dunnett’s or Games Howell post hoc tests as appropriate were used to statistically compare quantitative measures of each treatment group to the injured vehicle control. For selected outcomes of interest, multiple comparisons using Bonferroni post hoc tests were performed and noted in the Results text. ANOVA F-text and degrees of freedom (df), as well as p value from post hoc tests are given. All statistical tests required p ≤ 0.05 for significance.

Results

Disruptions to axonal and oligodendrocyte proteins in secondary degeneration

Structural axonal and oligodendrocyte proteins were examined in ventral optic nerve vulnerable to secondary degeneration, following partial optic nerve transection. Note that the focus of the current study is on regions of the optic nerve vulnerable to secondary degeneration, rather than areas directly impacted by the primary injury. A significantly higher ratio of immunoreactivity of Tau p[S396] and Tau p[T205] relative to total Tau was observed in ventral optic nerve at day 1 following injury (F = 16.03, df = 2, p ≤ 0.01 and F = 7.08, df = 2, p ≤ 0.01 respectively), compared to normal optic nerve (Fig. 1a, c). While the ratio of Tau p[S396] to total Tau remained significantly higher at day 7 (Fig. 1a, b; p ≤ 0.001), the ratio of Tau p[T205] to total Tau was significantly reduced (Fig. 1c; p ≤ 0.001), returning to normal optic nerve levels. Changes in the ratio were driven by trends towards decreases in total Tau levels and increases in the levels of Tau phosphorylation, neither of which reached statistical significance (p > 0.05). There was no significant
difference in the ratio of Tau p[S262] relative to total Tau in ventral optic nerve (Fig. 1d; F = 2.59, df = 2, p > 0.05), nor differences in Tau p[S262] immunoreactivity alone (p > 0.05). Immunoreactivity of acetylated tubulin was also significantly elevated in optic nerve vulnerable to secondary degeneration at day 1 following injury, returning to normal levels at day 7 (Fig. 1e, g; F = 16.49, df = 2, p < 0.001). In contrast, NogoA immunoreactivity was significantly decreased at day 1 post injury in ventral optic nerve, and returned to normal levels at 7 days post injury (Fig. 1f, h; F = 13.79, df = 2, p < 0.001).

No acute effects of ion channel inhibitor combinations on behavioural deficits

Partial optic nerve transection resulted in a significant reduction in the number of optokinetic nystagmus responses at 3 days after injury (Fig. 2a; F = 2.54, df = 5, p ≤ 0.05). Despite a strong trend to increasing function with more inhibitors, treatment with a selection of combinations of ion channel inhibitors had no significant effect on behavioural responses at this acute phase following injury, when compared to vehicle treated animals (p > 0.05), in contrast to our reported preservation
of visual function with the three inhibitors in combination at 3 months after injury [49]. Animals treated with more than one ion channel inhibitor made an intermediate number of responses, neither significantly improved above vehicle control nor different from normal animals (p > 0.05). Note that throughout the current study, outcomes of the different treatment combinations are not compared to each other. Furthermore, no detrimental effects of the inhibitor combination on animal welfare were observed.

**Effects of ion channel inhibitors on axonal and oligodendrocyte proteins**

Similarly to findings at day 1 after injury, the ratios of Tau [pS396] to total Tau and [pT205] to total Tau immunoreactivities were calculated using mean ± SEM area above an arbitrarily set threshold for each protein. Similarly, d mean ± SEM area above threshold of acetylated tubulin, e NogoA and f mean ± SEM intensity above threshold of MBP immunoreactivity. Significant differences compared to vehicle are indicated by *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

![Figure 2: Mean ± SEM responses in the optokinetic nystagmus test of visual function and immunoreactivity of axonal and oligodendrocyte proteins, 3 days following partial transection of the optic nerve. a Total number of smooth pursuits and fast resets/minute engaged in the task by normal, or injured vehicle or inhibitor treated animals. b Effects of injury ± combinations of ion channel inhibitors on ratio of Tau [pS396] to total Tau and c ratio of Tau [pT205] to total Tau immunoreactivities were calculated using mean ± SEM area above an arbitrarily set threshold for each protein. Similarly, d mean ± SEM area above threshold of acetylated tubulin, e NogoA and f mean ± SEM intensity above threshold of MBP immunoreactivity. Significant differences compared to vehicle are indicated by *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.](image-url)
significant decrease in NogoA immunoreactivity in ventral optic nerve from vehicle treated animals, compared to normal optic nerve (Fig. 2e; F = 9.59, df = 5, p ≤ 0.05). Treatment of animals with all combinations of inhibitors significantly increased immunoreactivity of NogoA above vehicle treated animals (p ≤ 0.001). Interestingly, multiple comparison tests revealed that treatment with the triple combination of ion channel inhibitors resulted in significant upregulation of the expression of NogoA compared to normal nerve (Fig. 2e; p ≤ 0.02). There was a significant increase in MBP immunostaining intensity 3 days after injury in vehicle treated animals (Fig. 2f; F = 3.46, df = 5, p ≤ 0.05). Following treatment with selected ion channel inhibitor combinations, intermediate effects were observed, with only Lom, and Lom + oxATP treatments resulting in significant decreases compared to vehicle treated animals (Fig. 2f, p ≤ 0.05).

Effects of ion channel inhibitor combinations on structure of the node of Ranvier complex

Structural parameters of the nodes of Ranvier and of paranodes were quantified in ventral optic nerve, and the effects of the selected combinations of ion channel inhibitors assessed. Note that analysis is confined to node/paranode structures able to be visualised using the imaging parameters employed, likely to include those associated with the majority of optic nerve axons [56]. Both the paranodal gap (F = 4.19, df = 5, p ≤ 0.05) and the average paranodal length (F = 3.12, df = 5, p ≤ 0.05) were significantly increased 3 days following injury compared to normal nerve (Fig. 3a, b, d). All of the tested ion channel inhibitor combinations resulted in significantly decreased paranodal gaps in ventral optic nerve, compared to vehicle treated animals (Fig. 3a, d; p ≤ 0.05). Similarly, most ion channel inhibitor combinations resulted in decreased paranode lengths (Fig. 3b, d; p ≤ 0.05); although outcomes following treatment with Lom + YM872 were not significantly different from vehicle treated animals or normal (Fig. 3b; p > 0.05). The average length of AnkG immunoreactivity at the node of Ranvier was significantly increased 3 days following injury, compared to normal (Fig. 3c; d; F = 15.237, df = 5, p ≤ 0.05), indicating a spread of Na⁺ channels in optic nerve vulnerable to secondary degeneration. Following treatment with selected ion channel inhibitor combinations, intermediate effects of Lom + YM872 and Lom + oxATP were observed, with only treatment with Lom + oxATP + YM872 resulting in significantly decreased lengths of AnkG immunoreactivity compared to vehicle treated animals (Fig. 3c, d; p ≤ 0.05). A representative orthogonal z-projection from normal optic nerve is shown (Fig. 3e): all node/paranode complexes assessed fell within the visual sampling.

Effects of ion channel inhibitor combinations on microglia/macrophage numbers

The numbers of ED1⁺ activated microglia/macrophages and Iba1⁺ resident microglia/macrophages were quantified in both dorsal optic nerve directly impacted by the injury and ventral optic nerve vulnerable to secondary degeneration, as macrophage derived reactive species from dorsal nerve are thought to trigger secondary degeneration [6]. The numbers of ED1⁺ and Iba1⁺ cells were increased following injury, in both dorsal and ventral aspects of the optic nerve, as expected [52] (F = 13.902, F = 14.79 respectively, df = 5, p ≤ 0.05). However, there were no significant effects of any of the inhibitor combinations on numbers of ED1⁺ or Iba1⁺ microglia/macrophage (Table 1, p > 0.05).

Effects of ion channel inhibitor combinations on oxidative stress indicators and OPCs

An increase in the immunopositive areas of lipid peroxidation product acrolein (Fig. 4a, c; F = 2.57, df = 5, p ≤ 0.05) was observed in optic nerve of vehicle treated animals 3 days following partial transection. This was associated with a significant decrease in the number of Olig2⁺/NG2⁺ OPCs (Fig. 4d; F = 4.03, df = 5, p ≤ 0.05). There was a significant effect of injury on immunointensity of the lipid peroxidation indicator HNE at three days following injury (Fig. 4b, c; F = 2.86, df = 5, p > 0.05). However, HNE was significantly reduced following treatment with Lom + OxATP + YM872 (p ≤ 0.05): other inhibitor combinations were not significantly different from vehicle treated animals or normal (p > 0.05). Similarly, only treatment with Lom + OxATP + YM872 significant increased OPC numbers relative to vehicle control (Fig. 4d; p ≤ 0.05); Fig. 4e shows representative images of OPCs. There was an intermediate effect of all combinations of ion channel inhibitors on acrolein, with no statistically significant difference between normal or injured vehicle treated nerve with any ion channel inhibitor treatment group (Fig. 4a, p > 0.05).

Significant increases in the mean intensity of immunoreactivity of DNA oxidation marker 8OHDG (Fig. 5a, e; F = 3.82, df = 5, p ≤ 0.05), advanced glycation endproduct CML (Fig. 5b, f; F = 2.27, df = 5, p ≤ 0.05), protein nitration indicator 3NT (Fig. 5c, g; F = 5.23, df = 5, p ≤ 0.05) and antioxidant enzyme MnSOD (Fig. 5d, h; F = 2.33, df = 5, p ≤ 0.05) were observed in optic nerve vulnerable to secondary degeneration, 3 days following partial transection. MnSOD immunoreactivity displayed a punctate pattern, likely reflecting aggregates of cellular debris as previously described [50]. Following treatment with the selected ion channel inhibitor combinations, no significant reductions in 8OHDG or CML were observed, relative to vehicle...
Fig. 3 Effects of combinations of ion channel inhibitors on node/paranode complexes. Mean ± SEM a length of the paranodal gap, indicative of node length; b paranode length and c AnkG+ length were quantified from normal, or injured vehicle or inhibitor treated animals, 3 days following partial optic nerve transection. Significant differences relative to vehicle are indicated by *p ≤ 0.05. d Representative images show Caspr immunopositive paranodes (green) and AnkG immunopositive structures within the node of Ranvier (red); scale bar = 5 µm. e A representative orthogonal z-projection of Caspr immunopositive paranodes (green) and AnkG immunopositive structures within the node of Ranvier (red) from normal ventral optic nerve; scale bar = 5 µm.

Table 1 Numbers of ED1+ and Iba1+ microglia/macrophages in dorsal and ventral optic nerve following partial transection

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Vehicle</th>
<th>Lom</th>
<th>Lom/YM872</th>
<th>Lom/oxATP</th>
<th>Lom/oxATP/YM872</th>
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<tbody>
<tr>
<td><strong>ED1</strong></td>
<td></td>
<td></td>
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<tr>
<td>Dorsal</td>
<td>5.4 ± 0.8</td>
<td>34.5 ± 11.2*</td>
<td>33.5 ± 10.5</td>
<td>29.0 ± 5.8</td>
<td>470 ± 11.3</td>
<td>386 ± 11.0</td>
</tr>
<tr>
<td>Ventral</td>
<td>5.0 ± 1.1</td>
<td>22.5 ± 5.5*</td>
<td>16.8 ± 4.9</td>
<td>26.1 ± 8.6</td>
<td>320 ± 9.5</td>
<td>332 ± 9.7</td>
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<tr>
<td><strong>Iba1</strong></td>
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<td></td>
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<tr>
<td>Dorsal</td>
<td>5.2 ± 1.0</td>
<td>19.2 ± 4.5*</td>
<td>21.1 ± 4.9</td>
<td>29.6 ± 3.6</td>
<td>328 ± 4.7</td>
<td>29.2 ± 3.9</td>
</tr>
<tr>
<td>Ventral</td>
<td>4.8 ± 1.0</td>
<td>14.1 ± 1.7*</td>
<td>9.5 ± 2.7</td>
<td>27.4 ± 6.8</td>
<td>248 ± 4.7</td>
<td>28.9 ± 4.4</td>
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</table>

Mean ± SEM numbers of ED1+ and Iba1+ cells within the field of view; significant differences relative to vehicle are indicated by *p ≤ 0.05.
Fig. 4 Effects of combinations of ion channel inhibitors on oxidative stress and oligodendrocyte progenitor cells (OPCs). Mean ± SEM area above an arbitrarily set threshold **a** acrolein and **b** HNE immunointensity above an arbitrarily set threshold, in ventral optic nerve from normal, or injured vehicle or inhibitor treated animals, 3 days following partial optic nerve transection. Significant differences relative to vehicle are indicated by *p ≤ 0.05. **c** Representative images of acrolein and HNE immunointensity are shown; scale bar = 50 µm. **d** Mean ± SEM oligodendrocyte progenitor cell (OPC) counts in ventral optic nerve from normal, or injured vehicle or inhibitor treated animals, 3 days following partial transection. **e** Representative images illustrating OPC identification as NG2+/Olig2+ cells; scale bar = 5 µm.
Fig. 5  Effects of combinations of ion channel inhibitors on additional indicators of oxidative stress. Mean ± SEM intensity above an arbitrarily set threshold for a 8-hydroxy-2′-deoxyguanosine (8OHDG), b Carboxymethyl lysine (CML), and mean ± SEM area above an arbitrarily set threshold for c 3-nitrotyrosine (3NT); or d manganese superoxide dismutase (MnSOD) immunoreactivity were quantified at 3 days following partial optic nerve transection, in ventral optic nerve of normal, or injured vehicle or treated animals. Significant differences relative to vehicle are indicated by *p ≤ 0.05. Representative images of e 8OHDG, f CML, g 3NT and h MnSOD immunoreactivity in normal and injured vehicle or inhibitor treated ventral nerve, 3 days following partial transection; scale bars = 50 μm
treated animals (Fig. 5a, b, p > 0.05). Only Lom treatment significantly reduced 3NT immunoreactivity, whereas both Lom and Lom + OxA TP + YM872 treatment reduced MnSOD immunoreactivity (Fig. 5c, d; p ≤ 0.05). For 8OHDG, all inhibitor combinations other that Lom + YM872 + oxATP remained significantly increased from normal (p ≤ 0.05); other inhibitor combinations resulted in an intermediate effect for CML, 3NT and MnSOD, not significantly different from normal or vehicle (p > 0.05).

Discussion

Changes in Ca$$^{2+}$$ dynamics during secondary degeneration have been shown to be associated with oxidative stress and disruptions to myelin structure. Here we demonstrate acute increases in Tau protein phosphorylation, tubulin acetylation and decreases in NogoA in optic nerve exclusively vulnerable to secondary degeneration. Combinations of ion channel inhibitors were used as a tool to dissect out which ion channels are important for influx of extracellular ions associated with the disruptions of secondary degeneration. It was found that inhibition of most ion channel combinations assessed, including VGCCs alone, led to restoration of the normal lengths of the paranodal gap, and paranode and normal levels of phosphorylated Tau and NogoA. However, only inhibition of VGCCs, P2X, Rs and AMPARs together, significantly restored AnkG length at the nodes of Ranvier to normal, limited HNE immunoreactivity and prevented OPC loss (see summary of outcomes in Table 2). Inhibition of all three ion channels also leads to long term preservation of visual function in this model [49], implying an important role for these latter components of the injury response in chronic deficits.

Increased Tau phosphorylation reduces the binding of Tau to microtubules in intact neurons [57] and phosphorylation of Tau at S262 reduces its affinity for and ability to stabilize microtubules [58]. While it is important to note that the number of specific phosphorylation sites recognised with the phosphorylation dependent antibodies we have used is limited, with over 25 phosphorylation sites in Tau that are associated with disease or injury [59] not assessed in the current study, some relationships can be explored. We observed no changes in the ratio of phosphorylated Tau at S262 in tissue vulnerable to secondary degeneration, but other isoforms exhibited increased phosphorylated Tau. Tau phosphorylation specifically at S262 has been reported to be independent of Ca$$^{2+}$$ whereas phosphorylation of other Tau proteins is Ca$$^{2+}$$ dependent [60]. Taken together, this suggests that the observed changes in Tau phosphorylation associated with secondary degeneration are induced by Ca$$^{2+}$$ influx. The importance of Ca$$^{2+}$$ influx in Tau phosphorylation in secondary degeneration is further supported by

<table>
<thead>
<tr>
<th>Outcome measure</th>
<th>Vehicle</th>
<th>VGCC</th>
<th>VGCC + Ca$$^{2+}$$ permeable AMPAR</th>
<th>VGCC + P2X, R</th>
<th>VGCC + P2X7R + Ca$$^{2+}$$ permeable AMPAR</th>
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<td>Visual function</td>
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<tr>
<td>Tau[pS396]/total Tau</td>
<td>↑</td>
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<tr>
<td>Tau[pT382]/total Tau</td>
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<tr>
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<td>–</td>
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<tr>
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</table>

Symbols for vehicle treated animals indicate direction of change from normal untreated animals. Following treatment with ion channel inhibitors, significant decreases relative to injured animals treated with vehicle are shown as ↓, significant increases as ↑, remaining significant differences to normal are indicated by— (p ≤ 0.05) and intermediate outcomes not significantly different from vehicle treated or normal as –(p > 0.05)
our findings of restored ratios of phosphorylated Tau to total Tau at [S506] and [T205] following treatment with all tested combinations of ion channel inhibitors. However, limiting flux of other ions such as Na\(^+\) through P2X-R and Ca\(^{2+}\) permeable AMPAR may also have contributed to observed effects. We have recently demonstrated that of the tested combinations of ion channel inhibitors, the Lom + YM872 and Lom + OxATP + YM872 combinations significantly reduced intracellular Ca\(^{2+}\) concentrations in mixed cortical cultures exposed to H\(_2\)O\(_2\) insult [61]. While the in vitro environment is very different to the injured nerve in vivo and results are not directly comparable, it is likely that limiting excess Ca\(^{2+}\) flux is contributing to beneficial outcomes for phosphorylated Tau and other measures. Acetylated tubulin increases have been linked to calcium changes and may reflect increased axonal stability [62] in optic nerve vulnerable to secondary degeneration, although this is not supported by observed increases in Tau phosphorylation. The restoration of normal tubulin acetylation only by two or three ion channel inhibitors together, likely reflects restored ionic homeostasis. Nogo-A inhibits axonal extension and has been shown to decrease early after spinal cord injury, followed by later increases [38]. Similarly, we observed an acute decrease in NogoA immunoreactivity, which was prevented by all of the ion channel combinations, indicating a generalised role for maintenance of intracellular ionic homeostasis in NogoA responses. Taken together, the data show that specific elements of axonal stability are regulated to differing degrees by altered ionic homeostasis.

An increase in the length of the node of Ranvier has been observed in many pathologies including multiple sclerosis [55], glutamate excitotoxicity [63], hypoxia [64]; and spinal cord injury [65, 66]. It has been suggested that this lengthening may be due to myelin retraction and a breakdown of the paranodal junction [63, 67]. The node lengths, whilst maintaining normal paranodal and juxtaparanodal structure, due to insertion of more membrane at the node [68]. Along the myelinated axon, an appreciable amount of Ca\(^{2+}\) influx is due to VGCCs, normally located and controlled at the axolemma [69, 70]. When the axolemma in these regions are exposed due to myelin retraction and splitting at paranodal regions, excessive influx of Ca\(^{2+}\) through axonal L-type VGCCs may occur [71]. Lomerizine inhibition of VGCCs, known to be present on myelinating mature oligodendrocytes [72], is associated with restoration of paranode structure. Inhibition of VGCCs, together with shielding of these channels by myelin, likely contributes to reduced Ca\(^{2+}\) mediated activation of Tau phosphorylation [60].

A generalised increase in MBP immunoreactivity was observed in optic nerve vulnerable to secondary degeneration, as previously described [52]. Increased MBP immunoreactivity has been shown to reflect a release of MBP into the surrounding milieu, followed by upregulated expression of MBP mRNA and protein [73], and/or conformational changes [74]. It is not yet clear why only Lom, and Lom + oxATP treatments resulted in reduced MBP immunoreactivity whereas other combinations of inhibitors did not. Disruption of myelin can cause increases in NG2 immunopositive cells [75]; however we have observed decreases in NG\(^{2+}/\text{olig}^{2+}\) OPCs acutely in the current study, and chronically [34]. OPC depletion can be due to the cytotoxic action of the lipid peroxidation product 4-HNE on OPCs [76], supported by the observation that reductions in HNE via treatment with the three inhibitors in combination decreased both HNE immunoreactivity and the loss of OPCs in nerve vulnerable to secondary degeneration. HNE is both a product of lipid peroxidation and a toxic metabolite, observed to increase in optic nerve vulnerable to secondary degeneration [6]. While HNE was not significantly increased compared to normal in the current study, the reduction with treatment with the three inhibitors indicates a shift in the balance of oxidative metabolism below normal homeostasis and may reflect a compensatory response. 4-HNE treatment increases intracellular Ca\(^{2+}\) levels [77] and 4-HNE is toxic to axons and oligodendrocytes [78], covalently binding cytoskeletal proteins [79, 80], disrupting cytoskeletal structure [81], conjugating proteins [82, 83], and inhibiting mitochondrial respiration [83]. The associative relationship between the protection of OPCs and reduction in HNE immunoreactivity as a consequence of inhibiting VGCCs, P2X-Rs and Ca\(^{2+}\) permeable AMPARs, supports the importance of influx of extracellular Ca\(^{2+}\) and associated lipid peroxidation in OPC vulnerability. OPCs are reported to be particularly vulnerable to oxidative damage, with maturation dependent vulnerability [33] and a lack of intrinsic antioxidants [84]. Indeed, we do not observe either acute or chronic loss of mature CC1\(^{+}\) oligodendrocytes in this model [34]. Acrolein elimination requires GSH, inherently low in OPCs [84], which may explain why the lipid peroxidation indicator acrolein was not similarly reduced. Intermediate effects of ion channel inhibition were observed with a range of other elements of oxidative damage, indicating complex contributions of ions including Ca\(^{2+}\) to generation of reactive species and subsequent secondary degeneration.

The preservation of OPC numbers observed in the current study may have been due to increased proliferation above the baseline proliferative response to the injury that we have already reported [34], or reduced OPC death, and further studies will be required to investigate this mechanism. OPCs are thought to contact axons at the node of
Ranvier and contribute to Na channel clustering [85]. In the current study, a continued spread of sodium channels, indicated by AnkG immunoreactivity [86] was observed, even when paranode structure was preserved by the less effective combinations of ion channel inhibitors. Our working hypothesis is that the protection of OPCs may contribute to AnkG clustering as a specific consequence of limiting HNE with the multiple ion channel inhibitors, thereby contributing to observed long term preservation of function [49]. The lack of significant preservation of acute function following treatment with various combinations of ion channel inhibitors, is associated with increased microglia/macrophages and alterations in MBP and selected oxidative stress indicators, some of which resolve as time passes [49]. Ca\textsuperscript{2+} influx modulated by Na ions and Na influx independent of Ca\textsuperscript{2+} [69, 87] may also contribute to acute pathology. In addition, potassium channels such as Kv2.1 can regulate Ca\textsuperscript{2+} influx [88] and have been shown to be oxidised following traumatic brain injury [89], which may further influence ionic flux. Internal Ca\textsuperscript{2+} stores released via ryanodine and inositol triphosphate receptors may also contribute to secondary degeneration [90]; ryanodine receptors are also oxidised by reactive species, thus leaking Ca\textsuperscript{2+}, and likely further contributing to pathology [91]. Visual responses are affected by secondary degeneration of the ventral optic nerve. Protection of ventral degeneration can preserve visual function at normal levels, as assessed by both the optokinetic nystagmus and Y-maze pattern recognition task [49, 92], and despite axotomy of dorsal axons. It is likely that the disparity between acute visual deficits and chronic visual function rescue reflects transient disruptions to axonal transport and function that were later rescued with the combination of three inhibitors.

Conclusions

Using ion channel inhibitors as a tool has allowed increased understanding of the multiple components of pathology that contribute to the acute phase of secondary degeneration.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All procedures were carried out in accordance with National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and approved by The University of Western Australia Animal Ethics Committee, Approval No. RA3/100/673.

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References


Chapter Five

Defining the effects of the optimised combinatorial ion channel inhibitor treatment strategy in a pre-clinical model of spinal cord injury

5.1 Overview of spinal cord injury study

To provide the pre-clinical data necessary to facilitate translation to the clinic, it was important to firstly investigate the optimised combination of ion channel inhibitors in a clinically relevant in vivo model of SCI. Human SCI often results from an initial mechanical insult to the spinal column that produces a contusion injury, resulting in both primary and secondary injury events (as discussed in depth in Chapter 1.3). Most human injuries do not involve complete transection of the spinal cord (Lee et al., 2014), therefore experimental models of incomplete (e.g. hemisection or contusion) SCI are commonly used for assessing the efficacy of various treatment regimes, rather than complete injuries (e.g. complete transection). Human SCI commonly results from blunt trauma, therefore in more recent years, there has been a major advance in the development of reproducible contusion models in animals. Several models in various animal species have been well developed and characterised. One such model involves the Infinite Horizon spinal cord injury device (Barker et al., 2009). Following a partial laminectomy, this device creates a reliable contusion SCI by rapid delivery to the spinal cord, of a force-defined impactor with a stainless steel-tipped impactor head. The Infinite Horizon device delivers very consistent and reproducible injury parameters (both primary and secondary), thus facilitating repeatable measurements of anatomical and functional outcomes (Scheff et al., 2003). Previous studies in our laboratory have established that there is an acceptable range of variability within cohorts of animals, subjected to the same location and force of injury. Therefore, careful exclusion criteria were employed to exclude animals from quantitative analysis, if they fell out of acceptable limits (discussed below).
5.2 Behavioural assessment following thoracic contusive SCI

The most common method for assessment of hindlimb motor function following SCI in rat models is the Basso, Beattie and Bresnahan (BBB) locomotor scale, ranging from 0, no observable movement; to 21, (uninjured) coordinated gait with parallel paw placement and trunk stability (Table 1) (Basso et al., 1995). The BBB test is reproducible and reliable, although entirely subjective (Metz et al., 2000). Therefore, in the current study, BBB scoring was performed by at least two independent researchers blinded to experimental group identity.

<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>0</td>
<td>No observable hindlimb (HL) movement</td>
</tr>
<tr>
<td>1</td>
<td>Slight movement of one or two joints, usually the hip and/or knee</td>
</tr>
<tr>
<td>2</td>
<td>Extensive movement of one joint or extensive movement of one joint and slight movement of one other joint</td>
</tr>
<tr>
<td>3</td>
<td>Extensive movement of two joints</td>
</tr>
<tr>
<td>4</td>
<td>Slight movement of all three joints of the HL</td>
</tr>
<tr>
<td>5</td>
<td>Slight movement of two joints and extensive movement of the third</td>
</tr>
<tr>
<td>6</td>
<td>Extensive movement of two joints and slight movement of the third</td>
</tr>
<tr>
<td>7</td>
<td>Extensive movement of all three joints of the HL</td>
</tr>
<tr>
<td>8</td>
<td>Sweeping with no weight support or plantar placement of the paw with no weight support</td>
</tr>
<tr>
<td>9</td>
<td>Plantar placement of the paw with weight support in stance only (i.e., when stationary) or occasional, frequent, or consistent weight-supported dorsal stepping and no plantar stepping</td>
</tr>
<tr>
<td>10</td>
<td>Occasional weight-supported plantar steps; no FL-HL coordination</td>
</tr>
<tr>
<td>11</td>
<td>Frequent to consistent weight-supported plantar steps and no FL-HL coordination</td>
</tr>
<tr>
<td>12</td>
<td>Frequent to consistent weight-supported plantar steps and occasional FL-HL coordination</td>
</tr>
<tr>
<td>13</td>
<td>Frequent to consistent weight-supported plantar steps and frequent FL-HL coordination</td>
</tr>
<tr>
<td>14</td>
<td>Consistent weight-supported plantar steps, consistent FL-HL coordination, and predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance; or frequent plantar stepping, consistent FL-HL coordination, and occasional dorsal stepping</td>
</tr>
<tr>
<td>15</td>
<td>Consistent plantar stepping, consistent FL-HL coordination, and no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is pararele to the body at initial contact</td>
</tr>
<tr>
<td>16</td>
<td>Consistent plantar stepping, consistent FL-HL coordination, and toe clearance occurs frequently during forward limb advancement; predominant paw position is pararele at initial contact and rotated at lift off</td>
</tr>
<tr>
<td>17</td>
<td>Consistent plantar stepping, consistent FL-HL coordination, and toe clearance occurs frequently during forward limb advancement; predominant paw position is pararele at initial contact and lift off</td>
</tr>
<tr>
<td>18</td>
<td>Consistent plantar stepping, consistent FL-HL coordination, toe clearance occurs consistently during forward limb advancement; predominant paw position is pararele at initial contact and rotated at lift off</td>
</tr>
<tr>
<td>19</td>
<td>Consistent plantar stepping, consistent FL-HL coordination, toe clearance occurs frequently during forward limb advancement; predominant paw position is pararele at initial contact and lift off, and tail is down part or all of the time</td>
</tr>
<tr>
<td>20</td>
<td>Consistent plantar stepping and consistent coordinated gait, consistent toe clearance, predominant paw position is pararele at initial contact and lift off, and trunk instability; tail consistently up</td>
</tr>
<tr>
<td>21</td>
<td>Consistent plantar stepping and consistent coordinated gait, consistent toe clearance, predominant paw position is pararele throughout stance, and consistent trunk stability; tail consistently up</td>
</tr>
</tbody>
</table>

Table 1. The 21-point Basso, Beattie, Bresnahan locomotor rating scale (Basso et al., 1995).

The CatWalk computer software (Hamers et al., 2006) has also been developed for assisted gait analysis, allowing extensive analysis of gait parameters with adequate temporal resolution, including interlimb coordination, stride length and swing duration (Hamers et al., 2001). The Catwalk gait analysis can be used in conjunction with BBB analyses and ladder walk testing (Metz and Whishaw, 2002) to assess functional recovery in depth following SCI. The “Ratwalk” software (developed by Giles Plant, Stuart Hodgetts and Iain Sweetman (Godinho et al., 2013) is based on the Catwalk software
from Hamers’ lab and provided objective analysis of multiple locomotive parameters, thus strengthening assessment of efficacy of treatment regimens such as the combinatorial ion channel inhibitor treatment employed in the current study. Ratwalk and ladderwalk data collection was conducted in this study, but due to time constraints were not incorporated into the thesis. This quantitative data will however, be incorporated into publications as appropriate.

5.3 Delivery of Lom, YM872 and oxATP in the SCI model

Unlike Lom and YM872, oxATP does not efficiently cross the blood brain barrier, therefore a direct delivery method was required to ensure reagents reached the site of injury in the spinal cord. This study utilised ALZET Brain Infusion Kits, designed specifically for use with ALZET pumps for targeted delivery of therapeutics to the CNS, thereby allowing for direct microperfusion of ion channel inhibitors into the injury site. The ALZET brain infusion kits and pumps can only remain in situ for approximately 2 weeks following SCI, as previous studies have demonstrated reduced efficiency in delivery (Hodgetts et al., 2013) and sometimes, moderate compression damage caused by tubing, at later time points (Jones & Tuszynski, 2001). It was important to analyse the therapeutic effects of all three of the ion channel inhibitors following SCI, at a time point at which the risk of re-damaging the cord was limited. Our treatment regime also ensured that the timing of delivery was similar to the Savigni et al (2013) study conducted in our laboratory, allowing us to generate complementary data regarding the efficacy of the selected ion channel inhibitors. As noted in Chapter 4, in the partial ON transection model, it appears that therapeutic intervention(s) during the acute phase of CNS injury, could translate to improvements observed during the later or chronic time phase of injury. For example, acute reductions in lipid peroxidation were associated with increased OPC numbers with Lom + oxATP + YM872 treatment at 3 days. Given that OPCs (and oligodendrocytes) have been shown to have a critical role in myelin integrity and node/paranode structures (Dupree et al., 1998; Poliak and Peles, 2003; Miyata et al., 2016; Brivio et al., 2017), we speculate that acute improvements in these processes lead to chronic reductions in node/paranode abnormalities and preserved functional outcomes at 3 months following injury (Savigni et al., 2013). Thus, to explore these possibilities further in the current study, we assessed both acute and chronic outcomes. We assessed effects of 14 days of combinatorial treatment with Lom + oxATP + YM872, assessed at
14 days. In addition, a second cohort of animals also underwent 150kd contusion SCI, were treated with the combination of three ion channel inhibitors for 2 weeks, and following surgical removal of pumps, were further treated with Lom only for 56 days following injury (i.e. 10 weeks total), and then assessed at 10 weeks.

5.4 Experimental design and limitations

Experimental design in pre-clinical models of SCI treatment is extremely important for the successful interpretation of data, and translation of results to clinical studies. The original study was designed to include three groups in each cohort; Normal/sham (laminectomy only), Control (SCI + vehicle treatment) and Treated (SCI + inhibitor treatment), with 15 animals in each group. Unfortunately, this final study of the PhD candidature was hindered by limited animal availability (from the Animal Resource Centre, Murdoch) and multiple hardware and software failures of the Nikon Inverted Microscope used for much of the immunohistochemical analyses. These failures meant that capturing of z-stack images for all planned experimental groups and outcomes was not possible during the time remaining. Therefore, as my candidature drew to a close, it was necessary to restructure the experimental design, to allow for pilot data collection and interpretation, whilst maintaining a reasonable completion date. Imaging was conducted by Ryan O’Hare Doig, Sreya Santhakumar, Brooke Fehily and Carole Bartlett. Various morphological and immunohistochemical markers were selected based on physiological changes previously observed following CNS injury. Particular consideration was given to those outcomes previously assessed in Chapters 2-4. Thus, the experiment was redesigned to compare SCI + PBS vehicle treated control animals, to SCI + ion channel inhibitor treated animals, at 2 weeks and 10 weeks following injury. Nevertheless, the data are presented in draft manuscript format, to aid in publication of the data once additional experimental groups and outcomes are incorporated.

5.5 Effects of Lom, oxATP and YM872 in SCI

Following injury, animals demonstrated significant locomotor deficits in the hind limbs, which over the course of 10 weeks, slowly improved (as indicated by BBB score). Any animal that demonstrated weight support on the hindlimbs (BBB score ≥9) 24 hours following SCI was excluded from both behavioural and histological analysis. These
observations typically correlate with an unsuccessful impact of the cord, likely caused by a laminar process interfering with the downward trajectory of the impactor device. Fortunately, this only occurred once in each cohort, of the vehicle treated groups. This meant one animal from 2W + PBS and 10W + PBS groups, were removed from the analysis. Animals treated with ion channel inhibitors, showed a statistically significant improvement in locomotor capacity as early as 1 day following SCI, compared to control animals. Once pumps were removed, and treatment with oxATP and YM872 ceased, the significant effect of treatment on function was no longer observed (3-10 weeks post SCI).

Upon completion of the behavioural analysis, it was clear that whilst all three ion channel inhibitors were present, there were statistically significant effects of treatment. However, with the current study design it was not possible to ascertain if the lack of effect on behavior at chronic timepoints was due to the removal of oxATP and YM872. Previous positive in vitro (O’Hare Doig et al., 2016) and in vivo outcomes (Savigni et al., 2013; Yates et al., 2017), in conjunction with the described positive 2 week outcomes below provide evidence that acute combinatorial treatment of Lom, oxATP and YM872 is effective at limiting damage in cell culture, both acutely and chronically following partial ON transection and acutely following SCI. However, sustained delivery is likely needed for successful treatment of SCI, during chronic time phases. These issues are discussed, in part, in Chapter 6, and we suggest the use of alternative drugs, delivery methods and/or larger animal models. We also report that unexpectedly within this study, a treated animal at 10 weeks demonstrated consistent plantar stepping and coordinated gait, consistent movement of toes; predominantly parallel paw position relative to its body during the whole support stage; consistent trunk stability and tail elevation (BBB score = 21). In contrast no other animal, control or treated, demonstrated this level of locomotor recovery. All parameters of this individual animal were crosschecked to ensure this animal had received an appropriate injury including exact impactor force (156kD) and displacement of the cord following impact. Upon review, there were no means to exclude this animal. Therefore, we believe that this is potentially the first report in rodent models of SCI, of an animal improving this greatly following combinatorial therapeutic treatment.

Upon gross examination of tissue, injured spinal cords had cystic, fluid filled cavities, at the level of injury. Animals treated with ion channel inhibitors in combination, had significantly less cystic formation and greater overall sparing of tissue at both 2 weeks
and 10 weeks following SCI. Although significant tissue sparing was demonstrated with treatment, there was no direct association between tissue sparing and behaviour, as 10 week treated animals showed no significant behavioural improvements compared to controls. We then performed immunohistochemical analysis of key markers known to change following CNS injury, as demonstrated previously in our laboratory, and others (Curtis et al., 1993a; Graz, 1997; Brook et al., 1998; Luo et al., 2005b; Kerr et al., 2010; Godinho et al., 2013; Hodgetts et al., 2013; Payne et al., 2013; Giacci et al., 2014; O’Hare Doig et al., 2014). We examined GFAP, β-III tubulin, SMI32, GAP43, MBP, HNE and Acrolein immunoreactivity, to assess and semi-quantify the effects of the ion channel inhibitors on GFAP reactivity, sparing and growth of neurons, myelin integrity and lipid peroxidation, 2 and 10 weeks following SCI. Intensity analysis data has not yet been normalized as per Chapter 4. Instead, threshold analysis was conducted on control spinal tissue to determine arbitrary threshold intensities for each antibody. Analysis was averaged across 6 regions of interest in a single tissue section, per animal. We also examined the density of two oligodendrocyte populations, CC1 \textsuperscript{+ve} mature oligodendrocytes, and NG2 \textsuperscript{+ve}/PDGF\textalpha \textsuperscript{+ve} OPCs. We acknowledge the use of different OPC antibody markers in comparison to Chapter 3, however, Olig2 was found to produce inadequate quality and inconsistent staining in the spinal tissue. Therefore, we used PDGF\textalpha as an appropriate alternative marker of OPCs, as discussed previously in Chapter 1 (Pringle et al., 1992).

At 2 weeks, animals treated with ion channel inhibitors demonstrated significantly less GFAP immunoreactivity surrounding the injury site, suggesting therapeutic efficacy in reducing astrocyte reactivity following SCI. β-III tubulin immunointensity was significantly increased with treatment, however it was unclear as to whether this was due to increased axonal sparing, and/or increased regeneration. It is also important to note that the lack of Normal/sham tissue in the current analysis precludes clear demonstration that increases are in fact preserving the tissue at a more ‘normal’ phenotype. Nevertheless, we assessed the regenerative capacity following treatment. Examination of GAP43 immunoreactivity demonstrated no significant effects of treatment within any time point, suggesting that although not neuronal specific, the increase in β-III tubulin is likely due to preservation and/or upregulation of tubulin within spared axons, and not necessarily regrowth or regeneration of injured axons. Following 2 weeks of treatment, animals were observed to have a significant increase not in the immunointensity of MBP, but in the
total area of MBP immunoreactivity. This is likely indicative of differences in focal myelin lesions (demyelination) around the injury site that are often observed following injury (Blight, 1985; Guest et al., 2005a; Totoiu and Keirstead, 2005), and the significant effects of these inhibitors previously observed on myelin changes following CNS injury (Savigni et al., 2013). We further explored changes in myelin by quantifying the density of mature oligodendrocytes (CC1^{+ve} cells) and their progenitor cells (OPCs; NG2^{+ve}/PDGFα^{+ve} cells). Following SCI, 2 week treated animals had significantly higher densities of both mature oligodendrocytes and OPCs than vehicle treated injured animals, which may reflect differences seen in MBP immunoreactivity between 2 week treated and vehicle control treated SCI animals. Interestingly, the significant effect of treatment was not maintained during the 2-10 week after injury, with mature oligodendrocytes densities relatively similar between animal groups. In fact, treated SCI animals at 10 weeks had significantly lower densities of OPCs present around the lesion site than vehicle treated SCI animals. This may be due to the proliferative turnover of OPCs into mature oligodendrocytes following SCI, leading to the similar CC1^{+ve} densities in 10 week treated and vehicle control SCI animals. Lipid peroxidation by-products, acrolein and HNE were significantly reduced in 2 week treated animals compared to vehicle treated controls, however, at 10 weeks these effects were no longer observed.

It is important to reiterate that the changes observed are not necessarily reflective of a therapeutic effect, as we do not have data from completely normal animals to provide a desirable baseline level. Nevertheless, they provide interesting and in some cases promising pilot indications of efficacy. Whilst all three ion channel inhibitors are present following SCI, several significant changes were observed, indicating potential efficacy of the combinatorial treatment as a therapeutic strategy for SCI. The pilot data generated in this SCI study follows, and is presented in draft manuscript format (Author contribution 80%).
5.6 Introduction

Spinal cord injury (SCI) is a seriously debilitating event that can quickly lead to paralysis or even death (Ditunno and Formal, 1994). In the United States alone, it is estimated that the annual incidence of SCI injury is 17,000 new cases each year, with approximately 282,000 persons currently living with a SCI (White and Black, 2016). Following SCI, there is an injury severity dependent disruption of axonal pathways (Fehlings and Tator, 1995), impairing motor, sensory and autonomic function at and below the site of injury. Although significant understanding of the etiology and pathophysiology of SCI has been attained, an effective therapeutic treatment strategy for SCI remains elusive. Despite promising outcomes from some studies assessing a number of pharmacological agents in pre-clinical SCI and other neurotrauma models, successful translation to improved outcomes in controlled, randomized human clinical trials is limited (see review Varma et al., 2013).

When the central nervous system (CNS) is contused, or damaged by a penetrative or compressive force, a plethora of damaging molecular and cellular cascades described as secondary degeneration, exacerbate neurological damage and functional impairment (Ahn et al., 2006; Fleming et al., 2006; Fitzgerald et al., 2010; Wang et al., 2012). The acute phase of secondary degeneration in the CNS can occur minutes to weeks following injury, with the chronic phase continuing for months to years following injury (Dihné et al., 2001; Nashmi and Fehlings, 2001; Chen et al., 2003; Fitzgerald et al., 2010; Payne et al., 2012; Petersen et al., 2012). It is understood that the biochemical events associated with secondary degeneration include, but are not limited to glutamate excitotoxicity (Szydlowska and Tymianski, 2010; Tsutsui and Stys, 2013), disruption of ionic balance of K⁺, Na⁺ and Ca²⁺ (Choi, 1987; Stys, 2004b), free radical formation and lipid peroxidation (Kontos and Wei, 1986; Liu et al., 2004; Vaishnav et al., 2010), with apoptosis of various cell types (Beattie et al., 2000). Specifically following SCI, damaged neurons release high concentrations of the neurotransmitter glutamate (Park et al., 2004), resulting in Ca²⁺ dysregulation, compromising cellular machinery (Herrero-Mendez et al., 2009) and increasing cell death (Duchen, 2012) along the apoptotic-necrotic continuum (Cheung et al., 1998).

Ca²⁺ entry into neurons and glia in the CNS is mediated by a number of channels, including but not limited to: voltage-gated Ca²⁺ channels (VGCCs) (Sattler et al., 1996;
Agrawal et al., 2000); ionotropic P2X\(_7\) receptors (Hollmann et al., 1991; North, 2002; Matute et al., 2007b) and \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors lacking the GluR2 subunit (Hollmann et al., 1991). Excessive Ca\(^{2+}\) influx or Ca\(^{2+}\)-mediated intracellular Ca\(^{2+}\) release following injury can activate a number of Ca\(^{2+}\)-dependent processes leading to the overproduction of mitochondrial free radicals, and oxidative damage such as lipid peroxidation (Camello-Almaraz et al., 2006; O'Hare Doig et al., 2014). Free radical production and lipid peroxidation following SCI plays an important role in secondary injury (Hall and Braughler, 1982; Barut et al., 1993; Springer et al., 1997; Lewen et al., 2000; Kamencic et al., 2001; Christie et al., 2008). Lipid peroxidation occurs when reactive oxygen species react with polyunsaturated fatty acids, to form lipid radicals such as 2-propenal (acrolein) and 4-hydroxynonenal (HNE) (Hamann and Shi, 2009; Vaishnav et al., 2010), consequently disturbing cellular machinery (Refsgaard et al., 2000).

Changes in myelin architecture are a neuropathological change contributing to deficits in locomotor function following SCI (Guest et al., 2005a). Changes seen in myelin structure may reflect disturbances in the oligodendrocyte lineage, resulting in perturbed axoglial support (Nave, 2008). We have previously demonstrated increased nitrosative and oxidative damage in CC1\(^{+}\) mature myelinating oligodendrocytes in vivo following partial injury to the optic nerve (O'Hare Doig et al., 2014), temporally and spatially associated with changes in node of Ranvier and paranode axoglial structure (Szymanski et al., 2013). Oligodendrocytes and their progenitors (oligodendrocyte progenitor cells; OPCs) are reported to be quite vulnerable to oxidative events, initiating necrotic and apoptotic pathways during CNS injury and neurodegenerative disorders such as multiple sclerosis (Bunge et al., 1993; Thorburne and Juurlink, 1996; Juurlink et al., 1998b; Gilgun-Sherki et al., 2004; Jana and Pahan, 2007), associated with myelin abnormalities (Payne et al., 2012; Payne et al., 2013; Szymanski et al., 2013), demyelination (Griffiths et al., 1998; Matute et al., 2001; Antony et al., 2004; Norenberg et al., 2004; Doan et al., 2013) and delayed degeneration of axons (Crowe et al., 1997; Warden et al., 2001; Irvine and Blakemore, 2008).

To facilitate functional recovery following CNS injury, in particular SCI, therapeutic strategies must overcome the volatile environment, preserve neuronal and glial cell numbers, and limit the changing Ca\(^{2+}\) dynamics, oxidative stress and myelin abnormalities, following SCI. It is increasingly recognized that combinatorial treatment
strategies are likely to be required to limit the multiple detrimental facets of neurotrauma (Tuszynski, 2005; Kelso et al., 2011). We have previously assessed the efficacy of combinations of ion channel inhibitors: Lomerizine (Lom) 2,3-dioxo-7-(1H-imidazol-1-yl)6-nitro-1,2,3,4-tetrahydro-1-quinoxalinyl] acetic acid monohydrate (YM872), and adenosine 5′-triphosphate periodate oxidized sodium salt (oxATP) to inhibit VGCCs, Ca^{2+} permeable AMPA receptors, and P2X_{7} receptors, respectively; both in vitro and in vivo. Combinations of ion channel inhibitors were shown to reduce intracellular Ca^{2+} concentration, increase cortical cell viability, and preserve astrocytes and neurons following peroxide insult in vitro (O’Hare Doig et al., 2016). Application of combinations of the inhibitors in vivo following partial injury to the optic nerve limited chronic myelin decompaction and node of Ranvier abnormalities, associated with preservation of optokinetic reflex, indicating preservation of function, in this model (Savigni et al., 2013). Similarly, we have demonstrated efficacy of this ion channel inhibitor combination in acute partial CNS injury, beneficial outcomes including reduced: hyperphosphorylation of tau, acetylated tubulin, lipid peroxidation; increased Nogo-A immunoreactivity, and preservation of AnkG lengths and OPC numbers (O’Hare Doig et al., Unpublished).

Therefore, this study was designed to further assess the efficacy of the described ion channel inhibitor combination of Lom, oxATP and YM872 on key events of secondary degeneration, during the acute and chronic time phases following SCI. To mimic the pathophysiology of SCI seen in humans, the Infinite Horizon impactor device was utilised to provide a clinically relevant, and reproducible contusion model of SCI in rodents (Anderson and Stokes, 1992).

5.7. Materials and Methods

5.7.1 Animals

Female Fischer rats (F344) (150-180g) were bred at the Animal Resources Centre (ARC; Murdoch, WA), housed under a standard 12h light/dark cycle, fed wet and dry rat chow and water ad libitum. Procedures performed complied with the “Principles of Laboratory Animal Care (National Institutes of Health Publication no. 86-23, revised 1985), and were approved by The University of Western Australia’s Animal Ethics Committee (AEC, approval number RA/3/100/1405). Animals were of adult age at the time of experimental testing (12-15 weeks old).
5.7.2 Treatments

Two cohorts of animals were used: Cohort 1 (2 Weeks; 2W); SCI plus PBS vehicle (2W control; n=6) or SCI plus ion channel inhibitors (2W treated; n=7); Cohort 2 (10 Weeks; 10W), SCI plus PBS vehicle (10W control; n=6), SCI plus ion channel inhibitors (10W treated; n=7). Treated animals were administered with three ion channel inhibitors in combination: Lom (LKT Labs), oxATP (Sigma), and YM872 (LKT Labs). Choice of treatment concentrations were based on previously published studies using these agents individually. Lom mixed in butter vehicle (30 mg/kg body weight) (Tamaki et al., 2003), or butter vehicle alone was administered orally to all animals using a spatula, whilst animals were gently held. Lom treatment began on the day of surgery, after recovery from anesthesia and continued twice daily for 2-10 weeks (cohort dependent), oxATP (1mM) (Matute et al., 2007b) and YM872 (240µM)(Savigni et al., 2013) were delivered for the first 2 weeks after injury via osmotic mini-pump (see below).

5.7.3 Anesthesia & surgery

Adult Fisher rats were anaesthetized with 2.5% (v/v) isoflurane (BoMac Attane Isoflurane) combined with 60% nitrous oxide (v/v) and 38% oxygen (v/v). A longitudinal incision through the skin and spreading of the underlying muscle tissue was performed, revealing the spinal column. Rats were positioned on a surgical plate for spinal cord impact using flexible armatures and Adson forceps (spinal cord stabilizing forceps). Partial laminectomy at vertebral level T9-T10 exposed the spinal cord underneath without disruption of the meninges. Using an Infinite Horizon impactor device, animals received a moderate contusion (150kd) injury to the dorsal surface of the exposed spinal cord. Following contusion injury, the dura was incised longitudinally, and a pre-filled osmotic mini-pump (Model 2002; 0.5µL/hr; ALZET) attached to a brain infusion kit (Kit 3; ALZET) were attached, and stabilized via subcutaneous sutures. The vinyl catheter was guided underneath the skin, the brain infusion cannula was placed above the dorsal aspect of exposed spinal cord, and the 30-gauge stainless steel tube was inserted directly into the injury site. The cannula was sutured in place, muscles were closed in layers and the incision closed with wound clips. Rehydrating saline (2mL subcutaneously) was administered in conjunction with Buprenorphine (Temgesic, Provet; 0.01ml/100g body weight, 300U/mL) immediately following surgery, and daily until ~day 5 post SCI. To prevent wound and bladder infections, Benacillin [0.02ml/100g body weight, 300 U/mL (150mg/ml procaine penicillin, 150mg/ml benzathine penicillin, 20mg/ml procaine penicillin)
hydrochloride); Troy Laboratories Pty. Ltd., Glendenning, NSW, Australia] was administered immediately after and at 2, 4 and 6 days following surgery. Pumps were surgically removed at 2 weeks under anesthesia (as described above) after injury (Hodgetts et al., 2013).

5.7.4 Behavioural analyses - Open field recovery (BBB)

Functional assessments of animals in all experimental groups were performed on days 1-7, then weekly from weeks 2-10 following surgery, for Cohort 2, and consisted of open field locomotion assessment, as described below. Handling and habituation of animals to behavioural apparatus were performed one week prior to surgery.

The Basso, Beattie & Bresnahan (BBB) locomotor rating scores were used to assess the range and type of forward locomotion (Basso et al., 1995). Rats were recorded using digital videography in an open field for 2-3 minutes on days 1-7, and weekly from weeks 2-10 post injury. Scoring of animals ranged from 0-21 based on the criteria outline (Basso et al., 1995). At least 2 independent investigators conducted the assessments, all blinded to experimental group identity.

5.7.5 Tissue preparation

At day 2 or 10 weeks post injury, animals were euthanized by lethal injection of sodium pentobarbitone (Provet; 50mg/100g) and transcardially perfused with heparinised PBS, followed by 4% (w/v) paraformaldehyde (PFA; Sigma) in 0.1 M phosphate buffer (pH 7.2). The vertebral columns were dissected from each animal and postfixed in PFA for 24 hours before cryoprotection in 15% (w/v) sucrose in phosphate buffered saline (PBS; pH 7.2). A 20mm segment was cut from the spinal cord, ensuring the injury site was at the midpoint, and embedded in 10% (w/v) gelatin (Sigma) in PBS. Gelatin blocks were trimmed and frozen at -20°C, then embedded in optical cutting temperature (OCT) compound (Tissue-Tek) and a cryostat (CM1900, Leica) used to cut longitudinal spinal cord sections (35µm). Consecutive series of sections were transferred to 24-well plates containing PBS + 0.01% (w/v) sodium azide (Sigma) and stored at 4°C until processed for immunohistochemistry.

5.7.6 Cyst size

Quantification of cyst size and tissue sparing was performed by analysis of toluidine blue stained spinal cord sections. Every seventh longitudinal section of spinal cord was
mounted on slides and air-dried overnight. For all animals, each section was from the same relative location, confirmed by histological analysis. Sections were submerged in 0.05% (w/v) toluidine blue (in 0.0055 w/v sodium tetraborate; Sigma) for 1 min then rinsed and dehydrated sequentially in 70%, 90% and 100% (v/v) ethanol for three minutes each. Slides were then submerged in xylene for 1 minute, cover-slipped using DPX slide mounting medium (Sigma), and then stored at room temperature before imaging. Each seventh section was analysed and then combined to give a total cyst area measurement, and total tissue area measurement, for each animal. Cyst area was defined as the outlines of the cysts within the spinal cord section, whereas tissue area was defined as the outline of the entire spinal cord section. Analysis of cyst area was carried out using Image J software where the total area of cystic structures was calculated as a percentage of total tissue area.

5.7.7 Immunohistochemical assessments

Selected sections surrounding the lesion epicentre were washed in PBS and immunohistochemical analyses conducted according to established procedures (Fitzgerald et al., 2010; Hodgetts et al., 2013) using primary antibodies recognising: astrocytes, glial fibrillary acid protein (GFAP; 1:1000; Abcam); neurons, β-III-tubulin (1:1000; Abcam); nonphosphorylated neurofilaments of mature motor neurons, (SMI-32; 1:1000; Calbiochem); growth associated protein 43 (GAP43; 1:500; Sigma); myelin basic protein, (MBP; 1:500; Merck Millipore); mature oligodendrocytes (CC1; 1:500; Merck Millipore); oligodendrocyte progenitor cells (OPCs); platelet-derived growth factor alpha (PDGFα; 1:500; BD Biosciences) and neural/glial antigen 2 (NG2; 1:500; Merck Millipore); lipid peroxidation markers acrolein (1:500; Abcam); 4-Hydroxynonenal (HNE; 1:500; Jomar Bioscience), and Hoechst nuclear stain (1:3000; Invitrogen). Secondary antibodies were species specific AlexaFluor® 488, 555 and 647 (1:400; Life Technologies).

5.7.8 Immunohistochemical analysis and semi-quantification

Immunohistochemical labelling was visualized in a single selected section of spinal cord, in the area surrounding the lesion site for each animal, and photographed using either an upright Eclipse E800 microscope (Nikon Corporation, Japan) or, where co-localisation and cellular densities were quantified, a Nikon Eclipse Ti inverted microscope (Nikon Corporation, Japan). Images were collected at set constant exposures in a single sitting
where feasible, and immunointensity analyses were conducted on six 200µm x 200µm regions of interest (ROI) using ImageJ/Fiji analysis software. Constant arbitrary threshold intensities for all images for a marker relative to background were set, and mean intensities and areas above set threshold were semi-quantified. Note that pilot analyses comparing outcomes between the six ROI for analysed tissue sections within each experimental group did not reveal differences and so data for the six ROI for each tissue section (or animal) were averaged and these means used for further statistical interpretation. Only mean rather than area of immunointensities are shown in the following results, unless stated within text. Cell type specific densities were quantified through a series of optical images at 6 individual ROIs at 1µm increments along the z-axis, acquired from the middle 9µm of each cord section. All images were taken in areas surrounding the injury site, where no evidence of cystic structures was present. This ensured any observable changes in immunopositive areas and cell population densities were not due to physical disturbances of spinal cord tissue. Secondary antibody only controls were processed concurrently to ensure selectivity of staining (data not shown).

**5.7.9 Statistical analyses**

All statistical analyses were performed using SPSS® Version 20 (IBM©) software. Kruskal Wallis Test for non-parametric data was used for open field locomotion testing (BBB analysis) (Hodgetts et al., 2013). Statistical analyses of cyst size and immunohistochemical assessments were analysed using an independent Students t-test, separately for each cohort. All data assumes equal variance, unless otherwise stated. All data were presented as the mean ± standard error of the mean. P values ≤ 0.05 were considered statistically significant, comparing vehicle treated control SCI injured animals to ion channel inhibitor treated SCI injured animals.
5.8 Results

5.8.1 Short term combinatorial ion channel inhibitor treatment facilitated significant early hindlimb functional recovery that was not sustained

Two cohorts of rats were scored daily for 7 days, and then once weekly thereafter on their ability to generate spontaneous, forward movement, from the day of surgery until endpoint: 14 days (2W: acute) or 70 days (10W: chronic) following SCI. During the 2 weeks of continuous combinatorial administration of ion channel inhibitors, treated animals demonstrated significant improvement in locomotor patterns compared to control as early as 1 day, and sustained until up to 14 days post SCI (p≤0.05). No significant differences in locomotor movements were observed between control and treated groups from 21 days to 70 days post SCI (p>0.05; Figure 1).

5.8.2 Ion channel inhibitor treatment reduced cyst formation and acute reactive gliosis following SCI

Cyst formation was calculated as a percentage of the damaged area relative to spared tissue, within each spinal cord segment, 2 and 10 weeks following SCI. Treatment with ion channel inhibitors significantly reduced cyst area, compared to the relative control groups at 2W (t (11) =2.623, p=0.024) and 10W post SCI (t (11) =2.337, p=0.039; Figure 2A-B). To determine whether the combination of ion channel inhibitors modified the
inhibitory environment surrounding the injury site, we assessed the effects of ion channel inhibitors on reactive astrogliosis. Semi-quantification of GFAP immunoreactivity demonstrated that treated animals at 2 weeks had significantly reduced intensity above threshold compared to 2 week controls ($t (11) =2.819, p=0.017$). However, no effects of treatment were observed between treated and control animals at 10 weeks post SCI (Figure 2C-D).

5.8.3 Ion channel inhibitor treatment promoted changes in tubulin immunoreactivity within spared axons, but not regenerative capacity, during acute SCI

To determine whether the combinatorial ion channel inhibitor therapy preserved axonal integrity and/or promoted axonal sprouting/regeneration following SCI, βIII-tubulin
(Roskams et al., 1998), SMI32 (Carriedo et al., 1996) and GAP43 (Aigner et al., 1995) immunoreactivity were semi-quantified. Following SCI, 2W treated animals had a significant increase in βIII-tubulin intensity above threshold compared to 2W controls (t(11) = -3.926, p=0.02). This effect was not observed in 10W treated animals (Figure 3A-B). To further determine whether this significant effect was due to sparing of β-III-tubulin axons, the area above threshold, indicative of the number of axons within an area (each ROI), was also analysed. However, no significant changes were observed between treated or control animals (data not shown). Therefore, it is unlikely that the inhibitors spared axons, but instead there were significant increases in tubulin immunoreactivity within spared axons, perhaps due to conformational alterations. There were no significant differences observed between groups in intensity or area above threshold of SMI32 immunoreactivity at 2W (t(11) = 1.469, p=0.173), or 10W following SCI (t(11) = 1.025, p=0.328; Figure 3C-D). Similarly, there were no significant effects of treatment observed between animals in GAP43 immunointensity, 2W (t(11) = 1.145, p=0.276) or 10W following SCI (t(11) = 1.197, p=0.257), suggesting no effect of treatment on axonal regeneration (Figure 3E-F).
Figure 3. Effects of Lom, oxATP and YM872 on βIII-tubulin, SMI32 and GAP43, at 2W and 10W post SCI. (A-F) Mean ± SEM of βIII-tubulin (A), SMI32 (C) and GAP43 (E), immunointensity above threshold (n=6-7; *p≤0.05). Representative images show areas of βIII-tubulin⁺ve (B), SMI32⁺ve (D) and GAP43⁺ve (F) immunoreactivity surrounding the injury site (scale bars = 50µm).

5.8.4 Preservation of MBP associated with density changes in CC1⁺ve and NG2⁺ve/PDGFα⁺ve oligodendroglia during acute SCI

To determine the effects of combinatorial ion channel inhibitor therapy on myelin, oligodendrocytes and their progenitors following SCI; MBP immunoreactivity was assessed, and the densities of CC1⁺ve mature oligodendrocytes and NG2⁺ve/PDGFα⁺ve OPCs were quantified. Following treatment, 2W animals had no significant changes in
MBP immunointensity (t (11) =-0.501, p=0.626) (Figure 4A), but instead were observed to have a significant increase in MBP immunopositive area above threshold, when compared to 2W control animals (t (11) =-4.115, p=0.002) (Figure 4B). However, no significant differences in MBP intensity or area above threshold were observed between 10W treated and control animals (Figure 4A-C). It is unlikely that MBP changes were associated with changes to axon density, as we saw no change in βIII-tubulin or SMI32. Quantification of CC1<sup>+</sup> cells indicated a significant increase in mature oligodendrocyte numbers in treated animals compared to 2W controls (t (11) =-6.202, p<0.001). However, no significant differences in the density of mature oligodendrocytes were observed at 10W, between treated animals and controls (t (11) =-1.373, p=0.200). Interestingly, the density of OPCs were significantly increased at 2W, following treatment with ion channel inhibitors (t (11) =-2.928, p=0.014). But, at 10W following SCI, NG2<sup>+</sup>/PDGFα<sup>+</sup> treated animals had significantly lower OPC densities, in treated animals (t (11) =3.794, p=0.004; Figure 4D-F).
**Figure 4.** Effects of Lom, oxATP and YM872 on myelin basic protein (MBP) and oligodendroglia sub-populations, at 2W and 10W post SCI. (A) Mean ± SEM intensity above threshold, and (B) mean ± SEM area above threshold of MBP (n=6-7; *p≤0.05) (C) Representative images show areas of MBP\textsuperscript{+} immunoreactivity surrounding the injury site. White arrows demonstrate large area regions where MBP was not detected. (D) Mean ± SEM mature oligodendrocytes cells per mm\textsuperscript{2} (n=6-7; *p≤0.05). (E) Representative images show the density of CCI\textsuperscript{+} mature oligodendrocytes (indicated by white arrows) surrounding the injury site. (F) Mean ± SEM OPCs per mm\textsuperscript{2} (n=6-7; *p≤0.05). (G) Representative images show the density of NG2\textsuperscript{+}/PDGF\textalpha\textsuperscript{+} OPCs (indicated by white arrows) surrounding the injury site (scale = 50\textmu m).

### 5.8.5 Ion channel inhibitor treatment limited lipid peroxidation during early SCI

The effects of combinatorial ion channel inhibitor therapy on oxidative by-products caused by lipid peroxidation were also assessed. Immunohistochemical analysis of lipid peroxidation by-products revealed 2 week treated animals had significant reductions in both acrolein (t (11) =2.720, p=0.020) and HNE (t (11) =3.151, p=0.009) immunoreactivity compared to 2W control animals (Figure 5A-D). However, at 10W, no significant effects of treatment on acrolein (t (11) =2.720, p=0.579) or HNE (t (11) =0.023, p=0.982) immunoreactivity were observed between treated and control animals following SCI (Figure 5A-D).
Figure 5. Effects of Lom, oxATP and YM872 on lipid peroxidation by-products, HNE and acrolein, at 2W and 10W post SCI (A-D) Mean ± SEM intensity above threshold of lipid peroxidation by-products HNE (A) and acrolein (C) (n=6-7; *p≤0.05). Representative images show areas of HNE+ve (B) and acrolein+ve (D) immunoreactivity surrounding the injury site (scale bars= 500µm).
5.9 Discussion

The current study was designed to assess the efficacy of a combination of ion channel inhibitors; Lom, oxATP and YM872, in a clinically relevant rodent model of contusion-injured spinal cord. This combination of inhibitors has been previously assessed in well established, high throughput in vitro and in vivo models of acute and chronic CNS injury, consistently demonstrating significant beneficial effects on Ca\(^{2+}\) influx, cell death, oxidative stress, myelin and node of Ranvier changes, as well as visual function (Savigni et al., 2013; O’Hare Doig et al., 2014). This study aimed to assess the effects of the ion channel inhibitor combination on key events of CNS injury, during the acute (2 weeks) and chronic time phases (10 weeks) following SCI. Following treatment with ion channel inhibitors, there was a significant increase in acute functional hindlimb recovery, reduced formation of cystic structures and reactive gliosis surrounding the injury site. These changes were associated with acute increases in tubulin and myelin protein immunoreactivity, as well as significant effects on oligodendrocytes and their progenitors, and significant reductions in lipid peroxidation products, acrolein and HNE.

In this study, treatment with Lom, YM872 and oxATP together caused a significant increase in the intensity of βIII-tubulin\(^{\text{ve}}\) profiles surrounding the injury site, in thoracic spinal cord. This was associated with reduced formation of cystic structures, as well as improved functional recovery. However, following 10 week treatment, despite reduced cyst formation, the changes in immunointensity of β-III tubulin staining, and animal locomotor recovery were no longer significant. β-III tubulin is a protein associated with dynamic microtubule extension in neurons, and although not neuronal specific, is generally a good indicator of regrowing axons. However, there was no significant indication of increase in GAP43, a marker expressed in elevated levels during development and regeneration, and shown to be up-regulated in growing axons following compressive SCI (Curtis et al., 1993b). Thus, it is unlikely that the combination of ion channel inhibitors promoted the regeneration of axons, or altered the endogenous regenerative capacity of these neurons. Instead, it is likely that the treatment resulted in changes in tubulin profiles within spared axons, independent of GAP43 upregulation. Interestingly, no significant effects of treatment were observed in SMI32\(^{\text{ve}}\) profiles (large, mature motor neurons). This may further highlight a selective vulnerability to damage and/or stress of different sub-type populations of neurons, shown previously (Morrison et al., 1996; Saroff et al., 2000; Greene et al., 2005; Ren et al., 2005; Wang
and Michaelis, 2010). Compared to bipedal primates, quadrupedal mammals typically have a much greater reliance on sensory input from limbs to drive load-bearing spinal locomotor movements. Therefore, rodents typically are much more capable of supported hindlimb stepping in the absence of supraspinal input (Côté et al., 2017). Given the final BBB scores of the current study are in the range of 12-14, we speculate that there is likely little preservation of supraspinal control driving hindlimb functional recovery. Therefore, final BBB scores are not entirely reflective of the amount of total tissue preservation that was achieved with combinatorial treatment. Inclusion of other functional tests such as the Louisville Swim Scale (Smith et al., 2006), where tactile inputs from fore- and hindlimb are diminished could confirm the extent of supraspinal damage. In conjunction, future studies should look to assess the density of sub-populations of neurons and their axonal projections, utilizing additional neuronal markers including, but not limited to: calcitonin gene-related peptide to assess sensory neurons (Wiesenfeld-Hallin et al., 1984), and tryptophan and tyrosine hydroxylase to assess dopaminergic and serotonergic neurons, respectively. Changes in the population of individual subpopulations of cells may reflect specific effects of ion channel inhibitors on particular neuronal subtypes (Van Den Bosch et al., 2000), and may provide greater insight and additional utility for the treatment of SCI. While effects of the inhibitor combination on axons are not yet fully elucidated, there is a clear association observed between myelin, oligodendrocyte populations, and lipid peroxidation surrounding the injury site.

We have previously demonstrated a host of pathophysiologica events that occur during secondary degeneration, including the formation of free radicals, associated with lipid peroxidation (O’Hare Doig et al., 2014). HNE has been shown to rapidly accumulate and consequently inhibit glutamate uptake, following SCI, causing neuronal dysfunction and apoptosis (Springer et al., 1997). Similarly, protein bound acrolein has also been observed to accumulate, up to 7 days following compressive SCI (Luo et al., 2005a). In a recent acute study of partial CNS injury, we showed that treatment with Lom, oxATP and YM872 in combination reduced various elements of oxidative stress, including lipid peroxidation (O’Hare Doig et al., Unpublished), associated with preservation of OPCs. In the current study, the acute effects of these inhibitors on by-products of lipid peroxidation were associated with acute changes to MBP immunoreactivity in the spinal cord, 2 weeks following SCI. Oligodendrocytes are known to be vulnerable to oxidative damage following neurotrauma, particularly during proliferative phases and the genesis
of myelin sheaths (Juurlink et al., 1998b; Roth and Nunez, 2016). ROS, hypochlorous acid and hydrogen peroxide along with reactive nitrogen species, hydroxyl radicals and peroxynitrite are likely contributors to the destruction of oligodendroglia following CNS injury (O’Hare Doig et al., 2014). The current study demonstrated a significant increase in the density of OPCs and mature oligodendrocytes following 2 week treatment with inhibitors, interestingly however, at 10 weeks, there was a significant reduction in the number of OPCs observed in treated animals, compared to 10 week control animals. It has been established previously that the differentiation of OPCs into mature oligodendrocytes occurs in response to demyelination (Fancy et al., 2004). Therefore, the increased density of OPCs observed in 2 week treated animals may be a response to changes in myelin proteins (e.g. MBP), indicated by changes in MBP immunopositive areas. The increased number of CC1\(^{+}\) acutely may reflect the early survival of OPCs, which then differentiate into mature oligodendrocytes. Previous studies have demonstrated changes in proliferation and migration of the oligodendrocyte and OPC populations in response to demyelination following SCI (Keirstead et al., 1998; Totoiu and Keirstead, 2005). However, it is as yet unclear whether the observed changes in mature oligodendrocytes are the cause of MBP changes in the present study, in turn driving the accumulation and differentiation of OPCs (Hornet et al., 2000; Franklin, 2002). Alternatively, a selective vulnerability of oligodendroglia to oxidative damage may be the underlying cause of myelin changes, and observed density changes of oligodendrocytes, and their progenitors, at 2 weeks following SCI treatment (Back et al., 1998; Back et al., 2001). Oxidative damage in the sub-population of oligodendroglia may account for the decrease in numbers of OPCs observed in treated animals at 10 weeks following SCI, compared to controls. However, this interpretation remains a matter of speculation.

This is the first study to assess the effects of Lom, oxATP and YM872 in a clinically relevant model of SCI and provides early indications of efficacy of acute treatment. However, like many injury and disease state models, differences in treatment delivery, anatomy, physiology and relative scale between humans and other mammals leads to difficulties in interpretation. Rodent models of SCI provide a plethora of advantages compared to non-human primate models, with several reproducible methods well described, including established behavioural assessments (see review Hodgetts et al., 2009). There are a number of studies which have shown the successful treatment of CNS
injury using osmotic mini-pumps in rodents. However, many of these studies do not demonstrate efficacy following chronic implantation in the spinal cord, either due to experimental design (only measuring acute time points), complications exacerbated by the implantation of the pump, such as tissue ablation, catheter failure, dislodgment or obstruction and inconsistent/adverse drug delivery (Garcia et al., 1987; Bear et al., 1990; Lu and Hagg, 1997; Angel et al., 1998; Hodgetts et al., 2013). These caveats demonstrate the difficulty of microinfusing Bbb impermeable agents which must be given directly to the spinal cord and/or injury site. Despite such difficulties, we have demonstrated that the presence of Lom, oxATP and YM872 is associated with acute effects following SCI. However, it is difficult to determine whether chronic administration of oxATP and/or YM872 is needed to maintain the significant changes, such as functional improvements, seen at 2 weeks. Due to experimental design, we do not know if surgical procedures involving the removal of osmotic mini-pumps administering these reagents, exacerbated the secondary events following SCI, potentially hindering further functional improvements that may have resulted from the acute treatment. Literature review indicates that the longer term effects of surgically removing osmotic mini-pumps has not been examined in depth (Jones and Tuszynski, 2001) and needs to explored further. Similarly, future studies should incorporate plasma/serum sampling to ensure longitudinal functionality of the osmotic mini-pumps and/or inhibitor stability.

The current study was designed to assess the particular combination of inhibitors shown to be efficacious following partial optic nerve transection, in a clinically relevant model of SCI. However, in order to overcome the difficulties of microinfusion, blood brain barrier-permeable drug alternatives are worth pursuing. Peng et al., (2009) found that an analogue FD&C blue dye No. 1, Brilliant Blue G (BBG) can be used to selectively antagonise P2X7 receptors in vivo, due to its low toxicity (Remy et al., 2008), high selectivity (Jiang et al., 2000) and ability to cross the Bbb (Peng et al., 2009). Administration of BBG reduced cord damage, and improved motor recovery, associated with reduced inflammatory and glial activation, and infiltration (Peng et al., 2009). Therefore, future studies should assess the effects of systemic delivery of Bbb permeable Lom and YM872 (Nishiyama et al., 1999b) with BBG, following SCI.
5.10 Conclusion

This study provides empirical evidence for the utility of combinatorial ion channel inhibitor treatment regimens to facilitate acute improvements in hindlimb functional recovery, following contusive SCI. We have provided evidence that significant positive changes in early functional recovery and pathophysiology can be achieved with a combinatorial treatment employing ion channel inhibitors following SCI, including reduced cyst formation and glial reactivity, increased tubulin, reduced demyelination and lipid peroxidation by-products and preservation of mature oligodendrocytes, and their progenitors. Very few studies show similar dramatic improvements in locomotor function at such early timepoints. Therefore, oxidative stress modulation may set the foundation for other acute injury regimes that have shown, or show promise, utilising a combinatorial approach. However, these effects appear to be governed by method and/or length of delivery of the treatment concerned. Therefore, future studies should further assess the efficacy of ion channel inhibitor combinations in chronic models of SCI, incorporating the latest drug delivery mechanisms and/or Bbb crossing properties.
Chapter Six

General discussion

This PhD candidature is presented in a series of chapters and was designed as a therapeutic optimisation approach study, utilising combinatorial ion channel inhibitor therapy for the treatment of CNS injury. We first characterised the time course of oxidative events, at a range of times at which therapy may be administered and in nerve regions specifically vulnerable to secondary degeneration following acute CNS injury; providing insight not previously clearly described within the literature. Utilising an established in vivo model of secondary degeneration, LC/MS/MS and fluorescent labelling we identified several previously undescribed increases in oxidative indicators and specific reactive species following partial transection to the ON of rodents. Fluorescent indications of reactive oxygen and/or nitrogen species, as well as oxidative products and antioxidant responses, increased at 1, 3 and 7 days after injury in ventral ON. Secondly, we defined the effects of various combinations of ion channel inhibitors, previously shown to have significant effects on myelin abnormalities, node of Ranvier/paranode structure and visual function (Savigni et al., 2013), directly measuring changes in intracellular Ca\(^{2+}\) concentration and cell viability in vitro and then in vivo. Specifically, the effects of the ion channel inhibitors Lom, oxATP, YM872 and Mem on Ca\(^{2+}\) concentration and viability of primary mixed cortical cultures exposed to a H\(_2\)O\(_2\) insult were assessed. These data indicated that following H\(_2\)O\(_2\) insult, limiting intracellular Ca\(^{2+}\) entry via P2X\(_7\) receptors was generally associated with increased cell viability. Data in vivo indicated that inhibiting any of a range of ion channels preserved certain elements of axon and node structure and limited some oxidative damage following injury, whereas ionic flux through all three channels must be inhibited to prevent, lipid peroxidation and preserve AnkG distribution and OPCs. Based on the significant results of both in vitro and in vivo studies, demonstrating the effects of various combinations of Lom, oxATP and YM872, it was clear that the combination Lom + oxATP + YM872 was the most efficacious. We were the first group to assess this combination in a clinically relevant model of SCI. At least while all three inhibitors were present, there was a significant effect of the inhibitor combination on function, cyst formation, glial reactivity, tubulin sparing, myelin (MBP) immunoreactivity, density of oligodendrocyte lineage
cells, and lipid peroxidation. Although these effects were generally no longer observed during the later time course of SCI, following the removal of treatment, this study highlighted many key issues that may need to be considered for both future therapeutic approaches, and chronic administration of therapeutic drugs.

6.1 The importance of early Ca\textsuperscript{2+} inhibition following CNS injury

To develop a successful therapeutic treatment strategy for CNS injury, including SCI, it is imperative to understand the multi-faceted pathophysiology of the injury. The current thesis demonstrated the key importance of Ca\textsuperscript{2+} mediated mechanisms following injury, including oxidative stress and cell death. As highlighted in earlier chapters, Ca\textsuperscript{2+} is a double-edged sword, important for the homeostatic mechanisms involved in the proper development and control of the CNS, whilst a key player in the death of neurons and glia following injury. Traumatic injuries and neurodegenerative diseases of the CNS share common underlying features, prominent among which is the dysregulation of Ca\textsuperscript{2+} homeostasis conditions (Doble, 1999; O'Neill et al., 2001; LaFerla, 2002; Arundine and Tymianski, 2003; Giaume et al., 2007; Bezprozvanny, 2009; Dong et al., 2009b; Weber, 2012). Under stress conditions, Ca\textsuperscript{2+} storage and transport can be inhibited or reversed. Thus, Ca\textsuperscript{2+} influx is considered to be the final common pathway of injury in a variety of cell types (see review Schanne et al., 1979), including those in white matter (Imaizumi et al., 1997; Brown et al., 2001; Tekkok and Goldberg, 2001). An excess rise in cytosolic Ca\textsuperscript{2+} concentration stimulates multiple Ca\textsuperscript{2+} -dependent pathways, leading to Ca\textsuperscript{2+} entering cells via a number of channels including VGCCS, ionotropic AMPA receptors and purinergic P2X\textsubscript{7} receptors, as discussed throughout the thesis.

In Chapter 3, we demonstrated and highlighted the ‘source specificity hypothesis’ whereby the death of CNS cells was mediated not only by Ca\textsuperscript{2+} concentration, but also the pathway/channel by which Ca\textsuperscript{2+} enters various cell types. Our previous findings also support this hypothesis. For example Lom alone has been previously shown to preserve chronic myelin compaction, but not node of Ranvier length changes following partial CNS injury (Savigni et al., 2013). In Chapter 3 we demonstrated the importance of oxATP and the inhibition of early excessive Ca\textsuperscript{2+} flux through the P2X\textsubscript{7} receptor. P2X\textsubscript{7} receptors are expressed in perinodal astrocytes and oligodendrocytes (including their processes), as well as inflammatory cells (see review Alcaraz et al., 2003). Their importance in mediating Ca\textsuperscript{2+} -dependent cell viability mechanisms in a range of cell
types indicates that they are an important therapeutic target for the treatment of CNS injury. Future investigations utilising oxATP (or P2X$_7$ antagonism) or other selected ion channel inhibitors should further explore their effects on the M1/M2 and A1/A2 activation states, of microglia and astrocytes, respectively. Although P2X$_7$ receptor inhibition appears important for Ca$^{2+}$ influx and cell viability, it is likely that inhibition of other channels, particularly VGCCs and AMPA receptors is needed for limiting acute increases in oxidative stress measures, such as MnSOD and HNE, and associated AnkG changes (as seen in Chapter 4). Once again, the data highlights the multifaceted physiology of CNS injury, likely requiring a multifactorial approach in treatment.

6.2 Oxidative stress and oligodendrocytes, a therapeutic target?

Glial cells, and their proper functioning and survival are critical in the pathophysiology of CNS disease and injury (Barres, 2008). In particular, multiple studies have shown that oligodendrocytes, OPCs and myelin are vulnerable to glutamate excitotoxicity, both in vivo and in vitro (Matute et al., 1997; Back et al., 1998; McDonald et al., 1998; Deng et al., 2003; Rosenberg et al., 2003). It is understood that secondary damage to the CNS following injury is due to a number of mechanisms, including excitotoxicity, Ca$^{2+}$ influx/overload, and oxidative stress. As demonstrated in this thesis, Ca$^{2+}$ entry and oxidative stress is a key acute event of secondary degeneration. It is thought that alleviating oxidative stress may be effective for the treatment of SCI, given its significant role in the pathophysiology of neurotrauma and neurodegenerative diseases (see reviews Juurlink and Paterson, 1998b; Katoh et al., 2014; Radi et al., 2014). In Chapters 3-5, we demonstrated the significant effects of various combinations of selected ion channel inhibitors in 3 different models of CNS injury. We also demonstrated the selective vulnerability of various cell types, in particular oligodendrocytes and their progenitors.

Following neurotrauma, studies have reported chronic demyelination following injury (Waxman, 1989; Bunge, 1993; Guest et al., 2005a), whilst others report a thin layer of myelin following injury, likely due to remyelination (McDonald and Belegu, 2006; Franklin and Ffrench-Constant, 2008). Although only immunohistochemical analysis was performed, it is clear from both Chapters 4 and 5, that changes in MBP were evident. Given the rich lipid content of phospholipids and galactocerebrosides in CNS myelin, it is highly vulnerable to lipid peroxidation (Bongarzone et al., 1995). Oxidative stress and
lipid peroxidation is almost certainly linked to loss of oligodendrocyte processes and oligodendrocyte death, reflected in changes to MBP immunoreactivity.

As discussed previously, during oxidative stress, cells undergoing Ca$^{2+}$ mediated overproduction of ROS produce NO. High levels of NO lead to S-nitrosylation of one of the major myelin proteins, PLP, resulting in a disturbance of homophilic bonding of PLP molecules between adjacent myelin lamellae, and decompaction of myelin at the level of the intraperiodic line (Bizzozero et al., 2001b; Bizzozero et al., 2004). The multiple effects of Lom, oxATP and YM872 on mature CC1$^{+ve}$ oligodendrocytes and NG2$^{+ve}$/PDGFα$^{+ve}$ OPCs and the lack of controls made interpretation of the data of Chapter 5 difficult. During the early phase (2 week) of SCI, both mature oligodendrocyte and OPC densities were increased following treatment, however due to the lack of normal uninjured controls, we were unable to ascertain whether this was due to survival, or proliferation and differentiation. It is also difficult to determine whether the changes in myelin following treatment were due to the effects directly on mature oligodendrocytes, or whether the observed myelin change resulted in the changes in OPC and mature oligodendrocyte numbers. We have previously shown associated structural alterations in the node of Ranvier, the paranode and myelin compaction, during both acute and chronic time phases following partial CNS injury (Szymanski et al., 2013). We have also observed chronic myelin decompaction and increased numbers of intraperiodic lines, indicative of remyelination during secondary degeneration (Payne et al., 2012). However, the driving mechanisms of these changes are yet to be clearly identified. Increased extracellular glutamate levels following injury lead to the over-activation of these receptors on oligodendrocytes and myelin sheaths, leaving oligodendrocytes susceptible to Ca$^{2+}$ influxes and subsequent excitotoxicity (Nakamura et al., 2010; Dutta and Trapp, 2011). However, blockage of Ca$^{2+}$ influx in the myelin sheath has been shown to be prevented by NMDA antagonists (Micu et al., 2006). Salter and Fern (2005) showed similar findings in mouse ON, whereby Ca$^{2+}$ dependent loss of oligodendrocyte processes was prevented by NMDA receptor antagonism. Thus, myelin disruption is likely to occur through the loss of oligodendrocyte processes via excessive Ca$^{2+}$ influx, however, how the loss of oligodendrocyte processes occurs, remains unclear. Currently within our laboratory, we are comprehensively investigating oxidative damage within the oligodendroglial subpopulations and OPC progeny.
6.3 Alternative experimental design

6.3.1 Large animal SCI models as a tool for testing ion channel inhibitor efficacy

Animal models (particularly rodent models) of SCI are used to develop the understanding of both acute and chronic phases following injury, incorporating cellular and molecular biology to assess the consequences that occur following injury. This understanding allows basic scientists and clinicians to identify potential therapeutic targets and establish and trial therapeutic treatment strategies to promote anatomical and functional recovery. The difficulty of modelling SCI is the non-heterogeneous types of injury that occurs in the human population. Demographic studies of SCI reveal that in humans, cervical injury is more common than thoracic, lumbar or sacral injuries, typically resulting in incomplete tetraplegia (Lee et al., 2014). However, modelling complete cervical injuries in rodents can be extremely difficult due to the physiological and behavioural consequences that occur following injury, typically requiring intense around the clock animal husbandry, leading to significant, and undesirable costs (Hodgetts et al., 2009). Consideration of animal care is crucial in the choice of animal model as the model must be reproducible, whilst offering affordable facilities and equipment, for both surgery and post-care administration.

In Chapter 5 we demonstrated the acute effects of ion channel inhibitor treatment, however the chronic effects were less clear, primarily due to the necessary removal of the osmotic-mini pump containing oxATP and YM872. We further highlighted the potential use of alternative delivery methods to assess the efficacy of chronic administration of Lom, oxATP and YM872. However, this still does not overcome the potential for the pump exacerbating injury in the cord, when physical implantation is required. Rodents not only have a small-scale CNS, they also lack a large, CSF perfused intrathecal space (Maikos et al., 2008), thus limiting the space for implantable catheters for injury site delivery. Furthermore, between rodents and humans, there is a considerable difference in cord diameter, ~2-3mm versus ~8-9mm, respectively (Ko et al., 2004). This important size difference between mammalian SCI and human SCI may be a crucial factor in the disparity in successful translation between rodent and human SCI therapies, particularly where the delivery of therapeutics is directly to the injury site. The sheer size difference of the spinal cord and surrounding CSF-filled intrathecal space (Jones et al., 2012a) and
the pathophysiological response to injury are important factors, which need to be taken into account. (Jones et al., 2012a) These factors of anatomical differences may confound regeneration studies, whereby the distance axons are required to regenerate, differ between species (Courtine et al., 2007). Interspecies differences also include body size and weight, which will affect both weight bearing parameters and gait. There are also a number of differences in ascending and descending tract projections, as a consequence of bipedalism (Bortoff and Strick, 1993; Rouiller et al., 1996; Lemon, 2004). Larger mammals such as cats, dogs and pigs have been less frequently used to model SCI, when compared to the plethora of rodent experiments. Primate experiments are the most ideal, however are extremely limited due to substantial costs associated, as well as strong ethical implications.

In the rodent, the intrathecal space is of negligible size, making it less suitable for chronic insertion of surgically placed cannulas for the delivery of therapeutic drugs, as highlighted in Chapter 5, nor other parameters like measuring intrathecal pressure changes following SCI. In contrast, porcine models are an ideal candidate for a large animal model of SCI as they have comparable cord size and intrathecal space to adult human’s, providing a larger space for cannula insertion, meaning re-injury due to surrounding tissue of the cord is less likely. Pigs are also of an experimentally workable size at neurological maturity, easy to train, and have a number of documented similarities to humans in the neurotrauma field, including in stroke (Donati et al., 2008; Mordasini et al., 2010), TBI (Browne et al., 2011; Teranishi et al., 2012) and SCI models (Zurita et al., 2008; Kuluž et al., 2010; Lim et al., 2010; Jones et al., 2012b; Zurita et al., 2012). The use of intrathecal drug delivery and implantable drug-device combinations are currently used in the clinical setting (see review Follett et al., 2004). The use of porcine models in future pre-clinical SCI experiments, may allow us to overcome the limited delivery time of oxATP and YM872 used in the current work, and provide a more comparable model to the clinical situation in humans. There is clearly an acute effect of these inhibitors, however the chronic effects, at least in SCI, remain relatively unclear. Therefore, utilising the porcine model, we can first ensure the acute effects of these inhibitors persist, and then assess alternative chronic administration modalities of these inhibitors, including use of Bbb crossing alternatives. This will give a clearer understanding of the effects of combinatorial ion channel inhibitor treatments, and efficacy for use in SCI treatment in the future.
6.3.2 Further neurological and behavioural testing parameters

SCI (or any type of traumatic CNS injury for that matter) is a pathological state of great misfortune that can lead to paralysis, bladder and bowel dysfunction, dependence on family members and personal aid, mobility limitations and increased secondary risks (pressure ulcers, infections etc.). As medicine has advanced, so has the survival rate and life expectancy of individuals suffering a traumatic CNS injury. When 1000 SCI individuals from the UK, Germany, Austria and Switzerland were asked to self-measure their current needs, the areas of highest community needs across all four countries included occupation, sexual activity and pain relief (Kennedy et al., 2006). In a similar study, a systematic review of electronic databases was conducted from 1948 to 2011. This study also aimed to determine the priorities of 5262 individuals. Functional priorities were identified as motor function (e.g. arm/hand function), bowel, bladder and sexual function. Additional health and relationship domains were also identified (Simpson et al., 2012). Of course, the ultimate aim of any treatment strategy following SCI is to give complete motor and sensory neurological recovery, ultimately leading to the ability of performing daily tasks (eating, drinking, walking etc.) and unperturbed occupational and sexual activities. We know that this currently is very unlikely given the lack of successful therapeutic treatments for SCI. However, we recognise from a therapeutic point of view, that even partial restoration of injured spinal tracts could result in improvements in upper/lower limb (motor and/or sensory), bladder and bowel, and sexual function(s). Therefore, future studies looking to employ treatment strategies should look to implement a suite of neurological/behavioural tests. Although a plethora of both injury model and sensorimotor assessments for animals are available, particularly in rodents (see review Hodgetts et al., 2009), there are a number of other outcomes that should be assessed when determining efficacy of a treatment. This includes, but is not limited to: Durham scale (Vargas, 2014), reaching tasks (Girgis et al., 2007), Louisville Swim Scale assessment (Smith et al., 2006), grooming responses, thermal (Hargreaves et al., 1988), tactile (Chaplan et al., 1994), pain/escape threshold testing (Vierck Jr et al., 2000), Von Frey filaments for hypersensitivity (Woolf, 1983), microstimulators for the assessment of urinary retention and bladder function (Walter et al., 2005), and sexual behaviour and function (see review McMurray et al., 2006). (Smith et al., 2006). The addition of such assessments in determining the behavioural effects of therapeutics may allow us to
determine the subtle efficacies of said treatments, which could in fact have a significant and substantial positive effect on the quality of life of CNS injured persons.

6.4 Conclusion

We have examined the effects of Lom, oxATP and YM872 in limiting Ca$^{2+}$ entry into cells via VGCCs, purinergic P2X$_7$ receptors, and Ca$^{2+}$ permeable AMPA receptors, respectively, in various models of CNS injury, both in vitro and in vivo. The importance of studying the multi-faceted pathophysiology and applying a combinatorial therapeutic strategy for the treatment of CNS injury is highlighted. We propose that future studies build on the promising findings of this thesis, providing further mechanistic information, and examining the positive effects of combinatorial treatment on CNS injury. Specifically, the chronic administration of Lom, oxATP and YM872 using either, or the combination of, larger animal models and alternative drug delivery, including use of BBG. We further propose that oxidative stress, particularly lipid peroxidation, and the oligodendrocyte lineage should be further investigated as potential therapeutic targets for the treatment of CNS injury. Such strategies further detailing the effects of combinatorial treatment strategies following SCI, could provide the key data necessary for the successful translation toward clinical trials.
Table 1. Proteins identified by redox proteomics determined to have undergone oxidative changes following partial ON injury.
Table 2. Summary of oxidative peptide changes identified following partial ON injury (PT), in Normal (sample A), 1 Day PT (sample B) and 7 Day PT (sample C) animals.

<table>
<thead>
<tr>
<th>Total Numbers</th>
<th>58 Proteins</th>
<th>330 peptides</th>
<th>132 peptides with no result</th>
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<tbody>
<tr>
<td></td>
<td>1 peptide with no results for sample A but has results for samples B and C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>17 peptides showing reduced oxidation levels across all three comparisons</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>47 peptides showing reduced oxidation levels in sample B &amp; C compared to A only</td>
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<td></td>
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<tr>
<td></td>
<td>11 peptides showing reduced oxidation levels in sample C compared to A &amp; B only</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 peptides showing reduced oxidation levels in sample B compared to A and sample C compared to B only</td>
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<tr>
<td></td>
<td>17 peptides showing reduced oxidation levels in sample B compared to A only</td>
<td></td>
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<tr>
<td></td>
<td>0 peptides showing reduced oxidation levels in sample C compared to A only</td>
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<tr>
<td></td>
<td>35 peptides showing reduced oxidation levels in sample C compared to B only</td>
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<tr>
<td></td>
<td>71 peptides showing increased oxidation levels across all three comparisons</td>
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<td></td>
<td>35 peptides showing increased oxidation levels in sample B &amp; C compared to A only</td>
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<td>17 peptides showing increased oxidation levels in sample C compared to A &amp; B only</td>
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<td>0 peptides showing increased oxidation levels in sample B compared to A and sample C compared to B only</td>
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<td></td>
<td>11 peptides showing increased oxidation levels in sample B compared to A only</td>
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<td></td>
<td>0 peptides showing increased oxidation levels in sample C compared to A only</td>
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<td>47 peptides showing increased oxidation levels in sample C compared to B only</td>
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<tr>
<td></td>
<td>peptides showing &gt;3 fold increase in oxidation levels across all three comparisons = 9 peptides</td>
<td></td>
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<tr>
<td></td>
<td>peptides showing &gt;3 fold decrease in oxidation levels across all three comparisons = 0 peptides</td>
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Figure 1. Oxidised/Non-oxidised peptide ratio following partial ON injury (PT).

Following partial ON injury, several oxidised peptides were identified including but not limited to: tubulin beta-2A chain (LAVNMVPFPR, ISEQFTAMFR), vimentin (LGDLYEEMR), dihydropyrimidinase-related protein 2 (MVPGGIDVHTR), haemoglobin subunit beta-1 (YFDSFGDLSSASAIMGNPK), sodium/potassium-transport ATPase subunit alpha-3 (AMVALIDVHQYSGR) and protein S100-b.
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