Changes in Gap Junction Expression and Function Following Ischemic Injury of Spinal Cord White Matter

by

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Abstract

The role of gap junctions in modulating the dynamics of axonal dysfunction in spinal cord white matter injury remains uncertain; hence, I examined the functional role and changes in expression of gap junctions following CNS injury. I hypothesized that inhibition of gap junctions improves axonal conduction during oxygen and glucose deprivation (OGD) in vitro. Carbenoxolone and octanol, gap junction blockers, did not change CAP amplitude in non-injured tissue, yet they significantly reduced the extent of its decline during OGD. No difference in mRNA expression of connexins 32, 36 was found. However, during OGD in the presence of gap junction blockers, expression of connexins 30, 43 was downregulated. Immunohistochemistry confirmed the presence of connexins in spinal cord slices: connexins 30, 43 overlapping with GFAP, connexin 32 with MBP and connexin 36 with CC1. Thus, blocking gap junctions enhances axonal conduction during OGD and promotes dynamic changes in connexin mRNA expression.
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Chapter 1
Introduction

1 Background Information

1.1 Secondary Spinal Cord Injury

One of the biggest obstacles to SCI treatment has been dealing with the effects of secondary injury that occurs after the initial fracture, compression, contusion, dislocation, transection, or mechanical deformation has occurred. Often, the primary injury does not completely sever the spinal cord; however, this does not prevent a total loss of function, because such injury worsens with time as a myriad of secondary molecular effects takes place (Tator and Fehlings, 1991). Some of the changes resulting from a primary spinal cord injury include hemorrhage, edema, necrosis, and demyelination (Tator and Fehlings, 1991). Typically, the gravest trauma resulting from secondary injury involves a vascular injury characterized by arterial disruption and compounded by anoxic and hypoxic events. The vascular injury creates ischemic zones in which large portions of both white and gray matter incur major reductions in blood flow, progressively worsening within hours of the original injury (Tator and Fehlings, 1991). As a result, ample effort has been spent focusing on eliminating the effects of secondary injury in order to effectively treat the primary injury and allow the spinal cord to heal. However, identifying the mechanisms involved is further complicated by a cascade of events happening as a result of a vascular injury, including reductions in axonal conduction and glial-neuronal interactions that propagate the injury - topics which will be the focus of this thesis.
1.2 Role of Anoxia and Ischemia

The spinal cord is supplied by three spinal arteries, one located on the anterior side and two on the posterior that carry blood rich in oxygen and nutrients throughout the spinal cord. A restriction in blood supply, also known as ischemia, prevents oxygen and glucose from being delivered to the tissue and subsequently diminishes the energy source and currency for cells: ATP. On the larger scale, ischemic incidents disrupt corticospinal tracts (descending motor pathways) and spinothalamic tracts (sensory pathway that transmits touch and pain and temperature sensation to the brain), often resulting in the loss of pain and temperature sensation, loss of proprioception, paraplegia or quadriplegia. The molecular basis of such debilitating injuries has been under investigation and scrutiny, however, no treatment to date has been able to reverse the effects of spinal cord injury (SCI).

During ischemia, the lack of blood flow to the CNS results in a reduction in ATP levels and loss of ionic homeostasis through failure of the $\text{Na}^+/\text{K}^+$ ATPase (Alix, 2006). As a result, extracellular levels of $\text{K}^+$ rise and non-specific $\text{Na}^+$ channels open, depolarizing the axonal membrane. This depolarization and subsequent $\text{Ca}^{2+}$ influx result in further damage such as mitochondrial swelling, retraction of paranodal myelin, reduction in microtubules and formation of submyelin vacuoles (Waxman et al., 1995).

In vivo, the effect of anoxic and ischemic injury on axons cannot be separated from the surrounding milieu, i.e. glia. White matter glia plays an important supporting role to axons, not only impacting axonal conduction through myelination, but also in supporting the energy requirements of myelinated axons via lactate (Stys, 1998). The biggest damage to axonal function occurs when anoxia and aglycemia (lack of glucose) coincide, as is the case in in vivo
ischemia. Restricting glucose alone does not contribute to as much damage as that seen with anoxia, because with aglycemia astrocytes still continue to produce lactate from their glycogen stores and relay it to axons in order to substitute for the lack of axonal ATP (Stys, 1998).

The CNS is particularly afflicted with the secondary injury effects. White matter is dependent on oxidative metabolism to maintain tissue excitability and myelinated axons are less able to withstand anoxia compared to unmyelinated axons (Waxman et al., 1990), possibly due to the continuous distribution of Na\(^+\) channels in the latter. Previous work shows that a relatively short hypoxic period of 30 minutes reduces the magnitude of the compound action potential (CAP) in the CNS, while having no effect on axonal conduction in the periphery: peripheral axons are more resistant to hypoxia (Utzschneider et al., 1991). However, the biggest differences occur during longer exposures to anoxia and ischemia. CAPs experience a full recovery with a short injury and little recovery with longer exposures to the lack of oxygen and glucose. With a short (10-15 min) period of anoxia, only electrogenesis or initiation of action potentials is affected; as a result, during recovery and reoxygenation, the necessary ATP is available to restore the ion gradient needed to initiate action potentials leading to a full recovery in signaling (Stys et al., 1990). This acute loss of excitability of CNS axons is due to energy failure. However, during longer periods of insult, such as 60 minutes of anoxia, the marked effect of Ca\(^{2+}\) influx sets in. With a large internal accumulation of Ca\(^{2+}\) even with reoxygenation and reinstitution of oxidative phosphorylation, only further damage to axons ensues, involving irreversible destructive mechanisms not limited to free radical formation and activation of pathways leading to necrotic cell death (Stys, 1998). Ultimately, studying white matter injury separately from gray matter or periphery is important as the two react differently to anoxia and ischemia; however, although highly clinically relevant, studies in vivo are confounded by gray matter and vascular
effects and to-date in vitro studies are key in distinguishing the effects of injury on white matter alone.

1.3 White Matter Axonal Injury

After spinal cord injury (SCI) a series of secondary ischemic and biomolecular events exacerbate the initial physical and mechanical trauma, resulting in white matter injury that is characterized by damage to the myelin sheath (Park et al., 2003, Park et al., 2004). Damage to the myelin sheath is an important aspect of the secondary injury because it inevitably results in disruption to signal transmission in the spinal cord. Myelin is an electrically insulating material that forms from oligodendrocytes in the CNS and is found wrapped around an axon. Myelin increases the electrical resistance across the cell membrane and prevents electrical current from leaving the axon. Different oligodendrocytes have the capability of wrapping around a single axon and the spaces of axolemma left unwrapped occur at regular intervals and have been termed the Nodes of Ranvier. The morphology of a myelinated axon dictates the distribution of ion channels and transporters, causing a high segregation of channels at the nodes while only $K^+$ channels remain dispersed under the myelin sheath. This distribution of channels plays an important role in generation and propagation of action potentials, allowing transmembrane ionic currents to be carried down an axon via saltatory conduction, action potentials “jumping” from one node to another, – a process that increases the speed at which an impulse propagates. This saltatory conduction is strongly dependent on the axonal morphology with ion channels being separated by tight paranodal myelin seals.

During an anoxic and ischemic secondary injury myelinated axons suffer from a halt in oxidative phosphorylation and subsequent lack of production of ATP that is needed to power the pumps that create the ionic gradients across the axonal membrane. The ATP dependent pumps
instrumental to the onset and propagation of injury include the Na\(^+\)-K\(^+\) pump, Ca\(^{2+}\)-ATPases, and the Na\(^+\) - Ca\(^{2+}\) antiporter (Stys, 1998). As a result, the lack of new ATP and the onset of rapid energy depletion lead to failure of the Na\(^+\)-K\(^+\) ATPase and accumulation of Na\(^+\) inside the axons largely through non-inactivating voltage gated Na\(^+\) channels. The newly depolarized axonal membrane suffers a severe internal depletion of K\(^+\) ions, as they escape down the electrochemical gradient to the extracellular space through K\(^+\) channels. Furthermore, the exceedingly high concentrations of Na\(^+\) ions in the axolemma cause a reversal in the Na\(^+\)- Ca\(^{2+}\) antiporter and a resulting Ca\(^{2+}\) overload ensues. Ca\(^{2+}\) also appears to enter via Na\(^+\) channels (Stys, 1998). The Ca\(^{2+}\) ion entry is of particular importance since it activates Ca\(^{2+}\) dependent enzymes such as calpain, phospholipases and protein kinase C, which exacerbate the initial injury and further promote its irreversibility.

As already mentioned, the vast majority of channels is located at the Nodes of Ranvier as opposed to being under the myelin, and plays a role in axonal conduction. There is a high density of Na\(^+\) channels and transporters at the nodes to respond to membrane depolarizations, while internodal K\(^+\) allows for the axon to remain responsive to stimuli and thus maintains a hyperpolarized resting membrane potential. Under normal conditions, the nodal Na\(^+\)-K\(^+\) ATPase serves as an active ion transporter that maintains the transmembrane gradient of Na\(^+\) and K\(^+\) ions. Under conditions like ischemia, the transporter halts and both ions go down their electrochemical gradients, resulting in increased intracellular Na\(^+\) and extracellular K\(^+\) (Ransom et al., 1992). The rise in intracellular Na\(^+\) pushes the axon into Ca\(^{2+}\) overload by reversing the Na\(^+\)- Ca\(^{2+}\) exchanger, whereby the high intracellular concentration of Na\(^+\) ions exits the axon and allows for Ca\(^{2+}\) entry into the axolemma. The rise in Ca\(^{2+}\) is not limited to the exchanger and may come from intracellular Ca\(^{2+}\) stores (Leppanen and Stys, 1997), Ca\(^{2+}\) channels, channels selective for other ions such as Na\(^+\), and directly through the cell membrane (Stys, 1998).
Treatments targeting the underlying molecular machinery, particularly the Na\(^+\) channels which have been implicated in the initiation and propagation of the injury, have not been fully successful. A vast number of uncharged and even specific blockers for voltage gated Na\(^+\) channels, such as tetrodotoxin TTX, depress the overall excitability of the axons and do not counteract the K\(^+\) ions leaving the axons (Stys, 1998). Furthermore, TTX applied during anoxia causes a reduction in water content inside the axolemma (Stys and LoPachin, 1996). This occurs due to the impact TTX has on the Cl\(^-\) ion. By blocking the Na\(^+\) channels, instead of the usual Na\(^+\)-K\(^+\) exchange taking place, the K\(^+\)-Cl\(^-\) water efflux occurs, leading to axonal shrinkage and later detachment of paranodal loops (Stys and LoPachin, 1996). However, TTX appears to be more effective than other methods, such as zero Na\(^+\) perfusate, as it is able to block not only Na\(^+\) influx but also Ca\(^{2+}\) that seeps through these channels (Stys and LoPachin, 1998). As an alternative, charged Na\(^+\) channel blockers, such as QX-314 have been attempted and found to be neuroprotective in vitro, yet their administration in vivo is hindered by the lack of permeability of these particles across the blood brain barrier (Stys, 1998).

Removing or impeding Ca\(^{2+}\) entry was another tactic used to counteract the effects of white matter injury. On the positive side, as a result of blocking voltage gated Na\(^+\) channels, TTX helped to reduce the direct entry of Ca\(^{2+}\) ions, which tend to seep through along with rapid Na\(^+\) influx during the injury (Stys, 1998). Another strategy was to remove Ca\(^{2+}\) from in vitro bathing solutions with chelators such as EGTA or BAPTA, and such a strategy was able to prevent permanent injury to the axons (Stys et al., 1990). However, this method of treatment did not prove to eliminate all the detrimental effects stemming from white matter injury. Removal of Ca\(^{2+}\) did not protect the mitochondria from swelling (Waxman et al., 1993). Neither did external Ca\(^{2+}\) removal improve the axonal recovery process, since internal mitochondrial Ca\(^{2+}\) levels increase dramatically during re-oxygenation through reactivation of the oxygen-dependent
electron transport that allows mitochondria to restore its proton gradient and membrane potential that act as driving forces for Ca\(^{2+}\) uptake by an electrophoretically driven mitochondrial uniporter (Stys and LoPachin, 1996; Stys, 1998). The internal accumulation of Ca\(^{2+}\) occurs at the expense of ATP synthesis, through a process involving extrusion of protons by the electron transport chain and dissipation of the mitochondrial electrochemical proton gradient used by the ATP-synthase in production of ATP (Stys and LoPachin, 1996; Stys, 1998). Further wasteful cycling of Ca\(^{2+}\) across the mitochondrial membrane is driven by the mitochondrial Na\(^{+}\)-Ca\(^{2+}\) exchanger at the expense of resuming ATP production (Stys, 1998).

One proposed mechanism involves glutamate excitotoxicity, which involves AMPA and Kainate glutamate receptors (Agrawal and Fehlings, 1997; Li et al., 1999; Kanellopoulos, 2000; Park et al., 2003); another is the reversal of Na\(^{+}\)-dependent glutamate transport (Li et al., 1999; Li et al., 2001; Agrawal and Fehlings, 1996). The myelin sheath, abundant in AMPA receptors, is a target for glutamate excitotoxicity (Li et al., 1999) and AMPA/Kainate blockers protect spinal cord white matter (Kanellopoulos, 2000). However, glutamate antagonists in general are plagued by clinically intolerable side effects (Alix, 2006), and hence alternative approaches to influence secondary injury cascades are desirable. Since little is known regarding the signaling between astrocytes and oligodendrocytes and in particular the functional role of gap junctions, in secondary spinal cord injury these areas represent important subjects for investigation.

### 1.4 Interaction between Astrocytes and Neurons

Ca\(^{2+}\) imaging studies show that presynaptic neurons release neurotransmitters that bind to nearby receptors on astrocytic processes, which typically surround the synaptic terminals of neurons and in turn, astrocytes release gliotransmitters as feedback that modulates synaptic transmission (Haydon and Carmignoto, 2006). Binding of neurotransmitters to astrocytic
receptors causes an increase in the internal levels of \( \text{Ca}^{2+} \) and induces a release of excitatory neurotransmitter glutamate that modulates synaptic transmission, and promotes neuronal excitability and synchrony (Haydon and Carmignoto, 2006). Furthermore, it is well known that a large portion of astrocytic communication occurs via intercellular communication channels: gap junctions. These channels promote synchronization by allowing small molecules and metabolites to pass from one astrocyte to another. Likewise, astrocytes use the same gap junction mechanism for potassium spatial buffering, whereby excess potassium ions released during neuronal activity are removed from the extracellular space, particularly near neuronal synapses, and redistributed to neighbouring astrocytes (Vukelic et al., 1991; Wallraff et al., 2006; Rash, 2010).

A key difference between astrocytes and neurons is their individual response and ability to cope with ischemic and anoxic injuries. For example, astrocytes are more resistant to injury than neurons. Partially the reason for this is the lack of a net accumulation of \( \text{Ca}^{2+} \) in astrocytes (Stys, 1998) following a prolonged (60 min) anoxic insult, characterized only by a rise in \( \text{Na}^+ \) and loss of \( \text{K}^+ \) ions. Even then, the \( \text{Na}^+ \) and \( \text{K}^+ \) changes in glia only become significant after an anoxic period of 60 minutes. However, entry of extracellular \( \text{Ca}^{2+} \) (either by internal release or through \( \text{Ca}^{2+} \) channels) does occur in glia yet the method is not fully understood (Stys, 1998). A proposed mechanism likely involves the glial AMPA/Kainate receptors (Agrawal and Fehlings, 1997) and their activation by glutamate that is released from axons during ischemia via the \( \text{Na}^+-\text{K}^+-\text{glutamate transporter} \) (Chiu and Kriegler, 1994). Glutamate does appear to have an even wider role in the ischemic anoxic injury. During ischemia, astrocytic gap junction channels open and release glutamate to the extracellular space, which readily binds the oligodendrocytic AMPA/Kainate receptors and further propagates the injury with a subsequent \( \text{Ca}^{2+} \) entry (Kanellopoulos, 2000; Ye et al., 2003).
More importantly, astrocytic channels appear to be modulated by neurons, and one such subset of channels is the gap junction (Vukelic et al., 1991; Hossain et al., 1994; Koulakoff et al., 2008). Studies using kainic acid to destroy neurons and other similar methods of inducing neuronal degeneration have shown a concurrent decrease in the immunoreactivity of the astrocytic connexin 43 in the striatum, thalamus and the hippocampus (Vukelic et al., 1991; Hossain et al., 1994). Further support linking expression of astrocytic connexins to neuronal regulation comes from a study showing that the two most abundant astrocytic connexins 30 and 43 are modulated by neurons in co-culture models (Koulakoff et al., 2008). The study went on to show that an excitotoxic brain injury impacting neurons results in mRNA downregulation of both connexins 30 and 43 (Koulakoff et al., 2008). However questions as to the interaction of astrocytes and neurons in vivo, the impact of other supporting cells such as oligodendrocytes, and the overall influence of the interactions on axonal conduction have not been explored. This study aimed at investigating the link between connexins, including astrocytic connexins, and axonal function after injury, in order to better understand the connection between underlying astrocytic mRNA changes and their impact on spinal cord white matter.

1.5 Structure, Function, Regulation and Expression of Gap Junctions

Gap junctions are ion channels that allow direct metabolic and electrical communication between most cell types by mediating the intercellular transfer of various ions and molecules less than 1000 Da, such as nutrients, second messengers (inositol-1,4,5-triphosphate (IP-3) and possibly Ca$^{2+}$), metabolites, cations and anions (Söhl et al., 2005). Each gap junction is composed of an array of six protein subunits, collectively called a connexon, on each side of the adjoining
membranes. Typically, the distance between the two membranes, and the length of the gap junction channel, is 2-4 nm. Individual connexin proteins making up this channel weigh anywhere between 20 and 60 kDa and are on average 380 amino acids long. Connexin subunits are typically abbreviated as Cx, followed by a number that indicates their molecular weight in kDAs. These channels have four transmembrane domains, two cysteine-rich extracellular loops, one intracellular loop, and both amino and carboxy terminal regions inside the cytoplasm (Fig. 1b) (Söhl et al., 2005). Collectively, gap junctions composed of one type of connexin subunit, i.e. gap junctions composed of connexins that are all the same weight, are termed homotypic; meanwhile, gap junctions made of different connexins are heterotypic. Hexameric connexons that do not find binding partners on another membrane are termed hemichannels. These hemichannels are able to function in a manner similar to full gap junctions, i.e. they have open and closed states; however, they are not able to relay ions and small molecules directly into the axoplasm of a neighbouring cell. Hemichannels have their own sub-designation: hemichannels with one type of connexin are homomeric and those with various connexins are heteromeric (Fig. 1a). Overall, gap junction channels are abundant in CNS tissues and often many gap junctions are formed side by side, resulting in gap junctional plaques – groups of gap junctions that are able to synchronize and relay molecules in close proximity to each other.

After translation by the ribosomes, connexins are inserted into the endoplasmic reticulum (ER), where they are folded and begin forming hemichannels. Once connexins exit the ER they oligomerize, and continue to pass through the Golgi network after which point they are inserted into the plasma membrane (George et al., 1999). The transport to the membrane occurs via two different pathways, depending on the connexin type, either via vesicles 100-150 nm or through direct integration with the membrane post-translation via a poorly understood mechanism (Saez et al., 2003). These connexons are then able to dock with adjacent hemichannels through
cadherins (Jongen et al., 1991), forming functional gap junction channels. Extra connexins are kept in intracellular stores in the ER/Golgi complex and growth factors are used to mediate shuttling of connexins between these stores and the gap junctions embedded in the membrane (Nadarajah et al., 1998; George et al., 1999). The transport of these membrane proteins occurs quite often in order to maintain coupling between cells, as the half life of connexin subunits is fairly short 1.5-3 hours (Musil et al., 1990) and connexins have high turnover rates – they are quickly removed from the plasma membrane (George et al., 1999).

Gap junction regulation occurs over both short and long term. Long term regulation involves the general maintenance of gap junctions, such as changes to the number of gap junction plaques, the number of individual gap junction channels within these, gap junction internalization and degradation. Short term regulation refers to immediate maintenance of each individual channel such as changes to their open and closed states, opening probabilities, conductances and functionality (Rouach et al., 2002). The latter is what gap junction blockers have an immediate effect on, with a possibility of altering long term regulation as well. Moreover, in addition to internal regulation of gap junctions, a number of external factors not limited to neurotransmitters, growth factors, peptides, cytokines and endogenous bioactive lipids alter connexin expression and intercellular gap junction communication (Rouach et al., 2002). As an extension, it appears that a variety of pathways and tissues may be involved in gap junction regulation and different tissues can exert effects on the other, e.g. neurons may regulate glia via various secreted neuronal transmitters and vice versa (Rouach et al., 2002). To further complicate the matter, the location of the gap junction is key, as the same neurotransmitter is able to both inhibit and activate the gap junction current depending on what tissue the gap junction is located on (Rouach et al., 2002).
The four connexins of interest for the proposed project were chosen to represent the three major tissues within the CNS that are particularly targeted during secondary spinal cord injury. In particular: connexin 32, connexin 36, and connexins 30 and 43. In the spinal cord, the first connexin (connexin 32) appears in the oligodendrocyte soma, processes, along the myelin sheath and in both gray and white matter, and it typically forms gap junctions with other oligodendrocytes (same connexin subunit) or with astrocytes (which have connexins 30 and 43) (Nagy et al., 2004). The gap junctions that the oligodendrocytic connexins form with astrocytes also tend to couple with other oligodendrocytes, thus forming an O-A-O channel that uses astrocytes as intermediates for communication with other oligodendrocytes. This coupling likely allows distant oligodendrocytes to be attached to a network with other oligodendrocytes further away (Nagy et al., 2004). The existence of gap junctions in neurons remains controversial because, due to the small amount of connexins spread out on neuronal tissue, detecting and imaging them has been challenging; nonetheless, a prominent connexin subunit, connexin 36 was found predominantly in neurons (Söhl et al., 2005; Nagy et al., 2004). Neuronal gap junctions may facilitate neuronal synchrony, particularly between neurons of the same type (Nagy et al., 2004). However, not only its small amount on neurons but also its possible location in oligodendrocytes (Dobrenis et al., 2005; Parenti et al., 2002) adds to the controversy surrounding connexin 36, and to-date its location remains elusive. Finally connexins 30 and 43 are very abundant in the CNS and are located exclusively in astrocytes. Astrocytic gap junctions, avid binding partners to connexin 32, typically include all three connexins 26, 30 and 43 (Nagy et al. 2004). In addition to binding to oligodendrocytes, astrocytes use gap junctions to form networks with each other and function to synchronize their activity and for potassium buffering as described earlier (Vukelic et al., 1991; Wallraff et al., 2006; Rash, 2010).
1.6 Gap Junctions in Healthy Central Nervous System

In healthy CNS, gap junctions serve an important role in electrochemical and metabolic coupling by mediating intercellular transfer of various ions and molecules. Half gap junctions or hemichannels also mediate the transfer of molecules; however, unlike gap junctions between two cell membranes, hemichannels extrude molecules to the extracellular space and also uptake small nutrients from the extracellular space, and although typically closed to prevent dissolution of ionic gradients and leakage of cytoplasmic constituents, hemichannels are known to open during normal physiological conditions in astrocytes and other cell types (Saez et al., 2003). However, the probability of these channels opening under physiological conditions is extremely low, and their opening is usually a sign of pathological conditions (Saez et al., 2003). As such, conditions typical of normal physiological functioning: negative membrane potentials, high extracellular $\text{Ca}^{2+}$ concentrations and intracellular $\text{H}^+$ ions, as well as protein phosphorylation, keep hemichannels in a closed state. Conversely, positive, depolarized membrane potentials, dephosphorylation and low extracellular $\text{Ca}^{2+}$, which are conditions occurring during anoxia and ischemia, keep the hemichannels in an open state (Saez et al., 2003). The gating of hemichannels has been shown to occur by two voltage gating mechanisms: fast transition of less than 1 ms between open and intermediate states, or slow transition of more than 5 ms between open and closed states, with a possibility of several intermediate states (Saez et al., 2003).

In order to test the usefulness of gap junctions in a biological system, studies using targeted knock-outs of particular connexin subunits have been implemented to derive which functions would be affected and hence, which functions benefit from gap junction communication in healthy CNS. Neuronal connexin 36 (Cx 36) knockouts, reduced the synchrony of gamma oscillations in neocortical slices (Rouach et al., 2002). Apart from information derived from
knockout studies, Cx 36 is a relatively sparse connexin in the CNS, which makes it difficult to study; as a result, non-synaptic and metabolic roles of this connexin in neurons are poorly understood. On the other hand, astrocytic connexins 30 and 43 have been thoroughly studied due to their abundance in the CNS. Studies using knockouts for glial connexins have shown gross anatomical deficits, including myelin swelling from the lack of K\(^+\)/H\(_2\)O removal from the innermost myelin to astrocytic endfeet mediated by connexins (Menichella et al., 2006, Rash, 2010). Indeed, this concurs with the line of thought that glial gap junctions, particularly those in astrocytes, are involved in spatial buffering of K\(^+\) and assist in its removal from areas of high concentration (near active axons) to areas of lower concentration propagated through astrocytic networks. Additionally, various blockers have shown that in normal tissue, blocking gap junctions in astrocytes increases their uptake of glucose (Tabenero et al., 1996), a finding agreeable with a hypothesis that astrocytes provide neurons with energy-producing compounds to fuel the Na\(^+\)-K\(^+\) ATPase and they are well equipped to do so, being located between oxygen-rich blood capillaries and neurons (Rouach et al., 2002). As a whole, there appears to be an unequivocal need for connexins in the healthy tissue since their deletion only harms various functions in the CNS.

Another function of astrocytes in the healthy CNS that depends on gap junctions has been under direct scrutiny: propagation of intercellular calcium waves between astrocytes in the syncitium. Gap junction channels are large enough and permeable to allow for Ca\(^{2+}\) ions and second messengers like IP-3 to move between astrocytes. Moreover, studies using various gap junction blockers showed a blockade of the Ca\(^{2+}\) propagation process and cell lines that do not have gap junctions also do not exhibit Ca\(^{2+}\) wave propagation, however, once transfected with connexin 43 the cell lines exhibit Ca\(^{2+}\) waves (Rouach et al., 2002). Like evidence in favour of Ca\(^{2+}\) propagation, there is evidence refuting this phenomenon. Gap junction blocker oleamide had no
effect on the propagation of Ca\(^{2+}\) waves and in knock-out models of connexin 43 only partial
decrease in Ca\(^{2+}\) propagation was observed (Naus et al., 1997; Rouach et al., 2002).

Although controversial in its methodology and the final outcome, astrocytes do appear to be able
to self-regulate themselves in normal CNS. Inhibiting gap junctions via glycyrrheticin acid
caused an increase in the rate of proliferation of astrocytes (Tabernero et al., 2001). However,
knocking them out completely, as is the case with connexin 43 knockouts, caused a decrease in
proliferation of astrocytes (Naus et al., 1997). In the former finding, gap junction blockers may
have caused an effect on all gap junctions since they are rarely specific and the latter finding may
be explained from the point of view of compensation by other astrocytic connexins, such as
connexins 26 and 30. A study at the transcriptional level suggests that the Cx 43 protein is able
to selectively regulate the recruitment and alter the ratio of preferential binding of transcription
factors sp1 and sp3 to connexin response elements (CxREs), which directly translate into strong
gene expression if there is a high sp1 to sp3 ratio or repress transcription if sp3 preferentially
binds (Stains et al., 2003). An occlusion of Cx 43 by the oleamide blocker caused a
dephosphorylation of sp1, acetylation of sp3, and preferential recruitment of sp3 to CxREs
resulting in transcription repression (Stains et al., 2003). The ability to modify and regulate
transcription, so far demonstrated only in gap junctions containing connexin 43, may be an
important task that defines how gap junctions react during injury, and may be an important
dynamic factor that can be targeted to reduce the impact of secondary spinal cord injury.

1.7 Gap Junctions in the Injured Central Nervous System

While there is an emerging consensus regarding the role of gap junctions in the normal CNS,
there is uncertainty as to whether gap junctions function to aid in recovery following SCI by
equalizing ions between coupled cells or hinder recovery by propagating cell injury through
degradation of ion gradients and subsequent release of glutamate to the extracellular space (Table 1) (Ye et al., 2003) during anoxia and ischemia. Similarly, the effect of opening hemichannels in injured CNS is two-fold. On the one hand, opening leads to collapse of electrochemical and metabolic gradients across membranes of cells leading to cell swelling and cell death. On the other hand, open gap junctions and hemichannels may try to alleviate the injury and save the dying cells by transferring ions and metabolites to them (Rouach et al., 2002). Evidence suggests that ischemia modeled by oxygen-glucose deprivation (OGD) opens neuronal hemichannels (Thompson et al., 2006) and astrocytic hemichannels via connexin 43 dephosphorylation (Ye et al., 2003). Opening of astrocytic hemichannels has been shown to release glutamate extracellularly and reduce glutamate uptake (Ye et al., 2003). Generally, astrocytes in normal tissue maintain low extracellular levels of glutamate, thus providing a good environment for synaptic signal transmission and preventing neuronal overexcitation (Ye et al., 2003). As a result of OGD, opening of hemichannels and glutamate release is implicated in excitotoxicity during ischemia.

Further complications come from the methodology used to study astrocytes, neurons, oligodendrocytes and the surrounding milieu. In order to compare and draw conclusions on each cell type separately it is necessary for studies to be conducted in similar conditions, however due to intricacies in experimental design to-date, most astrocytic studies have been conducted in primary cultures, while neurons tend to be studied in brain slices ex vivo (Rouach et al., 2002). As a result, in primary cultures, apart from astrocytes being studied outside their typical surroundings that regularly have a large effect on gap junctional communication and coupling, astrocytes are more highly coupled in cultures than they would be in the CNS (Rouach et al., 2002). Neurons also behave differently; specifically, in the ex vivo preparation they do not appear to couple at all, compared to in vivo (Rouach et al., 2002). Differences in studying gap
junction communication and coupling also vary depending on the methodology used, which further complicates findings implicating gap junctions in injury and the corresponding clinical relevance.

Akin to studies of gap junctions in normal CNS, connexin models have been used to also study their function in injured CNS. In injuries involving rapid proliferation of cells, such as tumor genesis and proliferation, gap junctions control cell growth by acting as tumor suppressor proteins (Yamasaki and Naus, 1996). Knocking out connexin 43 and inducing ischemia through the occlusion of the middle cerebral artery caused a larger volume of damage than ischemia alone in their wild-type counterparts, showing that the presence of this connexin in injured CNS is actually beneficial (Siushansian et al., 2001; Rouach et al., 2002). Moreover, during injury involving swelling of astrocytes from hyposmotic solutions, gap junction communication allows astrocytes to regulate and decrease their volume (Rouach et al., 2002). However, during an ischemic injury these astrocytic connexins do not play a beneficial role, they open and propagate the injury to adjacent cells through astrocytic networks, thus spreading and amplifying the initial injury (Lin et al., 1998; Rouach et al., 2002). As a result, the utility of astrocytic gap junctions during injury is paradoxical and the effect on other functions, such as axonal conduction, remains to be explored.

Certain connexin subunits have also been implicated in diseases. In the periphery, defects in oligodendrocytic connexin 32 were linked to Charcot-Marie-Tooth X-linked peripheral neuropathy that results from damage to the myelin sheath and affects motor nerves (Bergoffen et al., 1993). In the cerebral cortex, increased expression of connexin 43 has been linked with amyloid plaques in Alzheimer’s disease (Nagy et al., 1996). Whether the role of connexin 43 is beneficial or detrimental in this disease is not clear since the astrocytic gap junction can function
both to maintain tissue homeostasis and compromise it (Nagy et al., 1996). Other diseases have also been attributed to increases in connexin 43, namely: Huntington’s disease, Parkinson’s disease and epilepsy (Rouach et al., 2002). Although connexins have been implicated in a variety of diseases, the task to differentiate whether modified connexin expression is a cause or a consequence of the disease remains. Perhaps, further experiments with another astrocytic connexin, connexin 30 would help differentiate between cause and consequence, especially since to-date, the main connexins detected and studied in the CNS in this manner have been limited to Cx 26, Cx 32, Cx 36, and Cx 43.

Of particular interest to this thesis and the study of secondary spinal cord injury are disease models featuring ischemia as the focal component. It is still unclear whether ischemia is correlated with increases or decreases in connexins and it appears that some of the key factors include whether the ischemia is local or global and its location. In the hippocampus, global ischemia selectively increased protein levels of connexins 32 and 36 but not connexin 43; after 48 and 72 hours connexin 43 mRNA expression was increased in CA1, while mRNA expression of connexins 32 and 36 remained unchanged at all time points (Oguro et al., 2001). As a result, an increase in the amount of proteins without an increase in mRNA may mean that regulation of those two connexins occurs at the translational or post-translational level (Oguro et al., 2001). Connexin 43 appears to vary with injury since a mild ischemia caused an increase in connexin 43 protein immunoreactivity while a more severe ischemia caused its decrease (Hossain et al., 1994). However, studies tend to agree that gap junctions mediate cell injury after ischemic and oxidative stress (Lin et al., 1998), incur reduced gap junctional coupling (Rouach et al., 2002) and their blockade decreases the infarct volume and reduces neuronal death (Siushansian et al., 2001; Rouach et al., 2002). At this point, little is known of connexin changes during and after a short period of ischemia, particularly in spinal cord white matter.
1.8 Gap junction blockers: Carbenoxolone and 1-Octanol

There are numerous gap junction blockers varying in their selectivity for gap junctions and efficiency with which they bind, such as carbenoxolone, octanol, heptanol, oleamide, halothane, flufenamic acid, and 18 alpha-glycyrrhetinic acid. To date there is no indication that all of the above gap junction blockers work solely to block gap junctions and hemichannels, and although described as specific channel blockers, many have effects on other channel types. Likewise, blockers not typically designed or used to block gap junctions, such as antipsychotic drugs, have been shown to alter gap junction communication between neurons (Rouach et al., 2002). Of the known gap junction blockers, carbenoxolone (Cbx), often described as a relatively specific yet non-selective gap junction and hemichannel antagonist, apart from blocking gap junctions, has been shown to also block NMDA evoked responses in hippocampal cells (Chepkova et al., 2008). Carbenoxolone is derived from Glycyrrhizic acid (GA), which unlike Cbx, is devoid of gap junction blocking activity (Davidson and Baumgarten, 1988). An indication that carbenoxolone is not specific to only gap junctions comes from a study showing that at a concentration of 100 and 200 µM carbenoxolone depressed action potentials after a 10-15 min perfusion period in hippocampal slices (Chepkova et al., 2008). Like carbenoxolone, application of GA, known to be able to block NMDA responses, also caused a depression in action potential conduction; hence, by inference, carbenoxolone was not specific to solely blocking gap junctions. Further, in this study, blocking gap junctions did not appear to be responsible for action potential depression (Chepkova et al., 2008). Looking further in potential targets for carbenoxolone, carbenoxolone also inhibits 11 beta-hydroxysteroid dehydrogenase type 1, the enzyme that activates cortisol from cortisone (Sandeep et al., 2004). Although it appears that carbenoxolone has other targets, it is still considered a prominent gap junction blocker in the
field. When applied, carbenoxolone has been used to successfully block gap junctions in the hippocampus and such a blockade was shown to decrease cell death (Frantseva et al., 2002)

The carbenoxolone concentration used to achieve a blockade of gap junctions or their uncoupling is usually (100µM), and at this concentration the drug is able to block synaptic connexin 26 hemichannels (Kamermans et al., 2001), uncouples gap junctions made of connexin 43 in heart tissue (Poelzing and Rosenbaum, 2004), suppresses spontaneous dentate waves in the hippocampus following a 30 min application of the blocker (Colgin et al., 2004), blocks spontaneous epileptic discharges in the hippocampus (Samoilova et al., 2003) and alters the motoneuron synchronization via a blockade of brain stem gap junctions (Solomon et al., 2003). Its application in the spinal cord white matter remains unknown and will be the subject of investigation in this thesis.

In order to aid in analysis and to draw conclusions based on gap junction blockers, it is useful to run experiments with a few blockers; as such, 1-octanol is another widely used gap junction blocker that appears to have minimal effects on other channels. Like carbenoxolone, octanol was shown to decrease damage (size of the infarct) in the CNS after focal ischemia (Contreras et al., 2004). However, octanol appears to be less specific for gap junctions than carbenoxolone and a much wider range in concentrations ranging from 300 µM (Solomon et al., 2003) to 1 mM (Mylvaganam et al., 2010) is used depending on the tissue. An intricate study examining octanol inhibition of astrocytic gap junctional coupling in six CNS tissues: hypothalamus, hippocampus, cortex, optic nerve, spinal cord and cerebellum has shown that at least 100 µM octanol is required to elicit 50% inhibition in those tissues; however, only hippocampus and optic nerve required near 1 mM concentrations to reach this effect (Lee et al., 1994). In fact, for the spinal cord tissue, 100 µM was enough to elicit 50% inhibition, 300 µM caused 80% inhibition and at a
1mM concentration the inhibition plateaued at 100% (Lee et al., 1994). Another important aspect of a gap junction blocker is its reversibility, i.e. the ability of gap junction channels to revert back to their functional mode. Both carbenoxolone and octanol are reversible given a sufficient washout period, however the degree of reversibility also varies depending on the tissue (Lee et al., 1994). It appears that reversibility of gap junction blockers in the spinal cord is small compared to other tissues such as the cerebellum, indicating that the initial coupling of cells surveyed (in this case astrocytes) is weaker in the spinal cord than the cerebellum (Lee et al., 1994). As a result of these differences, it is erroneous to extrapolate findings with gap junction blockers to all CNS tissues, and in particular relate them to general disease models, without thorough investigation of the tissue and cell types of interest. Hence, this study is the first to show what impact gap junctions and gap junction blockers have on the white matter tissue in the spinal cord during and after periods of ischemia and addresses the possible underlying mechanisms at work.
## Table 1. Role of gap junctions in CNS injury and involvement in disease

<table>
<thead>
<tr>
<th>Connexin/Gap Junction Type</th>
<th>Role in CNS injury</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astrocytic hemichannels</td>
<td>Extracellular release of glutamate and reduced glutamate uptake in ischemia</td>
<td>Ye et al., 2003</td>
</tr>
<tr>
<td>Connexin 43 knockout</td>
<td>Increases the infarct size after stroke</td>
<td>Siushansian et al., 2001</td>
</tr>
<tr>
<td>Astrocytic gap junctions</td>
<td>Regulate and decrease astrocytic volume in hyposmotic solutions</td>
<td>Rouach et al., 2002</td>
</tr>
<tr>
<td>Cx 43, Cx 32, Cx 26</td>
<td>Act as tumor suppressor proteins during tumor genesis and proliferation</td>
<td>Yamasaki and Naus, 1996</td>
</tr>
<tr>
<td>Astrocytic gap junctions</td>
<td>Propagate injury through astrocytic networks during ischemia</td>
<td>Lin et al., 1998</td>
</tr>
</tbody>
</table>

### Implication in Diseases

<table>
<thead>
<tr>
<th>Connexin/Gap Junction Type</th>
<th>Implication in Diseases</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx 32</td>
<td>Charcot-Marie Tooth X-linked peripheral neuropathy (myelin damage)</td>
<td>Bergoffen et al., 1993</td>
</tr>
<tr>
<td>Cx 43</td>
<td>Amyloid plaques in Alzheimer’s disease, Huntington’s disease, Parkinson’s disease, Epilepsy</td>
<td>Nagy et al., 1996; Rouach et al., 2002</td>
</tr>
</tbody>
</table>
Figure 1. Molecular organization of gap junctions
Fig. 1 Molecular organization of gap junctions

(a) Schematic diagram depicting a gap-junctional plaque in apposed plasma membranes composed of different connexin combinations. Each hemichannel on their respective membrane is composed of six connexin protein subunits. 1. Gap junction and hemichannels formed from connexins of the same molecular weight (homotypic and homomeric). 2. Hemichannels composed of connexin subunits of different molecular weights (heteromeric). 3. Gap junctions composed of connexin subunits of different molecular weights (heterotypic). (b) Composition of a connexin subunit. A connexin subunit is composed of four membrane-spanning transmembrane domains with one cytoplasmic loop, both amino and carboxy terminals in the cytoplasm and two cysteine–rich extracellular loops important for channel docking. Figure reproduced from (Söhl et al., 2005) with permission from the Nature Publishing Group.
Chapter 2
Hypothesis, Predictions and Aims

2 Hypothesis, Predictions and Aims

2.1 General Hypothesis

Inhibition of gap junctions, using gap junction blockers carbenoxolone and octanol, improves axonal conduction in spinal cord dorsal white matter during oxygen and glucose deprivation.

2.1.1 Specific Hypotheses

1. Gap junctions play a beneficial role in normal axonal conduction of CNS myelinated axons.

2. Gap junctions play a detrimental role in axonal conduction of injured CNS myelinated axons undergoing oxygen and glucose deprivation.

3. Connexin expression is altered by gap junction blockers during oxygen and glucose deprivation.

2.2 Predictions

1. Under “no injury” conditions, blocking gap junctions would likely cause a negative effect on the CAP, since keeping them in an open state allows for the passage of nutrients and small molecules between cells.

2. During an ischemic and anoxic injury, the information passed through gap junctions likely includes Ca$^{2+}$ or IP$_3$ and the opening of hemichannels destroys ionic gradients, as a result blocking gap junctions at this time would likely cause a positive effect on the CAP by avoiding injury propagation and tissue swelling.
3. Blocking gap junctions during injury may initiate a compensatory mechanism, increasing connexin mRNA expression to compensate for dysfunctional subunits or decreasing mRNA expression to further mitigate the damage following oxygen and glucose deprivation.

2.3 Aims

**Aim 1**: Determine the functional role of gap junctions in normal and ischemic spinal cords on axonal function using the sucrose-gap technique.

**Aim 2**: Determine the changes in connexin mRNA expression in ischemia and with application of gap junction blockers using RT-PCR.

**Aim 3**: Determine the location of connexins in white matter spinal cord tissue using Immunohistochemistry.
3 Materials and Methods

3.1 Animals

All experimental protocols have been approved and executed in accordance with the guidelines set out by the Animal Care Committee at the University Health Network and the Canadian Council of Animal Care.

3.2 Electrophysiology

Spinal cord slices were prepared from adult female Wistar rats (250 - 300 g). The animals were anaesthetized with a brief exposure to halothane (1 minute), followed by an injection of sodium pentobarbital (60mg/kg body weight). Rats were intracardially perfused with ice cold, 300 mOsm artificial cerebro-spinal fluid (aCSF), containing 400 µM L-Ascorbic Acid to protect the fragile spinal cord from free radicals during extrusion (Barabás et al., 1995; Straub et al., 2009). The aCSF contained (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 2 CaCl$_2$, 1.3 MgSO$_4$, 26 NaHCO$_3$ and 10 glucose, with approximate pH 7.4 after bubbling with 95% O$_2$ and 5% CO$_2$ gas mixture as we reported previously (Agrawal and Fehlings, 1997; Fehlings and Nashmi, 1996; Fehlings and Nashmi, 1997; Velumian et al., 2010). Rats were quickly decapitated and the spinal cord from upper cervical to lower lumbar level was rapidly removed through hydraulic extrusion (Velumian et al., 2010) and placed into ice-cold oxygenated aCSF, where it was cut in longitudinal slices, roughly 1mm thick (Fig. 2B). After the dissection, slices were stored at room temperature (~ 25°C) in an oxygenated chamber for at least 1hr preincubation.

The single sucrose gap apparatus modeled after Fehlings and Nashmi, 1996 and Sinha et al., 2006 was modified and custom designed to contain three Lexan polycarbonate chambers. The
isotonic KCl and oxygenated aCSF chambers were separated by an isotonic sucrose solution (Mert, 2007). Two silver wire chlorided electrodes (AgCl) placed in aCSF and KCl chambers respectively, were used as recording electrodes; similarly, a double insulated gold-plated copper wire electrode was used for stimulation. The solutions within each chamber were ensured to be isolated from each other. A single slice was placed across the three chambers of the sucrose-gap and held in place with pins on a thin layer of silicone covering the bottom part of the chambers. The edges between adjacent chambers were separated using custom made silicone dividers embalmed in Dow Corning Compound valve lubricant and sealant, and they sat tightly around the longitudinal spinal cord slice (Fig. 2A) to avoid shunting effects (Mert, 2007). The slices were perfused at the rate of 3-4 ml/min with warm (35-37 °C) and oxygenated aCSF, isotonic KCl and sucrose. Solutions exited the chambers via individual polyurethane tubing, thus avoiding any contact between solutions.

A 0.1 ms constant stimulating pulse current (1-15mA range) was applied every 15 seconds via the SIU-PSIV6 stimulus isolation unit of a Grass S88 stimulator (Velumian et al., 2010). Recording electrodes were connected to the Axoprobe 1A amplifier headstage (Axon Instruments, USA) and signals amplified 100x in DC mode, processed via Digidata 1200 and the amplitude of the evoked compound action potentials (CAPs) was analyzed with pClamp software (Axon Instruments, USA).

3.3 Oxygen-Glucose Deprivation

To model anoxia and ischemia the composition of aCSF was altered to deprive white matter slices of glucose and oxygen. In order to maintain the 300 mOsm osmolarity, 10mM glucose was replaced with 10mM sucrose in aCSF and the 95% O₂ and 5% CO₂ gas mixture was replaced with 95% N₂ and 5% CO₂.
3.4 Gap Junction Blockers

Gap junction blockers (100 µM carbenoxolone and 300 µM 1-octanol, Sigma) were administered 30 minutes prior to, during the injury (30 min) and post ischemic injury during the recovery period (1 hour post injury). They were added to the perfusing aCSF solution prior to the onset of the experiment involving the blockers.

3.5 Quantitative RT-PCR

After spinal cord isolation and 1hr preincubation, longitudinal white matter slices were transferred to a custom Styrofoam incubation chamber with petri dishes containing warm aCSF (35-37 °C), oxygenated with a 95% O₂ and 5% CO₂ gas mixture for 30 min. Then, 1/3 of each slice was collected for PCR analysis and homogenized in Trizol (Invitrogen) according to manufacturer’s instructions and the remaining 2/3 transferred to a second chamber for 30 min OGD. Upon completion of ischemia, ½ of the cord in the OGD chamber was homogenized in Trizol and ½ transferred to the first incubation chamber for recovery. Upon extraction, RNA was further purified using the RNeasy Micro Kit (QIAGEN) and converted to cDNA using 100 ng – 500 ng of RNA, 1µl Oligo (dT), 1 µl 10 mM dNTP mix, 4 µl 5x first strand buffer, 2 µl 0.1 M DDT and nuclease-free water, run on the PCR 2400 machine at 42 °C for 50 min and inactivated at 75 °C for 15 min as we previously described (Eftekharpour et al., 2005).

Gene-specific gap junction primers, as well as the β-Actin control (IDT, Table 2), have been designed for real-time PCR using Primer Express Software (PE, Applied Biosystems) and thoroughly tested for efficiency as previously reported by our laboratory (Mylvaganam, et., 2010, Eftekharpour et al., 2005).
PCR was conducted on the ABI Model 7,900 Sequence Detector TM (Perkin-Elmer PE Biosystems, USA). Each plate well contained 4 µl of diluted cDNA template, combined with a mixture of 1x SYBR Green PCR Master Mix – a double-stranded DNA-specific fluorophore (Molecular Probes, USA), 0.1 µM of each primer (F+R), and nuclease-free water. Each sample was tested in triplicate and following each PCR run, the product was checked for primer dimmers via a heat dissociation protocol and ensured to have one peak on the resulting melting curve (Eftekharpour et al., 2005). Relative expression levels of the connexin genes were analyzed as fold changes normalized to the ß-Actin control using the delta-delta CT method (Livak and Schmittgen, 2001).

3.6 Immunohistochemistry

Hydraulically extruded spinal cords were sectioned into 1 cm long pieces from the Cervical to the Lumbar regions and embedded in 4% agar. Each piece was contained in ice-cold aCSF, bubbled with 95% O₂ and 5% CO₂ and sectioned longitudinally to 150 µm thick slices on the VT1200 (Leica Microsystems), designed for fresh tissue slicing. These slices were kept in aCSF for 30 min, then placed in 4% paraformaldehyde (PFA) at room temperature for 1 hr, and left overnight at 4 °C in 1x phosphate buffered saline (PBS). The following primary antibodies were added to minimum essential medium (MEM) (15mM Hepes, 0.5% BSA, 2% FBS, 0.1% Triton X, 0.1% NaN₃) for overnight spinal cord incubation at 4 °C, Ms Connexin 43 (MAB3068, Millipore, 1:250), Rb GFAP (AB5804, Millipore, 1:1000), Rb Connexin 30 (Invitrogen, 1:100), Ms Connexin 32 (Chemicon International, 1:50), Rb Connexin 36 (Zymed, 1:50), Anti-APC (Ab-7) Ms (CC-1) (Calbiochem, 1:100), Ms NF200 (NO142, Sigma, 1:250), Rb MBP (AB980, Millipore, 1:1000), Ms GFAP (MAB3402, Millipore, 1:250). Slice sections were washed three times in 1x PBS and treated with fluorescent Alexa Fluor 488 goat anti-rabbit (Molecular Probes,
1:1000) and Alexa Fluor 568 goat anti-mouse (Molecular Probes, 1:300) secondary antibodies. After incubating with secondary antibodies overnight at 4 °C, slices were washed in 1x PBS and coverslipped with Mowiol containing DAPI. Specificity of staining was confirmed by omitting the primary or secondary antibodies (data not shown). Imaging of spinal cord tissues was conducted on the Zeiss LSM510 Laser Confocal Microscope using the 63x objective and colocalisation analyzed with ImageJ 1.44 Image Processing and Analysis in Java program.

3.7 Statistical Analysis

All data are reported as mean ± SEM. Statistical significance is concurred at (p<0.05) using student’s t-test and two-way repeated measures ANOVA with Student-Newman-Keuls Method for post-hoc analysis (SigmaPlot SPSS statistical package).
Table 2. Primers for real-time PCR

(Karimi-Abdolrezaee et al., 2004; Eftekharpour et al., 2005; Mylvaganam et al., 2010)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
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</table>
| Cx 30 | fw 5’-CTTCATCTTCCGAGTCATGATCCT-3’  
        | rev 3’-CCTGCTCATCTCCCCACACT-3’ |
| Cx 32 | fw 5’-CCTCCGCGATCTGCATTATC-3’  
        | rev 3’-GCCCGGATGAGGTACAC-3’ |
| Cx 36 | fw 5’-GGTGGGAAGCAAGCGAGAAG-3’  
        | rev 3’-TGGAGCACCCTTGACAA-3’ |
| Cx 43 | fw 5’-CCCCGACGACAACCAGAAT-3’  
        | rev 5’-GGCTAATGGCTGGAGTTCATG-3’ |
| ß-Actin | fw 5’-CGTGCCTGACATCAAAGAGA-3’  
        | rev 5’-GGCCATCTCTGCTCGA-3’ |
Figure 2. Sucrose-gap setup and spinal cord longitudinal slice
Fig. 2 Sucrose-gap setup and spinal cord slicing

(A) Schematic diagram of the single sucrose-gap apparatus showing three adjacent chambers each perfused with isotonic KCl, isotonic sucrose and aCSF respectively, across which lies a piece of longitudinally sectioned spinal cord. The chambers are separated by silicone dividers that sit tightly around the white matter slice. (B) Schematic diagram of white matter longitudinal sectioning. Longitudinally cut slices from cervical to lumbar regions, mostly composed of white matter, would be either subjected to electrophysiological manipulation or used for RT-PCR, whereby the slice would be further sectioned as Pre, OGD, and Post OGD pieces after each respective condition.
Chapter 4
Results

4 Introduction

Experiments were performed on adult rat spinal cord white matter longitudinal slices at biologically relevant temperatures between 35-37 °C, with the goal to discern the effects two prominent gap junction blockers (carbenoxolone and 1-octanol) have on spinal cord function with and without ischemic injury. The 100 µM carbenoxolone and 300 µM 1-octanol doses were in line with previously established sufficient doses for blocking of junctions (Samoilova et al., 2003; Thompson et al., 2006; Solomon et al., 2003). Initially a dose of 1mM was attempted for 1-octanol, however it was found that at this dose the CAP was suppressed during baseline conditions (preliminary experiments, data not shown) and it was hard to discern the effects of the drug from the effects of OGD. As a result the concentration of 300 µM octanol was used and was enough to block gap junctions (Lee et al., 1994) without decreasing axonal conduction.

4.1 General characteristics of compound action potential conduction in spinal cord white matter before and after the blockade of gap junctions

Recordings of CAPs from spinal cord white matter segments submerged in aCSF show an average amplitude of 8.5 mV ± 1.9 mV SEM (Fig. 3) using the sucrose-gap recording apparatus. This amplitude is comparable to amplitudes in the sub-maximal range cited previously by our laboratory using the double and single sucrose gap techniques (Velumian et al., 2010; Sinha et al., 2006). With the sucrose-gap technique, higher amplitudes of CAPs often result from better electrical isolation between the isotonic KCl and aCSF fluid compartments. In an ideal situation there would be no mixing of the three compartments (Fig. 2A); however, some mixing inevitably occurs since the fluid can permeate the membrane of the spinal cord and use it as a medium to
reach other compartments. In all the experiments stable recordings of baseline CAPs were first achieved, before any anoxic/ischemic or drug experiments have been carried out. A stable recording is characterized as having relatively low signal to noise ratio, good isolation between the fluid compartments (each apparatus compartment tested separately to ensure that the solution remains within its designated well, separated from other wells with silicone dividers greased with vacuum grease), and having a steady CAP amplitude (small deviation in mV).

After the initial baseline recording period of 10 minutes, adding 300 µM 1-octanol for 20 minutes to aCSF resulted in no significant change to the CAP amplitude (Fig. 3A, p>0.05). Similarly, 100 µM carbenoxolone did not significantly alter the CAP amplitude (Fig 3B, p>0.05). As a result, at these concentrations, the gap junction blockers carbenoxolone and 1-octanol do not appear to significantly alter the amplitude of the signal conduction in the spinal cord.

4.2 CAP amplitude during OGD and the effect of gap junction blockers

Under control conditions (no addition of the blockers), during 30 min OGD, the amplitude of the compound action potential decreases until the signal disappears or has a very small amplitude (Fig 4Aa) that may subsequently be partially restored after a recovery period of 50 min. Throughout the recovery period, the amplitude of the CAP does not reach the initial mV value it had prior to the injury condition. The addition of carbenoxolone (Fig 4Ab) and octanol (Fig 4Ac) gap junction blockers did not fully prevent the decline in the CAP evident during OGD, however, the addition of these gap junction blockers significantly reduced the decline in CAP during the anoxic and ischemic insult. The recovery after the injury under gap junction blockers also did not completely restore the CAP amplitude to the original baseline values.
Figure 4B represents continuous recordings of CAPs under the two drug conditions and one control (no drug condition) with an initial baseline period of 10 min, followed by a 30 min OGD, and completed with a 50 min recovery period, plotted as percent change of CAP amplitude. The compound action potential declined rapidly within a few minutes after the onset of OGD and plateaued at 20 percent of the original CAP amplitude in the control condition (Fig 4B), 20 minutes after the onset of OGD. Similarly, the CAP decreased in the spinal cord slices bathed in aCSF and carbenoxolone or octanol, albeit the extent of the decrease was significantly less than that in the control condition and CAP plateaued between 30 and 40 percent of the original amplitude, under octanol and carbenoxolone respectively. After 30 min of OGD, the CAP amplitude began recovering once glucose and oxygen were reconstituted into the solution. This phenomenon was witnessed under the control condition, as well as with the two gap junction blockers used. The recovery between the conditions was not significantly different (Fig 4B).

The percent change of CAP amplitude for the various conditions in Figure 4B are plotted in a bar graph for three time points during early OGD (after the first 5 minutes of ischemia), late OGD (near the end of the OGD period, 30 minutes into ischemia) and during recovery (at the end of the 50 minutes). The differences in CAP amplitude between the control and drug conditions were the most significant during the initial onset of OGD, within the first 5 minutes (Fig 4C), (p<0.01) between control and carbenoxolone and (p<0.05) between control and octanol. While CAP under control conditions decreased to less than 30 percent of the original amplitude, the CAP amplitudes under the effect of carbenoxolone and octanol decreased to only 60 and 50 percent of the original amplitude respectively. This difference continued throughout and until the end of OGD between control and carbenoxolone conditions (p< 0.05); however, the difference was not significant between control and octanol conditions (p>0.05) (Fig 4C). There was no significant difference in the amplitudes of CAPs between the three conditions during recovery (Fig 4C).
Upon closer examination, the control and blocker conditions had different rates of decline within the OGD period (Fig 4D), such that the rate of decline of CAP was greater under the control condition than with either blocker (Table 3); however, the differences were not statistically significant. Although not significant, the biggest differences in the rate of decline occurred during the first 20 minutes of OGD, particularly within 5 to 10 minutes of the onset of OGD (Table 3. Time points 15-20) with the control condition having the highest percent change of CAP, followed by octanol and carbenoxolone showing the lowest change. This is indicative that the CAP was declining slower in the carbenoxolone condition, in octanol it was declining faster than carbenoxolone albeit not as fast as control, in which the CAP at 10 minutes of OGD declined to 50% of the amplitude seen after 5 minutes of OGD (Table 3. Time points 15-20).

4.3 Gap junction blockers alter Cx 43 and Cx 30 expression during OGD

The effect of anoxic/ischemic injury and gap junction blockers on connexin mRNA expression was examined for connexins 32, 36, 30 and 43. Overall, it appears that OGD alone does not alter their connexin expression (Fig 5A, B, C, D first column). The connexin 32 and connexin 36 mRNA expression further does not change with the addition of the blockers carbenoxolone and octanol (Fig 5A, B). In contrast, the presence of carbenoxolone alters mRNA expression levels of connexins 30 and 43 by causing a significant downregulation during OGD and recovery compared to pre-injury levels with the drug (Fig 5C, D second column). Similarly, octanol causes a downregulation of connexins 30 and 43 during OGD (Fig 5C, D), meanwhile the recovery under this drug does not differ from the baseline levels.
4.4 Localization of Cx 32, Cx 36, Cx 30 and Cx 43 in spinal cord tissue

Using laser confocal immunohistochemistry on longitudinally sectioned spinal cord slices, the connexins were probed for against markers for cells believed to house an abundant amount of these connexin proteins. It has been found that these connexins overlap with various proteins that are markers for glia or neuronal tissues in the spinal cord. In particular, Cx 32 was found to strongly overlap with myelin basic protein (MBP) – a marker for oligodendrocytes (Fig 6A). Little co-localization was noted between Cx 36 and NF200 (Fig 6B) – a marker for neuronal tissue that is controversially believed to contain the connexin 36 protein (Rouach et al., 2002; Oguro et al., 2001; Nagy et al., 2004). Upon closer examination, co-staining for Cx 36 and CC1 (a marker for oligodendrocytes), showed a fairly strong overlap, particularly in the region of oligodendrocytic somata (Fig 7A, B). As expected, Cx43 co-localized with glial fibrillary acidic protein (GFAP) commonly found in astrocytes (Fig 6C), and appeared to be the most abundant connexin type found in astrocytes when compared to staining for connexin 30 (Fig 6D). Although connexin 30 also overlapped with GFAP (Fig 6D), this connexin type appeared to mostly localize within the astrocytic cell body.
Figure 3. Gap junction blockers do not change the amplitude of CAP in non-injured tissue

A  Effect of 300 µM 1-octanol on CAP

B  Effect of 100 µM carbenoxolone on CAP
Fig. 3 Gap junction blockers do not change the amplitude of CAP in non-injured tissue.

(A) Relative change of CAP amplitude over time (30 min) in response to the addition of 300 μM 1-octanol (20 min, n=6). Data are shown as the mean ± SEM. To the right, sample traces of evoked CAPs prior to and during 1-octanol treatment. (B) Relative change of CAP amplitude overtime (35 min) in response to 100 μM carbenoxolone (15 min, n=7). Data are shown as the mean ± SEM. To the right, sample traces of evoked CAPs prior to and during the administration of carbenoxolone. The variation in CAP amplitude in response to both drug treatments was not significant (p = 0.376).
Figure 4. OGD induced reduction in CAP amplitude is attenuated with gap junction blockers
Fig. 4 OGD induced reduction in CAP amplitude is attenuated with gap junction blockers. (A) Traces of Compound Action Potential recordings for a. control, b. carbenoxolone, and c. octanol treated spinal cord slices under Baseline, OGD, and Recovery conditions. (B) Relative change of CAP amplitude over time, while subjected to 30 min OGD and 1 hour recovery. A comparison of the effects of 100 µM carbenoxolone (n=9) and 300 µM octanol (n=8) on the rate and amount of decline in CAP relative to no drug control (n=5) condition. The extent of decline in CAP during OGD was significantly decreased (p<0.005) with the addition of carbenoxolone compared to control. (C) Comparison of relative changes in CAP amplitude +/- SEM during OGD and recovery periods, between control (n=5), carbenoxolone (n=9) and octanol (n=8) treatments. The differences between changes in CAP peak amplitudes during OGD were highly significant (p<0.01) between carbenoxolone and control. There was a significant difference between octanol and control during early OGD. (D) Comparison of the rate of decline in CAP amplitude during OGD. The data is fitted to a polynomial regression function with r² values 0.9922-0.9959. The rate of decline is the fastest in the control condition, compared to either drug treatment.
Table 3. Comparison of the rate of decline of CAP during OGD

<table>
<thead>
<tr>
<th>Time points corresponding to Figure 4D (minutes)</th>
<th>Condition</th>
<th>Percent Change (Decline) (%) ± SEM (%)</th>
<th>Significance (between Control and either Drug group)</th>
</tr>
</thead>
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<tr>
<td>12-15</td>
<td>Control</td>
<td>20.7 ± 9.0</td>
<td>p=0.20</td>
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<td></td>
<td>Carbenoxolone</td>
<td>8.7 ± 8.9</td>
<td></td>
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<tr>
<td></td>
<td>Octanol</td>
<td>20.4 ± 11.4</td>
<td>p=0.49</td>
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<tr>
<td>15-20</td>
<td>Control</td>
<td>49.8 ± 10.7</td>
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<tr>
<td></td>
<td>Carbenoxolone</td>
<td>22.2 ± 11.2</td>
<td>p=0.07</td>
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<tr>
<td></td>
<td>Octanol</td>
<td>28.1 ± 13.3</td>
<td>p=0.14</td>
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<tr>
<td>20-25</td>
<td>Control</td>
<td>33.4 ± 13.6</td>
<td></td>
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<td>Carbenoxolone</td>
<td>18.0 ± 15.0</td>
<td>p=0.26</td>
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<tr>
<td></td>
<td>Octanol</td>
<td>32.0 ± 21.5</td>
<td>p=0.48</td>
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<tr>
<td>25-30</td>
<td>Control</td>
<td>28.4 ± 19.0</td>
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<td></td>
<td>Carbenoxolone</td>
<td>20.1 ± 15.5</td>
<td>p=0.37</td>
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<tr>
<td></td>
<td>Octanol</td>
<td>19.5 ± 33.3</td>
<td>p=0.42</td>
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<tr>
<td>30-35</td>
<td>Control</td>
<td>5.3 ± 30.4</td>
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<td>Carbenoxolone</td>
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<td></td>
<td>Octanol</td>
<td>2.9 ± 10.3</td>
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<tr>
<td>35-40</td>
<td>Control</td>
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<td>Carbenoxolone</td>
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</tr>
<tr>
<td></td>
<td>Octanol</td>
<td>3.9 ± 37.3</td>
<td>p=0.42</td>
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</table>
Figure 5. RT-PCR comparison of fold changes during OGD and recovery periods relative to baseline conditions for control, carbenoxolone, and 1-octanol treatments. Average fold change +/- SEM is normalized to β-Actin
Figure 5. RT-PCR comparison of fold changes during OGD and recovery periods relative to baseline conditions for control, carbenoxolone, and 1-octanol treatments. Average fold change +/- SEM is normalized to β-Actin.

(A) Comparison of fold changes for connexin 32 under control (n=15,10,14), carbenoxolone (n=11,9,10) and 1-octanol (14,9,12) conditions. There were no significant fold change differences for all conditions. (B) Comparison of fold changes for connexin 36 under control (n=11,10,10), carbenoxolone (n=8,6,8) and 1-octanol (12,8,12) conditions. There were no significant fold change differences for all conditions. (C) Comparison of fold changes for connexin 43 under control (n=9,11,14), carbenoxolone (n=13,8,9) and 1-octanol (15,9,13) conditions. There was a significant downregulation of expression during OGD and recovery with carbenoxolone treatment and (p<0.05) and during OGD only with 1-octanol treatment (p<0.05). (D) Comparison of fold changes for connexin 30 under control (n=7,5,10), carbenoxolone (n=6,7,8) and 1-octanol (16,12,15) conditions. Similar to connexin 43, there was a significant downregulation of expression during OGD and recovery with carbenoxolone treatment and (p<0.05) and during OGD only with 1-octanol treatment (p<0.05).
Figure 6. Laser confocal immunohistochemistry on longitudinal slices showing distribution of connexins and their overlap with various proteins localized in glia and neuronal tissues in the spinal cord.
Fig. 6 Laser confocal immunohistochemistry on longitudinal slices showing distribution of connexins and their overlap with various proteins localized in glia and neuronal tissues in the spinal cord.

(A) Strong overlap of connexin 32 (red), Myelin Basic Protein (MBP green) and DAPI (blue). The overlap appears to be the strongest in the outskirts of the oligodendrocytic tissue labelled by MBP. (B) Weak overlap of connexin 36 (green) with NF200 (red) and DAPI (blue). The connexin does appear to overlap with NF200, however, it is also scattered throughout the white matter. (C) Very strong overlap of connexin 43 (red), astrocytic glial fibrillary acidic protein (GFAP, green) and DAPI; Cx43 is an abundant astrocytic protein. (D) Selective overlap of connexin 30 (green), GFAP (red) and DAPI. These connexins are not as abundant as Cx43 and appear to localize mostly in the astrocytic cell body.
Figure 7. Laser confocal immunohistochemistry on longitudinal slices showing distribution of connexin 36 and its overlap with oligodendrocyte marker CC1 in the spinal cord
Fig. 7 Laser confocal immunohistochemistry on longitudinal slices showing distribution of connexin 36 and its overlap with oligodendrocyte marker CC1 in the spinal cord.

(A) Strong overlap of connexin 36 (green), CC1 (red) and DAPI (blue). Most overlap is seen in the oligodendrocyte somata. (B) Strong overlap of connexin 36 (green) with CC1 (red) and DAPI (blue). Most overlap is seen in the oligodendrocyte somata and processes.
Chapter 5
Discussion

5 Introduction

The secondary effects of spinal cord injury, characterized by anoxia and ischemia appear to severely impact axonal conduction in the spinal cord. We have shown that a 30 minute OGD period causes a severe decrease in the amplitude of the compound action potential that is not fully reversible after a one hour recovery period. This finding is consistent with previous studies showing a decline in CAP amplitude resulting from 30 min ischemic periods in the CNS (Utzschneider et al., 1991) and in particular in the spinal cord (Velumian et al., 2010). Typically the CAP plateaus 30-40 minutes after anoxia, as reflected in our finding, and the shorter the anoxic period the more rapid and higher is the recovery of the CAP magnitude (Stys, 1998). A 60 minute induction of in vitro anoxia reveals a recovery of at most 20-30% of the baseline CAP and fails to improve further (Stys, 1998). Since our model involved a 30 minute OGD, in line with this principle, it allowed for a higher recovery compared to that previously seen with a 60 minute induction.

As introduced previously, the recovery period may give insight into the extent of the injury on a molecular level. Since none of the CAPs under all the conditions recovered fully, the extent of the injury must involve more than impairment of electrogenesis and acute energy failure (Stys et al., 1990). Likely Ca$^{2+}$ influx, by means of the sequential failure of the Na$^+$-K$^+$ ion pump culminating in the reversal of Na$^{2+}$-Ca$^{2+}$ exchanger also contributed to the injury, and at the point of reoxygenation irreversible damage occurred. During reoxygénation, the neuronal membrane is already depolarized and has high internal Na$^+$, thus, restoring ATP causes the Na$^{2+}$-Ca$^{2+}$ exchanger to operate at an even higher rate, driving more Ca$^{2+}$ entry into the axon that it
cannot recover from (Stys, 1998). Different axons also react differently during and after the injury. The decrease in CAP is likely an indication of some fibers functioning normally while others are impaired. In fact, it has been shown that axons larger than 2 microns in diameter are more resistant to the initial effects of anoxia and they become progressively more sensitive to the increases in the injury after reoxygenation, and conversely, smaller fibers tend to behave in the opposite manner, being more sensitive to the initial onset of the injury, yet functioning normally after reoxygenation (Stys and LoPachin, 1996). There is also a possibility of free radical formation and necrosis; and it has been shown that the generation of free radicals after reoxygenation may further increase injury, by acting on mitochondria, cell membranes and ion transporters (Stys, 1998). However, a 30 minute OGD period might be too short to activate apoptotic pathways and in vivo apoptosis can occur days or weeks after a spinal cord injury (Tator and Fehlings, 1991). In acute traumatic spinal cord injury neuronal and glial apoptosis, examined with terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate-biotin nick and labeling (TUNEL), positive marker for cell death, showed that neurons were TUNEL positive 4-24 hours after injury and glia showed necrosis between 4 hours and 14 days after a moderate spinal cord injury (Liu et al., 1997). The time course of the experiment was also too short to note any long-term effects of the injury, i.e. only a one hour recovery period was looked at. The reason for the one hour recovery time point is directly influenced by the equipment and methodology of the experiment: maintaining a spinal cord longitudinal slice ex vivo may lead to insufficient internal perfusion of the slice (given a 1 mm thickness) and confounding effects of reduction in signal conduction due to a dying slice as opposed to a transient hypoxic injury.

The underlying causes of the decrease in signal conduction (Ca\textsuperscript{2+} influx) are in part due to glutamate excitotoxicity involving AMPA and Kainate glutamate receptors (Agrawal and
During ischemia the glutamate is supplied by open astrocytic hemichannels (Ye et al., 2003). It is yet to be directly confirmed that the glutamate emitted by astrocytes during ischemia binds to AMPA and Kainate receptors on oligodendrocytes; however, it presents a plausible explanation for an irreversible damage occurring during ischemia and involving Ca$_{2+}$. Since there have been no conclusive studies regarding the role of gap junctions and their constituent components, connexins, on axonal function after spinal cord injury, this thesis presents evidence that they do play an important role even though their mechanism of action is still unclear.

5.1 The role of gap junctions in the healthy and injured spinal cord: gap junction influence on axonal conduction

The novelty in this study comes from it being the first study to examine the role of gap junctions through the application of gap junction blockers on axonal conduction in the normoxic and ischemic spinal cord. Analysis of CAP recordings showed that there was no significant effect of either carbenoxolone or octanol on axonal signaling in the healthy spinal cord slice. The fact that gap junction blockers had no effect on CAP during the baseline (no injury) condition does not indicate that gap junctions have no effect on axonal conduction in the normal CNS, and in fact, it has been previously discussed that gap junctions allow for the transport of nutrients and metabolites between cells. Merely, our finding suggests that gap junctions do not have a detrimental function under normal conditions since blocking them did not increase the amplitude of the CAP. It is possible that not all the gap junctions were blocked and new ones may have docked with the glial and neuronal membranes, thus still allowing nutrient exchange. Another explanation is the possibility that the time frame of 30 minutes was not long enough to detect any
detrimental effects that could ensue from the lack of transfer of nutrients from gap junctions being blocked under normal conditions. A final possibility may lie with the concentration of the blockers used, such that although the carbenoxolone and octanol blockers have often been used at the 100 µM and 300 µM concentrations respectively, these concentrations may have been too low to block all the gap junctions and as a result, enough gap junctions remained open to fulfill nutrient and metabolite transfer between cells.

This is the also the first study to show that connexins do play an integral role in axonal conduction during SCI; in particular they moderate the suppression of axonal conduction. Applying the gap junction blockers carbenoxolone and octanol during OGD significantly attenuated the decline in CAP amplitude. These gap junction blockers also slowed the rate of decline of CAP during OGD. Both gap junction blockers were not sufficient to eliminate the damage from OGD, which is likely because gap junctions are not the sole source of initiation or propagation of injury, and as previously mentioned the ion exchanger, Ca\(^{2+}\) influx via the Na\(^+\)-Ca\(^{2+}\) exchanger and through internal stores, as well as the role of AMPA and Kainate are the major components of the injury. The novelty comes from the fact that gap junctions directly negatively affect axonal function. The conclusions are drawn for gap junctions as a whole, since two gap junction blockers with varying gap junction blocking capabilities have both shown the same attenuation of the decline of CAP. This is an improvement over studies using a single gap junction blocker, whereby the effect may have been due to the particular blocker used rather than the family of blockers it belongs to. Based on electrophysiological data, it appears that carbenoxolone is either a better blocker of gap junctions than octanol at the concentrations used, or it may have alternative targets than that of octanol and hence, is able to attenuate the decline in CAP to a greater extent. However, since both blockers improved axonal function during OGD and both share gap junction blocking in common, it is likely that the main observation of
improvements in axonal conduction comes from the gap junction blocker effect on various gap junctions in the spinal cord tissue. In reference to the rates of decline of CAPs during OGD, although not statistically significant, they do appear to have a trend with just the OGD condition having the initial highest rate of decline and carbenoxolone the lowest. Perhaps if sampled at smaller time points such as minute to minute account of the rate of decline there could be statistical differences that have not been detected once the data was pooled into larger sampling intervals.

In order to explain the mode of action of octanol and carbenoxolone it is important to understand how gap junction blockers work in general. There are several methods to block a gap junction channel: through binding to the channel and affecting its conductance, altering the lipid environment around the channel and by changing the intracellular pH or Ca\(^{2+}\) concentrations (Rozenthal et al., 2001). Gap junction blockers typically utilize intracellular acidification which is enough to close gap junctions and is often achieved by acidic gap junction blockers, and cell uncoupling via lipophilic blockers like octanol (Rosenthal et al., 2001). Acidification closes gap junctions via the amino (N) terminal histidine residue in the cytoplasmic loop of connexins, which reacts to acidification –induced channel closure, and acts as a binding site for a region in c-terminus (Rosenthal et al., 2001). When choosing gap junction blockers it is also important to consider their efficacy (magnitude of the blockade) and their potency (concentration needed to uncouple gap junctions, usually reported as concentration of the drug at which it is half-maximally effective (Rosenthal et al., 2001). Since gap junction blockers use different mechanisms to uncouple gap junctions it is hard to compare their efficacies and potencies to each other if they lie outside of the same method of gap junction modification. For example, it is hard to compare carbenoxolone and octanol in terms of efficacy, while for example in the lipophilic alcohol group the efficacies can be compared to each other and octanol/halothane efficacies
surpass that of oleic acid (Rosenthal et al., 2001). Although controversial, some blockers may act by phosphorylating connexin proteins and as previously introduced this could be a functional way to block gap junctions since phosphorylation keeps gap junction channels in a closed state (Saez et al., 2003). Carbenoxolone acts on gap junctions by mediating concentration-dependent inhibition of junctional conductance and is efficient at blocking junctional current; however, even at its highest concentration typically used (100 µM) it does not fully block gap junctions and at concentrations above 75 µM has the potential of being irreversible (Rosenthal et al., 2001). However, the exact method that carbenoxolone uses to reduce junctional conduction, whether by binding to cytoplasmic loop or C-terminus or inducing connexin changes such as phosphorylation remains unclear; however, based on the results in this study, its method of altering gap junction function appears to work better than that of octanol.

New studies may shed light on the exact location gap junction blockers may be targeting and direct evidence that alteration of this location results in direct neuroprotection during injury. Intramolecular loop and tail interactions are essential for connexin 43 hemichannel activity and interference with the interaction between cytoplasmic loop and carboxy-terminal for Cx43 leads to channel inactivity (Ponsaerts, et al., 2010). A recent study showed that peptides corresponding to the last 10 amino acids at the carboxy terminal or to the cytoplasmic loop prevented inhibition of cx43 hemichannel activity resulting from an injury involving high intracellular Ca²⁺; as a result, this intracellular loop-tail interaction is essential for proper function of Cx 43 (Ponsaerts et al., 2010) and may be targeted by various blockers in order to inactivate the gap junction channels. It has also been shown that restoring the tail-loop interaction can also restore activity of non-functional hemichannels, thus showing that transiently limiting the interaction of intracellular gap junction components can reversibly block gap junction channels. A further recent study also implicates the c-terminal of gap junctions and shows a direct correlation of this
component to mediating injury (Kozoriz et al., 2010). Particularly, the C-terminal region was shown to be important for channel activity and protection from cerebral ischemia, and elimination of which (via truncated connexin 43 with no C-terminus), reduces coupling between astrocytes, and enhances injury after stroke by propagating cell death (Kozoriz et al., 2010).

Overall, it appears that the c-terminus and the intracellular loop are important for channel function in connexin 43 (Ponsaerts et al., 2010). The presence of the C-terminus is neuroprotective during injury (Kozoriz et al., 2010). Thus, it would seem that if the gap junction blockade was through inactivation of the link between the c-terminus and intracellular loop (which effectively halts channel activity) then based on Kozoriz et al., 2010, an exacerbation of ischemic injury should occur. However, there are a few caveats. Connexin 43 coupling and expression was reduced as a result of c-terminus ablation, which could mean that fewer astrocytic channels remained in the tissue. Knocking out connexin 43 completely results in a larger ischemic infarct (Nakase et al., 2003) and similarly Kozoriz et al., 2010 showed that a C-terminus ablation tends to cause the same injury. In agreement with this study we found that connexin 43 mRNA expression is reduced during ischemia with the addition of blockers; however, in contrast, this appears to be neuroprotective on axonal conduction. The difference between these findings could be that:

a. In the Kozoriz et al., 2010 study all connexin 43 subunits were dysfunctional while in our study, gap junction blockers rarely affect all gap junctions and a few functional gap junctions remained; these few could have balanced the passage of nutrients and lactate at the onset of injury showing a net beneficial effect.

b. The differences in the timeline and methodology. We used ex vivo longitudinal slices and OGD recordings to determine immediate changes resulting from the
injury; meanwhile, Kozoriz et al., 2010 looked at the infarct volume 4 days after middle cerebral artery occlusion and may perhaps indicate a more long-term effect.

c. In our study a broad range of all CNS gap junctions have been targeted and perhaps it is the blockade of interaction with other tissues that conferred neuroprotection as opposed to solely inactivating one gap junction type, as is the case with altering the composition of one connexin.

d. By the same mechanism as above, removing the coupling of astrocytic connexin 43 gap junctions, by inactivating all C-termini, could have left other binding partners (such as oligodendrocytes) uncoupled – causing a leak in ionic gradient and loss of homeostasis in those tissues.

e. By removing the functionality of Cx43 yet leaving Cx30 they have not eliminated the propagation of injury during ischemia and possibly Cx 30 was upregulated to compensate for the lack of functional Cx 43.

f. Gap junction blockers Carbenoxolone and Octanol may not be altering the C-terminus or its interaction of the connexin 43.

There are numerous possibilities for the differences in findings, especially since the animal model, method of injury, location of injury and timeline differed vastly; however, it is clear that connexin 43 is an important gap junction connexin in the CNS that is directly involved in ischemic injury and alterations of which directly impact the outcome of the injury. In order to be able to answer the question what aspect of connexin 43 is involved in protection or exacerbation of ischemic injury, the mode of blockade of gap junctions and the respective targets ought to be
explored. Those findings may shed light on the discrepancy between why some gap junction blockers may confer neuroprotection while others would exacerbate injury.

### 5.2 Changes in connexin mRNA

The underlying cause for protection derived from blocking gap junctions in the spinal cord is not well understood; however, the underlying molecular changes of mRNA expression may offer some insight. Connexin mRNA expression turnover has previously been shown to be quite rapid (Vanslyke and Musil, 2005; Hervé et al., 2007; Leithe and Rivedal, 2007). As a result, the potential underlying changes in connexin expression and regulation during injury may affect their function, which directly impacts axonal conduction.

We have found that there are no changes in mRNA expression of connexins 32, commonly found in oligodendrocytes, and connexin 36, believed to be proprietary to neuronal tissue (Oguro et al., 2001; Rouach et al., 2002; Rash et al., 2007a,b), both with and without injury with the application of gap junction blockers. This finding supports previous results (Oguro et al., 2001) showing that under all time points these two connexin subunits remained unchanged. One other previous study suggested that Cx 43 undergoes major changes in response to SCI and confirmed that Cx 32 and Cx 36 mRNA does not change (Lee et al., 2005). The lack of mRNA change in these connexins during ischemia or with the addition of blockers could signify their lack of involvement in OGD, lack of self-regulation, or the inability of the gap junction blockers to block these particular connexins, e.g. lack of access to connexin 36 if the connexin is situated in the paranodal region. However a likelier explanation may be that these connexins have higher turnover rates than the other connexins surveyed and the short time period of incubation in this study of less than 2 hours may not have been enough to detect any mRNA changes. Another
possibility is the lack of abundance of these connexins, particularly true for connexin 36, in the spinal cord slices and as a result any minute changes were not detected.

Interestingly, connexins 30 and 43 are unequivocally localized to astrocytic tissue (Parys et al., 2010; Koulakoff et al., 2008; Nagy et al., 1997), appear to have downregulated mRNA expression during OGD with the administration of gap junction blockers. This is a novel finding because until now there has been no consensus on whether connexin 43 is upregulated or downregulated during ischemia in the spinal cord (Hossain et al., 1994; Rouach et al., 2002). There are no similar studies showing Cx 30 involvement during injury, which could potentially reveal whether the changes are inherent to connexin 43 or could be general of the astrocytic population. Hence, our study not only offers evidence that SCI causes major changes to Cx 43 mRNA but further implicates Cx 30. Moreover, implicating connexin 43 in ischemic injury is greatly supplemented by concurring evidence from connexin 30 mRNA that is in agreement with connexin 43. Not only does this finding suggest that the mRNA downregulation is targeted to astrocytic connexins, but is also one of the first to show regulation of mRNA expression of connexin 30 in the spinal cord.

There may be some indication from previous studies that downregulating astrocytic connexins, particularly Cx 43, is beneficial during SCI (Mori et al., 2006; Cronin et al., 2008). We have found that blocking gap junctions using both carbenoxolone and octanol downregulates Cx 43 and Cx 30 mRNA expression in the spinal cord during OGD. We have also found that this blockade salvages CAP amplitude during the injury. It is possible that blocking the astrocytic gap junctions during injury has caused a downregulation in their mRNA expression, which appears to be beneficial to axonal conduction during SCI. To further support this hypothesis, it has been found that Cx 43 gap junctions are able to modulate transcription and under gap
junction blocker oleamide, alter gene transcription in favour of transcription repression (Stains et al., 2003).

The choice of gap junction blockers may also play a role in mRNA expression. The variable mRNA expression depending on the gap junction blocker used may reflect the impact gap junction blockers have on axonal function. As previously mentioned, carbenoxolone seems to have had a stronger effect on the attenuation of the decline of CAP than octanol. Carbenoxolone also caused a downregulation of connexin 30 and 43 mRNA during the recovery period, unlike octanol. Perhaps the stronger effect carbenoxolone has on CAP during ischemia may correlate with its ability to maintain downregulated expression of connexin 43 and connexin 30 mRNA following the injury. Although it’s not possible to directly compare the amount of downregulation between the two blockers to each other in this experiment, it is a possibility that carbenoxolone caused a bigger downregulation of astrocytic connexins than octanol. Further experiments directly examining the mechanism of gap junction blocking by octanol and carbenoxolone and a direct comparison of the amount of downregulation each blocker achieves under ischemic and post-ischemic conditions are required to be able to answer this question.

Overall, we still have limited knowledge of connexin expression during injury in the spinal cord and it is worth further investigating the role of other connexins, particularly another astrocytic connexin 26 to see whether the changes in astrocytic mRNA expression seen in these experiments can be replicated.

5.3 Location of connexins in spinal cord white matter

The final finding of our study centers around the location of the connexins 32, 36, 43 and 30. As expected, connexins 30 and 43 have co-localized with astrocytic marker GFAP and appear
throughout astrocytes. In particular, Cx 43 does appear to be dispersed and concentrated more than connexin 30, affirming the proposition that connexin 43 is the most abundantly expressed connexin in the spinal cord (Lee et al., 2005). Connexin 32, also as predicted, has co-localized well with MBP, confirming its location in oligodendrocytic tissue. The most interesting and novel finding relates to the location of connexin 36. Previously thought to be located in neuronal tissue (Oguro et al., 2001; Rouach et al., 2002; Nagy et al., 2004; Rash et al., 2007a,b), connexin 36 co-localized poorly with NF200 (a neuronal marker) and surprisingly, connexin 36 co-localized well with CC1 (a marker for mature oligodendrocytes). In favour of this finding, Dobrenis et al., 2005, has found that microglia express functional connexin 36, and Parenti et al., 2002, postulated their possible expression in immature oligodendrocytes. Since connexin 36 appears to form functional gap junctions between microglia and neurons (Dobrenis et al., 2005) it provides a link between glia and neurons that is worth investigating during injury. Such a link might affect the efficiency of CAP transmission in the spinal cord, especially since we have found astrocytic gap junctions to be an important link connecting their expression to signal conduction during OGD and there already are notable interactions between gap junctions of astrocytes and oligodendrocytes following SCI (Ye et al., 2003; Kanelloupolos et al., 2000).

5.4 Limitations of the Study

There are some limitations that need to be acknowledged and addressed regarding the present study. To begin, the exact mechanism of action of gap junctions during injury that underlies their effect on axonal conduction has not been determined. Nor is there a concrete correlation of the effect of gap junction blockers on axonal function and subsequent change in connexin mRNA expression. I hypothesized, based on previous research, that the mechanism involves glutamate excitotoxicity and interaction of open astrocytic hemichannels (Ye et al., 2003) with
oligodendrocytic glutamate receptors. The effects of excitotoxicity likely further amplify the damage following the dissolution of ionic gradients; however, the exact mechanism is not known and may instead involve ATP-induced excitotoxicity (Domercq et al., 2010). Perhaps a comparison of measurements of the amount of extracellular ATP versus extracellular glutamate following OGD with the addition of gap junction blockers, as well as the effect of combining either glutamate receptor antagonists or P2X7R antagonists (blocking ATP) (Domercq et al., 2010) with gap junction blockers on CAP could show which of the two mechanisms appears to have a greater role.

The sucrose-gap technique, used to record axonal conduction, provides an effective and practical method for long-lasting testing of the effects of drugs on membrane activity in vitro and retrieving quantitative measurements of action potentials in these preparations. However, there is a limitation worth noting: the sucrose-gap technique provides an estimate of the real values of CAP amplitudes, the accuracy of which greatly depends on the quality of separation between the KCl and aCSF compartments and the shunting effect (Mert, 2007). In order to reduce the mixing of solutions, silicone grease and silicone dividers were used to seal the spinal cord slice in position and to avoid the diffusion of solutions between the wells. To minimize the diffusion of solutions through the inside of the spinal cord white matter, the separating well had constant perfusion of non-ionic, isotonic sucrose solution of high specific resistance, which also allowed for CAPs and membrane resting potentials (MRPs) to be recorded separately from the KCl and aCSF wells respectively.

Studying the effect of blocking gap junctions on a molecular level is further complicated by the lack of specificity of known gap junction blockers. In the present study, I have attempted to reduce the contribution of other channels targeted by the blockers or the influence of the
properties of the drug itself on axonal conduction by testing more than one gap junction blocker and confirming their lack of effect on axonal conduction under control conditions. There is also a possibility of gap junction blockers having a different effect on spinal cord white matter \textit{in vivo} as opposed to \textit{in vitro}, and further studies can focus on exploring any differences between the two preparations.

\section*{5.5 Next Steps and Future Directions}

Characterizing the role of gap junctions in secondary spinal cord injury is an important first step to achieving a complete picture of the underlying molecular mechanisms of this injury. As alluded to previously, it is important to replicate these findings \textit{in vivo}, before extrapolating the results to clinical trials. If, indeed, the detrimental role of gap junctions during secondary spinal cord injury can be replicated \textit{in vivo}, a pharmaceutical drug, Tonabersat, with demonstrated activity as a gap-junction inhibitor (Silberstein, 2009), has already undergone clinical trials. The drug targets neuronal-glial interactions during cortical spreading depression in the genesis of migraine aura and was found to be well tolerated with no cardiovascular effects (Silberstein, 2009). Using this gap-junction inhibitor may solve the issue present with targeting the glutamate excitotoxic cascade, namely, that glutamate antagonists are plagued by clinically intolerable side effects (Alix, 2006).

This study can also be further extended to incorporate other connexins present in the spinal cord, such as those present in microglia or other connexins in oligodendrocytes, astrocytes and neurons. In particular, the present study has shown the importance of the astrocytic connexin 30 which can be a useful target for any studies implicating connexin 43. The role of pannexins during ischemia in addition to connexins can be further explored. In regards to methodology, the
effect of gap junctions on a single axon, as opposed to CAP derived from bundles of axons, may be looked at using the patching technique, \( \text{Ca}^{2+} \) imaging may be used to detect changes in \( \text{Ca}^{2+} \) levels in different cells as a result of the application of gap junction blockers, and dye coupling studies could hint at the cells involved in the excitotoxic injury cascade.

Further focus can be dedicated to devising a longer timeline for the acute injury, implementing chronic injury and incorporating other aspects of traumatic spinal cord injury such as models of demyelination with traumatic insults to the spinal cord (i.e. contusion, compression, transection or mechanical deformation of the spinal cord prior to induction of ischemia), models of dysmyelination involving the shiverer mouse model (MBP gene knock out) (Sinha et al., 2006) or Long Evans Shaker (LES) dysmyelinated rat model (Eftekharpour et al., 2005), and models of remyelination, such as inclusion of stem cells after injury. In addition, various connexin knockouts and siRNA will be complementary to studies using gap junction blockers, as they would unearth any compensatory gap junction mechanisms and the effect of particular connexin subunits on axonal function during secondary spinal cord injury.
Chapter 6

Conclusion

Secondary ischemic spinal cord injury severely impacts axonal conduction. We have found that gap junctions reduce axonal conduction during oxygen-glucose deprivation and gap junction blockers aid in recovery through improvements in axonal function. Although the mechanism is unclear, we observed that blocking gap junctions using carbenoxolone and octanol has been beneficial in restoring some of the CAP amplitude lost due to OGD, likely in combination with ongoing downregulation of mRNA expression of astrocytic connexins 30 and 43. This study indicates that in the spinal cord, during injury, astrocytic gap junctional communication hinders recovery. The mechanism of action possibly involves astrocytes propagating cell injury via degradation of ion gradients and glutamate release. Further studies should focus on deducing the interaction between astrocytic gap junctions and oligodendrocytes and neurons to better understand the reason why blocking gap junctions during SCI is beneficial.
References


