The Effects of Bilirubin and its Oxidation Products on the Structure and Function of White Matter

by

Katarina Lakovic

A thesis submitted in conformity with the requirements for the degree of Master of Science

Institute of Medical Science
University of Toronto

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University of Toronto
2012

Abstract

Intracerebral hemorrhage (ICH) results in secondary brain injury caused partially by blood and its metabolites. Survivors of ICH are often left with severe disabilities, therefore, decreasing the extent of this secondary injury may improve functional outcome of patients. Incubation of mouse brain slices with partially oxidized bilirubin, a neurotoxic blood breakdown product, caused a dose- and time-dependent decrease in axonal function, suggesting a reduced number of conducting myelinated axons. These effects did not occur when tissue was incubated with non-oxidized bilirubin. Injection of bilirubin into the corpus callosum of mice caused functional impairment of unmyelinated axons; however, immunohistochemical staining of the tissue showed evidence of structural damage to both oligodendrocytes and axons. This data provides evidence for functional and structural damage to white matter in the presence of partially oxidized bilirubin. Therefore, diminishing the duration of presence of bilirubin and its oxidation in the brain warrants study as a means of decreasing secondary brain injury after ICH.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AB</td>
<td>Amlodipine besylate</td>
</tr>
<tr>
<td>aCSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AMPA</td>
<td>2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
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<tr>
<td>BOXes</td>
<td>Bilirubin oxidation products</td>
</tr>
<tr>
<td>CAP</td>
<td>Compound action potential</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTI</td>
<td>Diffusion tensor imaging</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalogram</td>
</tr>
<tr>
<td>ICH</td>
<td>Intracerebral hemorrhage</td>
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<tr>
<td>ICP</td>
<td>Intracranial pressure</td>
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<tr>
<td>GCS</td>
<td>Glasgow coma scale</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>GUT</td>
<td>Glucuronyl transferase</td>
</tr>
<tr>
<td>HO</td>
<td>Heme oxygenase</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
</tr>
<tr>
<td>LTD</td>
<td>Long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MCAO</td>
<td>Middle cerebral artery occlusion</td>
</tr>
<tr>
<td>MSM</td>
<td>Methylsulfonylmethane</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MK-801</td>
<td>Dizocilpine</td>
</tr>
<tr>
<td>NBQX</td>
<td>2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione</td>
</tr>
<tr>
<td>NMDG</td>
<td>N-methyl-D-glucamine</td>
</tr>
<tr>
<td>OB</td>
<td>Oxidized bilirubin</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>SAH</td>
<td>Subarachnoid hemorrhage</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TBI</td>
<td>Traumatic brain injury</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
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Chapter 1 Introduction

Intracerebral Hemorrhage

Intracerebral hemorrhage (ICH) is a type of hemorrhagic stroke that is defined as bleeding into the brain parenchyma. Bleeding must due to a ruptured cerebral blood vessel. Spontaneous ICH has a prevalence of about 15 in every 100,000 people (Sacco et al., 2009) and although it accounts for only 15% of all strokes (Xi et al., 2006), it has a very high mortality rate with only about 41% of affected individuals surviving for a year (Sacco et al., 2009). Furthermore, ICH results in significant tissue damage in and around the hematoma. Thus, individuals who are fortunate enough to survive are often left with severe disabilities, with only 20% of them regaining full independence (Parker, Jr. et al., 2010).

Unfortunately, there are few evidence-based treatments for ICH. Translational research and clinical trials have not been successful in finding effective pharmacological therapies which can prevent secondary injury, or decrease morbidity and mortality (Kellner and Connolly, Jr., 2010). Therefore, although there are a number of ongoing clinical trials (for review see Keep et al., 2012), the mainstay of treatment is still currently limited to supportive therapy such as monitoring vital signs, controlling blood pressure, reducing edema, and preventing re-bleeding and other secondary brain injuries (Parker, Jr. et al., 2010) (Balami and Buchan, 2012).

However, a multitude of complications occur after ICH including increased intracranial pressure, expansion of the hematoma, edema, breakdown of the blood–brain barrier, inflammation, oxidative stress, hydrocephalus, apoptosis, necrosis, atrophy, activation of the complement system, spreading depolarization and ischemia (Keep et al., 2012). The toxicity of blood breakdown products has been recognized as one of the major contributors to secondary injury which occurs after ICH; much work has been done to understand the effects of haemoglobin and iron, as well as whether decreasing the quantities of these molecules after ICH improves functional outcome (Nakamura et al., 2003) (Wagner et al., 2003) (Wu et al., 2003) (Hua et al., 2006) (Hua et al., 2007) (Song et al., 2007) (Katsu et al., 2010) (Wu et al., 2010). However, the effects of bilirubin have largely been overlooked in the context of ICH, despite the fact that free (i.e., unconjugated) bilirubin is known to cause serious neurological injury in humans (Roy-
Chowdhury et al., 2008). Although it is important to consider how acute mortality can be decreased after ICH, this work focused on the potential contribution of bilirubin and its oxidation products to the secondary injury, which occurs in the days following this type of hemorrhagic stroke.

**Bilirubin**

**Overview**

The major source of bilirubin in the body is the breakdown of the heme moiety from aging red blood cells in the spleen (Roy-Chowdhury et al., 2008). Bilirubin IXα (1,8-dioxo-1,3,6,7-tetramethyl-2,8-divinylbiladiene-a,c-dipropionic acid) has a tetrapyrole structure which is able to form hydrogen bonds within itself making the molecule largely insoluble in aqueous solutions (Roy-Chowdhury et al., 2008). However, bilirubin is not a lipophilic compound but rather an amphipathic molecule which aggregates with phospholipids due to its polar characteristics (Brodersen, 1979). This may explain why there is bilirubin transport across membranes and deposition in tissues in patients with hyperbilirubinemia (Brodersen, 1979).

Hemoglobin breaks down through the same mechanism in the brain as in the spleen. Initially, heme is released and is first broken down into biliverdin by the enzyme heme oxygenase (HO); this step also releases an iron atom. Biliverdin is then further reduced to bilirubin by biliverdin reductase (Fig. 1). Since bilirubin is sparingly soluble in water it binds tightly to albumin in order to be transported to the liver where it is conjugated by glucuronyl transferase by breaking the internal hydrogen bonds and forming, most commonly, bilirubin IXα-diglucuronide (Roy-Chowdhury et al., 2008). Conjugated bilirubin is water-soluble and is the form excreted in bile and urine. However, when molar concentrations of bilirubin exceed the binding capacity of albumin, the concentration of free unconjugated bilirubin increases, which is known to have a multitude of toxic effects (Brodersen, 1979). In newborns, toxicity is seen at a concentration of bilirubin of 250 to 350 µM, or a ratio at or above 0.7 bilirubin:albumin (Brodersen, 1979).

The effects of unconjugated bilirubin on the brain have largely been studied in the context of neonatal hyperbilirubinemia, a condition where the liver of newborn infants is unable to conjugate bilirubin in sufficient amounts. The effects of bilirubin after ICH have not been
extensively explored, however, it seems appropriate to apply the knowledge gained from hyperbilirubinemia research to better understand the pathophysiological mechanisms which may occur after intracerebral hemorrhage. Of course, it must be kept in mind that there may be differences in how the immature and the adult brain respond to blood breakdown products, as well as that the effects of bilirubin may be different when there is a local rather than a systemic increase in concentration.

![Figure 1: Hemoglobin Breakdown](image)

In infants, light therapy is often used to treat hyperbilirubinemia, where the child is exposed to ultraviolet (UV) light. The Z,Z conformation of bilirubin has the strongest hydrogen bonds, however, when the molecule is exposed to light, the conformation changes to ZE, EZ, or EE isomers which lack the internal hydrogen bonds and can therefore be excreted in bile without conjugation (Roy-Chowdhury et al., 2008). Light therapy thus is an intriguing potential therapy for ICH, however, it cannot be as easily applied to these patients as the unconjugated bilirubin is not uniformly distributed throughout the blood stream, but rather concentrated in and around the hematoma and it is inside the closed skull that is not penetrable by UV light.

In infants, once the binding capacity of albumin is surpassed, unconjugated bilirubin crosses the blood brain barrier (BBB) and is deposited in the brain (Roger et al., 1993). Generally this is only seen in neonates, probably in part due to the immaturity of the BBB. If the bilirubin levels are elevated for extended periods of time it can lead to death or kernicterus, a severe neurological
condition characterized by mental retardation, seizures, movement disorders and speech difficulties (McCandless D.W., 2010). Autopsies show that bilirubin staining is present in the neurons in brains of infants, most commonly in the corpus striatum and hippocampus. Spontaneous ICH also most commonly occurs in the striatum (Xi et al., 2006). Whether extrapolation of data acquired from studies of neonatal hyperbilirubinemia may be appropriate for ICH in adults due to a common anatomical location is unknown. It has been found that in chronic kernicterus there is a significant loss of neurons which are replaced by astrocytes, creating a glial scar; this contributes to the various presentation of kernicterus symptoms depending on the anatomical location (McCandless D.W., 2010). Since ICH results in significant damage to the BBB, the effects of bilirubin may be even greater and more rapid, as it can access the neuronal tissue more easily. Furthermore, neonatal hyperbilirubinemia has been associated with cerebral palsy, and these individuals show white matter damage on magnetic resonance imaging (MRI) scans (Gkoltsiou et al., 2008).

Although uncommon, disorders of bilirubin metabolism resulting in hyperbilirubinemia can occur in older children and adults (McCandless D.W., 2010). There are four such conditions: Rotor syndrome, Gilbert disease, Dubin-Johnson syndrome, and Crigler-Najjar syndrome. Whereas Rotor syndrome is asymptomatic and characterized by conjugated hyperbilirubinemia, the latter three do involve increased levels of unconjugated bilirubin (McCandless D.W., 2010). Gilbert disease is marked by a decrease in hepatic bilirubin uptake, and a decreased ability to conjugate bilirubin due to a defect in glucuronyl transferase (GUT). Although no neurological symptoms have been associated with this disorder, adverse effects such as fatigue, abdominal pain, and muscular weakness do occur (McCandless D.W., 2010). In Dubin-Johnson syndrome, there is an increase in both conjugated and unconjugated bilirubin, which occurs as a result of a decreased ability to transport organic anions into bile, and hence a regurgitation or recycling of bilirubin from hepatocytes back into the blood (McCandless D.W., 2010). Fortunately, this disease is generally asymptomatic, although jaundice can occur.

Crigler-Najjar syndrome occurs as a result of a single base pair mutation in the gene coding for the GUT protein, however, there are two subtypes of the disease (McCandless D.W., 2010). Type I is characterized by a complete lack of GUT activity whereas in Type II the activity of the enzyme is significantly reduced, and therefore, often noticed only later in life, as it doesn’t
usually present with symptoms of kernicterus because the levels of bilirubin are generally lower. Although type I is usually diagnosed very early on, in rare cases it can present later in adulthood. In one case study, a woman who acquired jaundice a few days after birth without subsequent treatment, eventually developed a multitude of problems such as bradykinesia, hyperreflexia, rapid alternating movements, myoclonic jerks, as well as seizure activity (McCandless D.W., 2010). The patient’s serum bilirubin was between 25 and 34 mg/100ml and the fluctuations correlated with neurological deficits with which she presented. Although treatment was effective at temporarily decreasing the levels of bilirubin, jaundice was still prominent suggesting that bilirubin had been deposited in her tissues, which was confirmed at autopsy. However, it is interesting that the decreases in bilirubin levels correlated with an amelioration of symptoms as this suggests that the toxic effects of bilirubin may not be irreversible.

It has been hypothesized that bilirubin induces neurotoxic effects because of its ability to bind to neuronal membranes and alter the structure, and therefore function, of nearby neuronal receptors (Hoffman et al., 1996). One mechanism that was proposed was functional N-methyl-D-aspartic acid (NMDA) receptor modification as observed in newborn piglets which were exposed to bilirubin (Hoffman et al., 1996). However, toxicity caused by bilirubin may not occur specifically through the disruption of NMDA receptors. In one study performed in rat pups, NMDA receptor antagonist MK-801 did not protect the brain from bilirubin toxicity in vivo or in vitro (Shapiro et al., 2007). The discrepancy between these studies may have occurred for a number of reasons such as a difference in methodology or species. However, since it is unknown if and how bilirubin interacts with membranes, and considering its affinity for polar molecules such as phospholipids, it should not be discounted that bilirubin can interact with cellular membranes and cause toxicity by disrupting membrane proteins including ion channels and various receptors.

Animal research has shown that bilirubin may also have a toxic effect by inhibiting respiration of cerebral cells, incorporation of amino acids into proteins, and uncoupling of oxidative phosphorylation (Cowger et al., 1965). Additionally, bilirubin may have an inhibitory effect on glycolysis, as evidenced by a decrease in glycolytic intermediates (Katoh-Semba, 1976). Furthermore, there is evidence that bilirubin impairs mitochondrial functioning, which may be more severe in older animals (Jew and Williams, 1977). It is of note that ICH occurs more
frequently in older individuals, therefore, the effect of bilirubin theoretically may be more pronounced in the aged brain. Accumulation of glycogen is observed in mitochondria, which become enlarged and form vacuoles (Jew and Williams, 1977). This may be due to disruption of the mitochondrial membrane, and appears to occur even without obvious damage to the neurons, suggesting that this may be one of the earliest steps of bilirubin toxicity (Jew and Williams, 1977). This is consistent with findings that bilirubin primarily interacts with phospholipids as opposed to proteins thereby impairing membrane-linked functions by physically disrupting cellular membranes (Mustafa and King, 1970).

Interestingly, in clinical cases of ICH, it has also been found that mitochondrial dysfunction occurs in the perihematomal tissue (Kim-Han et al., 2006). Levels of oxygen consumption decreased on average by 40% in perihematomal tissue compared to normal tissue and this decline progressed with time with virtually no oxygen being consumed by the third day. These data, in conjunction with a large body of work that has not identified ischemia around human ICH suggests that the decrease in oxygen metabolism seen after ICH is due to mitochondrial dysfunction as opposed to ischemia (Kim-Han et al., 2006). The mechanism underlying this deficit has not been investigated, however we hypothesize that it is at least in part due to disruption caused by bilirubin.

**Functional Impairment**

Animal research has implicated unconjugated bilirubin in various functional disruptions. For example, effects of bilirubin, particularly on hippocampal activity and memory were found as evidenced by impairment of population spikes, long-term potentiation (LTP) and long-term depression (LTD) in rat hippocampi *in vivo* (Zhang et al., 2003). Further evidence indicated that these effects were found to be mediated through cleavage of NMDA receptors; these receptors are crucial for induction of synaptic changes that are associated with induction of LTP and LTD (Chang et al., 2009). The mechanism was shown to involve calpain-mediated proteolytic cleavage (Chang et al., 2009). These findings are interesting in view of the clinical deficits in memory found in individuals with bilirubin-induced kernicterus.
Furthermore, clinical findings of impaired sensory processing in kernicterus correlate with findings from animal models. For example, behavioral studies found differences in bilirubin-treated rats compared to control animals, suggesting a difference in stimulus processing (Hansen et al., 1987). The mechanism by which bilirubin causes this disruption is unclear, however it may be due to altered neurotransmission since bilirubin has been found to impair the presynaptic neurotransmitter reuptake mechanism and decrease the response to depolarizing stimuli (Ochoa et al., 1993). Similarly, cochlear nucleus neurons were found to be affected by bilirubin through enhanced release of neurotransmitters due to presynaptic protein kinase A activation (Lou et al., 2010).

**Cell death**

Bilirubin toxicity leads to death of neurons (Gao et al., 2011; Grojean et al., 2000; Grojean et al., 2001; Rodrigues et al., 2002), oligodendrocytes (Genc et al., 2003) and astrocytes (Kumral et al., 2005; Silva et al., 1999). Although the exact mechanism through which cell death occurs remains to be elucidated, various components that are implicated have been found.

Bilirubin decreased cell viability in a concentration-dependent manner when cultured primary neurons were incubated for 96 hours (Grojean et al., 2000). This toxicity was attenuated by glutamate receptor antagonist MK-801 but not NBQX, suggesting a role for NMDA - but not 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid (AMPA) - receptors in the mechanism of cell death induced by bilirubin. Cycloheximide and caspase inhibitors also provided protection, supporting the hypothesis that bilirubin induces apoptotic cell death (Grojean et al., 2000).

Neuronal apoptosis caused by bilirubin in cultured cells also was prevented when the cells were co-incubated with ursodeoxycholate, a known antagonist of apoptosis which acts by preventing mitochondrial permeability (Rodrigues et al., 2002). This was further confirmed by incubation of isolated mitochondria with bilirubin which resulted in membrane permeabilization and cytochrome c release, both of which were effectively prevented by ursodeoxycholate and cyclosporine (Rodrigues et al., 2002). Unconjugated bilirubin was found to decrease cytochrome c oxidase activity by 20% at concentrations greater than 60 nM (Malik et al., 2010). It also caused translocation of Bax protein from the cytosol to the membrane, and caused mitochondrial
outer membrane permeabilization (Rodrigues et al., 2002; See Figure 2). This was accompanied by a decrease in mitochondrial membrane potential, an increase in cytosolic levels of cytochrome c, as well as degradation of poly(ADP-ribose) polymerase (PARP). Furthermore, previous work suggests bilirubin may directly interact with cell membranes, causing a disruption in lipid polarity and protein order, resulting in impaired mitochondrial functioning (Rodrigues et al., 2002).

Furthermore, inducing hyperbilirubinemia in baby mice resulted in an increase in free intracellular calcium (Ca$^{2+}$) and proapoptotic protease caspase-3 in neuronal cells, which were attenuated via an unknown mechanism when the mice were pre-treated with taurine for 4 hours (Gao et al., 2011). However, it was also found that lower levels of unconjugated bilirubin were able to induce cell death without an increase in intracellular Ca$^{2+}$ suggesting that bilirubin may cause toxicity through more than one pathway (Gao et al., 2011).

![Figure 2: Bilirubin Disruption of Mitochondria](image)

**Figure 2: Bilirubin Disruption of Mitochondria** When Bax protein is translocated to the mitochondrial membrane, cytochrome C is released. Cytochrome C in turn binds to apoptosis activating factor (Apaf)-1 which activates procaspase-9, leading to the formation of the apoptosome, release of caspases and initiation of apoptosis.

The viability of cultured rat oligodendrocytes incubated with bilirubin has been shown to decrease in a concentration- and time-dependent manner, indicating a toxic effect of bilirubin on
oligodendrocytes (Genc et al., 2003). Likewise, incubation of murine astrocyte cultures with healthy or hyperbilirubinemic sera from infants showed that cultures exposed to bilirubin exhibited cytotoxicity and astrocytic cell death in a concentration- and time-dependent manner (Kumral et al., 2005). The authors suggested that the cell death was apoptotic and occurred through caspase-3 activation. They further suggested that astroglial impairment facilitates neurodegeneration because of the supportive role of astrocytes for neurons. This hypothesis is supported by previous work which showed that bilirubin inhibited glutamate reuptake by astrocytes, causing cell death as excess glutamate in known to be neurotoxic (Silva et al., 1999).

Inflammation

Recently, the effect of bilirubin on inflammatory responses after hemorrhagic stroke have been acknowledged and explored. There are three kinds of HO; HO-1 is inducible by heme and stress, HO-2 is constitutively expressed in the brain, whereas HO-3 generally functions as a heme binding protein with little catalytic activity (Roy-Chowdhury et al., 2008). Since HO is necessary for reduction of heme to eventually produce bilirubin, it has been determined that blocking HO, such as with tin-protoporphyrin and tin-mesoporphyrin which irreversibly bind and inhibit HO, blocks the production of bilirubin (Roy-Chowdhury et al., 2008). Additionally, HO-1 knock-out mice were found to have significantly less leukocyte infiltration, microglia/microphage activation and free radical levels than control animals (Wang and Dore, 2007) suggesting that breakdown products of heme cause injury to the perihematomal tissue. Furthermore it has been found that treatment with unconjugated bilirubin results in excess glutamate levels, as well as proinflammatory cytokines interleukin(IL)-1β, IL-6 and tumor necrosis factor (TNF)-α (Fernandes et al., 2007).

Further experiments have elucidated that simulation of ICH in mice using autologous whole blood combined with unconjugated bilirubin, relative to blood with a control vehicle, resulted in concentration-dependent neutrophil activation (Loftspring et al., 2011). In cultures of isolated human neutrophils, it was found that low concentration of unconjugated bilirubin enhanced activation of neutrophils, promoted their degranulation, and increased levels of phosphorylated protein kinase C (Loftspring et al., 2011). Furthermore, intracellular adhesion molecule (ICAM), which is necessary for leukocyte migration, was increased on blood vessels after ICH (Loftspring
et al., 2011). Interestingly, it has been found that neutrophils significantly contribute to a multitude of secondary injuries after ICH such as BBB breakdown, axonal injury, and myelin fragmentation, as well as microglial and astrocyte activation (Moxon-Emre and Schlichter, 2011).

A mechanism through which bilirubin contributes to inflammatory processes has been put forth by Loftspring et al. (2010). They theorized that bilirubin and bilirubin oxidation products (BOXes) activate microglia and astrocytes which release proinflammatory cytokines and chemokines, and recruit peripheral leukocytes which enter the cerebrum. In turn, cytokines, leukocytes and activated glia contribute to injury through the production of superoxide, which not only causes injury, but is also a precursor to hydrogen peroxide, the hydroxyl radical and peroxynitrite. Microglial and astrocyte activation also causes release of proapoptotic factors such as TNF-α and IL-1β, and the death of these cells causes further increase in leukocyte migration, which is facilitated by the breakdown of the BBB. Therefore, inflammation causes increased blood flow, which increases edema and free oxygen species, which in turn facilitate further production of bilirubin and BOXes, creating a positive feedback loop (Loftspring et al., 2010).

**Oxidative Stress**

As mentioned, HO-1 is required for the first step in the breakdown of hemoglobin. After ICH, a significant amount of HO-1 is detected in the perihematomal area, particularly in microglia and endothelial cells (Wang and Dore, 2007) and reaches a peak at day 3 and 7 (Wang et al., 2011). Although a decrease in edema was not observed in HO-1 knockout mice, injury was clearly ameliorated as evidenced by a decrease in microglial activation, leukocyte infiltration, and levels of free radicals (Wang and Dore, 2007). Although this does not provide direct evidence for involvement of bilirubin, based on other findings that suggest that bilirubin plays a role in inducing inflammation, reduction of HO-1 levels may be therapeutic because it decreases the production of bilirubin.

Furthermore, the antioxidant copper-zinc superoxide dismutase (Cu/Zn-SOD) was also increased and correlated with HO-1 levels during the first 3 days after the ICH, whereas an increase in malondialdehyde (MDA) was detected and correlated with levels of HO-1 between 7 and 14
days post ICH. This suggests that HO-1 may trigger an antioxidant response initially after ICH, whereas prolonged increased levels of HO-1 result in oxidative damage (Wang et al., 2011). Again, although this study does not test the effect of bilirubin directly, if levels of HO-1 can be assumed to be directly correlated with the amount of bilirubin that is produced, these findings suggest a dual, time-dependent effect of bilirubin, with initial antioxidant properties, but toxicity with prolonged exposure and possible oxidation of bilirubin into BOXes.

**Antioxidant Properties**

Antioxidant properties of bilirubin have long been postulated. Hemoglobin is toxic to cells, therefore the body has evolved a mechanism to break it down (Everse and Hsia, 1997). The first breakdown product is biliverdin which can readily be excreted in bile, yet the human body expends more energy to further break it down into bilirubin, which as mentioned before, needs to be modified (using even more energy) in order to be excreted. A potential evolutionary benefit of this mechanism is the antioxidant properties of bilirubin (Sedlak and Snyder, 2004). Some evidence exists to support this prediction; for example, it has been shown that bilirubin is able to alleviate oxidative stress in mouse neuron cultures at a concentration less than 10,000 times the concentration of hydrogen peroxide (Dore and Snyder, 1999). One mechanistic hypothesis is that hydrogen peroxide oxidizes bilirubin to biliverdin, which can readily be reduced back to bilirubin by biliverdin reductase. This is supported by experiments which show that depleting cells of biverdin reductase triples the levels of reactive oxygen species (Sedlak and Snyder, 2004).

Evidence for bilirubin being an effective scavenger of reactive oxygen and nitrogen species has also been found in cultured human endothelial cells where silencing HO-1 and biliverdin reductase genes resulted in higher levels of oxidative stress (Jansen et al., 2010). It has further been found that bilirubin, but not biliverdin, induced the expression of guanosine triphosphate (GTP)-cyclohydrolase, a protective protein (Jansen et al., 2010). On a much larger scale, a longitudinal study looking at serum bilirubin levels for 10 years found that higher serum levels, although within normal range, were associated with lower cancer-related mortality, which the authors suggest may be due to antioxidant properties of bilirubin (Temme et al., 2001).
Although this evidence suggests that bilirubin expresses antioxidant properties, it is unlikely that these effects outweigh the toxicity caused in pathological conditions where local levels of bilirubin are abnormally high, such as in ICH, especially if such dramatic increases are not accompanied by increases in biliverdin reductase.

## Bilirubin Oxidation Products

Bilirubin oxidation products (BOXes) are organic compounds which form as a result of bilirubin oxidation. BOXes can be produced by nucleophilic attack of biliverdin and bilirubin with hydrogen peroxide, superoxide, nitric oxide or peroxynitrite radicals. Additionally free iron, released by heme degradation, interacts with hydrogen peroxide to produce hydroxyl free radicals which can also interact with bilirubin to generate BOXes (Clark and Sharp, 2006). These compounds were described by Clark and colleagues (Kranc et al., 2000)(Wurster et al., 2008). They include 4-methyl-5-oxo-3-vinyl-(1,5-dihydropyrrrol-2-ylidene)acetamide (BOX A), 3-methyl-5-oxo-4-vinyl-(1,5-dihydropyrrol-2-ylidene)acetamide (BOX B) both of which have a molecular weight of 179.2 m/z, and 4-methyl-3-vinylmaleimide (MVM) with a molecular weight of 135.9 m/z. MVM is not biologically active (Kranc et al., 2000)(Wurster et al., 2008).

Furthermore, inflammatory cells which are recruited to areas of tissue damage, such as after hemorrhagic stroke, act to clear the blood and cellular debris, however they are substantial sources of superoxide and peroxynitrite anion radicals (Clark and Sharp, 2006). The conditions that are required to form BOXes (namely HO, bilirubin, and oxidative stress) are present in the brain after hemorrhage, therefore their effects on the cerebral and vascular tissues are important potential pathological mechanisms and may warrant investigation.

![Figure 3: Bilirubin Oxidation Products (BOXes)](image-url)
Limited work has been done to examine the production and effects of BOXes after ICH; however, their presence in and around the hematoma has recently been acknowledged (Clark et al., 2008). Since BOXes were isolated and identified (Kranc et al., 2000), their structure and their role in subarachnoid hemorrhage (SAH) has been investigated. It has been confirmed that BOXes are found in the CSF of patients with SAH and their presence is correlated with occurrence of vasospasm (Pyne-Geithman et al., 2005). Further work elucidated that both BOXes, as well as cerebrospinal fluid (CSF) from SAH patients with vasospasm (CSF-v), have biological activity as evidenced by oxygen consumption and changes in vascular smooth muscle (Pyne-Geithman et al., 2008). The evidence suggests that these changes in smooth muscle occur due to effects of BOXes and CSF-v on expression of protein kinase-C (involved in muscle contraction) and Rho, a G-protein, which has been shown to mediate failure of muscle relaxation (Pyne-Geithman et al., 2008).

The mechanism of how they cause vasoconstriction has been partially elucidated. BOXes inhibit the Slo1 K\(^+\) channels, which are involved in vascular tone, by slowing the opening process of the channels (Hou et al., 2011). It is speculated that BOXes position themselves between the transmembrane helices and the cytoplasmic RCK1 domain, and stabilize the closed conformation of the ion conduction gate (Hou et al., 2011). They are likely further stabilized by the probable ability to partition well in membranes due to their planar and hydrophobic structure (Hou et al., 2011). Neither bilirubin nor MVM have vasoactive properties, unlike BOX A and BOX B, suggesting that the amine group is necessary for the alteration of the Slo1 channel (Hou et al., 2011). Although this finding is specific to vasospasm, the implications are of interest when considering the effects of BOXes on tissues after ICH.

In ICH, SOD, MDA, and BOXes are found in the hematoma within 8 hours of the ictus and these compounds are found at significant levels in the perihematomal white matter within 24 hours (Clark et al., 2008). Since BOXes can easily permeate membranes and have high biological activity, it is likely that they diffuse out of the hematoma. However, it is unclear whether they are diffusing as a result of edema and BBB breakdown, or if they are causing this damage (Clark et al., 2008). Therefore, simply removing the hematoma cannot be entirely successful unless performed very soon after the ictus, as the toxic chemicals will have already penetrated the surrounding tissue (Clark et al., 2008). It is of note that preliminary findings of the
clinical trial “minimally-invasive surgery plus rtPA for intracerebral hemorrhage evacuation” (MISTIE) has shown that this approach reduces the hematoma size significantly more than standard medical management (Morgan et al., 2008). Whether this intervention results in better patient outcomes will be evident in data obtained from the subsequent phases of the trial which are currently ongoing. It will be of interest how this therapy changes the cerebral levels of bilirubin and BOXes.

Although it is not clear exactly how BOXes affect the tissues within and surrounding the hematoma, the fact that they are able to permeate through membranes, interact with membrane channels, and that they are readily present after ICH warrants further investigation of whether and how BOXes are contributing to the damage, and whether their production can be reduced by decreasing the reactive oxygen and nitrogen (Lee et al., 2010) species which become elevated after hemorrhagic stroke.

**White Matter Damage**

Compared to ischemic stroke and other cerebral small vessel pathologies, patients with ICH show considerably larger volumes of white matter hyperintensities based on analyses of volumetric MRIs taken within 2 weeks of having a stroke (Rost et al., 2010). Furthermore, the extent of white matter damage is predictive of both early and long-term outcome, correlates well with the Glasgow Coma Scale (GCS) at admission, and with both 1 month and long-term mortality risk (Lee et al., 2010). This suggests that the white matter damage makes the brain more vulnerable to further insult as well as increasing the chances of mortality. White matter damage is associated with poorer cognitive outcome and a higher risk of stroke re-occurrence and post-stroke dementia (Lee et al., 2010). Additionally, white matter damage is of prognostic value as higher volumes of white matter hyperintensities correlate with greater ICH volumes and are associated with greater extent of hematoma expansion (Lou et al., 2010).

Neurons and white matter tracts within the hematoma are destroyed after ICH, and damage mainly occurs in the perihematomal area as opposed to more distal areas (Felberg et al., 2002). It has been shown in animal models that damage is most severe from 1-3 days after ictus, however there is also resulting long-term atrophy of the surrounding area which is manifest by ventricle
enlargement, with the degree of atrophy correlating with the size of the hematoma (Felberg et al., 2002). Although neuronal staining using the NeuN antibody did not reveal significant difference between the ipsi- and contralateral sides, it has been suggested that this may be because ICH is primarily disrupting axons leading to function impairment while leaving the cell body intact. Furthermore, white matter tracts were morphologically affected; a decrease in diameter was observed likely due to stretching as the tract migrated towards the scar tissue of the ICH, however it is yet to be determined whether and in what capacity this morphological alteration is affecting functionality of the neurons (Felberg et al., 2002).

Other work has shown that white matter damage around ICH in mice is observed both with and without demyelination (Wasserman and Schlichter, 2008). Additionally, the damage is worse in older animals, which correlates with the fact that they tend to have worse functional outcome despite having a similar extent of gray matter damage (Wasserman and Schlichter, 2008). This observation is of potential clinical significance as spontaneous ICH tends to occur more commonly with increased age. Microglial infiltration was found to occur after white matter was already disrupted suggesting that the inflammatory response was activated in response to the damage, and was not the initial cause of the damage even though it may serve to perpetuate it (Wasserman and Schlichter, 2008). It has subsequently been shown that neutrophil depletion reduces the extent of perihematomal axonal and myelin damage (Moxon-Emre and Schlichter, 2011), supporting the hypothesis that the inflammatory response contributes to white matter damage. However, the initial cause of damage to axons and myelin remains to be elucidated.

ICH produces an oxidizing environment and toxic metabolites are quickly found in the perihematomal white matter, suggesting that removal of the clot may not be sufficient to protect surrounding white matter from damage (Clark et al., 2008). Various parts of the brain including white matter areas, namely the corpus callosum and cerebellar white matter, show a reduction in glucose metabolic rate after intravenous injection of bilirubin in rats (Roger et al., 1993).

**Animal Models**

Animal models have been used to simulate human ICH in order to study its effects on the brain, the pathophysiological mechanisms that result, as well as possible pharmacological treatments,
which can prevent or reverse the damage. Various species of animals have been used but there are two commonly used models of ICH, usually targeting the striatum. The striatum is a common location for ICH in humans (MacLellan et al., 2010). Both models have been deployed in mice and rats. In one model whole autologous blood is directly injected into the brain, whereas in the other, bacterial collagenase is injected which breaks down the basal lamina of the surrounding vessels, leading to blood entering the parenchyma. The first model is characteristic of having an umbrella-like shape of lesion, lesser damage, faster resolution of the blood, and better recovery; however, it allows the investigator to control the for the amount of blood present in the brain. The collagenase model on the other hand, causes a larger lesion, greater and more continuous damage and functional loss, as well as long-term behavioural deficits. Although the collagenase model appears to recreate ICH more accurately, the presence of the collagenase, which does not occur in the natural milieu of ICH, cannot be discounted. Therefore, since there is no clear evidence suggesting that one model is superior to the other in recreating ICH, it has been suggested that both models should be used when conducting ICH studies (MacLellan et al., 2010).

Evidence exists in support of both models. Investigation of the collagenase model in mice, showed degeneration of neurons and oligodendrocytes in and around the hematoma (Masuda et al., 2010). The behavioral tests also showed significantly impaired motor abilities which correlated with decreased brain volume, suggesting that this model may be a good candidate for studying both the pathophysiological mechanism after ICH as well as the resulting physical impairments. On the other hand, experiments have shown that injection of whole blood into the striatum also can produce effects similar to those seen in patients including confluent hematoma volumes, cerebral edema, neurological deficits and perihematomal hypoperfusion (Belayev et al., 2003).

Most rodent experiments use only histological measurement of the effects of ICH. Since the correlation between histological damage and functional or behavioural outcome in animals is not widely explored, these models may not be idea for assessing the effectiveness of treatments. On the other hand, it is a reasonable assumption that brain injury is detrimental to outcome whether it is functionally measurable in rodents or not. Although it was found that no one test could effectively predict the group (mild, moderate or severe ICH), using the battery of functional tests
could reliably predict the severity of ICH (MacLellan et al., 2006). This suggests that animal studies should use numerous neurological and behavioural tests as an outcome measure in order to accurately assess the effect of treatments being tested. Unfortunately, functional assessments, just like using both models of ICH, are not commonly used. The events that occur after ICH are numerous and complex, therefore, they likely do not act in isolation but rather through interactions with one another. Furthermore, there is less white matter in rodents (Frantzias et al., 2011); therefore, any effects found in animal studies may actually be more severe in humans.

**Dimethyl Sulfoxide and Methylsulfonylmethane**

Dimethyl sulfoxide (DMSO) is a sulfur containing compound which is commonly used as a drug delivery vehicle because of its ability to solubilize drugs and to enhance permeability of biological membranes (Notman et al., 2006). However, DMSO is also a free radical scavenger (Panganamala et al., 1976) and has been shown to have protective properties when administered following brain injury, having beneficial effects in ischemia, traumatic brain injury (TBI), and excitotoxicity.

DMSO administration after middle cerebral artery occlusion (MCAO) in cats improved cerebral blood flow by 27% (de la Torre, 1991). After MCAO and subsequent reperfusion, DMSO reduced infarct size at 2 days (Nagel et al., 2007). Furthermore, DMSO administration prior to permanent MCAO in rats reduced the infarct volume at 24 hours compared to saline controls (Shimizu et al., 1997). DMSO treatment, even when administered after permanent MCAO, reduced infarct volume at 24 hours and 3 days (Bardutzky et al., 2005). DMSO also reduced CA1 pyramidal neuron death at 5 days of reperfusion after common carotid artery occlusion (Phillis et al., 1998).

Furthermore, some work has shown that DMSO may be able to protect against secondary cell death after TBI in rats (Di Giorgio et al., 2008) and against glutamate excitotoxicity. At concentrations of 0.5-1.0% DMSO inhibited the glutamate response in hippocampal neurons, suppressed Ca\(^{2+}\) influx induced by glutamate, reduced NMDA and AMPA receptor activation, and thus protected against excitotoxicity (Lu and Mattson, 2001). In addition, DMSO affects
membrane fluidity and enhances membrane resealing after cell injury (Howard et al., 1999). Therefore, it is also being studied in the context of spinal cord injury.

Furthermore, DMSO is a good solvent for bilirubin, with a solubility of bilirubin in DMSO of more than 10,000 µM. Another solvent is alkaline water in which bilirubin has a solubility of more than 10,000 µM (Brodersen, 1979). Therefore, the effects of DMSO on the effects of bilirubin on cells and tissues are difficult to predict. On one hand it may decrease injury due to its neuroprotective effects, but on the other it could enhance the damage caused by bilirubin by making it more available in solution to cause damage.

![Figure 4: Structure of DMSO and MSM](image)

**Figure 4: Structure of DMSO and MSM**

Methylsulfonylmethane (MSM) is another sulfur containing organic compound found in many fruits and vegetables, and marketed as a dietary supplement. When administered orally, it has been shown to readily cross the BBB (Rose et al., 2000). In rats MSM is absorbed well when administered orally, distributed fairly evenly throughout the body, and completely excreted by 120 hours (Magnuson et al., 2007). Oral administration of MSM decreased micro- and macroscopic colonic damage in rats. It also decreased the level of oxidative stress and proinflammatory cytokines (Amirshahrokhi et al., 2011). Furthermore, in mice, it downregulated nuclear factor-kappa B (NF-κB) signaling, which decreased secretion of pro-inflammatory mediators (Kim et al., 2009).

A randomized, double-blind, controlled clinical trial found that oral administration of MSM reduced pain and improved physical function in patients with osteoarthritis (Debbi et al., 2011).
Long-term daily administration of MSM was found to decease serum levels of markers of oxidative stress in untrained men after intense exercise (Rose et al., 2000). Furthermore, MRI spectroscopy of the brain of an older man who was taking MSM dietary supplements was found to have a brain concentration of 2.4 mmol/L of MSM (Rose et al., 2000). However, to the best of our knowledge, the effects of MSM on bilirubin have not been studied.

**Potential of Albumin Treatment**

Unconjugated bilirubin is insoluble in aqueous solutions; therefore, under physiological conditions it binds to albumin, which allows it to be transported to the liver for excretion. However, as mentioned, bilirubin can become toxic to the brain when its concentration exceeds that of albumin. Therefore, it seems reasonable to postulate that albumin therapy post-ICH may help reduce injury caused by the hematoma.

Based on recent studies in which albumin has shown some promise as an effective therapy for ischemic stroke, animal work has begun to examine its effects on hemorrhagic stroke as well. For example, in a dog model of SAH, administration of albumin was found to improve cerebral circulation which is impaired by vasospasm (Matsui and Asano, 1993). In humans with SAH, albumin was administrated to increase central venous pressure (CVP), and it was found that although it did not change the blood volume, it did prevent sodium and fluid loss (Mayer et al., 1998). It also improved sodium retention by decreasing glomerular filtration, and it is predicted to therefore decrease the occurrence of pulmonary edema by decreasing the amount of volume that needs to be administered to maintain the necessary level of CVP (Matsui and Asano, 1993).

Furthermore, a retrospective cohort study done at one institution found that patients with aneurysmal SAH who were treated with albumin had better clinical outcome after 3 months compared to a more recent cohort treated with plasma (Suarez et al., 2004). This lead the group to launch a multicenter phase 2a dose-finding clinical trial of increasing doses of albumin for improving outcome after SAH (Albumin in Subarachnoid Hemorrhage [ALISAH] study) to determine the safety of administration of 25% albumin to SAH patients with severe or life-threatening heart failure, as well as to obtain preliminary data on its effects on neurological status, rebleeding, hydrocephalus, delayed cerebral ischemia, and seizures (Suarez and Martin,
This study found that doses up to 1.25 g/kg/day for 7 days were well tolerate,

and suggests that albumin treatment may be neuroprotective (Suarez et al., 2012).

Much less work has been done to explore the potential of albumin therapy in ICH, however, some promising results have been shown. In rats, albumin treatment administered 60 minutes after injection of 50 µL of autologous blood improved neurological outcome (assessed 2 to 7 days post-injection) and decreased the extent of BBB damage 2 days after the surgery (Belayev et al., 2005). Improved neurological outcome in albumin-treated rats was confirmed by a later study, despite no evidence of a decrease in hematoma size (Belayev et al., 2007). Furthermore, in humans, albumin administered to patients with hemorrhagic stroke improved abnormal electroencephalograms (EEGs), possibly by reducing intracerebral pressure (ICP) and lowering the extent of edema (Huang et al., 2002).

Albumin administration prior to blood transfusion in infants with hyperbilirubinemia resulted in removal of an average of 41% more bilirubin (Odell et al., 1962). Interestingly, the amount of bilirubin removed varied between neonates even when serum bilirubin levels were similar prior to albumin treatment, which the authors suggest is a result of a difference in total body bilirubin (Odell et al., 1962). Similar results have been shown in a study many years later, which found that albumin treatment prior to blood transfusion in infants with hyperbilirubinemia resulted in lower total bilirubin levels by extracting some of the tissue-bound bilirubin into the circulation (Shahian and Moslehi, 2010). Considering the BBB is damaged after ICH, albumin’s ability to extract bilirubin from hematomal and perihematomal tissue may be even greater than in neonates. Furthermore, local, as opposed to systemic, administration of albumin may have an increased ability to remove bilirubin from the brain. Future studies in animals and humans are required to determine if albumin therapy has the potential to decrease secondary injury after ICH.
Chapter 2 Hypothesis and Research Aims

Secondary injury after ICH remains a significant hurdle in the recovery of individuals who survive the initial neural damage caused by the stroke. Although multiple pathways and mechanisms of damage occur, knowledge about how to effectively deal with each type of damage is limited. Therefore, studying each component individually is a rational initial step in elucidating the dynamic interactions of various biological factors that contribute to secondary injury after ICH. One mechanism, on which little work has been done, is the effect of the specific blood breakdown product bilirubin, on the structure and function of white matter. Bilirubin is known to be neurotoxic in high quantities, and it is therefore the focus of this project. Although bilirubin has been shown to cause damage to most cell types within the brain, due to limited scope of this initial project, we have limited the study to white matter, since hyperbilirubinemia has been correlated with clinical impairment, namely cerebral palsy, as well as white matter damage (Gkoltsiou et al., 2008). Second, many ICHs in humans occur in the white matter and are in direct proximity to neurons. Furthermore, BOXes have been identified in the hematoma and surrounding brain after hemorrhagic stroke, as well as to be biologically active; therefore, we chose to include these compounds in our investigation.

The two main aims of this work were to 1) determine the effect of bilirubin and its oxidation products on the structure and function of white matter in vitro, and subsequently 2) determine if these compounds have an effect on the structure and function of white matter in vivo. Based on previous knowledge about the effects of these molecules we hypothesized that bilirubin and/or its oxidation products cause functional loss in white matter tracts through direct or indirect structural disruption of myelin and axonal integrity.

In order to test our hypothesis, we sought to answer a number of specific questions. First of all, we aimed to elucidate whether bilirubin has effects in vitro on function and structure of white matter. To address this question we incubated brain tissue in various concentrations of bilirubin and recorded compound action potentials (CAPs). We also analyzed this tissue at high magnifications using electron microscopy to determine if any structural changes could be identified.
In order to explore potential mechanisms through which bilirubin and/or its oxidation products may produce effects on white matter, we co-incubated mouse brain tissue with bilirubin and NMDAR, AMPAR, and Ca\(^{2+}\) channels inhibitors, and tested the effects on CAPs. Furthermore, we tested the effects of co-incubation of bilirubin with DMSO and MSM on CAPs to determine if they had any protective effects on white matter.

In sequence, we aimed to determine whether bilirubin had different effects on CAPs when it was oxidized. The effects on CAPs from tissue incubated in fresh or oxidized bilirubin were compared. Since all the incubation had been for 7 hours, we examined whether bilirubin and BOXes had time dependent effects, by recording CAPs after increasing times of incubation. Additionally, fresh and oxidized bilirubin solutions were analyzed for presence of BOXes using UV spectrophotometry and mass spectrometry.

In the final set of experiments we focused on the second aim of this work, which addressed whether bilirubin and/or BOXes have any effects when present in vivo in the mouse brain. In order to test this we stereotactically injected bilirubin into the brains of mice and studied the brains at different time points. We analyzed this tissue electrophysiologically to test for effects on function, as well as using immunohistochemistry (IHC) to determine any effects on structural integrity of axons and myelin.
Chapter 3 Methods

Animals
All animal protocols were approved by the Animal Care Committee at St. Michael’s Hospital and complied with the regulations of the Canadian Council on Animal Care. We used CD-1 male mice 25-35g from Charles River. The animals were kept in a 12 hour light-dark cycle and had access to food and water ad libitum.

Sacrifice
Animals were anaesthetized with ketamine (120 mg/kg) and xylazine (30 mg/kg), intraperitoneally. Animals used for electrophysiological recording were decapitated and the brain immediately extracted and placed in oxygenated ice cold aCSF. Animals used for histology were perfused through the heart with 0.9% NaCl followed by paraformaldehyde (PFA). The brains were removed and placed in PFA at 4°C overnight. The tissue was prepared for histology by dehydration and paraffin embedding or it was frozen immediately at -80°C.

Tissue incubation
Unless otherwise specified, all tissue slices were incubated for 7 hours, and all solutions were made in artificial CSF (aCSF composition in mM: 124 NaCl, 5 KCl, 1.25 NaH₂PO₄, 2.5 CaCl₂, 1 MgSO₄, 26 NaHCO₃, and 10 D-glucose) and continuously bubbled with 95% O₂ and 5% CO₂. Sodium free aCSF was made similarly to regular aCSF however NaCl was replaced with an equivalent molar concentration of N-methyl-D-glucamine (NMDG; Sigma-Aldrich, St. Louis, MO, USA), and NaH₂PO₄ was replaced with KH₂PO₄. TTX (Accurate Chemical and Scientific Corporation, Westbury, NY, USA) was prepared and perfused at a concentration of 0.5 µM. Concentrated bilirubin stock solution was made by dissolving powdered bilirubin (Sigma-Aldrich, St. Louis, MO, USA) in distilled water and 2M NaOH. The pH of the solution was adjusted to 7.4 using commercially available HCl (adapted from Huang et al., 2002). The solution was either immediately diluted (from here on referred to as “fresh bilirubin”) to the appropriate concentration in aCSF, or placed in a tightly sealed glass vial and kept in the dark at 4°C (from here on referred to as “stock bilirubin” or “oxidized bilirubin”) until it was later used and diluted in the same way. Bilirubin was used at concentrations of 0.02 mM, 0.1 mM, and 0.5
mM. Previous work has shown that bilirubin reaches a maximum of 0.177 mM in perihematomal white matter in the porcine model of ICH 24 hours after ictus (Clark et al., 2008). All solutions with DMSO (Sigma-Aldrich, St. Louis, MO, USA) were prepared at a concentration of 0.001%. Solutions of 0.2 mM NMDAR antagonist D-AP5 (Tocris Bioscience, Ellisville, MO, USA), 30 μM AMPAR antagonist NBQX disodium salt (Tocris Bioscience, Ellisville, MO, USA), and 0.1 mM L-type Ca^{2+} channel antagonist amlodipine besylate (Tocris Bioscience, Ellisville, MO, USA), were prepared by dissolving the appropriate amounts of the compounds into the working concentration of bilirubin solution.

**Transmission Electron Microscopy**

Coronal brain sections, fresh or incubated for 7 hours in various conditions (normal aCSF, 0.1 mM bilirubin, 0.5 mM bilirubin, 0.1 mM bilirubin + DMSO, normal aCSF + DMSO) were immersed in 4% formaldehyde and 1% glutaraldehyde in phosphate buffer at pH 7.3 for one day. They were post-fixed in 1% osmium tetroxide for 1 hour, after which they were dehydrated in a graded series of acetone solutions. The sections were then cut sagittally through the midline and the area containing the corpus callosum embedded in epon-araldite epoxy resin. A microwave oven was used for the processing steps from post-fixation to polymerization of resin blocks (Pelco BioWave 34770, Pelco International, CA, USA). Sagittal brain sections were cut using a diamond knife on a microtome (Reichert Ultracut E, Leica Inc., Austria), and stained with uranyl acetate and lead citrate. The sections were examined using a transmission electron microscope (JEM-1011, JEOL Corp., Peabody, MA, USA). The images were acquired with a 1024 X 1024 pixel charge coupled device camera (AMT Corp., Denver, MA, USA).

**Analysis of TEM images**

The g-ratio, a commonly used index of structural and functional myelination, was calculated using the equation G-ratio = \sqrt{(A1/A2)} where A1 is the area within the inner lumen of the axon and A2 is the area of the entire cross-section of the axon including the myelin sheath (Chomiak and Hu, 2009). The axon density was calculated by averaging the number of axons per squared area.
**Electrophysiological recording**

Coronal sections 400 µm thick were cut from 2800 µm to 5200 µm from the most anterior point of mouse brains (and 3200 µm to 4400 µm from stereotactically-injected brains) using a vibratome (Leica, 1200, Leica Microsystem, Richmond Hill, ON, Canada) with a solid zirconia ceramic blade (Cadence, Staunton, VA, USA). Slices were placed in aCSF aerated with 95% O$_2$ and 5% CO$_2$. A single slice was transferred to the recording chamber, which was continuously perfused with aCSF at a rate of 1–2 ml/min at room temperature. Recordings were obtained within 8 h of sacrifice. Compound action potentials (CAP) were recorded extracellularly from the corpus callosum using a pulse range from 30 to 1000 µA, at a rate of 0.05 Hz. The tract was stimulated with a bipolar concentric tungsten electrode (FHC Bowdoinham, ME, USA), which was placed 750 µm away from the recording electrode (glass pipette filled with 150 mM NaCl). Signals were amplified with an amplifier (Multiclamp 700 A, Molecular Devices, Foster City, CA, USA), digitized (Digidata, 1320, Molecular Devices Foster City, CA, USA) and collected through a computer running Clampex 8.0 (Molecular Devices, Foster City, CA, USA). A digital stimulator was used and was controlled by a computer program (STG 1001, Multichannel System, Reutlingen, Germany).

![Figure 5: Recording of CAPs](image)
**Analysis of Electrophysiological Data**

Raw electrophysiological data was analyzed using Clampfit 9.0 software. As seen in Figure 6, amplitude was calculated by measuring the distance between the trough and the average of the two peaks. Latency was measured as the distance between onset of the stimulation and the trough of the curve, whereas the area under the curve was calculated using the formula \( AUC = \frac{a \times b}{2} \) where \( a \) is the amplitude and \( b \) is the time between the first and second peak of the curve.

![Figure 6: Analysis of CAPs](image)

**Mass spectrometry**

Mass spectrometry analysis was performed using an AB Sciex QSTAR XL ESI-Qq-TOF mass spectrometer in negative mode with a nano flow electrospray source. The samples were mixed with equal volume of 5% NH\(_4\)OH before loading to a glass capillary tip (Econotip, New Objective, Woburn, MA, USA) for static infusion.
**UV Spectrophotometry**

Solutions were prepared in aCSF at serially diluted concentrations (n=3/concentration) and were placed in triplicate into a 96-well plate (200 µL/well). The absorbance of BOXes at 320 nm (Loftspring et al., 2007a) was detected by spectroscopy using SpectraMax M5° (Molecular Devices, Sunnyvale, CA, USA) microplate reader and analyzed using SoftMax Pro 5.4.1 software.

**Surgeries**

Animals were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine and 20 mg/kg xylazine, and the scalp at the incision site was anesthetized with a subcutaneous injection of 0.5% bupivacaine (5 mL/kg). The cranium of the mice was then placed in a stereotactic frame. Body temperature was maintained at 37°C with a heating pad (Homeothermic systems, Harvard Apparatus, Holliston, MA, USA). A 2 cm midline incision was made in the scalp and the skull exposed. A burr hole was made with a 1 mm drill 1 mm posterior to the bregma and 1 mm lateral to the midline. A previously published protocol was used with slight modifications (Belayev et al., 2003). Briefly, a 50 µL glass syringe with an attached needle was lowered at a 15° angle 1.8 mm into the cranium, and a microinjection pump was used to inject saline or fresh 0.5 mM bilirubin intracerebrally. During the initial 3 minutes 5 µL were injected, which was followed by a 7 minute pause. Subsequently, 10 µL were injected over 5 minutes, and the needle removed 10 minutes later. Some previous studies have used smaller amounts of bilirubin (1 µL at 4.5 mM) however this was accompanied by 10 µL of whole blood (Loftspring et al., 2011). Others however, have used higher amounts of just bilirubin at 12 mg/mL (i.e., 205 mM) at a volume of 30 µL (Huang et al., 2002)-. All animals received subcutaneous buprenorphine (0.2 mg/kg) and 0.5 mL of saline twice daily for 48 hours.

**Immunohistochemistry**

IHC was performed according to the ABC protocol using Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). All antibodies were used at a dilution of 1:200. Degraded MBP was detected using rabbit anti-myelin basic protein primary antibody (Millipore, Temecula, CA, USA), and amyloid β precursor protein (βAPP) was detected using the rabbit anti-amyloid-β precursor protein primary antibody (Invitrogen, Camarillo, CA, USA). Both of these proteins
were detected using biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA) on paraffin sections. Activated microglia was detected on frozen sections with the primary rat anti-mouse CD68 antibody (Biolegend, San Diego, CA, USA) and biotinylated goat anti-rat IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA). Methyl green solution was prepared with 0.5 g of methyl green (Sigma Aldrich, St. Louis, MO, USA) in 0.1 M sodium acetate buffer (pH 4.2). All sections were counterstained in methyl green solution for 5 minutes then dehydrated quickly through increasing concentrations of ethyl alcohol. Images of the slides were taken using the Olympus DP72 microscope and Olympus cellSense software, and qualitatively assessed for presence of positive staining.

**Statistical Analyses**
All statistical analyses were performed using the SigmaStat 3.1 program. Student’s t-test was used when two groups were compared, whereas ANOVA was used to determine statistical difference between more than two groups. If the ANOVA was significant, pairwise comparisons were performed using the method suggested by SigmaStat.
Chapter 4 Results

I. Sodium channel analysis within the corpus callosum

We first confirmed that the CAPs recorded were consistent with prior descriptions (Baker et al., 2002).

![Figure 7: Effect of TTX on CAPs](image)

A) Myelinated and B) unmyelinated axons in the corpus callosum of brain tissue. The black bar indicates perfusion of the recording chamber with TTX (n = 3)

![Figure 8: Effect of Na\(^{2+}\) free aCSF on CAPs](image)

A) Myelinated and B) unmyelinated axons in the corpus callosum of brain tissue. The black bar indicates perfusion of the recording chamber with sodium-free aCSF (n = 3)

Stimulation of the corpus callosum with 300 µA showed a stable responding potential in myelinated (Fig. 7A) and unmyelinated (Fig. 7) axons, however after 5 minutes of perfusion of
the recording chamber with 0.5 µM TTX there was a steady decline in potential until there was no longer a response. Similarly, steady potential was observed in myelinated (Fig. 8A) and unmyelinated (Fig. 8B) axons until the recording chamber was perfused with sodium-free aCSF. The decrease in potential amplitude was gradual until it is no longer present, however, once sodium was reintroduced into the chamber, the potential response steadily increased. These results show the CAPs are due to electrical depolarization of the axons that activates only TTX-sensitive sodium channels and causes sodium influx, which is consistent with prior work.
II. Effect of bilirubin on CAPs in vitro

We next studied the effect of incubation of brain slices with varying concentrations of stock bilirubin solutions.

![Graphs showing the effect of bilirubin on CAPs](image)

**Figure 9: CAPs of brain slices incubated in 0.02 mM bilirubin**

A) Size of amplitude of myelinated axons in response to increasing stimulation. B) Size of amplitude of unmyelinated axons in response to increasing stimulation. C) Speed of conduction of myelinated axons. D) Speed of conduction of unmyelinated axons. (* p < 0.05)

Incubation of the brain tissue for 7 hours in 0.02 mM stock bilirubin did not result in any difference in the latency of action potentials compared to recordings from tissue incubated in aCSF (Fig. 9 C, D). However, there was a trend towards lower amplitude with the difference being significant (p < 0.05) at a stimulation of 290 µA in the myelinated axons (Fig. 9A), and at stimulations of 90 µA and 190 µA in the unmyelinated axons (Fig. 9B). These results suggest
that at such a low concentration bilirubin may have a slight effect on the amplitude of myelinated and unmyelinated axons, since the difference between the two conditions was only significant at a couple of stimulation strengths.

Figure 10: CAPs of brain slices incubated in 0.1 mM bilirubin.
A) Size of amplitude of myelinated axons in response to increasing stimulation. B) Size of amplitude of unmyelinated axons in response to increasing stimulation C) Speed of conduction of myelinated axons. D) Speed of conduction of unmyelinated axons. (* p < 0.05)

Incubation of the brain tissue for 7 hours in 0.1 mM bilirubin also showed a trend of lower amplitude in the bilirubin incubated tissue, which only reached statistical significance at a stimulation of 1000 µA in the myelinated axons (Fig 10B). However, there was also a trend towards a higher latency in both myelinated (Fig. 10C) and unmyelinated (Fig 10D) axons from tissue incubated with 0.1 mM bilirubin compared to tissue incubated in aCSF alone.
At this concentration, the effects of bilirubin appear to be similar to that of the lower concentration of 0.02 mM, suggesting that in vitro these concentrations are not sufficiently high to produce any significant changes in the electrophysiological properties of the corpus callosum.

**Figure 11: CAPs of brain slices incubated in 0.5 mM bilirubin**
A) Size of amplitude of myelinated axons in response to increasing stimulation. B) Size of amplitude of unmyelinated axons in response to increasing stimulation. C) Speed of conduction of myelinated axons. D) Speed of conduction of unmyelinated axons. (** p < 0.01).

The unmyelinated axons in tissue incubated for 7 hours in 0.5 mM bilirubin showed a trend towards a lower amplitude compared to those incubated in aCSF alone, with the only statistically significant difference being at a stimulation of 90 µA (Fig. 11B). There was no difference in latency of unmyelinated axons between the two conditions (Fig. 11D). However, myelinated axons had a statistically significantly lower amplitude at stimulations from 190 µA to 1000 µA (Fig. 10A) and higher latency at stimulations from 90 to 1000 µA (Fig. 11C). Although there
didn’t appear to be any noticeable differences between the myelinated and unmyelinated axons in tissue incubated with 0.02 mM or 0.1 mM or bilirubin, myelinated axons appear to be affected to a greater extent than the unmyelinated, in the tissue incubated in the highest concentration of (0.5 mM bilirubin), relative to the CAPs of aCSF incubated tissue.
III. Structural effects of bilirubin in vitro

Structural effects of bilirubin applied to brain slices were studied using transmission electron microscopy.

Figure 12: Axon density based on TEM
A) Transmission electron microscope images of cross sections of the corpus callosum after incubation in various solutions (aCSF, 0.1 mM stock bilirubin (SB), 0.5 mM OB) for 7 hours (magnification 5000x, scale bar = 2 µm). B) Analysis of axon density based on TEM images of OB treated tissue (n = 5 images/group).
Figure 12A presents representative TEM images (magnification of 5,000 x) of cross sections of the corpus callosum of tissue incubated in various concentrations of bilirubin. Qualitatively it appears that the diameter of axons increased and the thickness of the myelin decreased with increasing concentration. Furthermore, the axons were less compact and more debris was present with increasing bilirubin concentration. This trend was also observed quantitatively as the axon density decreased with an increase in bilirubin concentration (Fig. 12B).

Figure 13: TEM images of axonal mitochondria
A) Tissue incubated for 7 hours in aCSF. B) Tissue incubated for 7 hours in 0.5 mM bilirubin. (Magnification 100,000 x, scale bar (■) = 100 nm).

Furthermore, images taken at a magnification of 100,000 x showed differences in mitochondria (Fig. 13). Axons from tissue incubated in aCSF had a well-defined outer edge surrounded by myelin, and small mitochondria with compact, well-defined cristae (Fig. 13A). However, axons incubated in 0.5 mM bilirubin had significantly enlarged cristae, and vacuole-like bubbling, as well as loss of definition of the cristae (Fig. 13B). Furthermore, the outer membranes of the axon appear to have lost their integrity. This suggests that presence of
bilirubin, at a concentration of 0.5 mM, results in neuronal loss, as well as structural disruption of mitochondria. Although mitochondrial function was not assessed here, structural damage would presumably result in disrupted function, which in turn may contribute to the structural or functional effects of bilirubin on the white matter tract.

**IV. Role of NMDARs, AMPARs, and Ca\(^{2+}\) channels in the effects of bilirubin on CAPs**

![Figure 14: Effect of 0.1 mM amlodipine besylate on CAPs](image)


Incubation of brain tissue for 7 hours in 0.1 mM bilirubin or 0.1 mM bilirubin with 0.1 mM amlodipine besylate, an L-type calcium channel blocker, did not have statistically different
effects on the averages of amplitude or latency in either myelinated or unmyelinated axons (Fig. 14). However, there was a non-statistically significant trend suggesting that co-incubation with amlodipine besylate resulted in a larger amplitude in unmyelinated axons (Fig. 14B).

Figure 15: Effect of 0.2 mM D-AP5 on CAPs

Electrophysiologically, there was no difference between the tissue co-incubated with bilirubin and 0.2 mM of NMDA receptor antagonist D-AP5 (Fig. 15), or 30 µM AMPA receptor antagonist, NBQX (Fig. 16), when compared to the responses recorded from tissue incubated with bilirubin alone.
Figure 16: Effect of 30 µM NBQX on CAPs

Although there are limitations to this data (see Chapter 5: Discussion), it suggests that NMDA and AMPA receptors are not involved in the effects of bilirubin in vitro on the structure and function of white matter. The non-statistically significant trend observed in co-incubation with amlodipine besylate indicates that L-type Ca$^{2+}$ channels may be involved, and thus warrants further investigation.
V. The effect of DMSO on structure of the corpus callosum and CAPs in vitro

Figure 17: Effects of DMSO on CAPs

A comparison of electrophysiological recordings from tissues incubated in various solutions (aCSF, aCSF with DMSO, 0.1 mM bilirubin, and 0.1 mM bilirubin with DMSO) indicates that there were no differences between latencies recorded in these groups (Fig. 17C, D) However, a one-way ANOVA indicated that there are significant differences between the amplitudes of these groups in both myelinated and unmyelinated axons (Fig. 17A, B). More specifically, myelinated...
axons had differences in amplitude at stimulations from 150 µA to 310 µA. There do not appear to be differences between aCSF, DMSO, and 0.1 mM bilirubin groups, whereas the amplitude of 0.1 mM bilirubin with DMSO was significantly larger. However, in the umyelinated axons which had statistical differences at 230, 270 and 290, there does not seem to be a difference between the aCSF, DMSO, or 0.1 mM bilirubin + DMSO conditions, but the 0.1 mM bilirubin condition had lower amplitude across most stimulations.

Figure 18: TEM Images of Corpus Callosum Cross Sections
A) Fresh tissue. B) Tissue incubated for 7 hours in 0.1 mM bilirubin + DMSO. C) Tissue incubated for 7 hours in aCSF + DMSO.

Figure 18 shows representative images of cross sections of the corpus callosum from fresh tissue (A), as well as tissue incubated in 0.1 mM bilirubin (B) and aCSF with DMSO (C). The images of fresh tissue show a dense population of round myelinated axons of varying sizes, with few unmyelinated axons filling in the gaps between the myelinated ones. The tissue incubated in bilirubin and DMSO looks similar to the fresh tissue, with slightly more space not occupied by any axons. However, the tissue incubated in aCSF and DMSO had a number of oddly shaped axons as well a significantly more space where there were no axons present.
Figure 19: G-ratio of axons
Myelin thickness analysis based on TEM images (n=103/group). Overall ANOVA significance = p < 0.001.

The myelin thickness after exposure of brain slices to the various conditions was assessed based on a calculation of the G-ratio as measured on TEM images of axon cross sections. The G-ratio = √(A1/A2) where A1 is the area of the axons and A2 is the area of the axons and myelin. Each condition had a sample size of 103 axons, which were measured from images taken at the same magnification. The following were the calculated G-ratio values for each condition: Fresh tissue (normal 0 hours) = 0.71 +/- 7.8e-3, tissue incubated in aCSF (normal 7 hours) = 0.70 +/- 7.0e-3, tissue incubated in aCSF and DMSO (normal + DMSO) = 0.73 +/- 7.7e-3, tissue incubated in bilirubin (0.1 mM bilirubin) = 0.66 +/- 0.01, and tissue incubated in bilirubin and DMSO (0.1 mM bilirubin + DMSO) = 0.73 +/-6.9e-3.

A one-way ANOVA indicated a statistically significant overall difference between all of the conditions with a p < 0.001. A Tukey Test was then performed for all pairwise comparisons. Normal 0 hours was significantly different from 0.1 mM bilirubin, but not any other condition. Although 0.1mM bilirubin was not significantly different from normal 7 hour, it was significantly different than normal + DMSO and 0.1 mM bilirubin + DMSO. The two DMSO
conditions were not significantly different from one another; however, the normal 7-hour condition was different than both of the DMSO conditions.

This data indicates that DMSO may protect the corpus callosum from the damaging effects of bilirubin on its structural integrity and electrophysiological properties. However, presence of DMSO alone (i.e., without bilirubin) may be damaging to the white matter tract, as the electrophysiological recordings show worse performance in the DMSO only group, compared to the tissue incubated just in aCSF.
VI. The effect of MSM in vitro

Figure 20: Effect of 2.4 mM MSM on CAPs

The effects of methylsulphonylmethane (MSM) were tested on tissue incubated in bilirubin (Fig. 20). There were no differences in the amplitude between the tissue incubated in 0.5 mM bilirubin and tissue incubated in 0.5 mM bilirubin with 2.4 mM MSM in myelinated (Fig. 20A) or
unmyelinated (Fig. 20B) axons. However, there were also no amplitude differences between aCSF incubated and 0.5 mM bilirubin incubated tissue (Fig. 20A,B). Furthermore, there was no difference in latency between aCSF and 0.5 mM bilirubin incubated tissue (Fig. 20C,D). Co-incubation of tissue in bilirubin and MSM did not result in any statistically significant difference in latency in myelinated (Fig. 20C) or unmyelinated (Fig. 20D) axons, however there was trend for a longer latency in the unmyelinated axons compared to the other two groups.

This data may suggest that MSM actually has adverse effects on the electrophysiological properties of white matter, more specifically unmyelinated axon. However, its effects are important to assess relative to the effects of bilirubin, not just to the electrophysiological properties of healthy and undamaged neurons. Since there was no effect of bilirubin on the electrophysiology of the white matter in these recordings, further experiments need to determine if presence of MSM can prevent the effects of bilirubin.


**VII. Effects of oxidized bilirubin in vitro**

The effects of different ages of bilirubin solutions were tested on the electrophysiological response of the corpus callosum. More specifically, the effects of solution made immediately prior to the experiment (fresh bilirubin) was compared to the effects of bilirubin solution made 7 days prior and kept at 4°C (oxidized bilirubin).

![Graphs showing effects of fresh and oxidized bilirubin solutions on CAPs](image)

**Figure 21: Effects of Fresh and Oxidized Solutions of 0.1 mM Bilirubin on CAPs**
In Figure 21 we see no difference in the electrophysiological response of myelinated or unmyelinated axons between fresh and oxidized bilirubin at a concentration of 0.1 mM. Therefore, no difference is evident in the AUC either (Fig. 22). However, at a concentration of 0.5 mM bilirubin (Fig. 23) there are statistically significant differences in the response of myelinated axons. Tissue incubated in oxidized bilirubin showed a general trend of lower amplitude in myelinated axons (Fig. 23A), which reached statistical significance (p < 0.05) at stimulations of 90 \( \mu \text{A} \), 190 \( \mu \text{A} \) and 900 \( \mu \text{A} \). Similarly there was a general trend of higher latency of myelinated axons incubated in oxidized bilirubin which reached statistical significance (p < 0.05) at stimulations of 90 \( \mu \text{A} \), 190 \( \mu \text{A} \), 400 \( \mu \text{A} \), 700 \( \mu \text{A} \), 800 \( \mu \text{A} \), and 900 \( \mu \text{A} \). This data was further analyzed for the AUC. Fig. 24 shows that there was a trend of a smaller area of action potentials in myelinated axons (Fig. 24A), however this differences only reached statistical significance (p<0.05) at a stimulation of 900 \( \mu \text{A} \). The amplitude (Fig. 23B) and latency (Fig. 23D) of unmyelinated axons were not different between the two conditions at 0.5 mM bilirubin. Similarly, there was no effect on the curve of these axons (Fig. 24B).
Figure 23: Effects of Fresh and Oxidized Solutions of 0.5 mM Bilirubin on CAPs
A) Size of amplitude of myelinated axons in response to increasing stimulation. B) Size of amplitude of unmyelinated axons in response to increasing stimulation. C) Speed of conduction of myelinated axons. D) Speed of conduction of unmyelinated axons. (* p < 0.05).
Figure 24: Effects of Fresh and Oxidized Solutions of 0.5 mM Bilirubin on AUC
Area under the curve of brain slices incubated for 7 hours in 0.5 mM bilirubin solution made immediately prior to experiment (Fresh Bilirubin) or solution kept at 4°C for 7 days (Oxidized Bilirubin). A) Average area of action potentials of myelinated axons in response to increasing stimulation. B) Average area of action potentials of unmyelinated axons in response to increasing stimulation. (* p < 0.05).
Figure 25: Time Point Analysis of 0.5 mM Bilirubin Incubation Effects on CAPs
A) Size of amplitude of myelinated axons in response to increasing stimulation. B) Size of amplitude of unmyelinated axons in response to increasing stimulation. C) Speed of conduction of myelinated axons. D) Speed of conduction of unmyelinated axons (* p < 0.05).

Time point analysis of the effects of oxidized bilirubin is shown in Figure 25. Compound action potentials were recorded after every consecutive hour of incubation in 0.5 mM of oxidized bilirubin from 1 to 5 hours. There were no differences in the unmyelinated axons across the time points, in the amplitude (Fig. 25B) or latency (Fig. 25D) of the CAP recordings. Myelinated axons did not show a decrease in amplitude until the 5th time point, which was after 5 hours of incubation (Fig. 25A). Statistical analysis was performed using ANOVA, which showed an
overall statistically significant difference (p<0.05) from stimulations of 70 µA to 310 µA. Statistical analysis of the latency of myelinated action potentials did not reveal any significant differences. However, a trend of increasing latency with increasing incubation time is evident in Figure 25C.

Stock solution of bilirubin, at a concentration of 0.5mM, therefore has a damaging effect on myelinated axons’ amplitude and latency, and these effects are not immediate but rather take some time to occur. Furthermore, these findings suggest that bilirubin solution had changed during the 7 days it was kept at 4°C. Since fresh solution did not produce any effects on the electrophysiology, whereas the stock solution did, this indicates that whatever is changing or being produced during those 7 days, is what is causing the damage of the white matter tracts.
VIII. Analysis of Bilirubin Solutions

We used UV and mass spectrometry to assess the composition fresh and incubated solutions of bilirubin in order to determine if oxidation products were present and if they were BOXes.

Figure 26: Spectrophotometric Analysis of Bilirubin Solutions
Absorbance at 320 nm by freshly made bilirubin solution (Fresh bilirubin) and 7-day-old stock bilirubin solution (Oxidized bilirubin). (* p < 0.05 *** p < 0.00001)

BOXes have previously been quantified by assessing the absorbance of light at a wavelength of 320 nm (Loftspring et al., 2007). UV spectrophotometry analysis indicated that 7-day stock solution had significantly higher absorbency at 320 nm compared to freshly made bilirubin solution.
A

B
Figure 27: Mass Spectrometry of Bilirubin Solutions
Mass spectrometry of a pure solution of BOXes (Fig. 27 A) shows two major peaks, with the 188.94 m/z being the closest to the previously reported weight of 179.2 m/z (Kranc et al., 2000a). Analysis of the fresh bilirubin solution showed a large peak at 583.25 m/z, which is slightly different than 584.66 m/z, the reported molecular weight of the bilirubin purchased from Sigma-Aldrich and used to make this solution. Fresh bilirubin solution also showed presence of a small peak at 188.94 m/z. The analysis of 7-day stock bilirubin solution showed the opposite, with a small peak at 583.25 m/z and a large peak at 188.94. This is represented in Figure 27 D.

Therefore, there is a progressive decrease in the output at the molecular weight of bilirubin, and a progressive increase in the output at the molecular weight for BOXes, with 7 days of the compound being in solution. The maximum absorbance of BOXes has been previously determined to be 320 nm, therefore, the results of these experiments suggest that bilirubin oxidizes into BOXes when stored at 4°C. The data also suggests that significantly more BOXes are present in the bilirubin solution that had been oxidized for 7 days compared to 2 days.
IX. The effects of bilirubin on structure of the corpus callosum and CAPs in vivo

Bilirubin was stereotactically injected into the brain in order to examine its effects in vivo.

Figure 28: Effects of 0.5 mM Bilirubin in vivo on CAPs 2 Days after Injection
Figure 29: Effects of 0.5 mM Bilirubin *in vivo* on AUC 2 Days after Injection
A) Average area of action potentials of myelinated axons in response to increasing stimulation.  
B) Average area of action potentials of umyelinated axons in response to increasing stimulation.
Figure 30: Effects of 0.5 mM Bilirubin \textit{in vivo} on CAPs 7 Days after Injection
Figure 31: Effects of 0.5 mM Bilirubin in vivo on AUC 7 Days after Injection
A) Average area of action potentials of myelinated axons in response to increasing stimulation.
B) Average area of action potentials of unmyelinated axons in response to increasing stimulation.

The effects of bilirubin in vivo on the corpus callosum were assessed by stereotactic injection of 15 µL of 0.5 mM of bilirubin or saline (0.9% NaCl), and the subsequent extraction of brain tissue. Electrophysiological recordings from the tissue extracted 2 days after surgery are presented in Figures 28 and 29. Although there appears to be a trend towards lower amplitude in bilirubin-injected tissue, this did not reach statistical significance (Fig. 28A). There were also no significant differences in latency between the saline- and bilirubin-injected tissues in myelinated axons (Fig. 28C). Analysis of the AUC of the action potential also revealed a non-significant trend of smaller area of action potentials of myelinated axons (Fig. 29A). Unmyelinated axons were not significantly affected in terms of amplitude, latency, or AUC (Fig. 28BD; Fig. 29B).

However, in tissue extracted 7 days post-surgery there were no effects on amplitude or latency of myelinated axons (Fig. 30A,C), and thus no effect on AUC (Fig. 31A).

However, unmyelinated axons from tissue injected with 0.5 mM bilirubin had significantly lower amplitude than the saline injected tissue, from stimulations of 170 µA to 310 µA (Fig. 30B). There was no effect on latency of the unmyelinated axons (Fig. 30D), however, assessment of the AUC of unmyelinated axons from tissue injected with bilirubin had a trend of a smaller area which reached significance at stimulations of 210 µA, 250 µA, and 290 µA (Fig. 31B).
Figure 32: Effects of 0.5 mM Bilirubin in vivo on Structure of the Corpus Callosum
Representative images of immunohistochemical stains of tissue from 0.5 mM bilirubin injected mice 7 days after injection. Yellow colour is the bilirubin clot, blue-green is nuclei stained with methyl green, and dark purple is positive staining for the respective antibodies.

Immunohistochemical analysis of the structural effects in vivo revealed no positive staining for any of the antibodies in saline-injected control mice. However, mice injected with bilirubin had significant positive staining for axonal damage (β-APP), degraded myelin (dMBP), and activated microglia (CD68). The contralateral side of the bilirubin-injected brains were also positive for β-APP and CD68.
Chapter 5 Discussion

As previously mentioned, the two main aims of this work were to 1) determine the effect of bilirubin and its oxidation products on the structure and function of white matter in vitro, and subsequently 2) determine if these compounds have an effect on the structure and function of white matter in vivo.

Incubation of brain tissue in bilirubin showed that at a concentration of 0.5 mM, there is a decrease in amplitude and increase in latency of myelinated axons. TEM analysis of this tissue showed structural differences with lower density of axons and disruption of mitochondrial integrity in the bilirubin incubated tissues. However, we did not find any statistical differences in the CAPs when tissue was co-incubated with bilirubin and NMDAR, AMPAR, and Ca\(^{2+}\) channel inhibitors. Co-incubation of DMSO and bilirubin, on the other hand, showed an improved electrophysiological response in myelinated axons, compared to tissue incubated in just bilirubin. We could not determine whether MSM had protective effects on the white matter as the bilirubin solution used in these experiments did not cause any significant damage to the CAPs.

The next set of experiments therefore addressed the differential effects of fresh and stock bilirubin solution. We found that incubation of tissue in 7 day old bilirubin solution resulted in poorer electrophysiological response compared to the tissue incubated in freshly made solution. This effect did not occur immediately, but could only be detected after 5 hours of incubation. Additionally, fresh and oxidized bilirubin solutions were analyzed using UV spectrophotometry and mass spectrometry, and we found evidence for increased presence of BOXes in the stock solution that had been refrigerated for days.

The final set of experiments focused on the in vivo effects of bilirubin. CAP recordings indicated that 7 days after injection, bilirubin impaired the electrophysiological properties of unmyelinated axons, whereas immunohistochemical staining at this time point suggests axonal, and to a lesser degree, myelin damage.
I. Sodium channel analysis within the corpus callosum

Figure 6 is evidence that the CAPs, which were being recorded with this electrophysiological method, were exclusively carried by voltage-gated Na\(^+\) channels, since TTX blocked the CAPs and is known to selectively inhibit these channels. This is further supported by the data in Figure 7, which shows that the electrophysiological response is only possible when Na\(^+\) is present in the aCSF which is continuously perfused through the recording chamber.

All of the electrophysiological recordings in the experiments have been performed using the same methodology, therefore it can be inferred that the data presented is specifically of voltage-gated Na\(^+\) channel action potentials within the corpus callosum. As such, it is important to note that the findings and interpretations of the following data apply only to these conducting portions of axons within this particular white matter tract. Although some data may be extrapolated, no inference can be made about the effects of bilirubin on axonal conduction within the gray matter or on synaptic transmission.

II. Effect of bilirubin on CAPs in vitro

The size of the electrophysiological measure of amplitude of an action potential is proportional to number of conducting axons. Therefore, the data in Figures 8-10 suggest that incubation of mouse brain tissue results in decreased amplitude of the potential in the white matter tract, an effect that appears to increase with the concentration of bilirubin. A similar trend can also be observed in the responding latency, or rather speed of conduction, of action potentials. Average recordings from tissue incubated in bilirubin have a slower speed of conduction compared to the tissue incubated in aCSF, an effect that also increases with concentration of bilirubin.

Furthermore, it is evident based on this data that the myelinated axons are affected to a greater extent, which would suggest that bilirubin affects the electrophysiological properties of white matter tracts by interacting with the myelinating oligodendrocyte cells. However, the TEM studies indicate that there is a lot of variation within groups, particularly in the unmyelinated axons, which is likely contributing to the lack of statistical significance. Although this may indicate that there is truly no effect of bilirubin on unmyelinated axons, another explanation may
be that bilirubin is affecting unmyelinated axons of particular size, or to a varying degree based on size but that this graded effect is not detectable when all of the axons are analyzed together.

Therefore, a decrease in amplitude suggests there is a loss of axons as a result of bilirubin incubation, as well as that bilirubin causes disruption of axons which results in slower conductance. Based on this data alone, no inference can be made about the mechanism of action through which bilirubin is causing these effects. Furthermore, it should be noted that although this data is intriguing, a concentration of 0.5 mM may be higher than what would be clinically observed after ICH. However, some effect is seen on the electrophysiological response of the white matter at the lower concentrations of bilirubin which would readily be reached after hemorrhagic stroke in the porcine model (Clark et al., 2008). These effects may be further exacerbated in the toxic milieu following ICH, and studying the effects may be easier with a higher concentration due to more pronounced effects. Also the duration of exposure may be important; therefore, assessing these effects further at a concentration of 0.5 mM bilirubin is rational.

**III. Structural effects of bilirubin in vitro**

Although there was no statistically significant difference in axon density between the tissues incubated in aCSF, 0.1 mM, and 0.5 mM bilirubin, there was a trend suggesting that there are progressively fewer axons present in the increasing concentrations of bilirubin. Statistical significance may not have been reached due to a true lack of effect of bilirubin on axon density or because of the small sample size. The former seems unlikely given the consistently observed effects. Although the average axonal density was based on 5 images, all of the images were form the tissue of one animal. Therefore, quantification of axonal density of tissue from more than one animal may give a more accurate representation of the effects of bilirubin on axon density. Decrease in axons density could be due to swelling of axons, giving the appearance of fewer cells, or could be due to cell death. However, qualitative analysis of the images (Fig. 11A) supports the latter hypothesis. Future analysis of cell death, such as performing TUNEL staining of the tissue, would provide further insight into the cause of the potential density decrease.
Furthermore, qualitative analysis of the images revealed differences at a subcellular level. Whereas the mitochondria from tissue incubated in aCSF appear normal with compact, regularly spaced cristae, within well-defined cells, mitochondria from tissue incubated in 0.5 mM bilirubin were considerably larger, and were within cells which seemed to have lost their membrane integrity. This effect was not quantified. Further work is needed to determine the full extent of mitochondrial damage secondary to bilirubin and its oxidation products and to determine whether it contributes to functional impairment of axons.

IV. Role of NMDARs, AMPARs, and Ca\(^{2+}\) channels in the effects of bilirubin on CAPs

Previous studies have shown evidence that bilirubin disrupts the function of NMDARs, whereas no effect has been observed on AMPAR function (Grojean et al., 2000). Therefore, we postulated that the effect on CAPs and structure of white matter induced by bilirubin may be, at least in part, through disruption of NMDARs. This hypothesis was not supported by our data; co-incubation of bilirubin with AMPAR antagonist (NBQX) or NMDAR blocker (D-AP5) did not have any effect, suggesting that bilirubin is not acting via these receptors.

In order to test for evidence of a role for Ca\(^{2+}\) channels, the L-type calcium channel antagonist amlodipine besylate was tested. Based on the electrophysiological analysis in Fig 13, amlodipine co-incubation does not result in any statistically significant difference when compared to tissue incubated in bilirubin alone. However, unmyelinated axons did have a trend towards a larger amplitude (Fig. 13B), suggesting that bilirubin may in fact have some effect on this sub-population of axons. Statistical significance may not have been reached for a number of reasons, such as small sample size, other mechanisms also contributing to the damage, as well as other types of Ca\(^{2+}\) channels being potentially involved. As such, further experiments would be needed to assess these factors or others contribute in order to draw more firm conclusions.

This exploratory data does have some limitations. One main concern is that we did not include a positive control, therefore, it also is possible that the antagonists were not effective at the concentrations employed or that there are none of these receptors involved in the responses. It is
also of note that these are field potential recordings and therefore may not be sensitive enough to
detect subtle differences in conduction. Furthermore, as seen in Figs. 9 and 10, 0.1 mM bilirubin
does not have as great of an effect on the CAP as a concentration of 0.5 mM; therefore, 0.1 mM
bilirubin may not cause significant enough damage to show any improvement with the addition
of the receptor and channel antagonists.

V. The effect of DMSO on structure of the corpus callosum and CAPs in vitro

We were interested in testing some non-soluble drugs in vitro, and in order to solubilize them we
intended on using DMSO. However, due to its known neuroprotective effects, and its ability to
solubilize bilirubin, we tested the effects of DMSO alone. Therefore we compared the effects on
CAPs and structure of tissue incubated only in aCSF, aCSF and DMSO, 0.1 mM bilirubin, and
0.1 mM bilirubin and DMSO. Although there were no significant differences between the speeds
of conduction in the different conditions, there were statistically significant differences in the
amplitude of both the myelinated and unmyelinated axons. The myelinated axons did not show
a large difference between the aCSF, aCSF + DMSO and 0.1 mM bilirubin, however amplitude
of the 0.1 mM and DMSO condition had a significantly large amplitude then the other three
conditions. Unmyelinated axons however, had a similar size of amplitude in the aCSF and 0.1
mM + DMSO conditions, whereas the aCSF + DMSO and 0.1 mM bilirubin conditions had
lower amplitude.

This data suggests that DMSO cannot be used as a solvent for other drugs in these bilirubin
experiments as it has significant effects on the CAPs. Interestingly, it appears that tissue
incubated in bilirubin and DMSO electrophysiologically performs at least as well as tissue
incubated in aCSF alone, and better than tissue incubated in just bilirubin, therefore suggesting a
neuroprotective effect. However, based on qualitative analysis of the CAPs, DMSO without
bilirubin may be impairing the electrophysiological functioning of axons. This would need to be
further examined, however, if additional evidence was to be found, DMSO would not be suitable
therapeutic treatment as it could cause damage to areas of the brain where bilirubin is not
present.
The electrophysiological findings were further supported by qualitative analysis of TEM of cross sections of the corpus callosum, which show similar density and appearance of axons and myelin in the aCSF only condition as well as the 0.1 mM bilirubin + DMSO condition. Also in accordance with electrophysiological data, tissue incubated in aCSF + DMSO showed sparser axonal density, thinner myelin, and more misshapen cells.

**VI. The effect of MSM in vitro**

Although co-incubation of tissue with bilirubin and DMSO resulted in better electrophysiological response and structure of axons compared to bilirubin incubation, DMSO may not be a clinically suitable drug. Therefore, we sought other compounds that may have
similar properties to DMSO and could potentially conjugate bilirubin, without having harmful or toxic effects on the human body. Fulfilling these criteria was MSM, which, as mentioned previously, is a molecule, found in many plants and animals, and is marketed as a natural food supplement. MSM is structurally similar to DMSO; however, it is a less reactive compound due to the second oxygen molecule, which oxidizes the sulphur atom into its highest oxidation state.

Unlike what we predicted, CAPs of tissue incubated in MSM and bilirubin, did not have higher amplitude compared to white matter incubated in bilirubin alone (Fig. 19A, B). In fact we observed a slower speed of conduction of unmyelinated axons with MSM incubation (Fig. 19D). This data suggested that not only was MSM not able to decrease the negative effects produced by bilirubin of electrophysiological properties of white matter, it actually contributed to the damage.

What was more intriguing, however, was that there were no differences between the aCSF and the bilirubin conditions, even though we used the highest concentration of 0.5 mM bilirubin, which had previously had highly significant differences. Although we considered that our previous results may not be replicable, we realized that there was a slight difference in methodology between the two sets of experiments, namely in the preparation of the bilirubin solutions.

**VII. Effects of oxidized bilirubin in vitro**

The experiments analyzing the effect of MSM let to the unexpected observation that bilirubin incubation does not cause electrophysiological changes in white matter. However, our hypothesis that this may be due to use of fresh as opposed to stock bilirubin solution was confirmed with the next set of experiments. Although at the concentration of 0.1 mM bilirubin there were no differences in the CAP response of tissue incubated in fresh versus stock solution, there was a decrease in amplitude and speed of conduction of myelinated axons incubated in 0.5 mM stock bilirubin, relative to the CAP of tissue incubated in fresh bilirubin. This suggests there are differences in the bilirubin solution that are contributing to the effects observed in CAPs.
Lower amplitude of CAPs could result from a lower number of axons conducting and contributing to the overall size of amplitude, or a redistribution of the speed of conduction of the population of axons. Therefore, in order to differentiate the effects contributing to the lower amplitude observed in these recordings, this data was additionally analyzed by measurement of the AUC. Since there were no differences in amplitude or latency of unmyelinated axons, there was also no difference in the AUC of these axons. However, there was a trend of a smaller AUC of myelinated axons incubated in stock compared to fresh solution, which only reached statistical significance at one stimulation. Therefore, a difference in amplitude, latency, and AUC suggests that incubation of brain tissue in 0.5 mM stock bilirubin results in less conducting axons, as well as a decrease in conduction speed of myelinated axons. Loss of conducting axons could be due to cell death, whereas the decreased speed of conduction could be due to demyelination.

Furthermore, time point analysis of the effects of stock bilirubin solution suggests that there is a time-dependent effect. Therefore, the effect on axonal function does not occur immediately but rather it takes, according to this data, 5 hours for the damage to be severe enough to be detected with field potential recordings.

This data is in accordance with our previous findings where we see preferential effects on myelinated, but not unmyelinated axons. More experiments are needed to gain insight into mechanisms that may be causing this axon-subtype specific damage.

**VIII. Analysis of Bilirubin Solutions**

In order to determine the cause of different effects of fresh compared to stock bilirubin solution, we analyzed these solutions using spectrophotometry and mass spectrometry. We hypothesized that the bilirubin was oxidizing while in a stock solution, despite being kept in a glass vial at 4ºC and protected from light. As previous work has shown evidence of BOXes after hemorrhagic stroke, we predicted that these compounds may be present in greater quantities in the stock than fresh solution, and that they may be responsible for the impairment of electrophysiological response of myelinated axons.
As BOXes absorb light maximally at a wavelength of 320 nm we tested the absorbance of the 2 different solutions at this wavelength and found that 7-day-old bilirubin solution absorbs this wavelengths significantly more than fresh bilirubin solution at the same concentration. Furthermore, analysis of the solutions for the molecular weight of bilirubin and BOXes revealed that fresh solution has significantly larger proportional peak signal at the molecular weight of bilirubin than BOXes, which is reversed at 7 days. This data supports our hypothesis and suggests that stock bilirubin has a higher concentration of BOXes than the freshly made solution. Therefore, higher concentration of BOXes could be contributing to the greater electrophysiological effects seen with stock solution.

However, there are some limitations to these analyses. First of all, the mass spectrometry provides evidence of whether a compound is present or not, with only relative amounts, therefore it is not the best quantification method. Furthermore, it is important to note that masses other than the molecular weight of bilirubin and BOXes were detected, suggesting that these are not pure solutions, and therefore some other compound may also be contributing to the electrophysiological effects. This may be difficult to prove given the lack of specific inhibitors of BOX formation.

In addition, it must be acknowledged that this is only correlational evidence. Mere presence of a higher concentration of BOXes does not indicate that they are necessarily contributing to the damage. Future experiments could therefore address this by incubating tissue in known concentrations of BOXes and performing a concentration analysis.

Finally, it is important to recognize that these are in vitro data, and that in vivo effects may not be the same. We would predict that there would be a higher concentration of BOXes in vivo due to a highly oxidizing environment following hemorrhagic stroke, compared to sealed glass vials in which the stock bilirubin solutions were kept for these in vitro experiments.

**IX. The effects of bilirubin on structure of the corpus callosum and CAPs in vivo**

Injection of 0.5 mM of fresh bilirubin resulted in a statistically significant decrease (relative to saline-injected controls) in amplitude of unmyelinated axons, 7 days after the injection. There
was no effect on the latency suggesting that there was a loss of conducting axons, not a decrease in conduction speed. This is further supported by the AUC analysis, which shows significant decrease in area of potential, confirming that the decreased amplitude is not a result of decreased speed of conduction.

This effect was not observed 2 days after injection, suggesting the damage was not immediate and takes time to occur. However, there was a trend suggesting decreased amplitude of myelinated axons 2 days after injection, which was not present 7 days after surgery. The effect was not statistically significant therefore, this data may be true evidence of lack of effect on myelinated axons in vivo, or there might be initial damage, which was repaired by the 7th day. However, since there was no difference in latency, and this lower trend was also seen in the AUC, the data suggests that the damage would be due to a decreased number of conducting axons. Thus, if the axons were being damaged, they would have to be damaged in such a way in which they are not able to conduct at 2 days post-op, but not be damaged irreversibly (e.g., due to cell death) since the damage seems to be reversed by 7 days after the injection. This seems unlikely; therefore further analysis is necessary to determine a more conclusive effect of bilirubin on myelinated axons in vivo.

The histological staining indicates that presence of bilirubin or BOXes resulted in axonal and myelin damage, as well as infiltration of activated microglia. Positive staining for axon damage and microglia in the contralateral side suggests that the effects occurred distally as well, which may be due to diffusion of bilirubin or BOXes, and/or may be detection of axonal degeneration from local damage. However, the structural damage has not been quantified, which would be important for confirming the results and for having a quantitative endpoint for testing potential treatments.

In light of the in vitro finding which suggests that BOXes may be contributing to the functional damage, it is important to determine their effects in vivo. However, the data presented here does not address this factor. Future experiments should aim to determine the concentration of BOXes in the white matter at the different time points after injection of bilirubin this model, and what the effects are electrophysiologically, structurally and functionally.
Chapter 6 Conclusions

This set of experiments aimed to determine the effect of bilirubin and its oxidation products on the structure and function of white matter in vitro, as well as their effects when present in the mouse brain in vivo. Based on previous knowledge about the effects of these molecules we hypothesized that these compounds cause functional loss in white matter tracts through direct or indirect structural disruption of myelin and axonal integrity.

To reiterate, evidence for concentration-dependent effects was seen in the progressively smaller amplitude and decreased speed of conduction, with increased concentration of bilirubin. In vitro, myelinated axons appear to be affected to a greater extent than unmyelinated. However, there was a large variation in the data, which limits the definitive conclusions that can be drawn. This data supports our hypothesis and is in line with previous work showing that bilirubin can cause functional disruptions in brain electrophysiological processes, such as in long-term potentiation and long-term depression.

We found that the tissue incubated in vitro in varying concentrations of bilirubin showed decreased axon density, qualitatively more misshapen axons, and enlarged mitochondria. Decreased axonal density suggests that the bilirubin or its breakdown products cause cell death, which has previously been found to occur (Gao et al., 2011; Grojean et al., 2000; Grojean et al., 2001; Rodrigues et al., 2002). However, we did not include any methods to detect cell death such as routine histological assessment for necrosis or staining such as TUNEL, which would have been potentially useful methods to quantify this damage. Structural mitochondrial changes, which were evident in TEM images, have also been shown to occur previously in response to bilirubin; however, we did not assess mitochondrial functioning or cytochrome c release. Although further investigation of this damage would be necessary to draw any firm conclusions, this data does suggest that one of the ways bilirubin or BOXes may cause electrophysiological dysfunction of axons is by initiating structural damage within mitochondria. Furthermore, it is of note that although structural changes were assessed in the in vivo experiments, we did not specifically look at whether there was evidence for mitochondrial damage.
In order to further explore potential mechanisms through which bilirubin and/or its oxidation products may be affecting white matter, we co-incubated mouse brain tissue with bilirubin and NMDAR, AMPAR, or Ca$^{2+}$ channel antagonists. Although NMDA receptors have previously been shown to be affected by bilirubin, this was not supported by this data as we did not find any significant differences in the CAP analyses. However, the data is limited as we did not include a positive control for the effects of the receptor and channel antagonists used, and the concentration of bilirubin may not have been sufficiently high to cause significant disruption in the CAPs. Only single concentrations and incubation times of antagonists were tested. Therefore, more experiments need to be done to understand how bilirubin or BOXes cause functional disruption of white matter tracts.

Furthermore, we tested the effects of co-incubation of bilirubin with DMSO, and found that it significantly protected the white matter function from bilirubin damage. Although we did not further examine how DMSO was neuroprotective, we hypothesize that it may be due to its ability to solubilize bilirubin. This seems counterintuitive, as one would expect that more bilirubin in solution would cause more damage. However, bilirubin has been shown to be neurotoxic when in its unconjugated state, whereas when the bilirubin is unconjugated by GUT, internal hydrogen bonds are broken and it becomes soluble and easily transportable throughout the bloodstream without causing any damage. Therefore, DMSO may be neuroprotective by similarly solubilizing bilirubin by breaking its internal hydrogen bonds (Fig. 32), and thus decreasing the polarity of the molecule and its ability to interact with phospholipids membranes.

As mentioned, since DMSO may have caused impairment of normal tissues (Figures 17 and 18), we tested a structurally similar, although less reactive compound, MSM. However, this data lead to the unexpected observation that there is a difference in the effects bilirubin was causing when it had been made into solution the same day, as opposed to having been made previously. Although this allowed us to discover something interesting, which we did not initially predict, we did not further examine the effects of MSM. Therefore, whether MSM is protective like DMSO remains to be elucidated.
Bilirubin is known to change properties when exposed to UV light and higher temperatures, therefore, these results were unexpected since the stock solution was kept protected from light and in a tightly sealed glass vial at a low temperature. To address this concern, we controlled the age of the stock solution in order to quantify these differences and found that, at a concentration of 0.1 mM bilirubin, there were no significant differences. However, at a concentration of 0.5 mM, lower amplitude and smaller AUC of myelinated axons in the tissue incubated in stock solution suggests fewer conducting axons, whereas the increase in latency suggests further damage through a decrease in the speed of conduction. The analysis of the two different solutions supported our hypothesis that bilirubin was oxidizing while in the stock solution, and suggests that BOXes have a significant contribution to the damage to white matter that has been observed in these experiments. Despite the interpretation of this relationship between BOXes and the damage being correlational, it is an important novel finding.

In the final set of experiments we focused on addressing the second aim, namely whether these compounds also have structural or functional effect \textit{in vivo} in the mouse brain. This was assessed by stereotactic injection of 0.5 mM bilirubin or saline near the corpus callosum; electrophysiological and IHC data supported our hypothesis for structural and functional damage. Interestingly however, there were some notable differences between the \textit{in vitro} and \textit{in vivo} CAP findings. \textit{In vitro} data showed almost exclusive damage to myelinated axons, but not unmyelinated axons, suggesting that bilirubin or BOXes are causing the functional damage by affecting oligodendrocytes. However, the decrease in amplitude of myelinated axons was only observed 2 days, but not 7 days, after surgery, and it did not reach statistical significance. Unmyelinated axons on the other hand did not show impaired electrophysiological functioning 2 days after the surgical injection of bilirubin, but they had lower amplitude and AUC at the 7 day time point. Furthermore, there was no change it the latency of the CAPs suggesting that the difference in function was due to fewer conducting axons. The concentration of BOXes was not quantified in the white matter, therefore, the differences observed at day 2 and day 7 post-injection may have been in part due to progressively increasing concentration of BOXes.

Analysis of the structural damage in the white matter shows some evidence of degraded myelin basic protein, a major component of oligodendrocytes, and this damage was only present
immediately adjacent to the bilirubin. However, axonal damage was far more prevalent and may be occurring near, as well as distally, from the bilirubin injection site. Furthermore, there was evidence of microglial activation along the majority of the corpus callosum. Although qualitative, this data is in accordance to what was observed electrophysiologically.

The different effects on myelinated and unmeylinated axons observed *in vitro* and *in vivo* could be due to different vulnerabilities of the different types of cells under the different conditions. For example, oligodendrocytes may be more sensitive than neurons when the tissue is kept alive artificially outside of the brain, as was done in the *in vitro* experiments. Conversely, non-myelinated axons may be more susceptible to damage *in vivo* due to other factors, which may only occur within the living organism, such as inflammatory responses.

The findings of the experiments described above largely support our hypothesis that bilirubin and/or its oxidation products cause functional loss in white matter tracts through direct or indirect structural disruption of myelin and axonal integrity. Presence of bilirubin and/or BOXes showed damaging effects on electrophysiology of CAPs *in vitro* and *in vivo* in mouse tissue. Furthermore, their presence also affected the structure of axons and myelin *in vivo* and *in vitro*.

To address our hypothesis, both function and structure were assessed, however, specific analysis was not performed to directly correlate the findings that the functional damage is due to the structural changes observed, as we predicted. Although, not conclusive as to whether it is bilirubin, BOXes, or a combination of both compounds, this data is the first, to our knowledge, to show that these compounds cause structural and functional damage to white matter tracts in mice in the context of ICH. Another important finding was that both *in vivo* and *in vitro*, the damaging effects seen on structure and function of the white matter were not immediate suggesting the damage takes time to occur and hence there is a therapeutic window during which the extent of the damage may be prevented or diminished.
Chapter 7 Future Directions

Most of the experiments presented here have been exploratory. This is necessary given that there is very limited work done on this topic, and despite some limitations, these findings certainly warrant further investigation into the effects bilirubin and BOXes on white matter after ICH. Since the data is largely focused on effects in vitro, subsequent experiments should include further assessment of the effects in vivo. For example, assessment of the BOXes concentrations at different time points after stereotactic injection of bilirubin would allow for correlational analysis as to whether they are associated with structural and functional damage. This would be important to determine as it would give a better idea as to how the damage can be prevented. For example, if BOXes are found to have a concentration dependent effect on the white matter, prevention of bilirubin oxidation would be the main therapeutic target as opposed to bilirubin removal from the hematoma and surrounding tissue.

Another experiment would be to determine if these electrophysiological and structural changes caused by presence of bilirubin and/or BOXes cause behavioral changes and impaired functional recovery of animals. Although this could easily be assessed with various behavioral tests of the mice at various time points after surgery, the findings may not properly reflect what occurs in human patients. Due to their smaller volumes of white matter, species differences in rate of physiological recovery, and significantly lower level of cognitive complexity required for normal functioning, behavioral tests may not be sensitive enough to pick up on any functional impairment. Furthermore, even very small behavioral or cognitive improvements in human patients may lead to a significant increase in their subjective quality of life. Finally, bilirubin has to have some effect on brains given the existence of kernicterus and brain injury at some point has to be detrimental to function whether it can be measured in rodents or not. Therefore, although behavioral rodent tests would add to the understanding of the effects of bilirubin and BOXes, lack of findings should not necessarily be evidence against further study of the effects of bilirubin in humans.

Even if no behavioral differences were found, testing potential therapeutic treatments could be performed on rodents since there are electrophysiological and structural changes that we and others have identified due to bilirubin, and these can serve as endpoints. There are a number of
potential treatments that may prevent formation and oxidation of bilirubin. For example, blocking HO-1 would prevent formation of biliverdin and thus bilirubin and BOXes, however, this would increase levels of hemoglobin which is toxic in and of itself (Everse and Hsia, 1997). Other treatments could include local or systemic administration of albumin in order to bind the free bilirubin and assist in removing it from the area of injury. Furthermore, although highly speculative, oral MSM intake may help alleviate the damage. However, in vitro effects on oxidized bilirubin would be necessary to determine if it may have some protective properties like the similar compound DMSO. Another intriguing concept relates to the ability of UV light to degrade bilirubin and abrogate its toxicity. Whether light applications could be used after surgical removal of ICH when there is a surgical access route is not beyond reason. Sophisticated targeted endovascular methods can also be envisioned.

Another important consideration is to determine if there is any evidence for presence of bilirubin and BOXes in perihematomal white matter and whether there is evidence of damage due to these compounds in humans. These compounds are known to be present in a porcine model of ICH, however this could be confirmed in the rodent models and in humans by autopsy or analysis of hematomas removed at surgery. Ultimately, if the concentrations of these compounds can be determined noninvasively in survivors of ICH, their levels could be correlated with measures of brain injury like MRI and diffusion tensor imaging analysis of white matter, as well as cognitive and behavioral performance, and reported quality of life. If evidence of damage was present and if any treatments were found to be effective in animal studies, they could then be testing in humans in hopes of also being able to alleviate the secondary brain injury after ICH.
Chapter 8 References


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