# IMMUNOLOGICAL AND SEROLOGICAL MEDIATORS OF CONDUCTION DEFICITS IN MAMMALIAN SPINAL CORD

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by

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The thesis by

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entitled:

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# ABSTRACT

This thesis embodies three studies that investigated immunological and serological mechanisms contributing to spontaneous recovery after spinal cord injury (SCI). In the first study, the effects of the proinflammatory cytokine tumor necrosis factor alpha (TNFα) on axonal conduction were tested. TNFα reduced the compound action potential (CAP) amplitude and depolarized the compound membrane potential (CMP) of axons from the guinea pig spinal cord. These effects were reversed upon washout of TNFα. In the second study, the effects of carbon monoxide (CO) on axonal conduction were tested in the same animal model; CO is a byproduct of the breakdown of heme by the heme oxygenase system. CO was observed to irreversibly reduce CAP amplitude, increase stimulus-peak latency, and increase the refractory period for repetitive axonal discharge. The third study examined the effects of hemoglobin (Hb) *per se* on axonal conduction and the ability of Hb to reverse the effects of CO. Treatment with Hb induced reversible reductions in CAP amplitude and increased the refractory period, blocked the effects of CO when coadministered, but did not reverse the effects of CO when administered after CO treatment.

These studies present novel, proof-of-principle, demonstrations that immunological and serological factors are capable of impairing axonal conduction in isolated mammalian spinal cord. It seems plausible that these factors contribute to the early onset neurological dysfunction, and its reversal, as the acute neuroinflammatory events resolve, following CNS trauma or

symptom exacerbation in other neuroinflammatory diseases in which spontaneous recovery or remission are observed.

**Key Words:**

Spinal Cord Injury, Tumor Necrosis Factor Alpha, Carbon Monoxide, Hemoglobin, Heme Oxygenase, Electrophysiology, Guinea Pig, Double Sucrose Gap, Compound Action Potential

# COAUTHORSHIP STATEMENT

This thesis, and all material contained herein, was written by the author, Andrew L. Davies.

The first study entitled "RECOMBINANT HUMAN TNFα INDUCES CONCENTRATION-DEPENDENT AND REVERSIBLE ALTERATIONS IN THE ELECTROPHYSIOLOGICAL PROPERTIESOF AXONS IN MAMMALIAN SPINAL CORD" was conducted by the author, Andrew L. Davies. It was conceptualized by Dr. Keith C. Hayes and the author. This study was conducted at Purdue University (West Lafayette, IN, USA) under the guidance of Dr. Riyi Shi. All animal surgeries were performed by Phyllis D. Zickmund. The author, Andrew L. Davies, completed all additional data collection, analysis, and writing presented in this study. Dr. Keith C. Hayes and Dr. Riyi Shi provided editorial support during the writing process. The feasibility of this line of inquiry was assessed in a pilot study presented in the M.Sc. thesis of Andrew L. Davies (2004).

The second study entitled "CARBON MONOXIDE-RELEASING MOLECULE CORM-2 INDUCES CONCENTRATION-DEPENDENT ALTERATIONS IN THE ELECTROPHYSIOLOGICAL PROPERTIES OF AXONS IN MAMMALIAN SPINAL CORD" was conducted by the author, Andrew L. Davies at Parkwood Hospital (London, ON). It was conceptualized by the author and Dr. Keith C. Hayes. All animal surgeries and data collection presented in this study were performed by the author with some assistance from John L.K. Kramer. All data analysis and writing presented in this study

were done by the author, Andrew L. Davies. Dr. Keith C. Hayes and John L.K. Kramer provided editorial support during the writing process. The feasibility of this line of inquiry was assessed in a pilot study done in collaboration with John L.K. Kramer and presented in his M.Sc. thesis (2007) with the permission of Andrew L. Davies.

The third study entitled "HEMOGLOBIN INDUCES REVERSIBLE ALTERATIONS IN THE ELECTROPHYSIOLOGICAL PROPERTIES OF AXONS IN MAMMALIAN SPINAL CORD AND IS PROTECTIVE AGAINST THE CONDUCTION BLOCKING EFFECTS OF CARBON MONOXIDE" was conducted by the author, Andrew L. Davies at Parkwood Hospital (London, ON). It was conceptualized by the author and Dr. Keith C. Hayes. All animal surgeries and data collection presented in this study were performed by the author with some assistance from John L.K. Kramer. All data analysis and writing presented in this study was done by the author, Andrew L. Davies. Dr. Keith C. Hayes and John L.K. Kramer provided editorial support during the writing process.

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# ABBREVIATIONS

| | |
|---|---|
| CNS | Central Nervous System |
| SCI | Spinal Cord Injury |
| Hb | Hemoglobin |
| HO | Heme Oxygenase |
| CO | Carbon Monoxide |
| cGMP | Cyclic Guanosine Monophosphate |
| NO | Nitric Oxide |
| TNFα | Tumor Necrosis Factor alpha |
| CORM-2 | Carbon Monoxide-Releasing Molecule, Tricarbonyldichlororuthenium (II) Dimer |
| i.m. | intramuscular |
| AUS | Animal Use Subcommittee |
| CAP | Compound Action Potential |
| CMP | Compound Membrane Potential |
| DMSO | Dimethyl Sulfoxide |
| $RuCl_3$ | Ruthenium (III) Chloride Hydrate |
| RBC | Red Blood Cell(s) |
| MS | Multiple Sclerosis |
| GBS | Guillain-Barré Syndrome |

# CHAPTER 1: INTRODUCTION

## 1.1. Spinal Cord Injury (SCI)

### 1.1.1. Epidemiology of SCI

Traumatic injury to the spinal cord is one of the most catastrophic and disruptive types of injuries to the central nervous system (CNS) and has significant emotional, physical, and financial consequences. The annual incidence rate of spinal cord injury (SCI) in Ontario varied from 37.2 to 46.2 per million based on patient samples taken between 1994 and 1999 (Pickett et al., 2003). A more recent and local analysis of medical records at the London Health Sciences Centre (London, Ontario, Canada) yielded an annual rate of 42.4 per million between the years of 1997 and 2001 in adults aged 15-64 years and 51.4 per million in patients 65 and over (Pickett et al., 2006). Extrapolating from these figures, there are more than 13,000 new incidences of SCI in North America each year, while at least ten times that many individuals are currently living with the sequelae of SCI in Canada and the United States (Tator et al., 1993; Sekhon and Fehlings, 2001). The annual cost of care for such individuals is considerable, in fact, SCI is the second most expensive condition treated in American hospitals with an average acute-stage cost of $53,000 US (Winslow et al., 2002).

### 1.1.2. Etiology of SCI

The majority of studies identify motor vehicle collisions as the most common cause of SCI in the general population and this is particularly true in young adults (Tator et al., 1993; Sekhon and Fehlings, 2001; Pickett et al., 2003; Pickett et al., 2006). Falls are also frequently reported mechanisms of injury and

often account for the majority of injuries among the elderly. Additional causes include "other" vehicular accidents (bicycles, snowmobiles, etc.), sports-related injuries, work-related accidents, and violence. The direct mechanical consequence of these events is flexion, rotation, compression, or extension of the vertebral column that is abnormal in its extent, direction, or both (Hughes, 1978; Iencean, 2003). Associated with mechanical deformation of the spinal column is compromise of the spinal canal and injury to the cord itself. Contrary to the popular misconception, the spinal cord is rarely severed (Kakulas, 1988) and this has important implications for clinical management, functional outcomes, and research seeking to ameliorate the neurological deficits typically associated with SCI.

## 1.2. Pathophysiology of SCI

The most common biomechanical mechanisms of SCI are compression or contusion of the spinal cord as a result of the protrusion of ruptured intervertebral discs or fractured vertebrae into the spinal canal (Kakulas, 1999). The structural damage to the bony vertebral column encasing the cord may or may not rupture the meninges, but does result in mechanical deformation of the cord parenchyma and its blood supply (Sekhon and Fehlings, 2001). This initial traumatic insult may vary in duration, surface area, and location depending on the specific circumstances of the injury, but the ensuing neurological consequences and pathophysiological sequelae are relatively consistent across individuals and even across species.

### 1.2.1 The Primary Injury

It is now common to categorize the pathology of SCI into the so-called "primary" injury and the subsequent "secondary" injury (Tator and Fehlings, 1991). The primary injury refers to the initial physical insult to the cord parenchyma and the accompanying mechanical disruption of axons, myelin, and vascular supply. This may also include persistent compression in some instances and therefore varies in duration from case to case (Sekhon and Fehlings, 2001). Regardless of mechanism, SCI typically results in disruption of descending and ascending tracts with associated complete or partial motor paralysis, sensory loss, and autonomic dysfunction below the level of injury. As noted previously, cord transection is a relatively unusual occurrence and most injuries are a result of a contusion i.e. "bruising" with bleeding into the cord parenchyma (Kakulas and Taylor, 1992).

### 1.2.2. Hemorrhage and the Heme Oxygenase (HO) System

Most instances of SCI involve appreciable hemorrhage and early patterns of intraparenchymal bleeding have been shown to correspond to the severity of the initial injury and cavitation at later stages (Noble and Wrathall, 1989a; Noble and Wrathall, 1989b; Mautes et al., 2000b); such observations are indicative of the importance of vascular disruption in injury progression. Hemorrhage is usually found in the gray matter at the epicenter of the lesion and spreads to a varying degree into neighbouring white matter both rostral and caudal to this site (Tator and Koyanagi, 1997; Mautes et al., 2000b). One of the major consequences of intraparenchymal bleeds is disruption of regular blood flow, resulting in ischemia, and even restoration of blood flow can exacerbate tissue

damage via reperfusion injury and free radical production (Tator and Koyanagi, 1997; Mautes et al., 2000b). Hemorrhage and edema, along with the mechanical injury, also often result in disruption of the blood-spinal cord-barrier, facilitating additional extravasation, and contributing further to inflammation (Popovich et al., 1996). As blood collects proximal to the lesion site, erythrocyte lysis occurs, and this results in the liberation of hemoglobin (Hb) into surrounding tissue (Yip et al., 1996). The oxygen-carrying constituents of Hb are its heme groups, and "free" heme is released as a consequence of Hb breakdown. Heme is subsequently broken down into biliverdin, iron, and carbon monoxide (CO) by the endogenous enzyme heme oxygenase (HO). Constitutive expression of the HO isoform HO-2 is measurable under normal conditions, however, the expression of the inducible isoform HO-1 is upregulated in neurons, astrocytes, and microglia following SCI (Mautes et al., 1998; Mautes and Noble, 2000; Mautes et al., 2000a). Long term exposure to high levels of hemoglobin, iron, and carbon monoxide are thought to be neurotoxic, but little is known about the effects of these byproducts of posttraumatic hemorrhage on neuronal activity in the short term and at lower or intermediate concentrations.

Ultimately, the primary injury does not necessarily result in immediate and catastrophic damage to the cord *per se*, although it may do so indirectly via hemorrhagic events and the ensuing sequelae; these events are collectively referred to as the secondary injury.

### 1.2.3. The Secondary Injury

The idea that additional events beyond the initial primary injury contribute significantly to tissue loss and ultimately the clinical manifestations of SCI is now

widely accepted (Sekhon and Fehlings, 2001). Secondary injury encompasses a number of cellular and molecular processes that are initiated by the initial insult and extend in duration from minutes to weeks. Recognition that the rapid and immediate primary injury was not entirely responsible for the damage associated with SCI and the identification of slower, more prolonged, secondary injury processes, served to identify a new therapeutic window that had not been considered previously. Despite this development, the inherent difficulty in identifying therapeutic targets for SCI is the large number of putative factors involved in the progression of secondary injury.

Sekhon and Fehlings (Sekhon and Fehlings, 2001) summarized the key components of secondary injury into 9 categories: vascular, ionic, neurotransmitter, free radical, opioid, edema, inflammation, ATP depletion, and apoptosis. Inflammation in particular has been extensively studied in recent years as it is now thought to play a prominent role in tissue damage associated with secondary injury.

### 1.2.4. Inflammation and the Immune Response after SCI

Traumatic SCI results in increased expression of proinflammatory cytokines, microglial activation, and extravasation of neutrophils, macrophages, and lymphocytes that begins immediately after injury and may persist for weeks, months, or even years (Hausmann, 2003; Donnelly and Popovich, 2008). The specific time course varies with the inflammatory mediator or cell type in question (Fleming et al., 2006), and better characterization of the temporal profile of the immune response to SCI is facilitating more specifically targeted anti-inflammatory therapeutic strategies. There has also been some suggestion that

the immune response contributes to regeneration, but whether this can be said to exceed the detrimental effects of inflammation is unclear and controversial (Bethea, 2000). In the short term, activated immune cells mediate tissue damage via induction of excessively high concentrations of substances such as free radicals, enzymes, chemokines, and cytokines; the high concentrations being cytotoxic. However, the injury eventually stabilizes as many of the secondary injury processes are resolved, although the extent to which the immune response returns to preinjury levels in individual cases is not always clear (Cruse et al., 2000). In fact, there is now evidence accumulating from our laboratory (Hayes et al., 2002a; Davies et al., 2007) and others (Segal et al., 1997; Frost et al., 2005) that many individuals with longstanding, i.e. chronic, SCI exhibit abnormal immunoactivity that may be attributable to several different factors, such as impaired immunoregulation, the presence of secondary medical complications, or persistent neuroinflammation.

Ultimately, all of the factors associated with secondary injury contribute in varying degrees to further tissue damage and functional loss, but one key commonality is that in many cases, even those in which functional loss is complete, a proportion of spinal cord tissue that traverses the epicenter of the lesion remains intact (Kakulas, 1988). Generally speaking, the degree of tissue sparing is proportional to the functional recovery (Fehlings and Tator, 1995), although this is not universally true as there are numerous instances where the functional deficits are disproportionate (i.e. greater) to the degree of spared tissue (Dimitrijevic, 1988; Hayes et al., 1991; Kakulas, 1999; Finnerup et al., 2004).

### 1.2.5. Current Treatment Options

Given the relative infancy of current research programs targeting various facets of secondary injury processes and the vast number of potentially key factors, no fledgling treatment options have consistently demonstrated functional benefits and efficacy. There was a considerable amount of optimism surrounding the use of methylprednisolone (Bracken et al., 1990; Bracken et al., 1992), for example, as a means of limiting posttraumatic inflammation in order to reduce damage due to secondary injury. Despite the fact that this treatment was the standard of care for some time, its initial promise was subsequently tempered by the realization that high-dose corticosteroids increase risk of infection, prolong hospitalization and attendant costs, and cause other severe complications (Short et al., 2000). Cellular replacement therapies, in the form of transplantation of tissue or stem cells, are also being investigated in animal models as a means of replacing cord tissue, bridging the gap resulting from the lesion, or providing a suitable environment for remyelination or regeneration (Thuret et al., 2006). Ultimately, even in the most "successful" of these studies, only modest improvements have been reported. Other treatment options that have been explored include neuroprotective strategies and pharmacological interventions. Neuroprotection has been moderately effective, but may be limited in terms of clinical practicality by a narrow therapeutic window in many cases depending on the specific treatment tested. Of the numerous restorative therapeutic pharmacological approaches that have been advocated, 4-aminopyridine has received the most publicity, but has yet to satisfy the requirements of the Federal Drug Administration for marketing and distribution (Hayes, 2007).

More recently, a number of rehabilitation training strategies have achieved prominence, in part due to the high-profile advocacy of Christopher Reeve, but also based on their intrinsic appeal as non-invasive and non-pharmacological treatment options for those with longstanding injuries. Bodyweight-supported treadmill training (Hicks et al., 2005) and functional electrical stimulation cycling (Griffin et al., 2008) are two of the most commonly used methods and numerous trials are currently underway intended to assess their therapeutic effectiveness in restoring function, quality of life, and general health status.

There are, therefore, no therapeutic options currently available to individuals living with SCI that have been proven capable of restoring neurological function to any appreciable extent or preventing damage to any notable degree following traumatic SCI. It is possible that such a therapeutic intervention is still years away from discovery and widespread implementation. Nevertheless, the study of SCI and the search for therapeutic targets has yielded a number of promising avenues of inquiry that may provide hope in the much nearer future. Specifically, the demonstration that neurological function can improve, even years after injury (McDonald et al., 2002; Kirshblum et al., 2004; Fawcett et al., 2007), provides hope for improving the quality of life of all individuals currently living with SCI.

## 1.3. Neurological Improvement Following SCI

Neurological improvement following SCI has been reported at various stages post-injury and attributed to various causes. These improvements are

manifest as "conversions" from one level of impairment on the American Spinal Injury Association (ASIA) Impairment Scale (AIS) to another. The majority of functional improvements due to spontaneous and treatment-induced effects occur within the first 12 months after injury, and roughly 20% of SCI patients improve from an AIS classification of A (no sensory or motor function at the S4-S5 sacral segment level) to B or greater, depending on the treatment received (Fawcett et al., 2007). Some functional recovery has been noted beyond the one year mark and this is generally referred to as "late-onset" recovery (McDonald et al., 2002; Kirshblum et al., 2004). When dissociating treatment-induced neurorecovery from spontaneous or naturally occurring recovery, it is critical to consider the acute onset "spinal shock" and its resolution, a clinical phenomenon that has been described extensively since the early 1900's (e.g. Riddoch, 1917) but remains poorly understood in terms of physiological mechanisms.

### 1.3.1. The Acute Stage and Spinal Shock

The term spinal shock refers to the period of areflexia and paralysis occurring during the first 24 hours after injury and the ensuing (partial) recovery of reflex activity and function over the days, weeks, or even months that follow (Ditunno et al., 2004); it is a prime example of functional impairment that is disproportional to frank neurological damage. The initial period of areflexia may be characterized by the absence of normal reflex activity or the presence of pathological reflexes. Paralysis is noted in muscles caudal to the lesion site and flaccidity is usually apparent. The extent to which reflex activity and function are recovered varies greatly with the severity and location of injury (Ko et al., 1999; Ditunno et al., 2004). The early resolution of paralysis and return of reflexes

typically occur between one and three days postinjury, and appreciable "spontaneous" functional improvements can occur from days to years after the initial injury. Here 'spontaneous' refers to naturally occurring processes as distinct from surgical, pharmacological, or other therapeutic interventions. Return of function may be influenced by a number of factors, but even early accounts of SCI and spinal shock describe increased impairment of reflex activity during infectious episodes, at which time "functional activity of the reflex arcs deteriorates" (Riddoch, 1917).

### 1.3.2. Putative Mechanisms of Spinal Shock

The early paralysis and areflexia of spinal shock have been attributed to neuronal hyperpolarization (Ditunno et al., 2004), loss of background excitation (Leis et al., 1996), and altered patterns of inhibition (Calancie et al., 1993). Edema and associated neuropraxia are considered, clinically, to be contributory, although experimental verification is lacking. Elevation of extracellular potassium as a result of mechanical disruption of cell membranes (Chesler et al., 1991; Chesler et al., 1994) has also been purported to result in alterations in ionic gradients resulting in impaired neuronal activity. The subsequent resolution of spinal shock and restoration of some function and reflex activity have been similarly attributed to increased neurotransmitter sensitivity and synaptic plasticity (Ditunno et al., 2004). All of these factors may play a role in the resolution of spinal shock, but currently represent theories rather than robust evidence, and may in fact be inaccurate. This is especially true given the rapid time course of resolution and the absence of synaptic activity likely to lead to plastic adaptations. Moreover, the loss of background excitation would not be expected

to resolve in the absence of restoration of connections and regeneration, which are not likely to occur in the first 1-3 days postinjury. Two of the more widely espoused mechanistic explanations, that spinal shock is the result of neuronal hyperpolarization and the accumulation of extracellular potassium, are, in actuality, contradictory, as extracellular accumulation of potassium would result in depolarization of the membrane potential. It is therefore not clear how any of these proposed mechanisms would spontaneously resolve and thus be permissive to functional restoration.

As indicated, one of the major shortcomings of existing mechanistic theories of spinal shock is that the time course of many of these processes is not consistent with its typical clinical progression. However, the depth and duration of spinal shock have long been known to be dependent on the presence or absence of infection (Riddoch, 1917). Further, the clinical time course of spinal shock does parallel the temporal profile of immunoactivity associated with SCI (Donnelly and Popovich, 2008). For example, functional deficits reported during the acute stage of SCI occur during the first 72 hours in which edema, elevated proinflammatory cytokine profiles, and hemorrhage are most pronounced, and resolution of acute inflammation, reduced swelling, and red blood cell (RBC) clearance coincide with restoration of motor function, sensation, and reflex return. The temporal correspondence among these processes raises the possibility of the involvement of immunological events or processes in reversible neurological dysfunction.

### 1.3.3. The Chronic Stage and Late-Onset Recovery

The concept of late-onset recovery has gained prominence in the last decade due in large part to the purported success of the activity-based therapy program in which Christopher Reeve was an active participant (McDonald et al., 2002). During the treatment program, which was implemented 5 years after injury, Reeve's condition improved such that his status was upgraded from AIS A to AIS C, reflective of improved motor and sensory function and decreased spasticity. Close inspection of the findings of this study reveal a pattern that was not commented on by the author but may be of great relevance to the interpretation of the results. Adverse events (requiring medical care or hospitalization, such as severe infection or pressure sores) were recorded throughout the study as were measures of motor function, touch sensation, and pinprick sensation. A large decrease (>50%) in the number of infectious events was apparent prior to improvement in all three of these areas of neurological function. This correspondence between resolution of immuno-challenge and neurorecovery may be circumstantial but may also be indicative of a causal relationship.

Despite its obvious notoriety and value as a means of promoting public awareness of SCI, a single case study is of little inferential value. Fortunately, a subsequent study retrospectively quantified late-onset recovery, in this case referring to recovery between one year and five years postinjury, in a more systematic manner (Kirshblum et al., 2004). Within the sample group in question, spontaneous neurological recovery from complete functional loss to incomplete status was noted in 5.6% of cases. Further, roughly 40% of subjects

demonstrated improved motor index scoring. The observation that individuals with SCI can recover some degree of function long after injury is cause for optimism; indeed, a minor functional improvement can have a profound impact in terms of quality of life. What these studies do not address are the underlying physiological mechanisms by which improvement is achieved.

### 1.3.4. Putative Mechanisms of Late-Onset Recovery

Neuronal plasticity has been advocated as a potential explanation for long-term recovery as reported years after SCI (Bareyre, 2008). Plasticity may involve formation of new synaptic connections, axonal sprouting, and even neurogenesis. Evidence in support of these concepts speaks against the long-held belief that the generation of new neuronal cells does not occur in the adult mammalian CNS. Certainly, all of these changes in cortical or spinal architecture may result in improved neuronal connectivity after SCI, in theory even across the site of the cord lesion. Proof of their existence in human cases of SCI is lacking although accumulating evidence is suggestive of plastic adaptations (Hiersemenzel et al., 2000; Mikulis et al., 2002; Hayes et al., 2004; Dietz and Colombo, 2004; Jurkiewicz et al., 2007).

Exploring other lines of inquiry, it has been reported, anecdotally, that individuals with SCI occasionally present with functional scores that vary with general health status. For example, sensory function for an individual with a serious urinary tract infection may be markedly lower than that noted during an asymptomatic period. This is not necessarily surprising from an intuitive perspective; it is widely recognized that cognitive (and therefore neurological) function and other mental faculties (such as motivation) are impaired during

periods of illness. In instances where such changes take place over very short periods of time the involvement of plasticity in alterations in function would likely be precluded. Given the access that peripheral (circulating) immune factors have to the CNS (Banks et al., 1995), it is plausible that immune mediators directly or indirectly influence axonal or glial function and neurological function.

Regardless of the extent to which various plastic, adaptive, or immunological factors influence the CNS, there must be some anatomical substrate for these factors to influence. Neuropathological studies provide insights about residual, spared but dysfunctional axons that may provide such a substrate.

## 1.4. Anatomical Basis of Recovery

It is likely an oversimplification to suggest that all recovery following SCI is attributable to one specific underlying mechanism; a combination of several different factors likely contributes in each case. This may account, at least in part, for the fact that the underlying mechanisms by which functional improvement occurs remain relatively unclear. In the absence of transection, most clinical presentations of SCI include a proportion of tissue that traverses the lesion epicenter. This residual tissue, typically including a subpial rim of white matter, may represent an anatomical substrate and starting point for functional recovery.

### 1.4.1. Residual Tissue After SCI

Unsurprisingly, given the great variability in the specifics of each particular injury, the location, extent, and quality of spared tissue following SCI is similarly variable (Kakulas, 1999). Kakulas (Kakulas, 1999), for example, has reported that one patient with a clinically complete injury retained 27.4% of cord tissue at the lesion site, yet another patient with a clinically incomplete lesion had less than 10% spared tissue at the level of injury. Such findings are not unusual and likely reflect the fact that the location of spared tissue, the degree of myelination, and other qualitative factors may be of greater cumulative importance than quantity. Simple quantification of spared tissue is implicitly unlikely to produce a direct and linear correlation with function based on the fact that testable functional outcome measures are dependent on underlying neuronal summation and patterned firing. Therefore, although a significant amount of tissue may be present, the net output may still fall below the necessary threshold required to elicit excitatory (or inhibitory) postsynaptic potentials, and therefore yield no functional outcome. This may account for the observation that the average numerical distinction between a functionally complete injury and an incomplete one is relatively small. In one particular study (Kakulas, 1999), the average number of intact axons at the level of the lesion in clinically complete SCI patients was 2,113 as compared to 3,173 in clinically incomplete patients (for comparison, the average in control subjects was 41,472). It is possible that the separation of the two groups is in part due to a numerical threshold required for summation that falls somewhere in between the two axon counts.

Nevertheless, this cannot account for all instances in which disparate levels of function are encountered in the clinical setting, nor in all animal models of SCI. For example, the extent of functional recovery and residual axon counts in some animal models of SCI do demonstrate a significant correlation (indicative of a general correspondence between the two), but functional differences of greater than 40% with identical axon counts also occur (Fehlings and Tator, 1995). Additionally, electromyographically recorded muscle responses to transcranial magnetic stimulation, motor-evoked potential responses, and conditioning of reflex activity have all revealed that functional connections persist across the lesion epicenter (Hayes et al., 1991; Dimitrijevic et al., 1992; Wolfe et al., 1996). Similarly quantitative sensory testing methodologies and somatosensory evoked potential studies have provided evidence of preserved sensory innervation in patients clincally classified as sensory complete (Hayes et al., 2002b; Finnerup et al., 2004). These and other findings have informed the development of a third classification of SCI in addition to complete and incomplete; "discomplete" SCI (Dimitrijevic, 1988). Discomplete injuries are those in which anatomical contiguity of cord tissue is demonstrable despite absence of overt functionality. Indeed, in some instances, cortical stimulation is able to modulate distal responses despite the inability of the individual to do so voluntarily. This has lead to the suggestion that tissue surviving injury in individuals with SCI has greater functional capability than is being realized; essentially, tissue that survives injury is often dysfunctional. A number of possible mechanisms are being put forth to explain why dysfunctional tissue

becomes functional, or why the anatomical substrate for recovery (tissue that persists after SCI) yields functional improvement in some cases.

## 1.5. Channelopathy

The term channelopathy refers to any condition in which altered ion channel function is known to occur as a consequence of a congenital defect or some type of acquired factor (Waxman, 2001; Rose and Griggs, 2001; Waxman, 2002; Hayes and Fehlings, 2002). Within the injured spinal cord, altered axonal ion channel conductance could impair conduction across the lesion site in tissue that has survived injury sufficiently to result in conduction block. This hypothetical channelopathic contribution to neurological dysfunction would compound the functional deficits known to result from axonopathy and myelinopathy and would represent a new mechanism by which neurological function and recovery following SCI is limited but may be reversible. The putative involvement of axonal channelopathy in SCI is consistent with several distinct but converging lines of evidence. First, several immune factors and other signaling molecules with well-documented neuromodulatory effects play a prominent role in SCI during the acute and subacute stages, and altered immune function and chronic inflammatory markers are present in individuals with longstanding SCI. Second, improved neurological function has been repeatedly demonstrated under varying conditions both immediately after SCI (resolution of spinal shock) and in the long term (late onset recovery); such changes parallel general health status, wound healing, and resolution of infection in time course. Finally, a considerable

amount of tissue sparing is evident in most cases of SCI and is often "dysfunctional" as demonstrated using electrophysiological techniques. The limited number of putative explanations for these and other related clinical phenomena all have limitations that indicate that other unknown mechanisms are likely involved. The novel suggestion that immune and other factors result in acquired channelopathy that masks preserved innervation after SCI is consistent with the existing evidence but until now has not been empirically tested.

## 1.6. Theoretical Framework

The three studies described in this thesis were designed to test elements of the working hypothesis that elevated extracellular concentrations of immune mediators or serological factors can induce axonal conduction failure. Moreover, the proposition that such conduction failure would be reversible, upon elimination of the factors in question, was also tested. This second postulate is integral to the contextual framework that reversible immune-mediated axonal conduction failure underlies some aspects of neurorecovery after SCI.

In particular, the studies outlined have focused on hemorrhagic events, including immunological factors involved in degradation of heme, during the acute stage following injury, and on the proinflammatory cytokine, TNF$\alpha$, that is upregulated at the time of acute insult, but may remain at persistently high levels in the chronic state (Davies et al., 2007).

## 1.7. Hypotheses

The three studies that comprise the body of this thesis were intended to evaluate a number of novel hypotheses regarding the overall suggestion that immune factors play some role in impairing neurological function after SCI in both the short and long term:

**Study 1:**

"Recombinant human tumor necrosis factor α (rhTNFα) induces concentration-dependent and reversible alterations in the electrophysiological properties of axons in mammalian spinal cord"

**Objective:**

The objective of the first study was to examine and characterize the putative axonal conduction blocking properties of the proinflammatory cytokine TNFα. This was formalized into the **hypothesis** that elevated concentrations of rhTNFα reduce compound action potential (CAP) amplitude in isolated guinea pig spinal cord preparations and that this effect is reversed upon washout of rhTNFα.

**Study 2:**

"The carbon monoxide-releasing molecule CORM-2 induces concentration-dependent alterations in the electrophysiological properties of axons in mammalian spinal cord"

**Objective:**

The objective of the second study was to examine and characterize the putative axonal conduction blocking properties of CO, a byproduct of the breakdown of heme by the HO system. This was formalized into the **hypothesis** that elevated concentrations of carbon monoxide (CO) reduce CAP amplitude in isolated guinea pig spinal cord preparations and that this effect is reversed upon washout of CO.

**Study 3:**

"Hemoglobin induces reversible alterations in the electrophysiological properties of axons in mammalian spinal cord and is protective against the conduction blocking effects of carbon monoxide"

**Objective:**

The objective of the third study was to examine the putative axonal conduction blocking properties of Hb, and to test the ability of Hb, a known CO scavenger, to block and reverse the effects of CO. This was formalized into the **hypotheses** that 1) Hemoglobin reduces CAP amplitude in isolated guinea pig spinal cord preparations and that this effect is reversed upon washout of Hb, 2) Hemoglobin blocks the effects of CO on axonal conduction when coadministered, and 3) Hemoglobin reverses the effects of CO on axonal conduction when administered after CO exposure.

## 1.8. Research Plan

To evaluate the hypotheses, the double sucrose gap recording chamber was used to directly expose isolated guinea pig spinal cord axons to a number of immunological or serological factors, thus permitting the study of the specific effect of these factors on axonal conduction. Several electrophysiological parameters were recorded, including CAP amplitude, compound membrane potential (CMP), stimulus-peak latency, and other measures of axonal response to repetitive stimulation and stimulus intensity; all were designed to characterize the effects of the respective factors on neuronal activity.

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# CHAPTER 2: RECOMBINANT HUMAN TNFα INDUCES CONCENTRATION-DEPENDENT AND REVERSIBLE ALTERATIONS IN THE ELECTROPHYSIOLOGICAL PROPERTIES OF AXONS IN MAMMALIAN SPINAL CORD

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## 2.1. Introduction

The role of the proinflammatory cytokine tumor necrosis factor alpha (TNFα) in immune signaling and the immune response has been well documented (Bradley, 2008), however, much less is known about the effects of this molecule within the central nervous system (CNS). At a behavioural level, there is considerable evidence to suggest that TNFα is involved in immune-CNS signaling based on reports of TNFα-dependent changes in various physiological functions including sleep (Krueger et al., 1998), appetite regulation (Vitkovic et al., 2000; Dantzer, 2001), and fever (Conti et al., 2004). In addition, TNFα, as well as other cytokines, can pass from the circulatory system through the blood-brain barrier (BBB) under normal conditions (Banks et al., 1995; Pan and Kastin, 2002), and may then interact with the two principal TNFα-receptor subtypes (TNF-R1 and -R2), both of which are constitutively expressed by neurons and glial cells within the CNS (Benveniste, 1992; Woodroofe, 1995; Benveniste, 1998; Sredni-Kenigsbuch, 2002).

Increased neuronal expression of TNFα and its receptors has been reported following traumatic brain injury (TBI) (Goodman et al., 1990; Fan et al., 1996; Knoblach et al., 1999; Vitarbo et al., 2004; Ahn et al., 2004) and spinal cord injury (SCI) (Yune et al., 2003; Reece et al., 2004). Although most evident in the acute stage following vascular accidents (Gruber et al., 2000; Zaremba et al., 2001) or trauma (Knoblach et al., 1999), persistently elevated levels of TNFα in CNS tissue have also been reported (Holmin et al., 1997; Holmin and Mathiesen, 1999). Increased neuronal expression of TNFα has been definitively established

in animal models (Knoblach et al., 1999; Wang and Shuaib, 2002; Yin et al., 2003; Vitarbo et al., 2004) and comparable observations have been made from studies of human CNS tissue following TBI (Holmin and Hojeberg, 2004) and SCI (Yang et al., 2004).

The functional consequences of increased neuronal expression of TNFα following trauma are multifaceted. TNFα is instrumental in the acute inflammatory cascade that follows neurotrauma (Feuerstein et al., 1994) and has been shown to have both proinflammatory and anti-inflammatory properties (Schmidt et al., 2004). TNFα also has neuroprotective (Liu et al., 1998; Shinpo et al., 1999; Liu et al., 1999), neurotoxic (MacEwan, 2002; Viviani et al., 2004), and neuroplastic properties (Covey et al., 2000; Beattie et al., 2002). Enduring expression of neuronal TNFα has been thought to reflect a chronic neuroinflammatory process (Kollias et al., 1999; Holtmann and Neurath, 2004; Kollias, 2005).

At physiological levels, TNFα has well-documented neuromodulatory properties that include modifying ion channel kinetics (Kagan et al., 1992; Koller et al., 1998; van der Goot et al., 1999; Hribar et al., 1999) and enhancing synaptic transmission (Tancredi et al., 1992; Beattie et al., 2002; Pickering et al., 2005). At high concentrations, and with prolonged exposure, TNFα can be cytotoxic, for example by inducing axonopathy (Talley et al., 1995; Sipe et al., 1996) or oligodendrogliopathy (Selmaj and Raine, 1988a; Selmaj and Raine, 1988b; Hisahara et al., 1997) through apoptotic pathways (Talley et al., 1995; Lee et al., 2000; Yune et al., 2003). TNFα-induced cytotoxicity is considered to

contribute to the pathogenesis of certain autoimmune neuroinflammatory diseases in which demyelination is a prominent feature (Glabinski et al., 1995; Matusevicius et al., 1996; Taupin et al., 1997; Sun et al., 2004). High cerebrospinal fluid (CSF) TNFα concentrations also correlate with magnetic resonance imaging-defined human white matter injury (Ellison et al., 2005).

While appreciable evidence exists as to the physiological effects of low or very high extracellular concentrations of TNFα (Blatteis, 1990; Soliven and Albert, 1992; Rothwell and Hopkins, 1995; Sorkin et al., 1997; Vitkovic et al., 2000; Leem and Bove, 2002; Pickering et al., 2005; Hermann et al., 2005), less is known about the effects of intermediate concentrations ie. elevated but not cytotoxic. We have previously introduced the possibility that elevated concentrations of TNFα in serum or CSF following CNS trauma may be surrogate markers of an immune-mediated "channelopathy" (ie altered axonal ion channel conductance) leading to conduction failure that compounds the neurologic deficits associated with axonopathy and myelinopathy (Hayes et al., 2002). This proposition of cytokine-mediated conduction deficits within the CNS following SCI or TBI has not previously been tested.

Preliminary data reported in my M.Sc. thesis (Davies, 2004) established the feasibility of using a double sucrose gap methodology to evaluate the effects of rhTNFα on axonal conduction in the guinea pig spinal cord. The present study was thus intended to systematically characterize the effects of recombinant human TNFα (rhTNFα) on the electrophysiological properties of isolated mammalian spinal cord tissue at a range of concentrations (0.001-5000 ng/ml).

Specifically, we tested the hypothesis that elevated extracellular concentrations of rhTNFα would result in a decrease in CAP amplitude and depolarization of the CMP, as might be predicted based on our pilot study data.

## 2.2. Materials and Methods

*Isolation of spinal cord tissue*

The surgical procedure for isolating the spinal cord tissue has been described previously (Shi and Blight, 1996; Shi and Blight, 1997; Shi and Borgens, 1999; Shi et al., 2002). Adult female guinea pigs of 350-500 g body weight (n=17, Harlan, USA) were anesthetized prior to surgery (80 mg/kg ketamine hydrochloride, 0.8 mg/kg acepromazine maleate, and 12 mg/kg xylazine, i.m.). Following anesthesia, animals were perfused transcardially with 500 ml cold (15°C), oxygenated Krebs' solution (NaCl 124 mM, KCl 2 mM, $KH_2PO_4$ 1.2 mM, $MgSO_4$ 1.3 mM, $CaCl_2$ 1.2 mM, dextrose 10 mM, $NaHCO_3$ 26 mM, sodium ascorbate 10 mM, equilibrated with 95% $O_2$, 5% $CO_2$ to a pH of 7.2-7.4), and the vertebral column was excised rapidly. The spinal cord was carefully removed from the vertebrae and placed in cold Krebs' solution. The cord was initially separated into two halves by midline sagittal division and the ventral white matter was then isolated (see Figure 2.1A). White matter strips were maintained

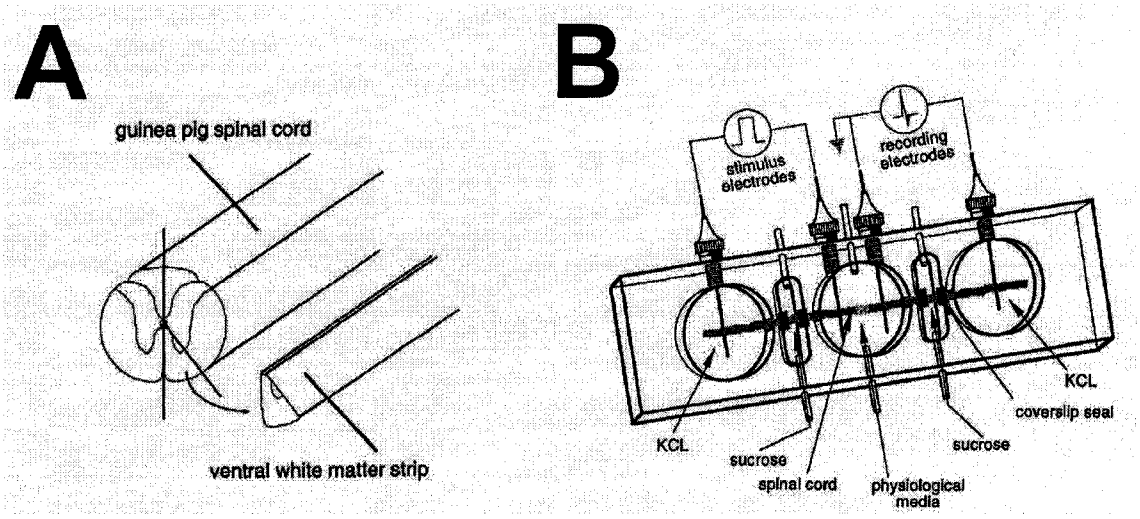


**Figure 2.1 Isolation of ventral white matter and double sucrose gap chamber for *ex vivo* recording of axonal conduction properties of excised guinea pig ventral white matter.** (A) Separation of ventral white matter strips for mounting in the double sucrose gap recording chamber. (B) Double sucrose gap recording chamber viewed from above showing the two outside compartments filled with isotonic KCl, a central compartment filled with oxygenated Krebs' solution, and two sucrose gap compartments maintaining electrical isolation between the KCl and Krebs. rhTNF$\alpha$ is added to the Krebs' solution to bathe the cord in the central compartment. The guinea pig cord is positioned in the trough that traverses the various compartments and is covered by the fluids. Stimulation electrodes (silver-silver chloride) with anode and cathode positioned in the KCL and Krebs' compartments, and bipolar recording (pin) electrodes in the opposite KCl compartment and the common (central) Krebs' compartment are also shown. The central recording electrode is referenced to ground. (Figure adapted with permission from Dr. Riyi Shi)

in continuously oxygenated Krebs' solution for at least one hour prior to mounting in the recording chamber to ensure recovery from dissection before experimentation. The experimental protocols were reviewed and approved by the Purdue University Animal Care and Use Committee (PACUC). All efforts were made to minimize the number of animals used and their distress.

*Electrophysiological Recording and Analysis*

The design and construction of the double sucrose gap chamber used for the electrophysiological recordings has been described previously (Shi and Blight, 1996; Shi and Blight, 1997; Shi and Borgens, 1999; Shi et al., 2002) and is illustrated in Figure 2.1B. The central compartment, 20 mm in diameter, was continuously superfused with oxygenated Krebs' solution at 2 ml/min into which the rhTNFα was introduced. The temperature of the central compartment was maintained at 36.0-37.0°C with an in-line solution warmer (Warner Instruments, Hamden, CT, USA), and monitored using a thermocouple probe. The two end compartments contained isotonic potassium chloride (120mM) and were electrically isolated from the central compartment by narrow channels through which sucrose solution (320 mM) continuously flowed.

The strip of tissue was stimulated through silver-silver chloride pin electrodes positioned within a side compartment and the central bath (Figure 2.1B). Constant current supramaximal stimuli were generated by a digital stimulator (Cygnus Technology, PA, USA) and delivered via a stimulus isolation unit (WP Instruments, Sarasota, FL, USA) in the form of 0.1 ms constant current rectangular pulses at a rate of 0.33 Hz. Compound action potentials (CAP) and

the compound membrane potential were recorded. The electrophysiological data were digitized and stored with a Neurodata Instruments Neurocorder for subsequent analysis. All the on-line recording and subsequent analysis was performed using LabView software (National Instruments, Austin, TX, USA).

*rhTNFα Treatments*

Commercially available rhTNFα was obtained from eBioscience (San Diego, CA, USA) for use in all experiments. As per the supplier, the rhTNFα was derived from Escherechia coli, with >98% purity by SDS-PAGE and maintained in a phosphate buffer with pH 7.2 (150mM NaCl, 1% BSA). This stock solution was diluted with oxygenated Krebs' solution to final concentrations of 0.001, 0.01, 0.1, 1, 10, 100, 1000, and 5000 ng/ml, respectively. The solution containing rhTNFα perfused the tissue in the central compartment for 30 minutes and was then washed out by perfusion with Krebs solution alone. Electrophysiological properties were monitored prior to introduction of the rhTNFα treatment, during treatment, and for an additional 60 minutes of washout. Fresh strips of tissue were used for each preparation and each condition was replicated 5 times (n=5) to establish reproducibility of the observations.

*Control Condition*

In order to dissociate the effects of rhTNFα from the effects of high concentrations of protein in solution, heat-denatured (by immersion in a >95°C water bath for 10 minutes) 5000 ng/ml rhTNFα in Krebs' solution was perfused through the central compartment for 30 minutes. A second control condition tested whether changing the perfusion solution altered the conduction properties

of the spinal cord preparation. To achieve this, the Krebs' solution used prior to rhTNFα treatment was replaced with a new Krebs' solution while all other aspects of the protocol remained identical.

*Statistical Treatment*

Linear regression analysis was used to characterize the association between rhTNFα concentration (natural log - ln transformation) and CAP amplitude. The CAP amplitude was expressed as a percentage of the baseline mean CAP amplitude for each preparation.

## 2.3. Results

The compound membrane potential stabilized over a mean 61.3 ± 8.5 (SD) minutes in the 45 preparations studied. Once stabilized, the membrane potential varied minimally (0.002-0.075 mV; SEM) prior to introduction of TNFα. Monophasic CAPs were recorded from each preparation. Stable recordings of the peak amplitude of the CAP were obtained prior to the introduction of rhTNFα treatment (SEM=0.005-0.015 mV, or 0.19-0.57% of the mean).

*0.001 ng/ml rhTNFα*

Application of rhTNFα at a concentration of 0.001 ng/ml (n=5) had no detectable effect on the CAP or membrane potential throughout the duration of the exposure phase (30 min) and the subsequent washout period (60 min).

*0.01 ng/ml rhTNFα*

Application of rhTNFα at a concentration of 0.01 ng/ml (n=5) had no detectable effect on the CAP or membrane potential throughout the duration of the exposure phase (30 min) and the subsequent washout period (60 min).

*0.1 ng/ml rhTNFα*

Application of rhTNFα at a concentration of 0.1 ng/ml (n=5) had no detectable effect on the CAP or membrane potential throughout the duration of the exposure phase (30 min) and the subsequent washout period (60 min).

*1 ng/ml rhTNFα*

Application of rhTNFα at a concentration of 1 ng/ml (n=5) had no detectable effect on the CAP or membrane potential throughout the duration of the exposure phase (30 min) and the subsequent washout period (60 min).

*10 ng/ml rhTNFα*

rhTNFα at a concentration of 10 ng/ml (n=5) resulted in a slow and persistent partial depolarization of the membrane potential in all preparations. After 20 minutes of exposure to rhTNFα, the mean CAP amplitude was reduced to 88.96 ± 10.26% of pre-treatment CAP amplitude and remained slightly depressed (92.62 ± 10.31%) at 30 minutes of rhTNFα exposure. Following 30 minutes of washout with oxygenated Krebs' solution, mean CAP amplitude returned to 96.16 ± 17.81% of pre-treatment levels and the membrane potential returned to baseline level.

*100 ng/ml rhTNFα*

Application of rhTNFα at a concentration of 100 ng/ml (n=5) again resulted in a small and prolonged depolarization of the membrane potential in the majority of preparations. After 20 minutes of exposure to rhTNFα, the mean CAP amplitude was reduced to 87.64 ± 31.68% of pre-treatment CAP amplitude and continued to decrease (79.07 ± 42.04%) at 30 minutes of rhTNFα exposure. Following 30 minutes of washout with oxygenated Krebs' solution, mean CAP amplitude had recovered to 89.70 ± 28.86% of pre-treatment levels. The membrane potential returned to its baseline level. In Figure 2.2A, the upper panel illustrates CAPs recorded prior to, during, and following bathing of the tissue in 100 ng/ml rhTNFα in a representative preparation. The inverse relationship between CAP peak amplitude and the membrane potential is shown in the lower panel.

*1000 ng/ml rhTNFα*

rhTNFα at a concentration of 1000 ng/ml (n=5) also resulted in a gradual and prolonged partial depolarization of the membrane potential, again persisting throughout the entire duration of the exposure phase. After 20 minutes of rhTNFα exposure, mean CAP amplitude was reduced to 72.71 ± 23.08% of pre-treatment amplitude. This decrease was maintained throughout rhTNFα application, and after an additional 10 minutes of exposure, CAP amplitude remained (73.81 ± 17.70%) below the pre-treatment baseline. Thirty minutes of

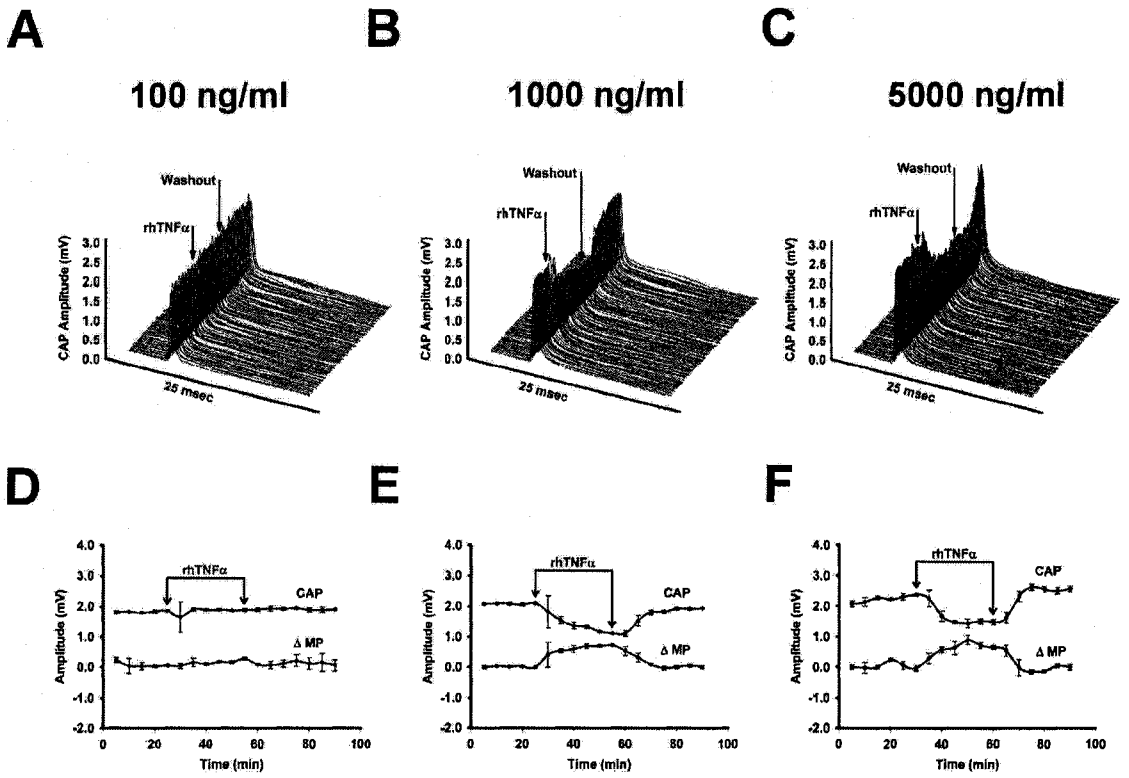


**Figure 2.2 Compound action potential (CAP) waveforms, mean CAP amplitude, and mean change in membrane potential before (30 min), during (30 min), and after (30 min) treatment with 100, 1000, and 5000 ng/ml rhTNFα.** (A) CAP waveforms recorded before, during, and after treatment with 100 ng/ml rhTNFα. The cytokine had little effect on CAP waveform or amplitude. The duration of each trace is 20 msec. (B) CAP waveforms recorded before, during, and after treatment with 1000 ng/ml rhTNFα. The cytokine reversibly blocked conduction and this effect was partially reversed on washout. (C) CAP waveforms recorded before, during, and after treatment with 5000 ng/ml rhTNFα. (D) Mean CAP amplitude and change in membrane potential before, during, and after treatment with 100 ng/ml rhTNFα. (E) Mean CAP amplitude and change in membrane potential before, during, and after treatment with 1000 ng/ml rhTNFα. (F) Mean CAP amplitude and change in membrane potential before, during, and after treatment with 5000 ng/ml rhTNFα.

washout with oxygenated Krebs' solution partially reversed the decrease, but the CAP amplitude remained depressed at 81.55 ± 16.42% of pre-treatment. The membrane potential also remained partially depolarized at this time. Figure 2.2B illustrates data for the CAP and membrane potential obtained from a preparation treated with 1000 ng/ml rhTNFα.

*5000 ng/ml rhTNFα*

Application of rhTNFα at a concentration of 5000 ng per ml (n=5) resulted in an immediate partial depolarization of the membrane potential in all five preparations that persisted throughout the duration of the exposure phase. On washout the change in membrane potential was partially reversed in three preparations; the membrane remained in its depolarized state in the other two preparations. Almost immediately following introduction of 5000 ng per ml rhTNFα the mean CAP amplitude was reduced to 43.98 ± 40.34% of the pre-treatment value. Mean CAP amplitude was depressed throughout rhTNFα application and remained at 59.82 ± 53.77% of the pre-treatment baseline after 30 minutes of exposure. Two of the preparations exhibited complete extinction of the CAP indicative of conduction failure and the CAP was not restored during washout. Thirty minutes of washout with oxygenated Krebs' solution partially reversed the decrease in those preparations where reduction in CAP amplitude was incomplete, and the overall mean CAP amplitude remained depressed at 63.19 ± 57.44% of the pre-treatment value. Figure 2.2C shows CAP and membrane potential data obtained from a preparation treated with 5000 ng/ml rhTNFα .and showing attenuation of the amplitude but not complete loss of CAP.

*Concentration Dependent Conduction Failure*

The concentration-dependent nature of the rhTNFα-induced conduction deficits across all preparations is illustrated in Figure 2.3. The normalized peak CAP amplitude (y) was found to reduce linearly with the ln-transformed rhTNFα treatment concentration ($y=1.06–0.06 \bullet \ln[rhTNF\alpha]$, $R^2=0.95$, $p<0.05$). As treatment concentration increased, reductions in CAP amplitude and depolarization of the membrane potential occurred at a faster rate.

*Control Conditions*

Replication of the treatment protocol using heat-denatured rhTNFα at the highest concentration tested (5000 ng/ml) yielded no reduction in peak CAP amplitude or change in membrane potential (n=5). Illustrative data are shown in Figure 2.4. Similar observations were made when a replacement Krebs solution containing no cytokine alone was used instead of the rhTNFα.

## 2.4. Discussion

Neuronal and glial expression of TNFα and its soluble receptors is increased following trauma to the brain (Knoblach et al., 1999; Holmin and Mathiesen, 1999; Vitarbo et al., 2004) or spinal cord (Lee et al., 2000; Wang et al., 2002; Yune et al., 2003) and a variety of potentially beneficial and detrimental consequences have been identified (Blatteis, 1990; Pan et al., 1997b; Lenzlinger et al., 2001; Barnhart and Peter, 2003). TNFα is also synthesized by circulating immune cells and is selectively transported across the blood-brain barrier and

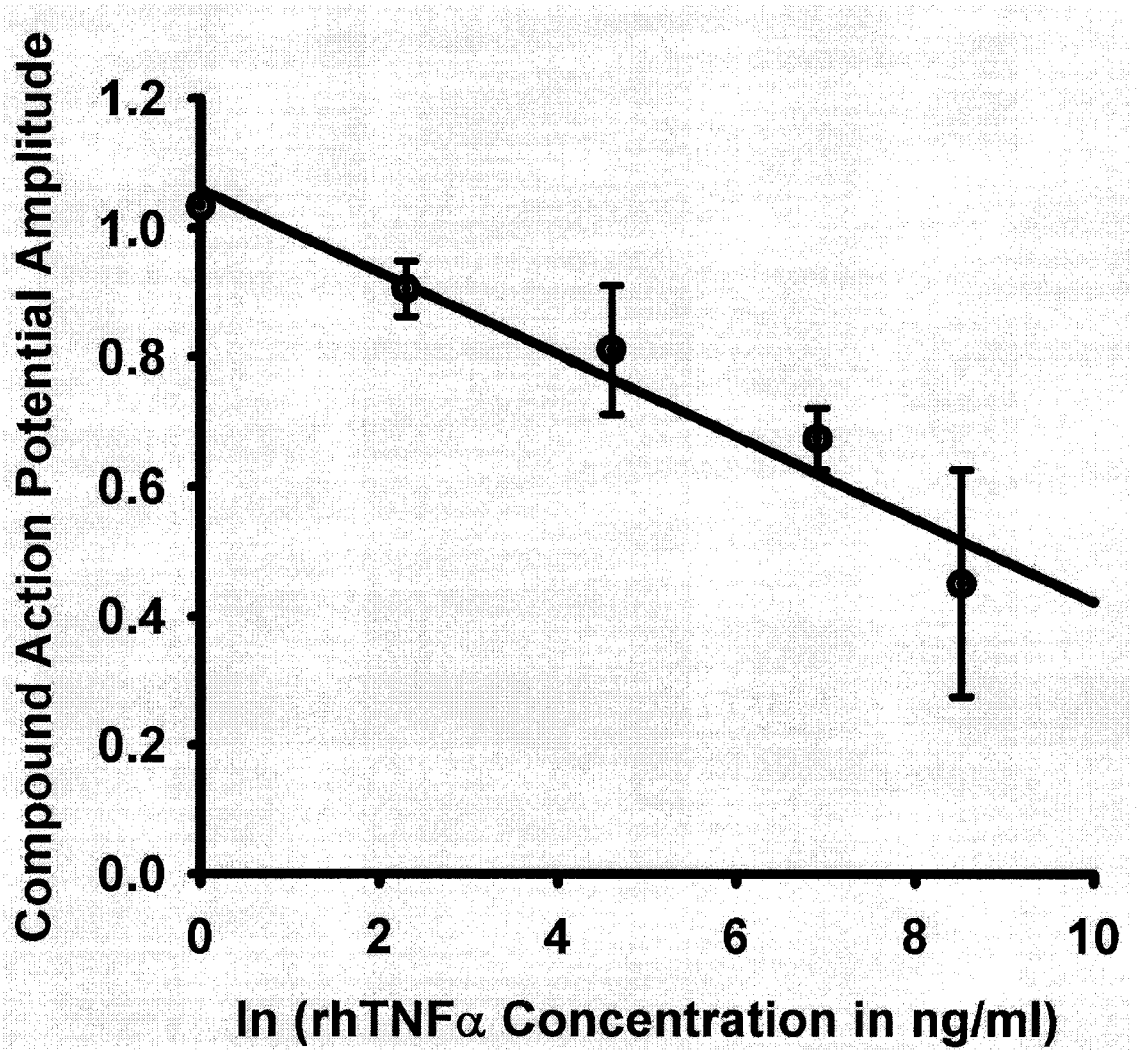


**Figure 2.3 Concentration-dependency of rhTNFα-induced conduction block.** A linear relationship existed between normalized mean CAP amplitude and the ln-transformed rhTNFα concentration (Mean (normalized) CAP amplitude = 1.06-0.06•ln[rhTNFα], $R^2$=0.95, $p<0.05$).

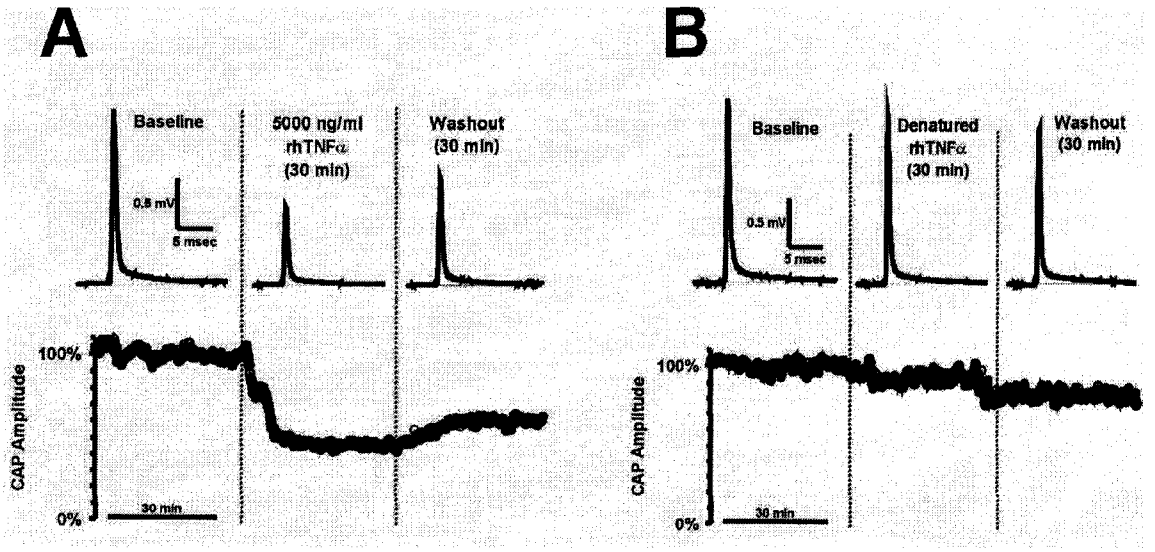


**Figure 2.4 Heat-denatured control condition.** Effects of denatured (control) 5000 ng/ml rhTNFα on CAP amplitudes. (A) Effects of 5000 ng/ml rhTNFα on CAP amplitudes recorded during baseline, treatment, and washout periods. Upper trace shows average CAPs. Lower trace shows peak amplitudes recorded throughout each period. (B) Effects of denatured (5000 ng/ml) rhTNFα on CAP amplitudes. Upper trace shows average CAPs. Lower trace shows peak amplitudes recorded throughout each period. Note the preservation of large amplitude CAPs (>80% of pretreatment condition) throughout the application and washout of denatured (control) rhTNFα solution.

blood-spinal cord barrier (Gutierrez et al., 1993; Banks et al., 2001). This selective permeability is increased following brain injury (Ott et al., 1994) and SCI (Pan et al., 1997a; Pan et al., 1999; Pan and Kastin, 2001a; Pan and Kastin, 2001b; Pan et al., 2003), and varies with time since injury, as well as the location and type of lesion (Pan et al., 1996; Pan and Kastin, 2001b; Pan et al., 2002). Elevated concentrations of TNFα and its receptors are evident in the CSF of patients with traumatic brain injury shortly after trauma (Goodman et al., 1990; Ross et al., 1994). The physiological consequences of increased neuronal expression and concentrations of TNFα within the extracellular fluid and CSF compartments of the CNS are gradually being elucidated (Campbell et al., 1997; Shohami et al., 1999; Wang and Shuaib, 2002; Tonelli and Postolache, 2005).

In the present study we examined the effects of elevated extracellular concentrations of rhTNFα on axonal membrane and action potential properties in the CNS utilizing an electrophysiological recording protocol that has been previously shown to yield sensitive yet stable indices of axonal conduction in excised guinea pig spinal cord tissue (Shi and Blight, 1996; Shi and Blight, 1997; Shi and Borgens, 1999; Shi et al., 2002). In this initial set of studies we examined the immediate effects of relatively brief (30 min) exposure of the tissue to rhTNFα and employed a wide range of concentrations that were considered to be relevant to the levels present in pathologic states. Our intent was to further explore the possibility that an immune-mediated "channelopathy" contributes to the neurologic deficits seen following CNS trauma (Hayes et al., 2002).

The main result of this study was that 30 minutes of exposure to rhTNFα induced a dose-dependent reduction in CAP amplitude and depolarization of the compound membrane potential in axons of guinea pig spinal cord. These electrophysiological changes occurred faster at higher concentrations; the reduction in CAP amplitude was linearly proportional to the natural log of the rhTNFα concentration and was largely reversed during 60 minutes washout with Krebs' solution. The reduction in amplitude of the CAP was, in the vast majority of cases, temporally associated with depolarization of the resting compound membrane potential. Heat-denatured rhTNFα (5000 ng/ml) did not lead to changes in CAP amplitude or membrane potential, thereby implying that it was the molecular structure of the rhTNFα per se rather than the presence of constitutive proteins that caused the altered electrophysiological properties. Collectively, these observations provide support for the notion that immune-mediated conduction deficits may contribute to neurologic dysfunction following trauma.

The mechanisms underlying this form of cytokine-mediated alteration of membrane and CAP properties remain to be determined. At the concentrations employed in the present study, and with the limited duration of exposure, the reduction in CAP amplitude and depolarization of the membrane potential were largely reversible. This implies that cytotoxicity was not a factor ie there was unlikely any appreciable TNFα-induced axonopathy or oligodendrogliopathy leading to demyelination-based conduction failure. Moreover, the rapid onset of membrane and CAP changes would likely be more consistent with changes in ion

channel kinetics rather than in slower acting receptor-mediated second messenger signaling.

TNFα has been shown previously to increase membrane permeability to $Na^+$ through direct interaction with endogenous ion channels or membrane proteins coupled with ion channels (van der Goot et al., 1999). Altered channel conductance resulting from exposure to extracellular TNFα is pH- and voltage-dependent, and receptor-independent (Hribar et al., 1999). Accumulation of axoplasmic $Na^+$ per se can induce membrane depolarization sufficient to cause conduction failure. Moreover, $Na^+$ accumulation is functionally coupled to transmembrane $Ca^{++}$ import. Increases in intracellular [$Na^+$] cause reverse operation of the $Na^+$-$Ca^{++}$ exchanger, which admits potentially disruptive quantities of $Ca^{++}$ into the cell (Stys et al., 1992; Li et al., 2000). Calcium accumulation, in turn, results in increased phospholipase and protease activity, and production of reactive oxygen species such as nitric oxide (Kristian and Siesjo, 1998; Paschen, 1999; Paschen, 2000; Paschen, 2003), which can also block conduction (Redford et al., 1997; Shrager et al., 1998; Kapoor et al., 1999).

TNFα also enhances (McLarnon et al., 1993; Ilschner et al., 1995; Houzen et al., 1997; Koller et al., 1998; Nietsch et al., 2000; McLarnon et al., 2001) or impairs (Sawada et al., 1990; Soliven et al., 1991; Sawada et al., 1991a; Sawada et al., 1991b; Diem et al., 2001) $K^+$ currents in excitable membranes. In rat cortical neuron cultures, prolonged (12-48hr) application of 10-100 ng/ml TNFα enhanced outward $K^+$ currents (Houzen et al., 1997). TNFα also modulates $Ca^{++}$ and $K^+$ channel activity in human microglia (McLarnon et al., 1993; McLarnon et

al., 2001). Changes in either $Na^+$, $Ca^{++}$, or $K^+$ conductances in axons or glia might therefore underlie the membrane and CAP changes observed in the present study.

Another candidate mechanism stems from the observation that elevated concentrations of TNFα directly increase expression of inducible nitric oxide synthase (iNOS) in macrophages and astrocytes within the CNS with release of greater amounts of nitric oxide (NO) (Yune et al., 2003). NO causes a dose-dependent and reversible axonal conduction failure that is activity-dependent; demyelinated axons are especially susceptible (Kapoor et al., 1999). Disruption of mitochondrial respiration and insufficient production of adenosine triphosphate (ATP) to support the membrane potential is thought to underlie this form of conduction deficit.

Central to the plausibility of immune-mediated conduction deficits *in vivo* is the question of how closely the concentrations of TNFα used in the present study approximate those found in the clinical state. Where there exists local neuronal expression of TNFα in the CNS, as in the case of a localized inflammatory response, the extracellular concentration (gradient) falls off rapidly as a function of both time and space; drainage into the larger volume CSF fluid compartment then results in a dilution effect, and CSF concentrations underestimate local extracellular concentrations (de Lange and Danhof, 2002; Shen et al., 2004). The problem of CSF as a surrogate marker of extracellular concentration is further complicated by the fact that circulating (serum) TNFα -secreting cells and the cytokine itself can pass through both the blood-brain barrier and the blood-

CSF barrier and into the extracellular space at rates that depend on disease state; thus neuronal and glial expression, serum and CSF sources all influence extracellular TNFα concentrations. Acknowledging these caveats, it follows that experimental (extracellular) concentrations needed to induce electrophysiological changes of the type reported here would need to be many times higher than the concentrations detectable in CSF. Abnormally elevated TNFα CSF concentrations of ~20 pg/ml have been reported following human TBI (Hayakata et al., 2004) and we have detected levels up to 37 pg/ml following human SCI (unpublished studies).

Also relevant to the concentration question is the cross-species protocol employed herein. Cross-reactivity between rhTNFα and rodent tissue, at various concentrations, has been demonstrated previously (Stephens et al., 1988; Mallick et al., 1989; Hocking et al., 1990; Catanzaro et al., 1991; Shibata and Blatteis, 1991a; Shibata and Blatteis, 1991b; Pennings et al., 1998; Fischer et al., 1999) and the amino acid alignment of TNFα is remarkably similar for human and guinea pig, particularly at signature sequences (Goetz et al., 2004). Nevertheless, it has been shown previously that higher (x1000) concentrations of human TNFα than rodent TNFα are required to induce equivalent changes in firing rates of rat neurons. This suggests that the *in vivo* extracellular concentrations of TNFα that will induce electrophysiological changes may be appreciably less than the concentrations employed in our cross-species *ex vivo* model.

The extracellular concentration of TNFα necessary to induce conduction deficits *in vivo* may well be dependent on the physiological status of the axons, glia, and surrounding milieu. For reasons noted previously we used uninjured tissue in the present study. Trauma to cord parenchyma introduces additional considerations most notably with respect to the astroglial scar. Astrocytes are a major immunoresponsive source of TNFα within the brain and spinal cord (Aschner, 1998; DeLeo et al., 2000), and a pathological increase in their density at a lesion site (astrocytosis or astrogliosis) may render injured tissue exquisitely sensitive to immune-mediated alterations in neurological function. The astrocyte marker glial fibrillary acidic protein (GFAP) (Du et al., 1999; Eng et al., 2000; Menet et al., 2003) which is upregulated following neurotrauma (Haghighi et al., 2004), and is elevated in the CSF of patients with TBI (Regner et al., 2001) and SCI (Guez et al., 2003), has been associated with acidification (Oh et al., 1995). TNFα-induced modulation of $Na^+$ channel activity occurs in a pH-dependent manner (Kagan et al., 1992; van der Goot et al., 1999), with increased activity at lower pH. A focal, astrocyte-induced, acidification of the axonal environment at a lesion site may therefore result in altered axonal conduction at relatively lower concentrations of TNFα following CNS trauma. The implication here is that injured CNS tissue, where reactive gliosis is present, may be even more sensitive to cytokine-mediated conduction deficits than the uninjured tissue used in the present study.

The potential clinical implications of the present findings are numerous. In the acute stage of CNS trauma when neuronal expression of TNFα is increased

(Vitarbo et al., 2004) and CSF levels of TNFα are high (Schmidt et al., 2004), there exists the possibility that TNFα-mediated impairment of axonal conduction contributes to the observed neurologic deficits. Immune-mediated deficits in conduction, of the type described here, would be distinguishable from those attributable to axonopathy or myelinopathy by virtue of their reversibility and their resolution may contribute to spontaneous neurologic recovery. Since elevated CNS levels of TNFα may endure during chronic neuroinflammatory processes (Kollias et al., 1999; Holtmann and Neurath, 2004; Kollias, 2005), because of infection, autoimmune disease, or elevated levels of TNFα passing through the blood-spinal cord barrier, there also exists the potential for a sustained impairment of axonal conduction that would only reverse on resolution of the infection or autoimmune response. Reversal of neurologic deficit of this type might contribute to elements of late-onset recovery. If this is the case, anti-inflammatory, anti-cytokine or anti-TNFα therapy may be expected to foster accelerated recovery or resolution of longstanding deficits. This recovery of function would occur earlier than could be reasonably attributed to remyelination.

Reversible, cytokine-mediated impairment of axonal conduction is thus a potentially new mechanism of central neurological deficit following CNS trauma. If attributable to altered membrane permeability to various ions, this form of neuroimmunomodulation would fit into the recently identified category of deficits termed "acquired channelopathy" (Waxman, 2001).

In summary, the results of the present study demonstrated concentration-dependent effects of rhTNFα on membrane and CAP properties in guinea pig

spinal cord tissue. Increasing concentrations of rhTNFα yielded progressively greater reductions in amplitude of the CAP accompanied by depolarization of the membrane potential. These effects were reversed on washout of rhTNFα and were not present when denatured rhTNFα was introduced. TNFα-mediated changes in the compound membrane potential and axonal conduction may underlie some of the neurologic deficits observed following various forms of neurotrauma and provide a target for future neuro-restorative therapies.

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# CHAPTER 3: CARBON MONOXIDE-RELEASING MOLECULE CORM-2 INDUCES CONCENTRATION-DEPENDENT ALTERATIONS IN THE ELECTROPHYSIOLOGICAL PROPERTIES OF AXONS IN MAMMALIAN SPINAL CORD

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## 3.1. Introduction

Petechial hemorrhage, vascular disruption, hyperemia with red blood cells present, hematomyelia and hemorrhagic necrosis of cord parenchyma are all prominent features of the pathology of traumatic spinal cord injury (SCI) (Hayes and Kakulas, 1997; Kakulas, 1999; Norenberg et al., 2004). Heme, the iron protoporphyrin constituent of hemoglobin (Hb), is a potent oxidative stressor and leads to formation of reactive oxygen species which contribute to secondary pathology (Bishop and Cashman, 2003). Degradation of heme, via the heme oxygenase (HO) system, represents an endogenous means of reducing oxidative stress and inflammation. Both the stress-inducible isoenzyme HO-1 and the glucocorticoid-inducible isoenzyme HO-2 are upregulated following SCI and appear to contribute to neuroprotection (Mautes et al., 1998; Mautes et al., 2000; Mautes and Noble, 2000; Panahian and Maines, 2001; Liu et al., 2002; Lin et al., 2007).

Catalysis of heme yields carbon monoxide (CO), free ferrous iron and biliverdin (Maines, 2005). CO normally functions as a signaling molecule and gaseous modulator of the second messenger cyclic guanosine monophosphate (cGMP) in neurons (Verma et al., 1993; Baranano and Snyder, 2001). At high concentrations and with prolonged exposure (48h) CO is neurotoxic; this toxicity is independent of hypoxia (Tofighi et al., 2006). CO shares several of the biological and chemical properties of the other principal gaseous neuromodulatory compound nitric oxide (NO) and parallels exist in the regulation of the CO and NO generating systems (Hartsfield, 2002). Both CO and NO are

involved in the inflammatory and immunological processes following parenchymal damage (Baranano and Snyder, 2001; Hartsfield, 2002; Freitas et al., 2006).

In a series of studies investigating the putative axonal conduction blocking properties of immune mediators that are known to be up-regulated following SCI, we have shown that NO (Ashki et al., 2006) and the proinflammatory cytokine tumor necrosis factor alpha (TNF$\alpha$) (Davies et al., 2006b) modify electrophysiological properties of axons in the guinea pig spinal cord. These neuromodulatory effects appear to impair axonal conduction and thereby compound the neurologic deficits associated with traumatic axonopathy and myelinopathy. They may also be contributory to the neurologic deficits of other neuroinflammatory disorders (Redford et al., 1997; Shrager et al., 1998). An important property of these neuromodulatory effects is that the conduction deficits are reversible; an observation which has ramifications for explaining various forms of neurologic recovery (Hayes et al., 2006) and providing insights for future immunomodulatory therapies (Redford et al., 1997; Ashki et al., 2006; Davies et al., 2006b).

In the present study we tested the hypothesis that elevated extracellular concentrations of CO, introduced by the CO-releasing molecule tricarbonyldichlororuthenium (II) dimer (CORM-2), induce concentration-dependent and reversible changes in the electrophysiological properties of excised long tract axons from the spinal cord of adult guinea pigs. Such neuromodulatory effects, if demonstrated, would reveal a new role for CO in addition to its previously demonstrated anti-inflammatory (Wu and Wang, 2005;

Zuckerbraun et al., 2007) and neurotoxic (Sener, 2003; Tofighi et al., 2006) properties.

## 3.2. Experimental Procedures

The methodological procedures were identical to those we have used previously to demonstrate the effects of immune mediators on axonal conduction properties (Ashki et al., 2006; Davies et al., 2006a). Briefly, the electrophysiological properties of excised guinea pig spinal cord axons were recorded in a double sucrose gap recording system while the tissue was perfused (30 min) with varying concentrations of CORM-2 or control solutions. After 30 min of CORM-2 exposure the tissue was perfused with Krebs' solution to eliminate CO; the electrophysiological properties were also monitored continuously throughout this 30 min washout period.

*Isolation of spinal cord tissue*

The surgical procedure for isolating the spinal cord tissue has been described previously (Shi and Blight, 1996; Shi et al., 1997; Shi and Borgens, 1999; Shi and Pryor, 2002; Shi and Whitebone, 2006; Ashki et al., 2006; Davies et al., 2006b). Adult female Hartley guinea pigs of 350-500 g body weight (n=12, Charles River, Wilmington, MA, USA) were anesthetized prior to surgery (80 mg/kg ketamine hydrochloride, 0.8 mg/kg acepromazine maleate, and 12 mg/kg xylazine, i.m.). Following anesthesia, animals were perfused transcardially with 500 ml cold (15°C), oxygenated Krebs' solution (NaCl 124 mM, KCl 2 mM,

$KH_2PO_4$ 1.2 mM, $MgSO_4$ 1.3 mM, $CaCl_2$ 1.2 mM, dextrose 10 mM, $NaHCO_3$ 26 mM, sodium ascorbate 10 mM, equilibrated with 95% $O_2$, 5% $CO_2$ to a pH of 7.2-7.4), and the vertebral column was excised rapidly. The spinal cord was carefully removed from the vertebrae and placed in cold Krebs' solution. The cord was initially separated into two halves by midline sagittal division and the ventral white matter was then isolated (see Figure 2.1A). Strips of white matter (2-4 per animal) were maintained in continuously oxygenated Krebs' solution for at least one hour prior to mounting in the recording chamber to ensure recovery from dissection before experimentation. The experimental protocols were reviewed and approved by the University of Western Ontario's Animal Use Subcommittee (AUS). All efforts were made to minimize the number of animals used and their distress.

*Electrophysiological Recording and Analysis*

The design and construction of the double sucrose gap chamber used for the electrophysiological recordings has been reported elsewhere (Shi and Blight, 1996; Shi and Blight, 1997; Shi and Borgens, 1999; Shi and Pryor, 2002) and is illustrated in Figure 2.1B. The central compartment, 20 mm in diameter, was continuously superfused with oxygenated Krebs' solution at 2 ml/min into which the treatments (CORM-2 and controls) were introduced. The temperature of the central compartment was maintained at 36.0-37.0 °C with an in-line solution heater (Warner Instruments, Hamden, CT, USA), and monitored using a thermocouple probe. The two end compartments contained isotonic potassium

chloride (KCl, 120 mM) and were electrically isolated from the central compartment by narrow channels through which sucrose solution (320 mM) flowed continuously.

The strip of excised cord tissue was stimulated through silver-silver chloride pin electrodes positioned in one of the end chambers (cathode) and in the central chamber containing Krebs' (anode) (Figure 2.1B). Constant current supramaximal stimuli were generated by a digital stimulator (Cygnus Technology, PA, USA) and delivered via a stimulus isolation unit (WP Instruments, Sarasota, FL, USA) in the form of 0.1 msec constant current rectangular pulses at a rate of 0.33 Hz. The evoked compound action potentials (CAP) were amplified using an intracellular recording DC amplifier (Cygnus Technology, PA, USA) and recorded continuously throughout the experiment using silver-silver chloride pin electrodes in the grounded central chamber containing Krebs' and in the opposite end chamber containing KCl. Signals were filtered using a Hum Bug (Quest Scientific, Vancouver, BC, Canada) to eliminate any 60 Hz noise. CAP waveform and amplitude, the compound membrane potential (CMP), and stimulus-peak latency were recorded. The electrophysiological data were digitized (25 kHz, 16-bit) and stored with a Neurodata Instruments Neurocorder for subsequent analysis. The electrophysiological properties of each preparation were allowed to stabilize prior to the introduction of CORM-2 or control treatment. At the end of each preparation tested, the tissue was transected in the central chamber. This resulted in a complete depolarization of the CMP which enabled quantification of any membrane potential changes that occurred due to the experimental

treatment. All the on-line recording and subsequent analysis was performed using LabView software (National Instruments, Austin, TX, USA).

*CORM-2 Treatment*

The transition metal carbonyl CORM-2 (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) and subsequently diluted with oxygenated Krebs' solution to a final CORM-2 concentration of 100, 500, or 1000 μM in 1% DMSO. Previous studies have shown that for each molecule of CORM-2, 0.7 moles of CO are liberated when the donor comes into contact with tissue (Motterlini et al., 2002), yielding experimental concentrations of 70, 350, and 700 μM of CO in the present study. The duration of tissue perfusion with CORM-2 was 30 minutes. After 30 minutes the CORM-2 was washed out by perfusion with Krebs' solution alone and the electrophysiological properties were monitored for an additional 60 minutes. Fresh strips of tissue were used for each preparation and each condition was replicated 5 times (n=5) to establish reproducibility of the observations.

*$RuCl_3$ Control Condition*

In order to dissociate the effects of CO from the effects of the donor molecule, ruthenium (III) chloride hydrate ($RuCl_3$, Sigma-Aldrich, St. Louis, MO, USA) was used. $RuCl_3$ does not yield CO in solution and therefore served as a negative control. $RuCl_3$ was dissolved in DMSO and diluted to a concentration of 1000 μM in 1% DMSO. This control solution perfused the tissue (n=5) in the

central compartment for 30 minutes; this was followed by a 60-minute washout period in which Krebs' solution alone perfused the tissue.

*DMSO Control Condition*

To ensure any electrophysiological effects observed were due to CO and not the DMSO solvent, a 1% solution of DMSO in oxygenated Krebs' solution was used. This control solution was perfused through the central compartment for 30 minutes and followed by a 60 minute washout period of Krebs' solution alone in a manner identical to the CORM-2 protocol.

*Statistical Treatment*

The effects of CORM-2 treatment on CAP amplitude and area, CMP, and stimulus-peak latency measured during pretreatment (immediately preceding treatment), treatment (at the conclusion of 30 min of treatment), and washout (following 30 min Krebs' washout) were analyzed using repeated measures ANOVA (SPSS 15.0 for Windows). Raw CAP amplitude values were normalized to the pretreatment values in order to facilitate aggregation of data. Linear regression analysis was used to characterize the relationships between CAP amplitude and CORM-2 concentration and stimulus-peak latency and CORM-2 concentration, respectively. Statistical significance was defined as $p < 0.05$.

## 3.3. Results

Prior to the introduction of CORM-2 the electrophysiological properties of the tissue were monitored and allowed to stabilize. During this time, the CMP recorded across the sucrose gap became increasingly polarized as the sucrose perfused the extracellular space, reducing the leakage current (Shi and Blight, 1996). The CAP amplitude progressively increased until it stabilized after a mean interval of 31.0 ± 6.5 (SD) min, the CMP by 37.0 ± 6.0 min, and the stimulus-peak latency by 16.0 ± 6.5 min in the 25 preparations studied. Stable recordings of the peak amplitude of the CAP were obtained prior to the introduction of treatment (SD=2.9% of the mean).

*100 μM CORM-2*

Application of CORM-2 at a concentration of 100 μM (n=5) had no detectable effect on the CAP, CMP, or stimulus-peak latency throughout the duration of the exposure phase (30 min) and the subsequent washout period (30 min). Representative recordings of the peak CAP amplitude over time are shown in Figure 3.1.

*500 μM CORM-2*

Treatment with CORM-2 at a concentration of 500 μM (n=5) resulted in a gradual and progressive reduction in CAP amplitude in all preparations ($p<0.05$) throughout the duration of the exposure phase (30 min). After 30 minutes of exposure to 500 μM CORM-2, the mean CAP amplitude was reduced to 83.2 ±

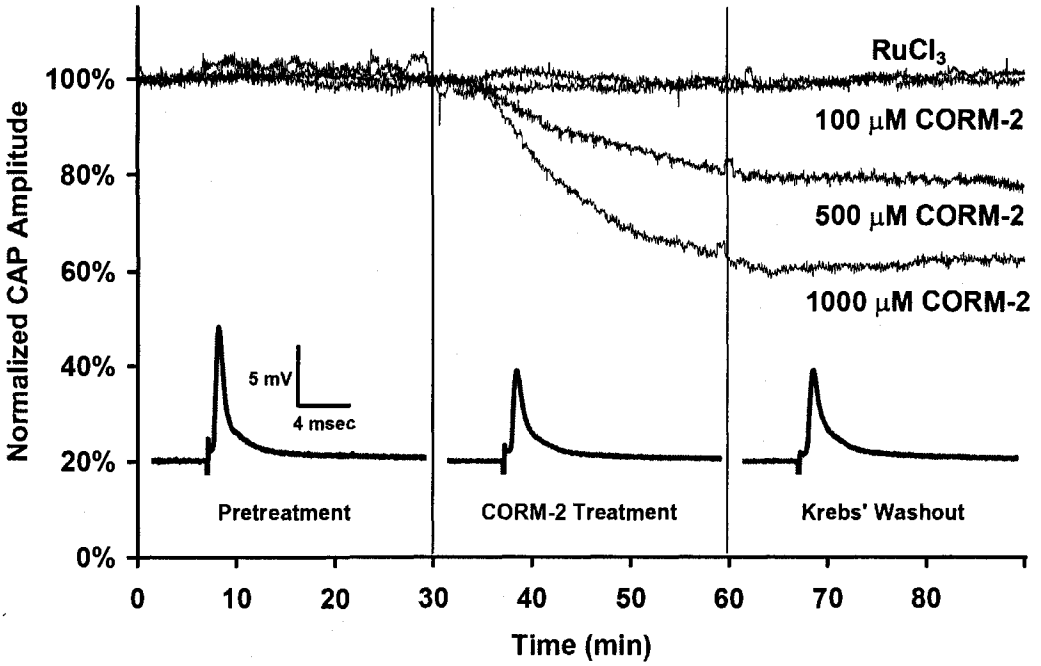


**Figure 3.1 Time course of normalized CAP amplitude changes, from representative preparations treated with 100, 500, and 1000 μM CORM-2, and 1000 M $RuCl_3$, over pretreatment (30 min), treatment (30 min), and washout (30 min) periods.** Treatment (CORM-2 or $RuCl_3$) begins at 30 min, and Krebs' washout begins at 60 min. CAP amplitude is recorded following application of each stimulus (0.33 Hz). Individual CAP waveforms (each an average of 10 consecutive CAPs) from the 1000 μM CORM-2 treatment preparation are inset.

6.3% of pre-treatment CAP amplitude, dropping further to 80.4 ± 6.0% immediately following cessation of treatment, and remained depressed (80.3 ± 5.3%) following 30 minutes of washout with oxygenated Krebs' solution. Stimulus-peak latency increased concomitantly with the observed decrease in CAP amplitude by 0.09 msec to 112.2 ± 6.9% ($p<0.05$) of pretreatment values, and remained prolonged (115.0 ± 7.9%) at the conclusion of the Kreb's washout. The CMP remained unchanged in all preparations throughout 30 min of treatment with 500 μM CORM-2 and during the additional 30 min of Krebs' washout. CAP waveforms and amplitude recorded from a representative preparation treated with 500 μM CORM-2 are presented in Figure 3.1.

*1000 μM CORM-2*

Treatment with CORM-2 at a concentration of 1000 μM (n=5) also resulted in a persistent, progressive reduction in CAP amplitude in all preparations ($p<0.05$) throughout the duration of the exposure phase (30 min) with a greater magnitude reduction than seen following treatment with 500 μM CORM-2. After 30 minutes of exposure to 1000 μM CORM-2 the mean CAP amplitude was reduced to 66.4 ± 8.9% of pre-treatment CAP amplitude, dropping further (61.7 ± 8.7%) immediately following cessation of treatment, and remained depressed (65.1 ± 8.4%) following 30 min of washout with oxygenated Krebs' solution. Stimulus-peak latency increased concomitantly with the observed decrease in CAP amplitude by 0.2 msec to 133.5 ± 15.7% ($p<0.05$) of pretreatment values and increased further during washout with Krebs' solution alone (144.9 ± 14.8%).

The CMP remained unchanged in all preparations throughout 30 min of treatment with 1000 μM CORM-2 and subsequent Krebs' washout. CAP waveforms and amplitude recorded from a representative preparation treated with 1000 μM CORM-2 are presented in Figure 3.1.

*Concentration-Dependent Electrophysiological Changes*

The concentration-dependent nature of the CORM-2-induced reduction in CAP amplitude and increase in stimulus-peak latency across all preparations is illustrated in Figure 3.2. The mean normalized peak CAP amplitude ($y_1$) decreased linearly with the CORM-2 treatment concentration ($y_1$=-0.0004*[CORM-2]+1.0609, $R^2$=0.46). The stimulus-peak latency ($y_2$) was found to increase linearly with increased CORM-2 treatment concentration ($y_2$=0.0002*[CORM-2]-0.0257, $R^2$=0.46).

CAP waveform area was measured as the time integral of the CAP waveform (area under the CAP voltage curve) from onset to 4 msec post-onset as illustrated in Figure 3.3A. The area under the curve decreased with CORM-2 treatment concentration ($p<0.05$), but this decrease in area was relatively less (Figure 3.3B) than the corresponding decrease in CAP amplitude (Figure 3.2). At 100 μM CORM-2, CAP waveform area remained at 99.0% of pretreatment area, but reduced to 93.6% following 500 μM CORM-2 treatment and 79.1% following treatment with 1000 μM CORM-2. The changes in waveform are shown in Figure 3.4.

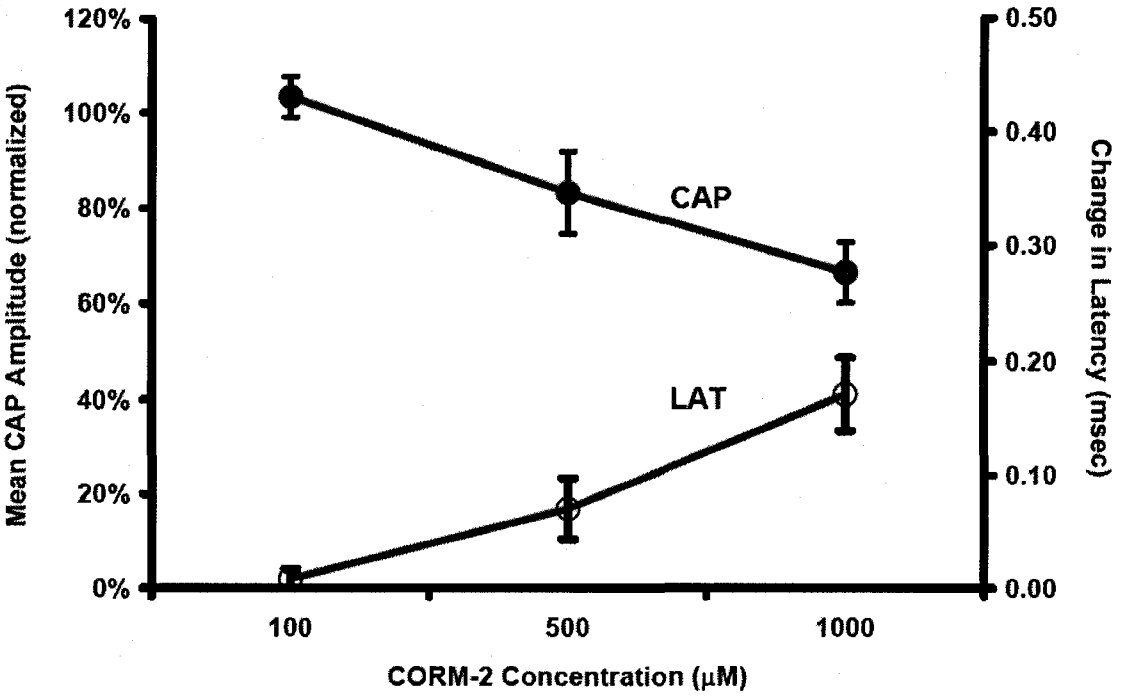


**Figure 3.2 CORM-2 concentration-dependent reduction in mean CAP amplitude (normalized, closed circles) and increase in stimulus-peak latency (msec, open circles).**

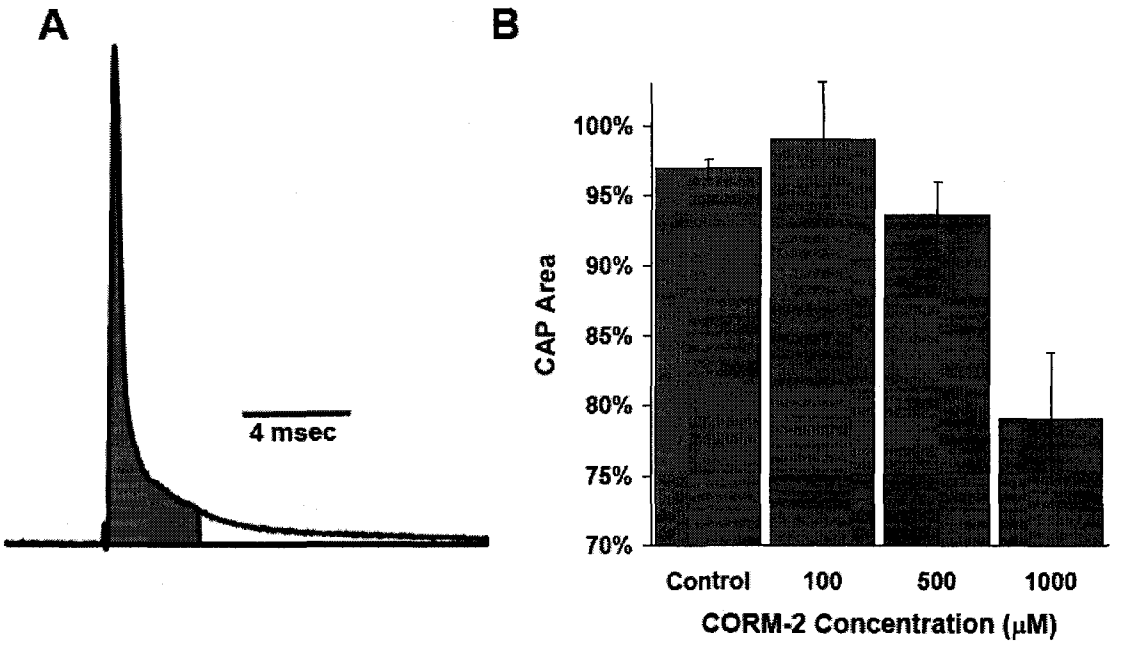


**Figure 3.3 Reduction in area of the CAP waveform following treatment with 100, 500, and 1000 μM CORM-2 and 1000 M $RuCl_3$.** (A) A CAP waveform illustrating the area under the curve from the onset of the CAP waveform to 4 msec following onset used for area determination. (B) Changes in area of the CAP waveform following treatment.

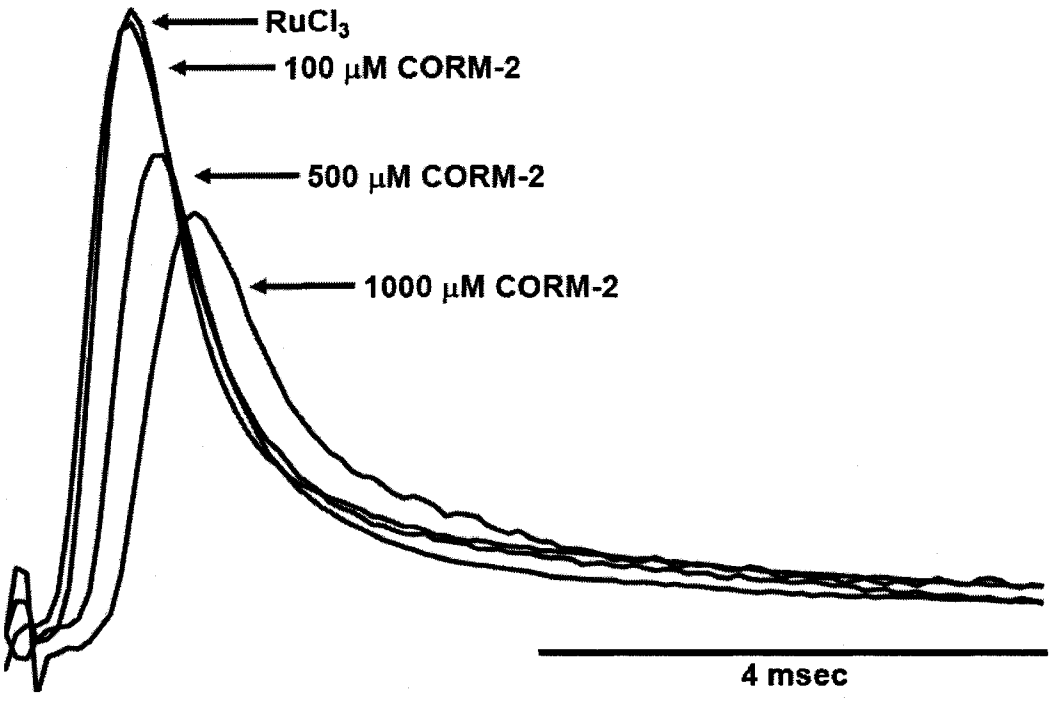


**Figure 3.4 Representative CAP waveforms (normalized) illustrating changes in CAP waveform following treatment with 100, 500, and 1000 μM CORM-2 and 1000 μM $RuCl_3$.**

*Inverse CAP Amplitude/Stimulus-Peak Latency Relationship*

An inverse power function characterized the relationship between mean normalized CAP amplitude (x) and stimulus-peak latency (y) following 30 min of treatment at a concentration of 1000 μM. This is illustrated in Figure 3.5 and is described by the equation $y=0.5265x-0.71$ ($R^2=0.98$).

*CORM-2 Paired-pulse stimulation*

Illustrative paired-pulse CAP waveforms from a single preparation for each interpulse interval are superimposed in Figure 3.6A. At two interpulse intervals, 2 and 4 msec, the amplitude of the second wave was reduced relative to the initial wave (mean=63.9 ± 13.0%, mean=90.2 ± 6.4, respectively). This profile was evident in all preparations studied.

Following 30 minutes of exposure to 500 (n=5) and 1000 μM (n=5) CORM-2 the amplitude of the second CAP elicited by a 2 msec delay paired-pulse stimulus was reduced by 3.5 ± 1.6% and 8.2 ± 4.4%, respectively ($p<0.05$). In contrast to the CORM-2 treatments, 30 minutes of exposure to $RuCl_3$ (n=5) resulted in a 4.7 ± 2.6% and 6.2 ± 4.1% *increase* in the amplitude of the second CAP to paired-pulse stimuli with interpulse intervals of 2 and 4 msec, respectively ($p<0.05$). All changes persisted throughout the Krebs' washout phase in all conditions ($p<0.05$). Paired-pulse stimuli with a delay greater than 4 msec did not result in significant changes in the amplitude of the second CAP relative to the first ($p>0.05$). Curves generated from representative preparations of the normalized amplitude of the second CAP at each interpulse interval for 100, 500,

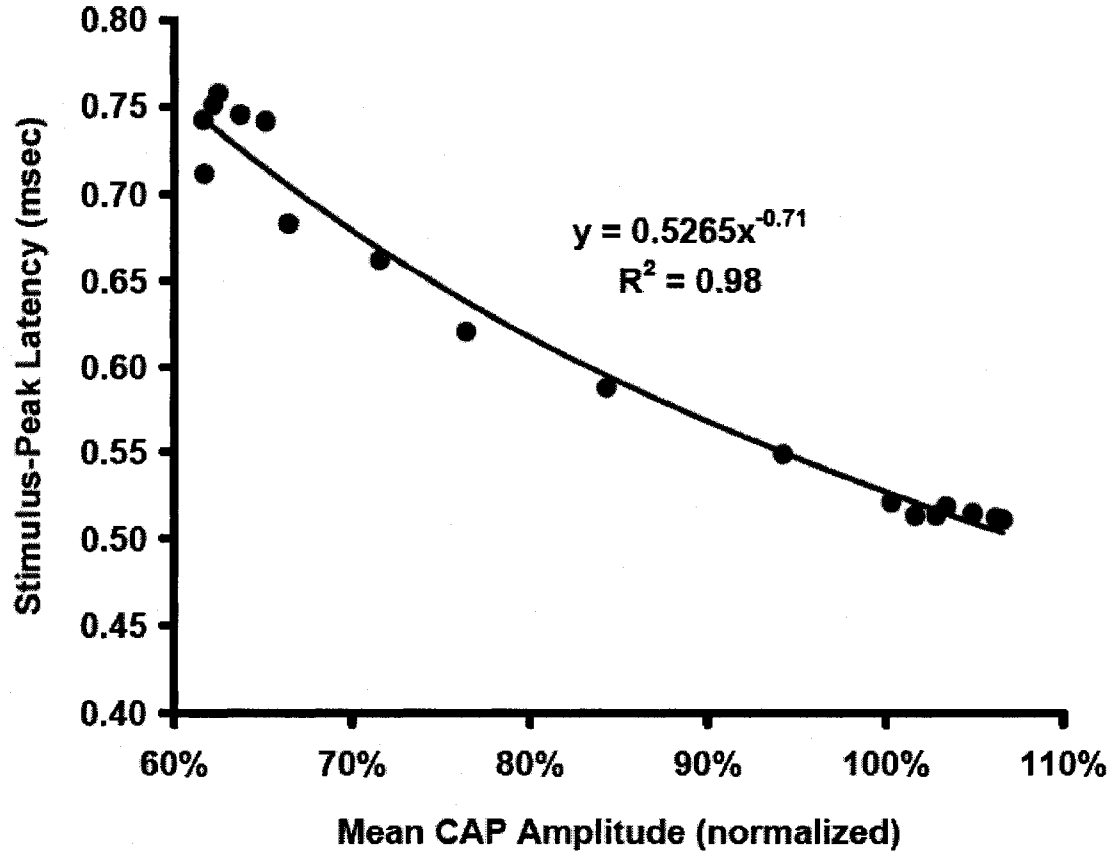


**Figure 3.5 The inverse relationship between stimulus-peak latency (ms) and mean CAP amplitude (normalized) following treatment with 1000 μM CORM-2.**

and 1000 μM CORM-2 and $RuCl_3$ treatments are shown in Figure 3.6B. Superimposed, normalized CAP waveforms during pretreatment, treatment with 1000 μM CORM-2, and Krebs' washout with an interpulse interval of 2 msec are shown in Figure 3.6C.

*$RuCl_3$ Control Condition/DMSO Control Condition*

Replication of the treatment protocol using the negative control $RuCl_3$ (n=5) at a concentration equivalent to that used in the highest CORM-2 treatment (1000 μM) yielded no reduction in peak CAP amplitude, CAP waveform, change in CMP, or increase in stimulus-peak latency. Similar observations were made when a DMSO solution containing no CORM-2 was introduced following stabilization of the electrophysiological recordings.

## 3.4. Discussion

Hemorrhage, including petechiae, rupture of postcapillary vessels or sulcal arterioles, hematomas, intravascular coagulation, and hemodynamic events such as ischemia, hyperemia, and reperfusion, are hallmarks of the pathology of traumatic injury to the spinal cord (Hayes and Kakulas, 1997; Tator and Koyanagi, 1997; Kakulas, 1999; Norenberg et al., 2004). While primarily focused within the central gray matter, the consequences of intraparenchymal bleeds are also expressed in the adjacent white matter where they likely contribute to long tract degeneration. Hb, an erythrocytic protein, has been shown to reach millimolar concentrations in hematomas and can be toxic to neurons

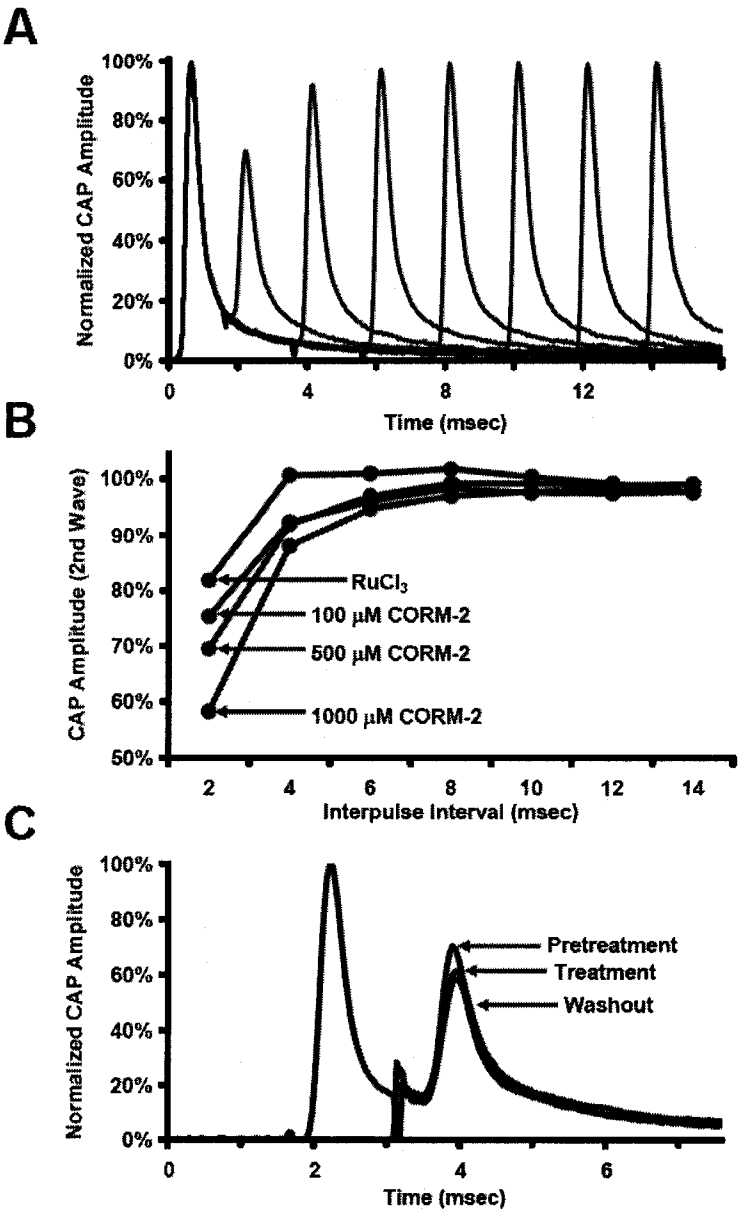


**Figure 3.6 The effects of CORM-2 treatment on CAP amplitude in response to paired-pulse stimuli.** (A) Representative, superimposed paired-pulse CAP waveforms recorded from an individual preparation with interpulse intervals of 2 to 14 msec. (B) Representative curves obtained from individual preparations of the normalized amplitude of the second CAP following treatment with 100, 500, and 1000 μM CORM-2 and $RuCl_3$ at each interpulse interval. The amplitude of the second CAP is normalized to the amplitude of the first CAP. (C) Representative normalized, superimposed CAP waveforms obtained from an individual preparation during pretreatment, treatment with 1000 μM CORM-2, and during Krebs' washout, following paired-pulse stimulation with an interpulse interval of 2 msec.

(Xi et al., 1998; Goldstein et al., 2003; Rogers et al., 2003; Chen and Regan, 2004; Regan et al., 2004; Chen-Roetling et al., 2005; Qu et al., 2005). The toxicity of Hb is driven by a series of reactions that oxidize deoxygenated Hb to methemoglobin, which subsequently releases hemin, the oxidized form of heme (Goldstein et al., 2003). In response to the release of free heme, there is an upregulation of the HO system in microglia, macrophages and white matter tracts above and below the epicenter of injury (Mautes et al., 1998). Neutrophils expressing HO-1 are also recruited to the site of injury (Mautes et al., 1998; Panahian and Maines, 2001; Liu et al., 2002). Increased HO activity contributes to the degradation of free heme and can be considered neuroprotective in this context by counteracting the cytotoxic properties of heme and free radicals (Panahian and Maines, 2001).

Uncertainty remains as to the potential detrimental effects of HO-mediated heme catalysis because of the increased local production of CO and the inability of the compromised blood flow to transport the CO. High extracellular concentrations of CO can potentially result in neuronal hypoxia in juxtaposed neurons due to the affinity of CO for hemoglobin and the formation of carboxyhemoglobin. The consequences of hypoxia on axonal conduction are well documented (Stys, 1998; Fern et al., 1998; Tekkok and Ransom, 2004). Less is known about the direct effects of CO exposure on axonal electrophysiology.

Under normal physiological conditions CO serves as a signaling molecule in intercellular signal transduction (Verma et al., 1993) and activates soluble guanylyl cyclase resulting in increased production of cGMP (Baranano and

Snyder, 2001). It has well documented neuromodulatory properties, influencing conductance in a variety of ion channels either directly or via signaling pathways. Until recently it was assumed that channel proteins lack any molecular domain that would validate a direct chemical interaction with CO. In fact, heme or metal centers in channel proteins are preferential targets for CO, making them the likely molecular switches by which cells transduce signals from CO (Varadi et al., 2007). CO-induced cytotoxicity, that is independent of hypoxia, has been reported in neuronal cells following prolonged (48h) exposure to high levels of the gas (Tofighi et al., 2006). Thus CO may well have neuromodulatory effects that become neurotoxic when concentrations exceed normal physiologic levels.

The present study was conducted to investigate the putative detrimental effects of CO on axonal conduction in isolated guinea pig spinal cord tissue. Exposing the tissue to varying concentrations of CORM-2 (yielding CO concentrations of 70-700 μM) for 30 minutes resulted in a concentration-dependent decrease in CAP amplitude and CAP waveform area, and an increase in stimulus-peak latency with no systematic changes in CMP. These effects were not observed following treatment with the negative control $RuCl_3$ indicating that CO was responsible for the observed changes in electrophysiological properties. In addition, a negative correlation was observed between CAP amplitude and stimulus-peak latency.

The attenuation in CAP amplitude was not accompanied by any prolongation of the duration of the waveform indicative of temporal dispersion although the CAP onset appeared to be delayed. This suggests that the reduced

amplitude of the CAP was attributable to recruitment of a fewer number of axons and the prolonged time to peak CAP amplitude implies that it was the faster conducting axons that were more susceptible to the effects of CO. The double-pulse stimulation protocol also revealed an increased refractory period (in the 2 and 4 msec interpulse interval conditions) that would likely extrapolate into a disrupted capacity for viable axons to support repetitive axonal discharge.

The present results provide the first reported evidence of CO-mediated impairment of axonal conduction in spinal cord tissue. The non-reversible effects of CO on CAP waveform, at least within the time frame of washout used in this study (30 min, and in some preparations in excess of 90 min), without concurrent depolarization of the CMP, may be indicative of CO-induced alterations in $Na^+$ channel function. Voltage-gated $Na^+$ channels emerge as the most likely candidate as they are critically involved in action potential electrogenesis, but minimally involved in the maintenance of the resting membrane potential. CORM-2 has previously been reported to modify transmembrane flux of $Na^+$ in other excitable tissues (Varadi et al., 2007) and CO exposure during development results in profound $Na^+$ channel modifications (Carratu et al., 1995). Alterations to voltage-dependent $Na^+$ channel activation and/or inactivation currents would be expected to reduce the number of axons reaching threshold and propagating action potentials, and therefore reduce the CAP amplitude and area while increasing stimulus-peak latency, as observed in the present studies.

Conduction deficits could also potentially be attributable to subtle myelin deficits, a known morphological consequence of elevated CO concentrations in the brain (Sener, 2003). However, if the concentration-dependent effects of CO

were a result of myelin disruption, then one would expect changes in the CMP due to exposure of a variety of $K^+$ channels (Nashmi and Fehlings, 2001; Judge and Bever, Jr., 2006). Changes in CMP were not evident following the short duration exposure of the tissue to CORM-2 used in the present protocol. Other known properties of CO, including the disruption of mitochondrial ATP by inhibition of $O_2$ on cytochrome C (Zuckerbraun et al., 2007), increased $K^+$ conductance via cGMP activation (Wu and Wang, 2005), and induction of nitrosative stress (Thom and Ischiropoulos, 1997; Thom et al., 1997) would all be expected to modify CMP and are therefore not considered to be primarily involved in the electrophysiological changes demonstrated in the present study. With this same line of reasoning, the reduction in CAP amplitude and other evident neuromodulatory influences do not appear to be dependent on anoxia-related mechanisms as these would also be expected to alter the CMP.

The results support the hypothesis that CO exposure reduces CAP amplitude and area in a dose-dependent manner that is not reversible in the short term. In the context of neurotrauma, the observation that elevated concentrations of CO produce axonal conduction deficits may be indicative of a role for CO in post-traumatic neuronal dysfunction. CO would be expected to contribute to neurological deficits during the time course that the HO system is upregulated. This period of increased HO activity may last for many weeks (Mautes et al., 2000). In the absence of effective clearance, eg because of impaired blood flow, persisting elevated levels of CO would be expected to contribute to neurotoxicity with permanent tissue loss and accompanying neurologic deficits.

## 3.5. Acknowledgements

Financial support for this work was provided by the Ontario Neurotrauma Foundation (A. Davies) and the Rick Hansen Foundation.

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# CHAPTER 4: HEMOGLOBIN INDUCES REVERSIBLE ALTERATIONS IN THE ELECTROPHYSIOLOGICAL PROPERTIES OF AXONS IN MAMMALIAN SPINAL CORD AND IS PROTECTIVE AGAINST THE CONDUCTION BLOCKING EFFECTS OF CARBON MONOXIDE

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Davies AL, Kramer JLK, Hayes KC, *Hemoglobin induces reversible alterations in the electrophysiological properties of axons in mammalian spinal cord and is protective against the conduction blocking effects of carbon monoxide*, Neuroscience, April, 2008

## 4.1. Introduction

Trauma to the spinal cord typically results in vascular disruption, rupture of post-capillary vessels or sulcal arterioles, hematomas and intravascular coagulation (Hayes and Kakulas, 1997; Kakulas, 1999; Norenberg et al., 2004). The associated ischemia contributes directly to axonopathy and myelinopathy through anoxic mechanisms (Stys, 1998) as well as indirectly through the induction of immunologic processes (Feuerstein et al., 1994; Sei et al., 1995; Saito et al., 1996). Accumulation of red blood cells (RBC) at or near the lesion site (Hayes and Kakulas, 1997), coupled with interruption of normal blood flow to the area, results in erythrocyte lysis, a degradative process most likely influenced by induction of the complement cascade (Hua et al., 2000). Following RBC lysis, hemoglobin (Hb) is released into the vasculature and parenchyma surrounding the lesion site (Yip and Sastry, 2000). Hb has neurotoxic properties (Regan and Panter, 1993; Regan and Guo, 1998) and its effective clearance is essential for neuroprotection. Degradation of heme, the functional constituent of Hb subunits, is catalyzed by the heme oxygenase (HO) system. The two isoforms of HO, HO-1 and HO-2, are upregulated following spinal cord injury (SCI) (Mautes et al., 1998; Mautes and Noble, 2000; Mautes et al., 2000a; Panahian and Maines, 2001; Liu et al., 2002; Lin et al., 2007) and the pattern of HO-1 expression corresponds to areas in which intra-parenchymal hemorrhage is evident (Mautes et al., 1998). The HO-catalyzed breakdown of heme in turn results in the release of carbon monoxide (CO), free ferrous iron and biliverdin (Maines, 2005).

We have recently shown that CO, introduced by the CO-releasing molecule Tricarbonyldichlororuthenium (II) dimer (CORM-2), induces conduction failure in axons of mammalian spinal cord (Davies et al., 2008). Hb is a well-documented scavenger of CO and the influences of the HO system on local concentrations of Hb and CO are likely critical to the progression or mitigation of CO-mediated cytotoxicity. Whether or not Hb per se influences the electrophysiological properties of long tract axons within the spinal cord is unknown. Similarly, the capability of Hb to modify the axonal conduction blocking properties of CO has not previously been examined.

In the present study we tested the hypothesis that elevated extracellular concentrations of Hb induce concentration-dependent and reversible changes in the electrophysiological properties of long tract axons from the excised spinal cord of adult guinea pigs. We also tested the hypotheses that Hb blocks the effects of CO on axonal conduction when Hb and CO are co-administered and reverses the effects of CO when administered after CO exposure.

## 4.2. Experimental Procedures

The experimental procedures were essentially identical to those we have used previously to demonstrate the effects of CO, nitric oxide, and the pro-inflammatory cytokine, tumor necrosis factor alpha, on axonal conduction properties (Ashki et al., 2006; Davies et al., 2006a; Davies et al., 2008). Briefly, the electrophysiological properties of excised guinea pig spinal cord axons were recorded in a double sucrose gap recording system while the tissue was perfused

(30 min) with varying concentrations of Hb (alone, or following CORM-2 treatment) or CORM-2+Hb solutions. After 30 min of treatment the tissue was perfused with Krebs' solution alone to eliminate treatment solutions (except where indicated); the electrophysiological properties were also monitored continuously throughout this 30 min washout period.

*Isolation of spinal cord tissue*

The surgical procedure for isolating the spinal cord tissue has been described previously (Shi and Blight, 1996; Shi et al., 1997; Davies et al., 2006b; Davies et al., 2008). Adult female Hartley guinea pigs of 350-500 g body weight (n=12, Charles River, Wilmington, MA, USA) were anesthetized prior to surgery (80 mg/kg ketamine hydrochloride, 0.8 mg/kg acepromazine maleate, and 12 mg/kg xylazine, i.m.). Following anesthesia, animals were perfused transcardially with 500 ml cold (15°C), oxygenated Krebs' solution (NaCl 124 mM, KCl 2 mM, $KH_2PO_4$ 1.2 mM, $MgSO_4$ 1.3 mM, $CaCl_2$ 1.2 mM, dextrose 10 mM, $NaHCO_3$ 26 mM, sodium ascorbate 10 mM, equilibrated with 95% $O_2$, 5% $CO_2$ to a pH of 7.2-7.4), and the vertebral column was excised rapidly. The spinal cord was carefully removed from the vertebrae and placed in cold Krebs' solution. The cord was initially separated into two halves by midline sagittal division and the ventral white matter was then isolated (see Figure 2.1A). The ventral quadrant is comprised mainly of descending long tract axons with diameters between 2.5 and 6 μm (Shi and Whitebone, 2006). Dissected strips of white matter (2-4 per animal) were maintained in continuously oxygenated Krebs' solution for at least one hour prior

to mounting in the recording chamber to ensure recovery from dissection before experimentation. The experimental protocols were reviewed and approved by the University of Western Ontario's Animal Use Subcommittee. All efforts were made to minimize the number of animals used and their distress.

*Electrophysiological Recording and Analysis*

The design and construction of the double sucrose gap chamber used for the electrophysiological recordings has been reported elsewhere (Shi and Blight, 1996; Shi and Blight, 1997; Shi and Borgens, 1999; Shi and Pryor, 2002) and is illustrated in Figure 2.1B. The central compartment, 20 mm in diameter, was continuously superfused with oxygenated Krebs' solution at 2 ml/min into which the treatments (Hb and CORM-2+Hb) were introduced. The temperature of the central compartment was maintained at 36.0-37.0 °C with an in-line solution heater (Warner Instruments, Hamden, CT, USA), and monitored using a thermocouple probe. The two end compartments contained isotonic potassium chloride (KCl, 120 mM) and were electrically isolated from the central compartment by narrow channels through which sucrose solution (320 mM) flowed continuously.

The strip of excised cord tissue was stimulated through silver-silver chloride pin electrodes positioned in one of the end chambers (cathode) and in the central chamber containing Krebs' (anode) (Figure 2.1B). Constant current supramaximal stimuli (except where indicated) were generated by a digital stimulator (Cygnus Technology, PA, USA) and delivered via a stimulus isolation

unit (WP Instruments, Sarasota, FL, USA) in the form of 0.1 msec constant current rectangular pulses at a rate of 0.33 Hz. The evoked compound action potentials (CAP) were amplified using an intracellular recording DC amplifier (Cygnus Technology, PA, USA) and recorded continuously throughout the experiment using silver-silver chloride pin electrodes in the grounded central chamber containing Krebs' and in the opposite end chamber containing KCl. Signals were filtered using a Hum Bug (Quest Scientific, Vancouver, BC, Canada) to eliminate any 60 Hz noise. CAP waveform and amplitude, the compound membrane potential (CMP), and stimulus-peak latency were recorded. To investigate the effects of Hb on axonal discharge properties, five paired stimuli were applied for each of seven interpulse intervals (2-14 ms), immediately preceding treatment, at the end of treatment, and at the end of the washout period. This enabled generation of paired pulse curves. Stimulus-response recruitment curves were also created by varying stimulus intensity (12.5-100% of maximum, 5 stimuli per stimulus intensity) immediately before treatment onset and at the conclusion of the treatment and washout periods. The electrophysiological data were digitized (25 kHz, 16-bit) and stored with a Neurodata Instruments Neurocorder for subsequent analysis. The electrophysiological properties of each preparation were allowed to stabilize prior to the introduction of treatment. At the end of each preparation tested, the tissue was transected in the central chamber. This resulted in a complete depolarization of the CMP which enabled quantification of any membrane potential changes that occurred due to the experimental treatment. All the on-line

recording and subsequent analysis was performed using LabView software (National Instruments, Austin, TX, USA).

*Hemoglobin Treatment*

Lyophilized human Hb (Sigma-Aldrich, St. Louis, MO, USA) was dissolved with oxygenated Krebs' solution to a final concentration of 100, 300, or 700 μM. As per the manufacturer, due to the ease with which native Hb is oxidized when exposed to air, the treatments may have been predominantly methemoglobin. For reference, in a previously published study we reported blood Hb concentrations of circa 1400 μM in SCI and able-bodied control subjects (Davies et al., 2007). All treatment solutions were prepared immediately prior to administration. The duration of tissue perfusion with Hb was 30 minutes. After 30 minutes the Hb was washed out by perfusion with Krebs' solution alone and the electrophysiological properties were monitored for an additional 60 minutes. Fresh strips of tissue were used for each preparation and each condition was replicated 5 times (n=5) to establish reproducibility of the observations.

*Hemoglobin and CORM-2 Co-administration*

In order to evaluate the hypothesis that Hb blocks the effects of CO, Hb was coadministered with CO. CO was delivered via the transition metal carbonyl CORM-2 (Sigma-Aldrich, St. Louis, MO, USA), dissolved in dimethyl sulfoxide (DMSO), and subsequently diluted with oxygenated Krebs' solution to a final CORM-2 concentration of 1000 μM in 1% DMSO. Previous studies have shown

that for each molecule of CORM-2, 0.7 moles of CO are liberated when the donor comes into contact with tissue (Motterlini et al., 2002), yielding experimental CO concentrations of 700 μM of CO in the present study. Hb was added to the CORM-2 solution to a final concentration of 700 μM immediately before introduction of the CORM-2+Hb solution to the central compartment bathing the tissue. The duration of tissue perfusion with CORM-2+Hb was 30 minutes. After 30 minutes the CORM-2+Hb solution was washed out by perfusion with Krebs' solution alone and the electrophysiological properties were monitored for an additional 60 minutes. Fresh strips of tissue were used for each preparation and each condition was replicated 5 times (n=5) to establish reproducibility of the observations.

*Hemoglobin Following CORM-2 Treatment*

To evaluate the hypothesis that Hb can reverse the electrophysiological effects of CO, Hb at a concentration of 700 μM was introduced (with Krebs' washout) following a 30 min treatment with 1000 μM CORM-2. Hb was perfused through the central compartment for 30 minutes and followed by a 60 minute washout period of Krebs' solution alone.

*Statistical Treatment*

The effects of Hb or CORM-2+Hb on CAP amplitude, CMP, and stimulus-peak latency measured during pretreatment (immediately preceding treatment), treatment (at the conclusion of 30 min of treatment), and washout (following 30

min Krebs' washout) were analyzed using repeated measures analysis of variance (SPSS 15.0 for Windows). Raw CAP amplitude values were normalized to the pretreatment values in order to facilitate aggregation of data. Linear regression analysis was used to characterize the relationships between CAP amplitude and Hb concentration and stimulus-peak latency and Hb concentration, respectively. In addition, recruitment curves and paired-pulse response curves were developed to further characterize any changes in electrophysiological properties resulting from treatment. Statistical significance was defined as $p < 0.05$.

## 4.3. Results

Prior to the introduction of any treatment, the electrophysiological properties of the tissue were continuously monitored and allowed to stabilize. During this pretreatment stabilization period, the CMP recorded across the sucrose gap became increasingly polarized as the sucrose perfused the extracellular space, reducing the leakage current (Shi and Blight, 1996). Treatment was introduced following 68.4 ± 14.9 (SD) min, following stabilization of CAP amplitude (31.8 ± 7.9 min), the CMP (37.5 ± 13.3 min), and the stimulus-peak latency (18.5 ± 13.7 min) in the 25 preparations studied. CAP amplitude varied minimally prior to the introduction of treatment (SD=3.6% of the mean).

*100 μM Hb*

Hb treatment at a concentration of 100 μM (n=5) had no effect on CAP amplitude, CMP, and stimulus-peak latency throughout the duration of the exposure phase (30 min) and the subsequent washout period (30 min). CAP amplitude remained at 100.7 ± 4.6% of pretreatment values after 30 min of treatment, and remained at 99.7 ± 1.5% following 30 min of Krebs' washout. The mean CAP amplitude over time is shown in Figure 4.1, panel A.

*300 μM Hb*

Hb treatment at a concentration of 300 μM (n=5) resulted in a reduction in CAP amplitude in all preparations ($p<0.05$) during exposure (30 min). CAP amplitude was reduced to 77.5 ± 4.4% of pretreatment values after 15 min of treatment and remained at 76.4 ± 4.6% after 30 min of Hb treatment. The observed decrease in CAP amplitude was reversed on Krebs' washout, reaching 97.0 ± 1.0% of pretreatment values by the end of the washout period (30 min). No change in CMP or stimulus-peak latency was observed during Hb treatment or Krebs' washout periods. The mean CAP amplitude over time is shown in Figure 4.1, panel A.

*700 μM Hb*

Hb treatment at a concentration of 700 μM (n=5) also resulted in a reduction in CAP amplitude in all preparations ($p<0.05$) during exposure (30 min). The magnitude of this reduction was greater than that seen following treatment

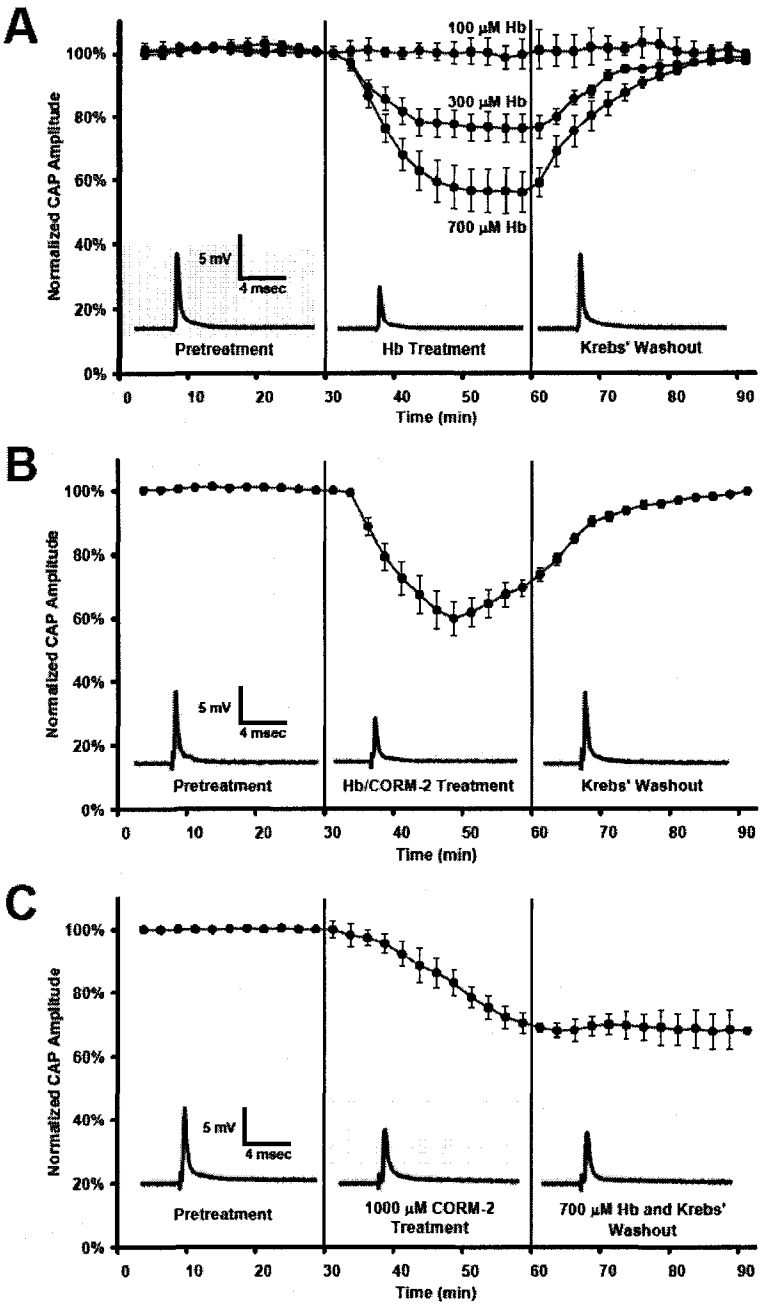


**Figure 4.1 Time course of mean, normalized CAP amplitude from preparations treated with (A) 100, 300, and 700 µM Hb, (B) 1000 µM CORM-2 and 700 µM Hb, or (C) 1000 µM CORM-2 followed by washout with Krebs' solution and 700 µM Hb over pretreatment (30 min), treatment (30 min), and washout (30 min) periods.** Treatment begins at 30 min, and Krebs' washout begins at 60 min. CAP amplitude is recorded following application of each stimulus (0.33 Hz). Individual CAP waveforms from preparations treated with (A) 700 µM Hb, (B) 1000 µM CORM-2 + 700 µM Hb, and (C) 1000 µM CORM-2 followed by washout with Krebs' and 700 µM Hb are inset.

with 300 μM Hb, dropping to 56.3 ± 6.7% of pretreatment values following 20 min of Hb treatment, and remained at 58.8 ± 6.5% following 30 min of Hb treatment. As with the preparations treated with 300 μM Hb, CAP amplitude began to increase following cessation of Hb treatment and the beginning of the Krebs' washout period, recovering to 98.6 ± 1.1% by the conclusion of the washout period (30 min). No change in CMP or stimulus-peak latency was observed during Hb treatment or Krebs' washout periods. The mean CAP amplitude over time and representative CAP waveform traces from a preparation treated with 700 μM Hb are shown in Figure 4.1, panel A. CAP waveforms at 5 minute intervals from an individual preparation treated with 700 μM Hb are shown in Figure 4.2.

*Cotreatment with 700 μ M Hb and 1000 μM CORM-2*

Simultaneous treatment with 700 μM Hb and 1000 μM CORM-2 (n=5) resulted in a reduction in CAP amplitude in all preparations ($p<0.05$) during exposure and a spontaneous recovery that preceded the onset of the Krebs' washout period. The reduction in CAP amplitude reached a maximum of 59.9 ± 5.8% of pretreatment levels following 17.5 min of treatment and recovered to 73.6 ± 2.7% by the end of the treatment period (30 min). The CAP amplitude continued to recover to pretreatment values following 30 min of Krebs' washout, reaching 99.7 ± 0.3% of pretreatment values by the conclusion of washout. No change in CMP or stimulus-peak latency was observed during Hb treatment or

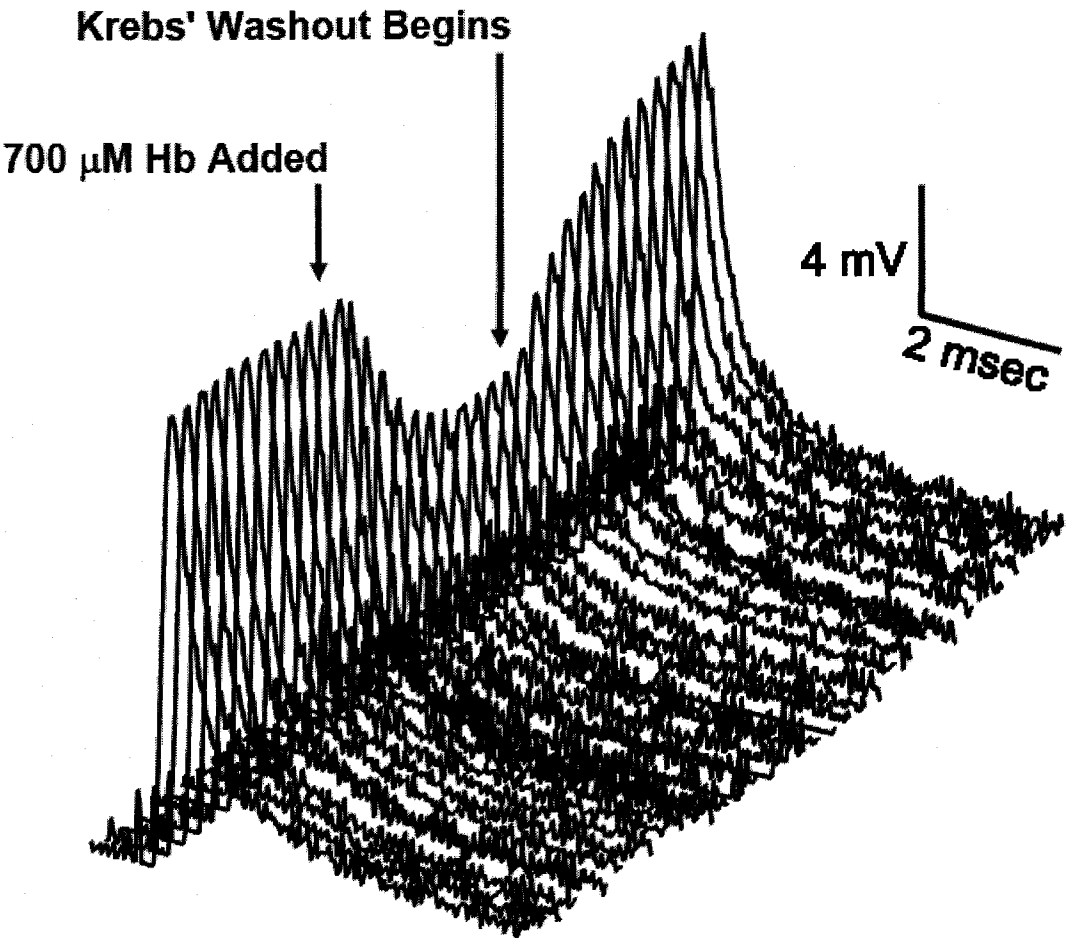


**Figure 4.2 Individual CAP waveforms at 5 min intervals from a preparation treated with 700 μM Hb. Hb treatment and Krebs' washout onset are indicated by arrows.**

Krebs' washout periods. The mean CAP amplitude over time and representative CAP waveform traces are shown in Figure 4.1, panel B.

*Treatment with 1000 μM CORM-2 and Washout with Krebs' and 700 μM Hb*

Treatment with 1000 μM CORM-2 (n=5) resulted in a reduction in CAP amplitude in all preparations ($p<0.05$) during exposure that did not reverse on washout with Krebs' and 700 μM Hb. The reduction in CAP amplitude was persistent and progressive, reaching 68.9 ± 3.1% of pretreatment values by the end of the 1000 μM CORM-2 treatment period (30 min). CAP amplitude remained depressed throughout washout with Krebs' and 700 μM Hb (30 min), and remained at 67.8 ± 5.8% of pretreatment values by the end of the washout period (30 min). Stimulus-peak latency increased following introduction of CORM-2 ($p<0.05$), reaching a maximum of 117.05 ± 2.8% of pretreatment values by the end of the CORM-2 treatment period (30 min), and remained elevated (113.7 ± 3.1%) at the end of the washout period (30 min). No change in CMP was observed during the 1000 μM CORM-2 treatment period or subsequent washout with Krebs' and 700 μM Hb. The mean CAP amplitude over time and representative CAP waveform traces are shown in Figure 4.1, panel C.

*Inverse CAP Amplitude/Stimulus-Peak Latency Relationship*

A linear function characterized the relationship between mean normalized CAP amplitude (x) and stimulus-peak latency (y) following 30 min of treatment with CORM-2 at a concentration of 1000 μM. This is illustrated in Figure 4.3,

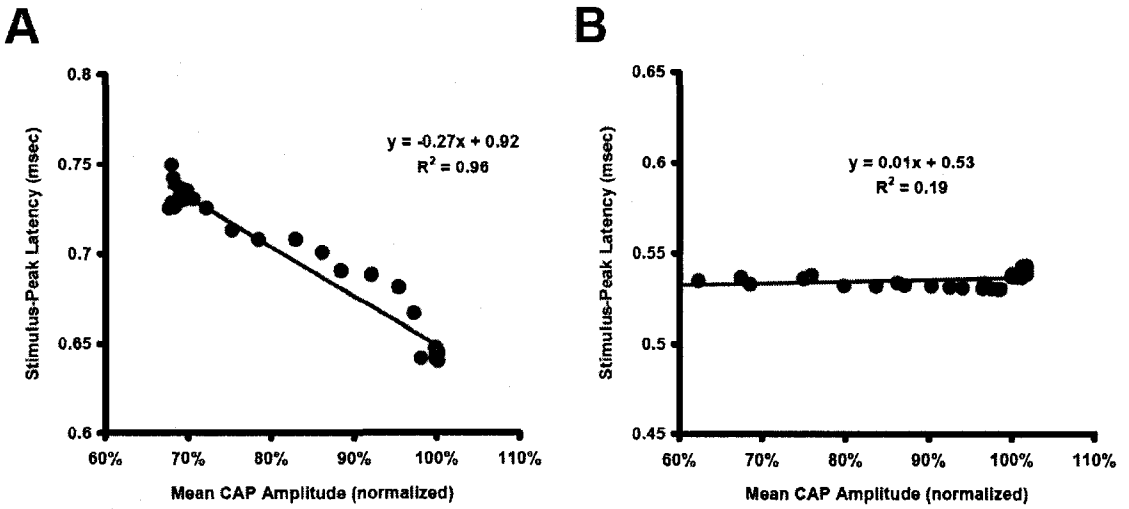


**Figure 4.3 (A) The inverse linear relationship between stimulus-peak latency (msec) and mean CAP amplitude (normalized) following treatment with 1000 μM CORM-2. (B) For comparison, no relationship was observed between stimulus-peak latency (msec) and mean CAP amplitude (normalized) following treatment with 700 μM Hb.**

panel A and is described by the equation $y=-0.27x+0.92$ ($R^2=0.96$). For comparison, the lack of association between mean normalized CAP amplitude and stimulus-peak latency following 30 min of treatment with 700 μM Hb is shown in Figure 4.3, panel B.

*Changes in CAP Waveform following Treatment*

No change in CAP waveform was observed following treatment with 100, 300, and 700 μM Hb, or following simultaneous treatment with 1000 μM CORM-2 + 700 μM Hb. After treatment with 1000 μM CORM-2, and prior to washout with Krebs' and 700 μM Hb, an increase in stimulus-peak latency was observed, and this was reflected by a change in waveform. A delay in CAP onset following application of the stimulus was observed following CORM-2 treatment but not Hb treatment, and is shown in Figure 4.4.

*Paired Pulse Stimulation*

A significant decrease ($p<0.05$) in the amplitude of the second CAP (expressed as a percentage of the 1st) was observed following treatment with 700 μM Hb at interpulse intervals of 2 and 4 ms; a reduction was also seen at interpulse intervals of 6 and 8 ms although this change was not statistically significant. At all interpulse intervals, the amplitude of the second CAP recovered to pretreatment levels following Krebs' washout. These changes are illustrated in Figure 4.5. No significant change in paired pulse response was seen following treatment with 100 or 300 μM Hb at interpulse intervals of 2, 4, 6, and 8 ms.

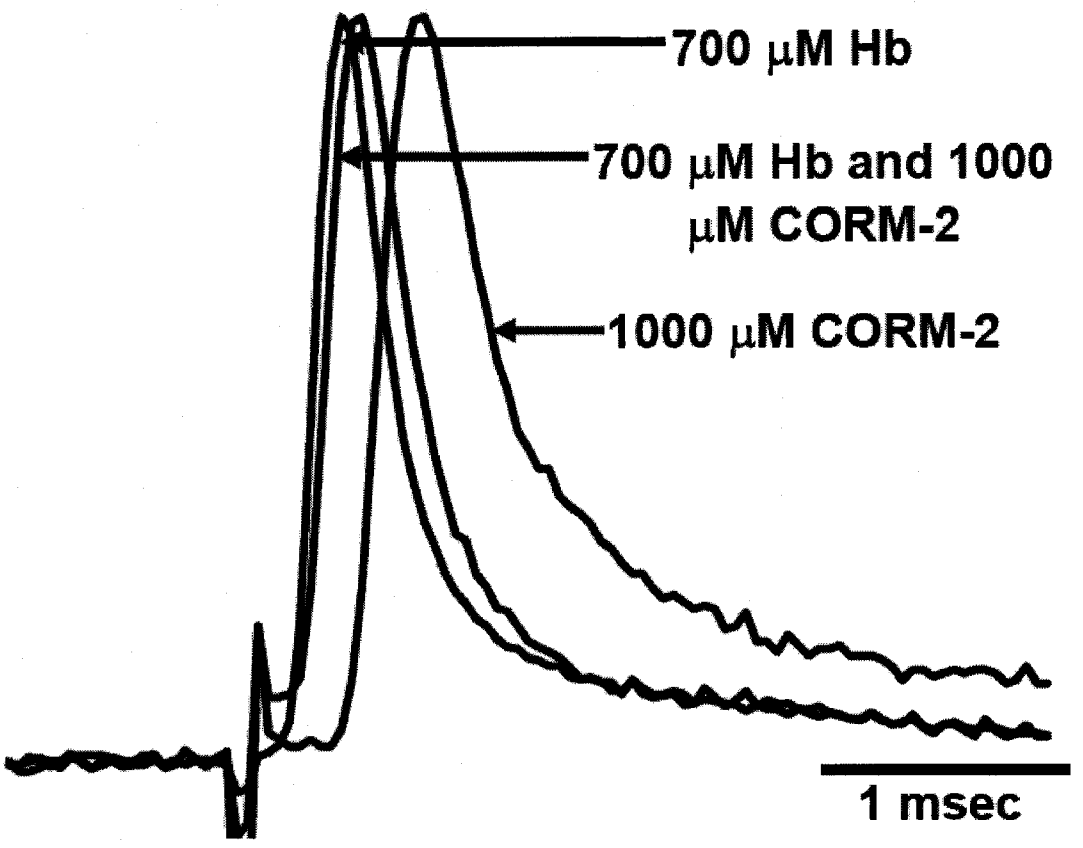


**Figure 4.4 Representative CAP waveforms (normalized) illustrating changes in CAP waveform following treatment with 700 μM Hb, 700 μM Hb and 1000 μM CORM-2, and 1000 μM CORM-2.**

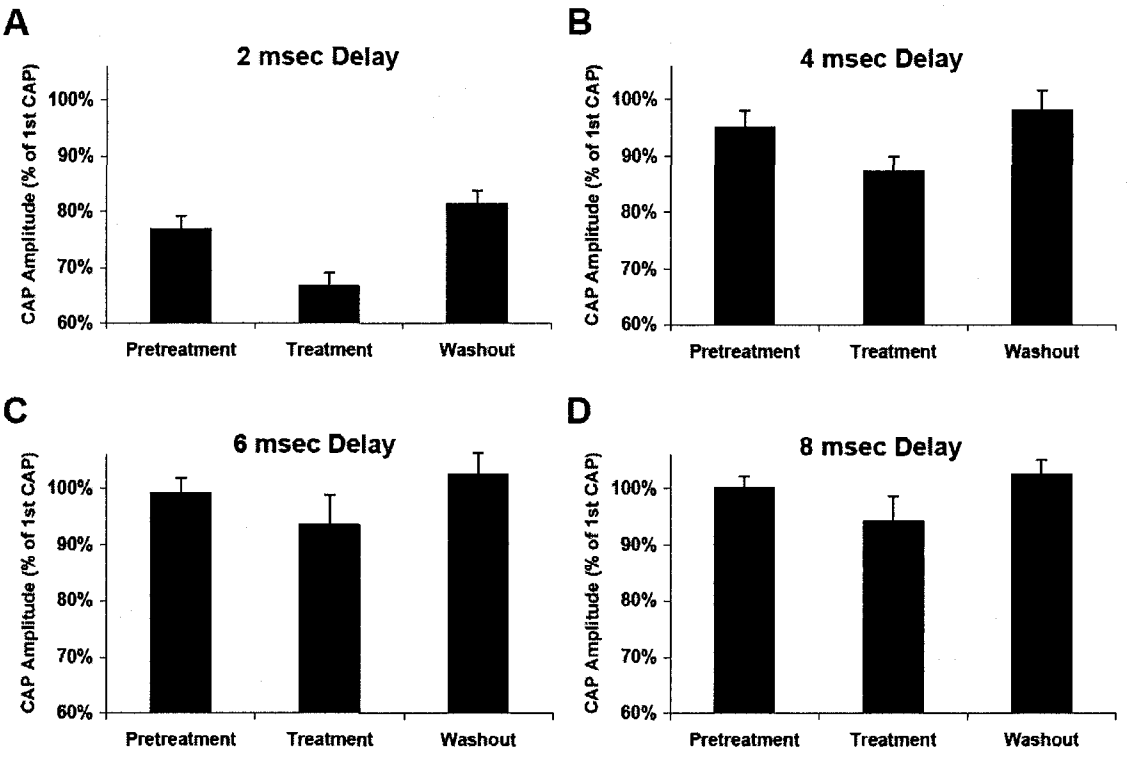


**Figure 4.5 Paired-pulse responses to treatment with 700 μM Hb at interpulse intervals of 2, 4, 6, and 8 msec.** Paired-pulse response is expressed as the amplitude of the second CAP as a percentage of the amplitude of the first.

*Stimulus-Response Recruitment Curves*

In all conditions in which a decrease in CAP amplitude was evident at supramaximal stimulus intensity (300 and 700 μM Hb, 1000 μM CORM-2 + 700 μM Hb, and 1000 μM CORM-2 followed by 700 μM Hb), a similar reduction in CAP amplitude was observed at all other stimulus intensities (12.5-100.0% of maximum). In preparations where CAP amplitude recovered to pretreatment levels during Krebs' washout (300 and 700 μM Hb, and 1000 μM CORM-2 + 700 μM Hb), CAP amplitude at all stimulus intensities also recovered to pretreatment levels. This is illustrated in Figure 4.6, which depicts stimulus-response curves prior to, during, and following Krebs' washout of a representative preparation treated with 700 μM Hb. No change in the *profile* of stimulus-response curves was evident following any treatment protocol tested.

## 4.4. Discussion

Traumatic injury to the spinal cord results in mechanical disruption of both neuronal tissue and the cord's intrinsic vascular supply (Mautes et al., 2000b). Trauma-induced hemorrhage within the CNS results in the exposure of cord tissue to Hb concentrations in the millimolar range (Rogers et al., 2003). Previous studies have linked Hb exposure with iron-dependent neurotoxicity (Regan and Panter, 1993; Regan and Panter, 1996; Regan and Guo, 1998; Rogers et al., 2003; Regan and Rogers, 2003; Chen-Roetling and Regan, 2006) based on the observation that iron-chelating agents are neuroprotective following Hb exposure (Regan and Guo, 1998). While the cytotoxic effects of long-term Hb

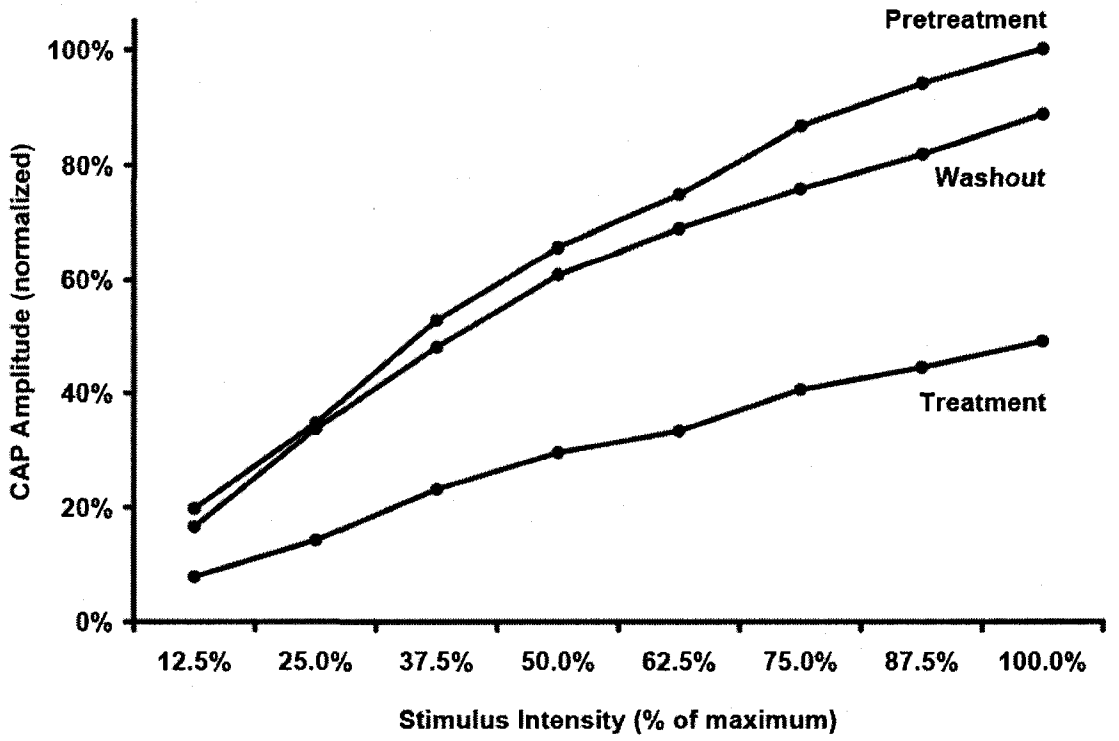


**Figure 4.6 Stimulus-response recruitment curves prior to, during, and following treatment with 700 μM Hb.** CAP amplitude is expressed as a percentage of the pretreatment CAP amplitude.

exposure are well-documented, less is known about the electrophysiological consequences of limited Hb exposure within the spinal cord.

The present study investigated the effects of Hb on the electrophysiological properties of isolated mammalian axons and the ability of Hb to antagonize and reverse effects of CO on axonal conduction (Davies et al., 2008). Hb treatment at concentrations of 300 and 700 μM resulted in a rapid reduction in CAP amplitude that was reversed completely on washout with Krebs' solution alone. No change in CMP, stimulus-peak latency, or CAP waveform was observed at any of the Hb treatment concentrations tested. Treatment with 700 μM Hb also resulted in a reduction in the amplitude of the second CAP following paired-pulse stimulation at delays of 2 and 4 ms that was reversed during Krebs' washout. This indicates that the electrogenesis of the CAP and the capability of axons to discharge repetitively were also impaired by Hb. These results provide the first evidence in support of the hypothesis that direct exposure of axons to Hb invokes neuromodulatory effects that impair axonal conduction. The fact that these impairments were reversible indicated that these effects of Hb are exclusive of its well-documented neurotoxicity.

The present results also confirm previously published reports of the conduction-blocking effects of the CO donor CORM-2 in this preparation (Davies et al., 2008); unlike Hb-induced changes in axonal conduction these effects were not reversible following washout with Krebs' solution. In order to establish whether the CO-scavenging properties of Hb could reverse CO-induced conduction deficits, Hb was added to the Krebs' washout solution. In the present

case, treatment with 1000 μM CORM-2 followed by washout with Krebs' solution containing 700 μM Hb, resulted in a reduction in CAP amplitude, but with an increase in stimulus-peak latency that did not reverse during the washout period. No corresponding change in CMP was observed, however a change in CAP waveform was evident and a delay in CAP onset was apparent. This change in waveform did not reverse during Krebs' washout. The observation that Hb treatment following CORM-2 exposure did not reverse the effects of the CO donor on axonal conduction may suggest that initiation of intracellular signaling events or intracellular accumulation of CO has already taken place, both of which would preclude the ability of Hb to antagonize CO.

In order to test whether the combined effects of Hb and CO were additive or antagonistic in their effects on axonal conduction, Hb and CO were coadministered. Simultaneous treatment with 700 μM Hb + 1000 μM CORM-2 (yielding a CO concentration of 700 μM) resulted in a rapid reduction in CAP amplitude that partially recovered during treatment (prior to the onset of Krebs' washout) and returned to pretreatment levels during the Krebs' washout period. As observed following treatment with Hb alone, this reduction in CAP amplitude was not accompanied by any change in CMP, stimulus-peak latency, or CAP waveform. These results establish that the neuromodulatory effects of CO and Hb were not additive; to the contrary, the Hb effects were attenuated by the presence of CO and vice versa. This is consistent with the well-known CO-scavenging properties of Hb and the previously documented high affinity of Hb for CO (Wu and Wang, 2005).

It was noted that the reduction in CAP amplitude reversed *prior to* the onset of washout during concomitant treatment with Hb + CO. This may indicate that the effects of Hb on axonal conduction were immediate, whereas CO release by the CORM-2 donor molecule requires tissue contact and therefore CO accumulation was delayed. This suggestion is supported by the lack of any observable change in stimulus-peak latency or CAP waveform following treatment with Hb + CORM-2 in contrast to the observed increased in stimulus-peak latency and change in CAP waveform following treatment with CORM-2 alone. This implicates Hb per se as the cause of the initial reduction in CAP amplitude.

The present results establish proof of principle that Hb induces rapid and completely reversible changes in CAP amplitude, independent of any observable change in CMP or stimulus-peak latency. Given that the cell membrane is impermeable to Hb (Yip and Sastry, 2000), these observations would be consistent with an Hb-induced effect on voltage-gated $Na^+$ channels or gradients. This speculation is further supported by the demonstration that Hb induced changes in CAP amplitude, which itself is largely governed by $Na^+$ conductance, conversely there were no changes in CMP, which is largely maintained by $K^+$ conductance. Previous studies have established the cytotoxic effects of Hb on neuronal tissue following long-term exposure (Regan and Panter, 1993; Regan and Panter, 1996; Regan and Guo, 1998; Rogers et al., 2003; Regan and Rogers, 2003; Chen-Roetling and Regan, 2006). The present findings suggest that brief exposure to Hb may exert neuromodulatory effects that precede any neurotoxic effects and reverse following effective Hb clearance.

The neuromodulatory and potentially detrimental effects of CO have been reported previously and the endogenous production of CO from the HO system with the degradation of Hb has been implicated in the secondary pathology of SCI (Sharma et al., 2000; Sharma and Westman, 2003; Davies et al., 2008). Antithetically, Hb serves as a natural scavenger for CO and the actions of the HO system governing the disposition of CO and Hb is complex and influenced by other key reactions (Maines, 2005). How progressively increasing local extracellular concentrations of CO affect axonal activity is not well known, although it appears likely that if the concentration is excessive and the scavenging properties of local Hb are inadequate, the effects will be detrimental. The factors influencing HO-catalyzed reactions are therefore critical in deciding the balance between the reversible neuromodulatory influences and neurotoxic effects of Hb and CO.

The most obvious clinical implication of the demonstrated reversible Hb-induced conduction block pertains to the acute phase of traumatic SCI, which is typically characterized by a period of spinal shock followed by spontaneous neurologic recovery. Although the clinical aspects of spinal shock are well-documented, the underlying mechanisms responsible for the temporary loss of reflex and motor function are not well-documented. The byproducts of hemorrhage and subsequent RBC lysis including Hb and its breakdown products, and their eventual clearance, may be contributory to the initial axonal conduction deficits and their subsequent reversal, thereby contributing to spontaneous neurologic recovery. Support for this proposition comes from the general

correspondence between the time course of transient paralysis (Ditunno et al., 2004) and temporal patterns of HO activity (Mautes et al., 2000a).

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# CHAPTER 5: DISCUSSION

## 5.1. Overview

Improved neurological function is observed in individuals with SCI at the acute stage with the resolution of spinal shock (Ditunno et al., 2004), in the first 30 days as other acute pathophysiological processes resolve, and in the longer term with late-onset neurological recovery (Fawcett et al., 2007). Similar phenomena are observed with animals that sustain SCI (Kaegi et al., 2002; Smith and Jeffery, 2005; Brown et al., 2005). To date, no physiological mechanisms have been identified that adequately account for the improvements in motor and sensory function seen in the context of these clinical phenomena. The identification of such mechanisms may permit targeted therapeutic interventions that result in accelerated recovery, optimization of neurological function in individuals with SCI and significant improvements in quality of life. To this end, research in our laboratory has been focused on evaluating possible physiological mechanisms that may contribute to changes in neuronal activity that are manifest as alterations in gross function. The three studies presented in this thesis were designed to identify potential neuromodulatory substances, characterize their effects on axonal conduction, and examine the reversibility of these effects. Each study evaluated entirely novel hypotheses and therefore each one represents a proof-of-principle study.

## 5.2. Study 1

Study one evaluated the hypothesis that elevated concentrations of rhTNFα reversibly impair axonal conduction in isolated guinea pig spinal cord preparations. This hypothesis was confirmed, as 30 minutes of exposure to rhTNFα induced concentration-dependent reductions in CAP amplitude, with concomitant depolarization of the CMP that reversed during washout with Krebs' solution alone (Davies et al., 2006). The findings of this study are consistent with previous reports of TNFα-induced increases in membrane conductance to $Na^+$ ions (Kagan et al., 1992; van der Goot et al., 1999) as this would result in an influx of $Na^+$, which would in turn depolarize the membrane potential and lead to conduction failure. TNFα-induced alterations in the membrane conductance of $K^+$ and $Ca^{++}$ have also been reported (McLarnon et al., 1993; McLarnon et al., 2001) and therefore these mechanisms cannot be ruled out entirely. It is also important to note that TNFα is a potent inducer of iNOS (Yune et al., 2003) and as NO has been repeatedly shown to induce conduction deficits (Redford et al., 1997) in conjunction with depolarization of the membrane potential (Ashki et al., 2006), the possibility exists that NO is the factor that is directly responsible for the effects reported in this study rather than TNFα *per se*. Although TNFα has been shown to trigger cytotoxicity and apoptosis (Lee et al., 2000), the exposure duration needed is far greater than that used in the present study and the reversibility of the deficits also speaks against this possibility. Recently published studies of animal models have provided support for the suggestion that TNFα is linked with impaired function after SCI by demonstrating improved functional

outcomes following TNFα inhibition (Genovese et al., 2006; Genovese et al., 2008), but contradictory evidence has also been presented (Chi et al., 2008). In human subjects with chronic SCI, new evidence has also emerged that demonstrates a correlation between decreased TNFα and improved ASIA scores following exercise therapy (Griffin et al., 2008), perhaps indicative of the role of this cytokine in neurological impairment. The results of study one of this thesis may thus have important implications for neurorecovery in the acute, subacute, and chronic stages for SCI in particular, and by inference, other forms of neurotrauma.

## 5.3. Study 2

Study two evaluated the hypothesis that elevated concentrations of carbon monoxide (CO) reversibly impair axonal conduction in isolated guinea pig spinal cord preparations. This hypothesis was refuted as CO, introduced via the donor CORM-2, induced concentration-dependent reductions in CAP amplitude, with concomitant increase in stimulus-peak latency and change in CAP waveform, that occurred independent of changes in CMP and did not reverse during washout (Davies et al., 2008). Examination of the paired-pulse response curves also revealed a CO-induced increase in the refractory period at interpulse intervals of 2 and 4 msec. The observation that CO exposure resulted in a decrease in CAP amplitude without concurrent depolarization of the CMP suggests $Na^+$ involvement based on its prominent role in electrogenesis and minimal contribution to the resting membrane potential; this is consistent with

previous reports of CO-dependent changes in transmembrane $Na^+$ flux (Varadi et al., 2007). The observed changes in waveform and refractory period are consistent with delayed onset of the action potential and this would also be compatible with alterations in $Na^+$ channel activity. Exposure to CO may also have an effect on cellular metabolic processes (Zuckerbraun et al., 2007), and disruption of neuronal metabolism could be contributory to the observed deficits, although the absence of any effect on CMP renders this possibility unlikely in this particular case. Similarly, neurotoxicity or general disruption of the membrane would be associated with membrane depolarization and CO-induced cytotoxicity has only been reported following prolonged (i.e. greater than 24 hours) exposure (Tofighi et al., 2006). In fact, it is possible that CO produced via the HO system has a protective rather than detrimental effect within the CNS (Parfenova and Leffler, 2008) by limiting production of destructive oxidants. If this is the case, temporary impairment of neuronal function may represent the cost of limiting permanent oxidative damage.

## 5.4. Study 3

Study three evaluated three hypotheses concerning the effects of CO and Hb on axonal conduction. The hypotheses were that 1) Hb reversibly impairs axonal conduction in isolated guinea pig spinal cord preparations 2) Hb blocks the effects of CO when coadministered 3) Hb reverses the effects of CO when administered after CO exposure. The first hypothesis was confirmed as Hb induced rapid and entirely reversible reductions in CAP amplitude and increases

in the refractory period. The second hypothesis was also confirmed as Hb blocked the effects of CO when coadministered. The third hypothesis was refuted, as Hb did not reverse the effects of CO when administered after CO treatment.

Hemoglobin has reported neurotoxic properties following prolonged exposure (Regan and Panter, 1993; Regan and Guo, 1998), but in the present study the effects of Hb on axonal conduction were completely and rapidly reversed following washout of treatment. This is consistent with the suggestion that Hb-induced toxicity is dependent on the breakdown products of Hb, such as free iron, rather than Hb itself (Regan and Rogers, 2003). Due to the inability of Hb to cross the cell membrane (Yip et al., 1996; Yip and Sastry, 2000), any changes in axonal conduction would have to be triggered from outside of the neuronal membrane. Coupled with the observation that no changes in any measure of axonal conduction were observed with the exception of a pronounced reduction in CAP amplitude, the effects of Hb may have been a result of disruption of ionic gradients. The inability of Hb to reverse the effects of CO is consistent with both the possibility that internalization of CO precluded Hb-CO interactions (and therefore any antagonistic effects) and their respective membrane permeabilities. Given the affinity of Hb for CO, it is not surprising that coadministration of the two substances lead to mutual antagonism.

## 5.5. Summary and Clinical Implications

The three studies contained herein provide, for the first time, proof of principle that proinflammatory cytokines as well as immune and serological factors are capable of reversibly altering axonal conduction in mammalian spinal cord. This may occur during the acute stage following trauma or possibly many years after injury.

Shortly after injury, in the hours or days after trauma, the cord is exposed to Hb as a result of hemorrhage (Hayes and Kakulas, 1997; Yip and Sastry, 2000) and its breakdown products such as CO following induction of the HO system (Mautes et al., 2000; Maines, 2005). These two factors interact in a mutually limiting manner and the relative contribution of each (which likely depends on modifiers of HO activity) may help to dictate whether temporary neurological impairment as seen during spinal shock resolves or extends to permanent cell loss and disability. It appears plausible that in more severe cases of injury, in which hemorrhage is more pronounced, the accumulation of Hb occurs to such an extent that effective clearance is delayed beyond the point at which Hb- or CO- induced cytotoxicity is initiated. At these early stages, the immune response to trauma also includes intraparenchymal immunoactivity as well as the extravasation of peripherally circulating immune cells (Fleming et al., 2006), and release of inflammatory mediators such as cytokines (Yang et al., 2004). We have demonstrated that elevated levels of the proinflammatory cytokine TNF$\alpha$ can depolarize the membrane potential and impair axonal

conduction (Davies et al., 2006), and therefore may also contribute to functional changes following SCI.

In the longer term, following stabilization of the injury and lesion site, fluctuations in functional status are also reported. There is some evidence that these changes correspond to infectious episodes, the presence of pressure ulcers, or other secondary medical complications involving an immune response (McDonald et al., 2002; Griffin et al., 2008). Decreases in sensory and motor function appear to be temporally associated with immune challenge, while functional improvement follows resolution of these complications in these studies. We have previously shown that elevated levels of TNF$\alpha$ and other immune mediators are present in the serum of individuals with longstanding SCI (Hayes et al., 2002) and are further elevated in those experiencing complications (Davies et al., 2007); inflammation and infection also trigger HO upregulation (Maines, 2005). The permeability of the BBB to these substances has been demonstrated (Pan et al., 1999; Pan et al., 2003), and therefore it is possible that they influence neurological function at the level of the spinal cord. Indeed, in individuals with longstanding injury this may be manifest as spontaneous late-onset recovery as a consequence of improved general health status, resolution of infection, and wound healing.

An important consideration regarding the potential implication of these findings for SCI research and treatment specifically is that the considerable loss of tissue inherent in SCI cases results in a loss of neuronal redundancy. This in turn may mean that the SCI population is particularly susceptible to immune-,

CO-, or Hb-mediated neurological impairment. Simply put, conduction block of 1000 axons in an individual with an intact spinal cord may represent a loss of roughly two percent of functional axons, but in an individual with an injured cord, this may represent one third of remaining axons at the level of a lesion. As reported by Kakulas, a difference of 1000 axons is the numerical distinction between a sample of individuals with incomplete injuries and those with complete loss of motor and sensory function (Kakulas, 1999). Therefore, although certain factors that are not conventionally considered to have neuromodulatory effects may in fact modulate neuronal activity in the absence of any pathology, these effects may simply be less apparent than they are following neurotrauma.

The results presented in this thesis may also be relevant to other disorders with neurological involvement including neuroinflammatory diseases such as Guillain-Barré Syndrome (GBS), multiple sclerosis (MS), or traumatic brain injury (TBI). There are also transient neurapraxias associated with cord concussion states. One element that these various conditions have in common is a degree of neurological impairment that changes in severity over relatively short periods of time. Dramatic functional changes with brief durations preclude many of the putative mechanisms that have been proposed to explain these clinical phenomena, leaving the underlying physiological causes of neuronal dysfunction unexplained. For example, neurapraxia involves transient motor and sensory dysfunction, or even paralysis, in the absence of any structural or radiological abnormalities (Torg et al., 1997). In some cases symptoms, such as bilateral paresthesias, can last from several minutes to more than 24 hours (Maroon et al., 2007). So-called "transient" SCI has also been documented (Scher, 1991; Perks,

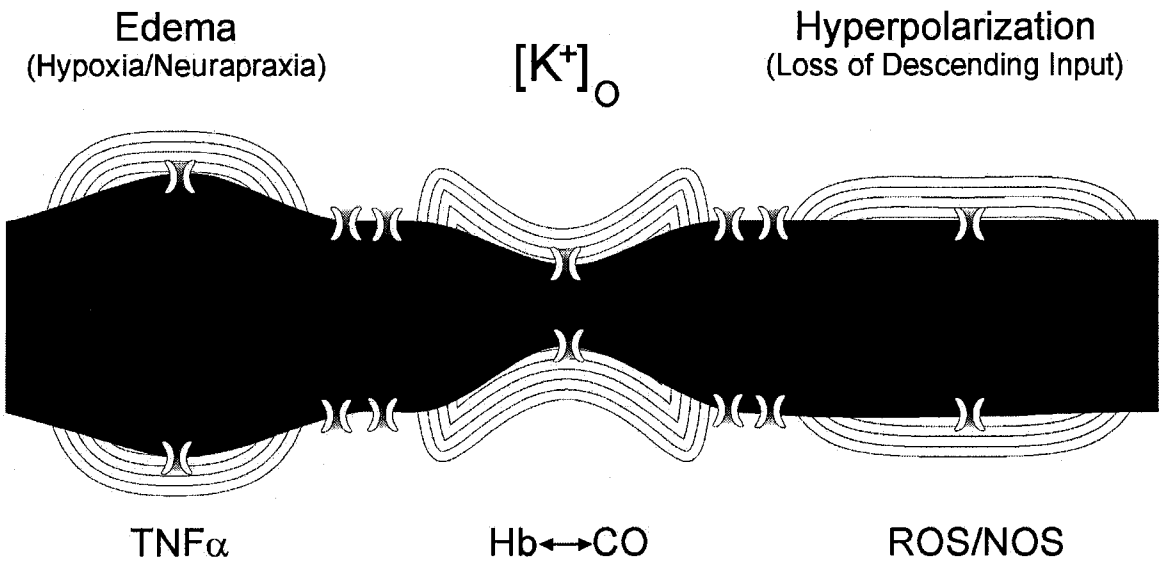
2005) and is distinct from SCI in that no evidence of structural damage is evident and complete functional recovery occurs within minutes to hours. These cases still necessarily involve a traumatic injury that is accompanied by an inflammatory response; however, permanent damage to the cord due to mechanical disruption of the vertebral column or parenchymal injury is notably absent. It is possible that the immune response contributes to transient neurological impairment in these cases. In MS and other neuroinflammatory diseases, this connection between inflammation and neuronal activity has been addressed. Autopsy and biopsy samples from human subjects with acute neuroinflammatory disease have revealed increased HO activity (Stahnke et al., 2007) and it has been suggested that although low levels of HO activity are neuroprotective, chronically high levels may result in tissue damage. In GBS, symptoms are often preceded by infection (Burns, 2008) and although some of the symptoms are consistent with demyelination, in most cases conduction abnormalities are apparent in the absence of any changes in myelination during the first week of symptoms (Gordon and Wilbourn, 2001).

In SCI, and other neurological conditions in which inflammation is prominent, it is apparent that numerous factors contribute to pathology and progression. Identification of the underlying mechanisms responsible for functional manifestations of altered neuronal activity is complicated by this problem, and perhaps this has hindered the discovery of satisfactory mechanisms to account for many of these clinical phenomena. Whether TNF$\alpha$-, CO-, or Hb-induced neuromodulation are involved in such mechanisms remains

to be seen; the three studies comprising the body of this thesis represent a starting point for future studies to improve our understanding of functional recovery after SCI.

## 5.6. Theoretical Model of Neurorecovery in the Acute Phase

Figure 5.1 illustrates a new conceptual model of the pathophysiological mechanisms thought to underlie transient neurological dysfunction following neurotrauma, incorporating the essential outcomes of the abovementioned studies. An idealized axon is depicted in the upper portion of the figure, illustrating internodal segments with intraaxonal swelling (left), extramural compression (center), and normal axonal configuration (right). The pathophysiological mechanisms that have previously been speculated to contribute to these factors include edema, which may be responsible for hypoxia and neurapraxia, elevated extracellular concentrations of $K^+$ leading to depolarization, and hyperpolarization due to the loss of facilitatory descending inputs (Ditunno et al., 2004). Beneath the axon, the potential contributions of cytokine-mediated conduction block, Hb-mediated conduction block, and other factors such as ROS and NOS (Smith et al., 2001; Ashki et al., 2006) that have now been shown to contribute to reversible axonal conduction failure are identified. In the lower part of the figure, representative time courses of these processes are shown in association with the time course of the resolution of neurological deficits. Since the magnitude and duration of these effects are contingent on many contributing elements only a conceptualization of the time



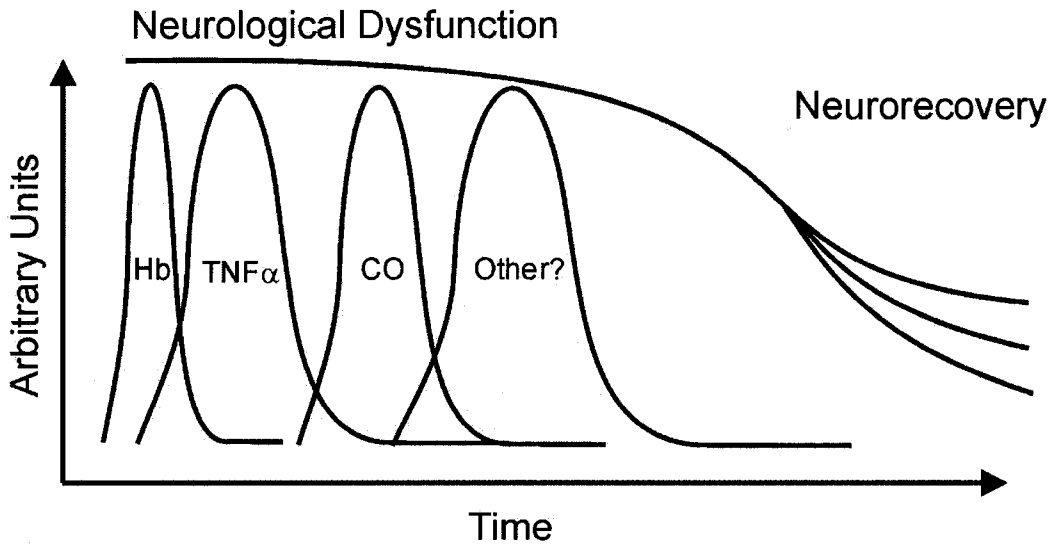




**Figure 5.1 Model of the Pathophysiological Basis of Transient Neurological Dysfunction/Neurorecovery Following SCI.**

course can be depicted at this time. For a more detailed summary of the time course of underlying immunological events that follow neurotrauma, see Donnelly and Popovich (Donnelly and Popovich, 2008). This model may help explain why certain acute phase surgical, pharmacological, and other interventions, e.g. cooling, are neuroprotective and accelerate neurorecovery. When extracellular concentrations of cytokines, CO, ROS, NOS, and Hb exceed the levels tested in the studies contained herein, they clearly contribute to neurotoxicity and irreversible neurological impairment.

## 5.7. Conclusions and Future Directions

The three studies contained herein represent a demonstration of the principle that the immune and serological factors TNFα, CO, and Hb can induce rapid impairments in axonal conduction in isolated mammalian spinal cord. In the case of both TNFα and Hb these deficits were rapidly reversible. Additional studies are required to further characterize the mechanisms by which these effects are produced and *in vivo* studies would be of great interest as a means of evaluating their relative contributions at a clinical level. These questions may be addressed using additional electrophysiological techniques, such as patch-clamp recording, and the use of blocking agents with *in vivo* animal models. If immunological and serological factors do substantially impair axonal conduction following SCI, functional improvement may be achieved via therapeutic interventions that selectively target these factors.

## 5.8. References

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# Ethics Approval:

Western

03.12.07
*This is the 3rd **Renewal of this protocol***
*A Full Protocol submission will be required in 2009

Dear Dr. Hayes:

Your Animal Use Protocol form entitled:

**Immune-Mediated, Reversible, Conduction Deficits in the Spinal Cord**

has been approved by the Animal Use Subcommittee.
This approval is valid from **04.01.07 to 03.31.08**
The protocol number for this project remains as **2005-012-03**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

**ANIMALS APPROVED FOR 1 YR. - Highest Pain Level: B**

| Species | Other Detail | Housing/Use Locations | Animal # Total for 1 Year |
|---|---|---|---|
| Guinea Pig | Hartley 350-500g Female | LHSC-SS | 40 |

**REQUIREMENTS/COMMENTS**
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

c.c. Approved Protocol K. Hayes, T, Kirkpatrick
Approval Letter - , T, Kirkpatrick

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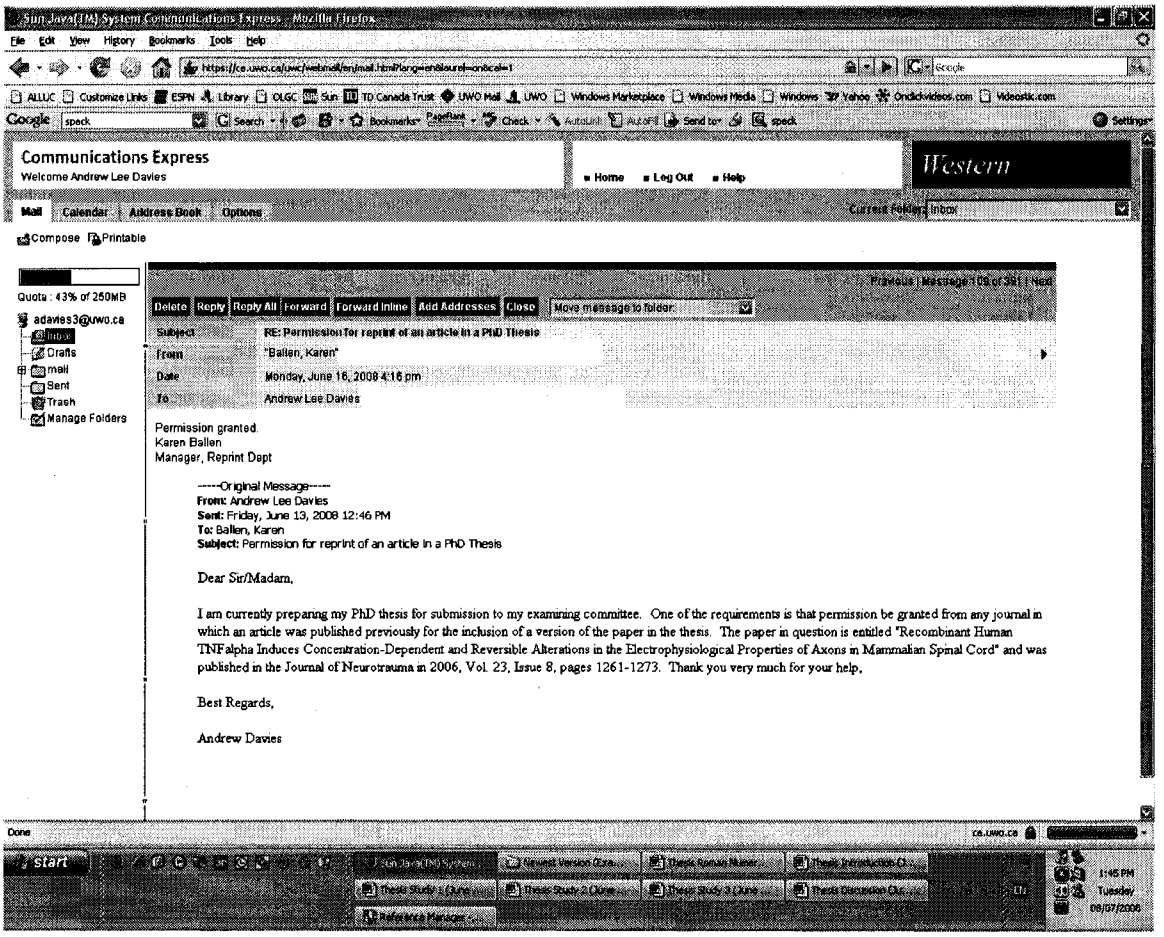


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21. **Other conditions:**

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**International Baccalaureate Diploma**
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## ACADEMIC AWARDS AND SCHOLARSHIPS

- Siskinds Studentship in Spinal Cord Injury Research (2008)
- CIHR Student Forum University of Western Ontario Representative (2006)
- Ontario Graduate Scholarship (2006-Present )
- National Neurotrauma Society Top Abstract Finalist (2005)
- University of Western Ontario Graduate Research Studentship (2005-Present)
- University of Western Ontario Teaching Assistant Award Nominee (2004-2005, 2007-2008)
- Ontario Neurotrauma Foundation Studentship (2004-2006)
- Ontario Graduate Scholarship in Science and Technology (2004)
- National Neurotrauma Society Top Abstract Finalist (2003)
- NIH Travel Grant (2003)
- University of Western Ontario Special University Scholarship (2002-2005)
- Gordon Cressy Student Leadership Award (2001)

## TEACHING AND RESEARCH EXPERIENCE

**Physiology Tutor**, First Nations Student Services ***Sept. 2007 – April 2008***
University of Western Ontario
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**Teaching Assistant**, Physiology and Pharmacology ***Sept. 2003 – April 2008***
University of Western Ontario
London, Ontario, Canada
- Phys 310 Laboratory in Mammalian Physiology
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**Research Assistant**, Molecular Genetics ***July 1996 - August 1996***
University of Calgary
Calgary, Alberta, Canada
- Laboratory of Dr. M. Bentley
- Assisting in *D. melanogaster* studies
- Molecular Techniques (PCR, Gel Electrophoresis)

## PROFESSIONAL SKILLS

Extensive Lab Work in Neuroscience and Biology:
- Animal Handling, Surgery, Sacrifice, Dissection, Perfusion
- Staining, Sectioning, Microscopy
- Electrophysiology (extracellular microelectrode recording, drug treatments, etc.)
- Cellular/Molecular Techniques (culture preparation, purification, ELISA, gel electrophoresis, etc.)
- Database Design and Statistical Analysis Software (SPSS, Labview, Matlab, Microsoft Access, etc.)

## PUBLICATIONS

1. **Davies AL**, Kramer JLK, Hayes KC. Hemoglobin induces reversible alterations in the electrophysiological properties of axons in mammalian spinal cord and is protective against the conduction blocking effects of carbon monoxide. *Submitted to Neuroscience, 2008.*

2. **Davies AL**, Kramer JLK, Hayes KC. Carbon monoxide-releasing molecule CORM-2 induces concentration-dependent alterations in the electrophysiological properties of axons in mammalian spinal cord. *Neuroscience*, 2008, 151(4): 1104-1111.

3. **Davies AL**, Hayes KC, Dekaban GA. Clinical correlates of elevated serum concentrations of cytokines and autoantibodies in patients with spinal cord injury. *Archives of Physical Medicine and Rehabilitation* 2007, 88(11): 1384-1393.

4. Hayes KC, **Davies AL**, Ashki N, Kramer JK, Close TE. Re: Ditunno JF, Little JW, Tessler A, Burns AS. Spinal shock revisited: a four-phase model. Spinal Cord 2004; 42: 383-395. *Spinal Cord* 2007, 45(5): 395-396.

5. **Davies AL**, Hayes KC, Shi R. Recombinant human TNFα induces concentration-dependent alterations in axonal conduction in mammalian spinal cord. *Journal of Neurotrauma* 2006, 23(8): 1261-1273.

6. **Davies AL**, Hayes KC, Shi R. Recombinant human TNFα induces concentration-dependent alterations in axonal conduction in mammalian spinal cord. *Journal of Neurotrauma* 2005, 22(10): 1164. (Published Abstract)

7. **Davies AL**, Hayes KC, Dekaban GA, Askes HK. Elevated Levels of Interleukin-1 Receptor Antagonist and Interleukin-6 in Serum of Chronic Spinal Cord Injured Patients. *Journal of Neurotrauma* 2004, 20(10): 1056. (Published Abstract)

8. Hayes KC, **Davies AL**, Potter PJ. Restorative Neurological Approaches to the Rehabilitation of Individuals with Longstanding Spinal Cord Injury. *Topics in Spinal Cord Injury Rehabilitation*, 2004, 10(1): 51-62.

## ACADEMIC CONFERENCE PRESENTATIONS

1. Hayes KC, **Davies AL**, Ashki N, Close TE, Kramer JLK. *Immunologic contributions to spontaneous recovery following spinal cord injury.* **Spinal Cord: Function, Repair, and Rehabilitation after Injury**, June 2008, Montreal, Quebec, Canada

2. **Davies AL** *Channelopathies of the central nervous system and spinal cord injury (Invited Presentation).* **Brock University**, Kinesiology Undergraduate Program, March 2008, St. Catharines, Ontario, Canada

3. **Davies AL** *The carbon monoxide-releasing molecule CORM-2 induces concentration-dependent alterations in the electrophysiological properties of axons in mammalian spinal cord.* **Neuroscience 2007**, November 2007, San Diego, California, USA

4. **Davies AL** *Understanding spontaneous recovery following neurotrauma (invited presentation).* **ARGC Research Centre**, October 2007, London, Ontario, Canada

5. **Davies AL** *Channelopathies of the central nervous system and spinal cord injury (Invited Presentation).* **Brock University**, Kinesiology Undergraduate Program, March 2007, St. Catharines, Ontario, Canada

6. Kramer JLK, **Davies AL**, Hayes KC *Potential conduction mediating properties of carbon monoxide and interleukin-6 in spinal cord white matter ex vivo: A proof of principle investigation.* **Lawson Health Research Institute**, March 2007, London, Ontario, Canada

7. **Davies AL** *Preliminary examination of cerebrospinal fluid cytokine concentrations in individuals with chronic spinal cord injury.* **ARGC Research Centre**, November 2006, London, Ontario, Canada

8. Hayes KC, **Davies AL**, Close TE, Ashki N, Kramer JLK *Immunological insights into neurologic recovery following spinal cord injury: spinal shock and conversions.* **The 2nd National Spinal Cord Injury Conference**, October 2006, Toronto, Ontario, Canada

9. **Davies AL**, Hayes KC, Trombly R, Kramer JLK, Close TE, Guest J *Cytokine concentrations in the cerebrospinal fluid of individuals with chronic spinal cord injury undergoing surgery for syringomyelia or detethering.* **Neuroscience 2006**, October 2006, Atlanta, Georgia, USA

10. **Davies AL** *Clinical and translational research studies toward enhancing the quality of life following SCI.* **Ontario Neurotrauma Foundation General Meeting**, June 2006, London, Ontario, Canada

11. **Davies AL**, Hayes KC, Shi R *Recombinant human TNFα induces concentration-dependent reversible alterations in axonal conduction in mammalian spinal cord.* **CIHR Student Research Forum**, June 2006, Winnipeg, Manitoba, Canada

12. **Davies AL** *Channelopathies of the central nervous system and spinal cord injury (Invited Presentation).* **McMaster University**, Kinesiology Undergraduate Program, March 2006, Hamilton, Ontario, Canada

13. Hayes KC, **Davies AL**, Ashki N, Close TE *Cytokine-mediated axonal dysfunction: a novel and reversible mechanism of neurologic deficits following spinal cord injury?* **National Neurotrauma Society Symposium**, November 2005, Washington, DC, USA

14. **Davies AL**, Hayes KC, Shi R *Recombinant human TNFα induces concentration-dependent reversible alterations in axonal conduction in mammalian spinal cord.* **National Neurotrauma Society Symposium**, November 2005, Washington, DC, USA

15. **Davies AL**, Hayes KC, Shi R *Recombinant human TNFα induces concentration-dependent alterations in axonal conduction in mammalian*

*spinal cord.* **Southern Ontario Neuroscience Association**, June 2005, Hamilton, Ontario, Canada

16. **Davies AL**, Dekaban GA, Askes HK, Hayes KC *Secondary medical complications and immune dysregulation in individuals with SCI.* **National Spinal Cord Rehab Conference**, September 2004, Toronto, Ontario, Canada

17. **Davies AL**, Hayes KC, Dekaban GA, Askes HK *Elevated levels of interleukin-1β receptor antagonist and interleukin-6 in serum of chronic spinal cord injured patients.* **National Neurotrauma Society Symposium**, November 2003, Biloxi, Mississippi, USA

18. **Davies AL**, Dekaban GA, Askes HK, Hayes KC *Elevated humoral immunoactivity in patients with chronic spinal cord injury.* **13th Interurban Spinal Cord Injury Conference**, October 2003, London, Ontario, Canada

19. **Davies AL**, Hayes KC, Dekaban GA, Askes HK *Elevated humoral immunoactivity in patients with chronic spinal cord injury: a double-edged sword.* Sister Mary Doyle Research Day, **Lawson Health Research Institute**, March 2003, London, Ontario, Canada

20. **Davies AL**, Hayes KC, Dekaban GA, Askes HK *Elevated humoral immunoactivity in patients with chronic spinal cord injury: a double-edged sword.* **Ontario Neurotrauma Foundation Provincial Conference**, January 2003, Toronto, Ontario, Canada

## ACADEMIC SERVICE

**Reviewer**
Clinical Chemistry and Laboratory Medicine

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