SENSORIMOTOR DEFICITS FOLLOWING CHEMOTHERAPY

A Dissertation submitted in partial fulfillment of requirements for the degree of Doctor of Philosophy

By

JACOB ADAM VINCENT
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Wright State University
I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Jacob Adam Vincent ENTITLED Sensorimotor Deficits Following Chemotherapy BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy.

Timothy C. Cope, Ph.D.
Dissertation Director

Mill W. Miller, Ph.D.
Director, Biomedical Sciences Ph.D. Program

Robert E.W. Fyffe Ph.D.
Vice President for Research and Dean of the Graduate School

Committee on Final Examination

Timothy C. Cope, Ph.D.

Mark M. Rich, M.D.,Ph.D.

Mill W. Miller, Ph.D.

David Ladle, Ph.D.

F. Javier Alvarez-Leefmans, M.D., Ph.D.
Abstract

Vincent, Jacob Adam. Ph.D., Biomedical Sciences Ph.D. Program, Wright State University, 2017. Sensorimotor Deficits Following Oxaliplatin Chemotherapy.

Neurotoxicity is one of the most significant side effects diminishing clinical efficacy and patient quality of life during and following chemotherapy. Oxaliplatin (OX) is a platinum based chemotherapy agent used in the treatment of colorectal cancer. Colorectal cancer currently ranks as the 4th most common cancer, and the majority of patients receive OX as a part of their adjuvant therapy. OX based adjuvant therapies significantly improve 5 year survival rates, however in many cases patients must stop treatment early because of the neurotoxic side effects. OX causes two clinically distinct forms of neurotoxicity. Acutely, within hours and for days following OX infusion patients experience positive symptoms including, paresthesia, cold induced pain, and muscle cramping/fasciculation. These symptoms typically subside in between treatments but often worsen with each successive OX infusion. Chronically, with accumulating doses of OX and following treatment cessation, patients experience negative symptoms including numbness, pain, and sensory ataxia. The symptoms persist in a substantial number of patients for years following cessation of chemotherapy and significantly diminish quality of life. While OX neurotoxicity has been widely studied, there are still fundamental gaps in knowledge regarding (a) the mechanisms underlying acute and chronic neurotoxicity, (b) the suggested causal link between acute and chronic OX, and (c) targeted treatment aimed at alleviating the neurotoxic side effects of acute and chronic OX. In order to address these gaps we applied behavioral and in vivo electrophysiological techniques and pharmacological manipulation in chronic OX-treated, acutely OX-treated, and control rats. Building on our recent discovery that following chronic OX-treatment
muscle proprioceptors cannot sustain repetitive firing necessary for normal encoding, we hypothesized that loss of proprioceptive signaling should result in behavioral deficits and that impaired encoding results from disruption of persistent inward Na⁺ currents (NaPIC). Using the methods above we confirmed these hypotheses and have developed a targeted treatment that restores proprioceptive encoding after chronic OX-treatment. Lastly, given the suggested link between acute and chronic neurotoxicity we sought to determine the location of changes in muscle proprioceptor excitability following acute OX-treatment.
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This work is dedicated to Shea, Raegan, Mom, and Dad

Thank you for your love and support
Chapter 1: Purpose & Aims

Purpose

Oxaliplatin induced neurotoxicity is a significant clinical problem that limits its clinical efficacy and negatively impacts patient lives. Study of acute neurotoxicity has failed to provide any meaningful treatment of acute symptoms and has failed to prevent development of chronic neurotoxicity. Similarly, chronic studies have focused solely on neuropathy and have failed to account for symptoms in patients without neuropathy. Studies undertaken in this dissertation seek to fill the gaps in the understanding of acute and chronic neurotoxicity. Changes in muscle proprioceptor function will be analyzed following acute and chronic OX and changes in motor function will be analyzed following acute OX. The purpose of these studies is to gain insight into changes that cause acute symptoms and chronic symptoms in the absence of neuropathy with the hope of identifying changes for targeted treatment.

Specific Aims

In order to address the specific aims in this dissertation, electrophysiological techniques will be applied in vivo. Studies are designed to characterize deficits in muscle proprioceptor encoding, how those changes correlate with behavior, and to identify the mechanism(s) responsible for those changes. Further studies will be undertaken to test the ability of different agents to improve muscle proprioceptor function following chronic OX. Lastly, muscle proprioceptor and motor function will be studied
following acute OX in order to identify and localize clinically relevant changes in excitability that result in patient symptoms.

**Specific Aim 1:** To test the hypothesis that OX treatment causes proprioceptive deficits by reducing ionic currents that support sustained repetitive firing necessary for sensory encoding

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**Rationale**

The recent observation that OX can produce sensory symptoms, including loss of cutaneous sensation, proprioceptive deficits, and pain in patients that have no evidence of neuropathy suggests that additional mechanisms are at play (Burakgazi et al., 2011). Support for this novel idea is found in that following chronic OX treatment muscle proprioceptors are unable to maintain repetitive firing necessary for sensory encoding. Specifically, Ia muscle spindle afferents fail to encode static muscle length, while effects on rapid stimuli are unaffected (Bullinger et al., 2011b). Other muscle proprioceptors, group II and Ib golgi tendon organ afferent were also affected but to a lesser extent. Importantly, the animals used in these studies showed no evidence of neuropathy (Bullinger et al., 2011b). Thus, disruption of sensory encoding by OX is a novel idea potentially explaining sensory symptoms in the absence of neuropathy.

The inability to sustain repetitive firing suggests that OX is disrupting an ionic current that supports repetitive firing during a sustained stimulus. In dorsal root ganglion neurons and other neuronal subtypes, persistent inward currents (PIC) underlie repetitive firing during sustained stimuli (Lee and Heckman, 2001; Harvey et al., 2006a;
Xie et al., 2011). In these various neuronal subtypes sodium persistent inward current (NaPIC) is essential for generating and maintaining repetitive firing. If OX is disrupting a NaPIC necessary for normal sensory encoding, then drugs given acutely that block NaPIC should reproduce the OX firing phenotype.

Specific Aim 2: To test the hypothesis that drugs that increase persistent inward currents can restore sensory encoding following OX treatment.

Rationale

Persistent inward currents have been identified in numerous neuronal subtypes including, motoneurons, dorsal root ganglion neurons, and cortical neurons (Crill, 1996; Lee and Heckman, 2001; Harvey et al., 2006a). Their role is best defined in the alpha motoneuron where they have been shown to be essential for the generation and maintenance of repetitive firing (Lee and Heckman, 1998, 2001; Theiss et al., 2007).

Preliminary evidence (Vincent et al., 2015) and results from aim 1 support the notion that chronic OX treatment disrupts a NaPIC resulting in encoding deficits in muscle proprioceptors. The failure of muscle proprioceptors to maintain repetitive firing during static and slowly changing muscle length represent a critical loss of signal to the central nervous system. These findings are the first suggestion outside of neuropathy to explain symptoms following OX. Therefore, NaPIC is a potential therapeutic target following OX. If NaPIC can be increased after OX, then there is potential to restore the muscle length and force signals arising from muscle proprioceptors.

Specific Aim 3: To determine if spontaneous activity following acute OX in muscle proprioceptors arises from sensory nerve terminals
Rationale

Within hours and for days following infusions of OX, greater than 90% of patients describe abnormal sensations, pain, muscle spasm, and muscle fasciculation (Argyriou et al., 2008; Argyriou et al., 2014). These symptoms typically last less than one week, but re-occur following every OX infusion (Krishnan et al., 2005; Park et al., 2009b; Park et al., 2011b; Park et al., 2013). The common mechanism attributed to this acute syndrome is sensory and motor axonal hyper-excitability (Krishnan et al., 2006; Park et al., 2009b; Park et al., 2011b). Support for this comes from several different studies showing OX alters the function of nodal voltage-gated Na⁺ channels either in vitro, or by clinically used techniques such as threshold tracking (Adelsberger et al., 2000; Grolleau et al., 2001; Benoit et al., 2006; Krishnan et al., 2006; Park et al., 2011b).

The importance of understanding OX acute effects is demonstrated by the correlation between the severity of acute symptoms and the likelihood of developing chronic neurotoxicity (Krishnan et al., 2005, 2006; Park et al., 2009a). Information gained from previous studies has been used to identify neuroprotective strategies targeted at blocking the acute syndrome in order to prevent the chronic neurotoxicity. Unfortunately, these strategies have failed and there is no current effective treatment for blocking the acute syndrome and providing neuroprotection against chronic neurotoxicity. Preliminary findings (Vincent et al., 2015) and results from aim 1 demonstrate that changes following chronic OX occur at the sensory nerve terminal. If acute and chronic neurotoxicity are linked then acute OX may also target the sensory nerve terminal.

In order to nail down the origin of hyperexcitability we will use in vivo intra-axonal recording, the gold standard for assessing axon parameters and novel spike triggered
averaging to localize spike origin. These *in vivo* techniques will allow us to study the intact proprioceptor system in a way that has not previously been done.
Chapter II: Significance/Background

Chemotherapy induced neurotoxicity is a poorly understood dose limiting side effect of chemo agents limiting the efficacy of cancer treatment:

Oxaliplatin (OX), a 3rd generation platinum chemotherapy drug, is one of the most commonly used adjuvant chemotherapeutic agents in the treatment of colorectal cancer. Colorectal cancer ranks as the 4th most common cancer, with nearly 140,000 new cases occurring each year (cancer.gov). Currently there are nearly 1.2 million people living with colorectal cancer (cancer.gov), the majority of these patients receive OX as part of their adjuvant treatment. The efficacy of OX treatment is limited by its neurotoxicity (Argyriou et al., 2014; Sereno et al., 2014), which forces many patients to stop treatment early (Argyriou et al., 2014). OX neurotoxicity can be divided into two different phases. Up to 95% of patients experience acute neurological symptoms including paresthesia, muscle spasms, and cold hypersensitivity (Argyriou et al., 2008; Argyriou et al., 2013a). Acute hyper-excitability following treatment is related to changes in ions channels. Acute OX in vivo administration alters voltage-gated Na⁺ current in sensory and motor axons (Krishnan et al., 2006; Park et al., 2009b; Park et al., 2011b). OX causes hyperpolarized shifts in the voltage dependent activation of voltage-gated Na⁺ channels (Adelsberger et al., 2000; Benoit et al., 2006). These changes in voltage-gated Na⁺ current are thought to arise via Ca²⁺ chelation by oxalate, a metabolite of OX (Grolleau et al., 2001; Benoit et al., 2006; Argyriou et al., 2008; Argyriou et al., 2014). Additionally, OX has been shown to increase TRPA currents in sensory neurons contributing to the cold hypersensitivity (Zhao et al., 2012; Nativi et al., 2013).
Patients who continue OX treatment experience chronic neurological symptoms that are primarily sensory, including decreased cutaneous sensation, pain, proprioceptive deficits, and sensory ataxia (Bennett et al., 2012; Beijers et al., 2014). Clinically, these symptoms are commonly referred to as chemotherapy induced peripheral neuropathy (CIPN) and their severity is correlated with cumulative dose of OX. In a self-report questionnaire patients reported feeling unsteady and clumsy, nearly falling over, difficulty walking and driving, and decreased sensation in their toes and fingers (Bennett et al., 2012). Similar to other chemotherapy induced neuropathies, OX induced neuropathy is length dependent with the longest axons being effected first (Cavaletti, 2014). The severity of neuropathy (peripheral axon loss) is scored using a combination of electrophysiology (reduced sensory nerve action potential amplitudes), epidermal punch biopsy (intra-epidermal nerve fiber density), and clinical assessment (sensory and reflex testing) (Burakgazi et al., 2011; Park et al., 2011a; Park et al., 2012b). These dose limiting neurotoxic effects are evident in up to 90% of OX patients, with over 50% of those cases being either moderate (grade 2) or severe (grade 3) (Park et al., 2011a). Neurological deficits can persistent for up to 2 years after treatment in up to 1/3 of patients (Park et al., 2011a). The common accepted pathogenic mechanism of OX’s chronic effects results from its accumulation in the dorsal root ganglion where it causes decreases in cellular metabolism, oxidative stress, and axonal transport (Argyriou et al., 2008; Park et al., 2013; Argyriou et al., 2014; Briani et al., 2014; Cavaletti, 2014; Avan et al., 2015; Carozzi et al., 2015). Within the dorsal root ganglion OX causes atrophy in large diameter neurons marked by parvalbumin (Jamieson et al., 2005), and morphometric changes indicative of cell damage (Cavaletti et al., 2001). Changes in the dorsal root ganglion are associated with loss of axons in the periphery (Cavaletti et al., 2001).
OX Chronic Neurotoxicity: Additional Mechanisms

Within the clinical literature there is disparity between the clinical signs of neuropathy and patient reported symptoms. Patients frequently report symptoms that are more severe than suggested by clinical measurements of neuropathy (Park et al., 2013). This disparity is supported by a recent clinical study that found subsets of patients that describe the same sensory symptoms in the absence of any clinical signs of peripheral neuropathy, i.e. reduced sensory nerve action potential amplitude (SNAP), or intraepidermal nerve fiber density (IENFD) (Burakgazi et al., 2011). If patients experience sensory symptoms in the absence of neuropathy then there must be additional mechanisms contributing to sensory deficits.

Sensory symptoms experienced by patients could potentially be caused by either problems in the periphery, i.e. encoding or by changes that limit the transmission of sensory information to the central nervous system (CNS). The focus of this dissertation is to investigate peripheral mechanisms other than neuropathy that could cause sensory deficits following OX treatment.

Novel Peripheral Mechanism: OX Impairs Muscle Proprioceptor Encoding:

Recent evidence demonstrates that following OX treatment muscle proprioceptor sensory encoding is impaired (Bullinger et al., 2011b). Muscle proprioceptors (muscle spindle and golgi tendon organs) lie within the muscle and supply the CNS with critical length and force feedback (Matthews, 1972). More specifically, Ia muscle spindle afferents are activated by the stretch of their parent muscle. These mechanical signals are encoded into action potentials out in the periphery and are conducted centrally where Ia muscle spindle afferents synapse with a wide variety of CNS neurons. Most notably Ia muscle spindle afferents form a monosynaptic connection with alpha motoneurons (Matthews, 1972). This connection forms the basis for the stretch reflex,
where stretching a muscle activates la muscle spindle afferents, which through their monosynaptic connection excite motoneurons, causing them to fire action potentials resulting in muscle contraction (Proske and Gandevia, 2009). This sensory feedback is essential for the sensorimotor control of locomotion (Proske and Gandevia, 2012).

In response to ramp-hold-release stretch (3 mm, 20 mm/s) normal rodent la muscle spindle afferents display a high frequency initial burst at the onset of stretch, followed by a rapid increase in firing until the peak of the ramp, and then a sustained discharge during the hold phase (Fig. 1 A). Normal la muscle spindle afferents are slowly adapting (Fig. 1A), firing >800 ms through the 1000 ms hold phase of muscle stretch (Bullinger et al., 2011a; Vincent et al., 2015). Following a 4-week OX (40 mg/kg cumulative dose) treatment regime and a 3-5 week coasting period, 50% of sampled la muscle spindle afferents fired ≤ 500 ms during the hold phase (Bullinger et al., 2011a; Vincent et al., 2015) (Fig. 1B). The loss of hold phase firing was not due to an inability to conduct action potentials centrally, as shown in Fig. 1C la muscle spindle afferents were capable of conducting superimposed vibration with high fidelity. The inability of la muscle spindle afferents to encode sustained muscle stretch results in the loss of critical length signal to the CNS. This encoding deficit occurred in the absence of any neuropathy (Fig. 1 D, E) making it distinct from any previously described form of chemotherapy neurotoxicity.

Muscle Proprioceptors: Muscle Spindles

Muscle spindles lie within the surrounding muscle, and are made up of intrafusal fibers surrounded by a capsule (Matthews, 1972; Hunt, 1974). The intrafusal fibers within spindles orient themselves in parallel with the surround extrafusal tissue. Their specific orientation with respect to the extrafusal muscle allows them to detect muscle stretch amplitude and velocity (Matthews, 1972; Hunt, 1974).
The spindle structure can be divided into two segments. Within the polar regions of the spindle central nuclei are sparsely spaced. Near the center of the spindle is the equatorial region, which contains densely packed nuclei (Banks et al., 1982). The intrafusal fibers within the equatorial region can be further divided into larger type 1 and type 2 nuclear bag fibers, and smaller nuclear chain fibers. Intrafusal fibers receive both sensory and motor innervation (Boyd, 1968; Boyd and Ward, 1975; Boyd et al., 1977).

The first type of sensory innervation is the annulosprial endings of Ia muscle spindle afferents. These endings form coils around the intrafusal fibers. The second type of ending is referred to as a flower spray ending of group II muscle spindle afferents, and is most often found in the juxta-equatorial position (Barker, 1948; Hunt, 1974; Banks et al., 1982; Banks et al., 2009). Typically, each spindle receives innervation from a single Ia muscle spindle afferent and multiple group II muscle spindle afferents (Banks et al., 1982).

Proprioceptive afferents that innervate muscle spindles encode changes in muscle length, and velocity (Matthews, 1972). As a group, given their in parallel orientation with the extrafusal fibers, muscle spindle afferents fail to firing during muscle shortening, such as during active muscle contraction (Matthews, 1972). The responsiveness of these different muscle spindle afferent types to different stimuli allows for their distinction. Ia muscle spindle afferents show a much higher dynamic stretch sensitivity, initial bursting at the onset of muscle stretch, and cessation of firing on release, and are uniquely sensitive to high frequency vibrations of 100-150 Hz (Matthews, 1972; De-Doncker et al., 2003; Haftel et al., 2004; Bullinger et al., 2011a). These properties allow Ia muscle spindle afferents to encode both muscle length and velocity changes. In contrast, Group II muscle spindle afferents are much less sensitive to dynamic stretch, lack initial bursting, are relatively insensitive to muscle vibration, and
continue to fire upon release from stretch. Group II muscle spindle afferents function as length sensors (Matthews, 1972; De-Doncker et al., 2003).

Muscle spindles are innervated by small motor axons arising from gamma motoneurons. Gamma motoneurons are interspersed within their associated muscle motor pools within lamina IX of the ventral horn (Burke et al., 1977). In comparison with alpha motoneurons, gamma motoneurons are much smaller, show less dendritic branching, and lack monosynaptic connection with 1a afferents and recurrent collaterals (Eccles et al., 1960; Burke et al., 1977; Lagerback, 1985). Gamma motoneurons are modulated by supraspinal structures, however this input and therefore gamma motoneuron output is greatly decreased during anesthesia (Haftel et al., 2004).

Gamma motoneurons can be subdivided into dynamic and static groups. The dynamic gamma axons innervate the type 1 nuclear bag fibers (dynamic Bag$_1$ fiber), while the static gamma axons innervate the type 2 nuclear fibers (static bag$_2$ fiber), and the nuclear chain fibers. Each type 1 nuclear bag fiber is innervated by one or two dynamic gamma motor axons, while each type 2 nuclear bag and chain fibers are innervated by three to seven static gamma motor axons (Matthews, 1962; Matthews, 1972; Boyd et al., 1977; Banks et al., 1982). Gamma motoneurons extrinsically modulate muscle spindle output by contracting intrafusal muscle fibers (Boyd and Ward, 1975). Their segregated innervation results in differential modulation of dynamic and static responsiveness. Dynamic gamma motoneurons cause an increase in the dynamic responsiveness, while static gamma motoneurons increase the static responsiveness of the muscle spindle afferent (Hunt, 1974; Boyd and Ward, 1975; Banks et al., 1997). This is accomplished by contraction of intrafusal muscle fibers, and compressing the sensory nerve terminal. At the onset of muscle lengthening, the shortened and taunt intrafusal muscle fibers stretch along with the sensory nerve terminal to a greater degree, allowing more current to flow into the sensory axon (Hunt,
This results in an increase in firing during dynamic or static stretch depending on which intrafusal fibers were stimulated, i.e. Bag1, or Bad2/chain.

**Muscle Proprioceptors: Golgi Tendon Organ**

In contrast to muscle spindles, golgi tendon organs consist of bundles of collagen oriented in series with the extrafusal muscle fibers (Hunt, 1974; Zelena and Soukup, 1983; Jami, 1992). Each golgi tendon organ receives innervation from a single group Ib golgi tendon organ afferent. Given their in series orientation, Ib golgi tendon organ afferents increase their firing during active muscle contraction, distinguishing them from muscle spindle afferents (Houk et al., 1971; Matthews, 1972; Zelena and Soukup, 1983; Jami, 1992). In fact, contraction of the motor unit in which a Ib golgi tendon organ afferent lies is considered their adequate stimulus. Thus, Ib golgi tendon organ afferents are considered force sensors (Houk et al., 1971; Jami, 1992). Their responsiveness to passive muscle stretch is thought to lie outside the physiological range of passive muscle stretch (Houk et al., 1971; Jami, 1992). Other studies though have suggested that Ib golgi tendon organ afferents do in fact respond to passive muscle stretch within the physiological range (Stephens et al., 1975).

Together Ib golgi tendon organ afferents and muscle spindle afferents project into the spinal cord where they ascend through the dorsal spinocerebellar tract and dorsal column medial lemniscal system to supraspinal centers, and synapse with multiple targets within the spinal cord; including interneurons, and motoneurons. Together the circuits that these afferents feed into provide essential feedback about limb position, allowing for control of locomotion (Proske and Gandevia, 2012).
Mechano-transduction and Encoding

The arrival of action potentials within the central nervous system relies on three processes in peripheral signaling: mechanotransduction, encoding of action potentials, and orthodromic conduction. Mechanotransduction and encoding has been best described in la muscle spindle afferents. Fig. 2 provides a diagrammatic representation of these processes. Signaling begins with the stretch activation of mechanically gated ion channels allowing sodium (and to a lesser extent calcium) ions to enter the afferent terminal producing a depolarizing receptor potential (Hunt, 1974; Hunt et al., 1978; Bewick and Banks, 2015). The size and shape of the receptor potential reflect the different dynamic and static components of mechanical perturbation. Recent evidence indicates that DEG/ENaC, and Peizo 2 channels may be responsible for the receptor potential (Hunt, 1974; Hunt et al., 1978; Bewick and Banks, 2015; Woo et al., 2015). After formation, the receptor potential spreads the unmyelinated heminode of the afferent where its features are encoded in firing patterns of action potentials by voltage-gated Na$^+$ and K$^+$ channels. The final step in signaling proceeds as action potentials propagate from the heminodes orthodromically along the afferent axon as it traverses the peripheral nerve and enters the central nervous system.

The output of la muscle spindles can be modulated intrinsically by Ca$^{2+}$ activated mechanisms (Bewick and Banks, 2015) and extrinsically by gamma motoneuron innervation of intrafusal muscle fibers (Taylor et al., 2006). Muscle spindle afferent firing is reduced by negative feedback initiated within the heminode by action potentials that open P/Q-type Ca$^{2+}$ channels. This Ca$^{2+}$ current suppresses afferent firing by activating large (BK) and small conductance (SK) K$^+$ channels (Bewick and Banks, 2015). This negative feedback mechanism suppresses afferent firing on a time scale of fractions of a second. Additionally there is a distinct intrinsic mechanism that increases or maintains
spindle firing. In this autogenic excitatory mechanism, increase Ca\(^{2+}\) causes glutamate release from synaptic like vesicles in the afferent terminal, which bind auto-genically to a PLD linked metabotropic glutamate. This activates an unknown second messenger cascade that serves to increase afferent firing over a time course of minutes to hours (Bewick et al., 2005; Bewick and Banks, 2015).
Fig. 1. Chronic OX disrupts muscle proprioceptor encoding (Bullinger et al., 2011b; Vincent et al., 2015). (A) Control slowly adapting Ia muscle spindle afferent firing response (gray lines are action potentials, black dots representation instantaneous firing rates) to ramp-hold-release stretch (bottom trace in B). (B) Following OX Ia muscle spindle afferents were unable to maintain hold phase repetitive firing, while encoding during the ramp phase was normal. (C) Superimposed 100 Hz vibration (bottom trace) restored firing during the hold phase. (D) Animals displayed no reduction in SNAP amplitude or changes in conduction delay. (E) Following OX treatment Ia muscle spindle afferents maintained normal innervation of muscle spindles. Staining the soleus muscle with PGP 9.5 demonstrates normal innervation and counts of muscle spindles as compared to controls. Note the intact annulospiral ending of a Ia muscle spindle afferent.
**Fig. 2.** Mechanosensory model of Ia muscle spindle afferent encoding adapted from Bewick and Banks 2015. Neural mechanisms in mechano-transduction and sensory encoding for Ia muscle spindle afferent fitted to muscle spindle structure including intrafusal muscle fiber (pink) wrapped by annulospiral ending (green) that extends unmyelinated followed by myelinated axon. Mechano-transduction involves mechano-sensitive sodium channels (MSSC) and metabotropic glutamate receptors (MGLUR) that act through a phospholipase D (PLD) mechanism to modulate glutamate release from synaptic like vesicles (SLV); Sensory encoding mechanisms include BK and SK potassium channels activated by Ca$^{2+}$; P/Q and T type Ca$^{2+}$ channels; and voltage gated Na$^+$ channels (NaV).
Chapter III: General Methods

Using in vivo electrophysiology in combination with behavioral analysis, this proposal will investigate the mechanism(s) underlying acute and chronic forms of OX neurotoxicity. The general methods utilized are described in detail below.

Animal Use

All animal procedures used are approved by Wright State University Laboratory Animal Care and Use Committee. Detailed behavioral and electrophysiological analyses were performed on adult female wistar rats (250–300 g, Charles Rivers Laboratory Wilmington, VA).

Anesthesia and Vitals

Rats were deeply anesthetized by isoflurane inhalation throughout the entire terminal experiment, beginning with induction in a closed chamber (5% in 100% O2) and continuing with delivery via a tracheal cannula (1.5–2.5% in 100% O2). Subcutaneous injections of lactated ringer solution were given to maintain adequate fluid levels and blood pressure. Respiratory rate, heart rate, oxygen saturation, and pCO2 were monitored to ensure anesthesia and overall animal health. Body temperature was recorded via a rectal probe, and maintained between 36 and 38 °C with heated water pads and a heat lamp.
**Surgical Technique**

Surgical procedures based on Haftel et al. 2004 and Bullinger et al. 2011 (Haftel et al., 2004; Bullinger et al., 2011b) were used to dissect the hind limb and spinal cord in order to record afferent firing resulting from electrical stimulation and muscle stretch. Briefly, the rat was placed in a rigid recording frame, and the legs were placed into stereotaxis with the knee and ankle joints between 90-120°. The left triceps surae muscles were isolated via their common Achilles tendon, and resting length was marked with suture. The tendon was cut at the calcaneus, and the triceps surae was attached to a force and length-sensing servomotor (Model 305B-LR, Aurora Scientific Inc.) in order to apply muscle stretches and record length and force changes. Triceps surae nerves were freed from the surrounding tissue and placed on a unipolar stimulating electrode (bipolar electrode in some instances), all other hind limb nerves including common peroneal, sural, and posterior tibial nerves were crushed. A laminectomy was performed from T10-S1, and the dura mater was removed to expose the spinal cord and dorsal roots. Skin flaps were tied up in the back and hind limb to create pools for mineral oil, in order to prevent the tissue desiccation. During certain experiments ventral roots were sectioned to eliminate gamma motoneuron influence on muscle spindles.

**Recording Preparation/ Data Collection**

Dorsal roots were isolated and placed on bipolar recording hook electrodes. Rootlets were tested for orthodromic action potential volley, and stretch sensitive activity. Within dorsal rootlets individual sensory axons were penetrated with sharp glass microelectrodes filled with 2M K⁺ acetate⁻. Sensory axons were randomly sampled and selected for recording when they displayed orthodromic action potentials with a
conduction delay of < 3 ms following peripheral electrical stimulation of triceps surae nerves.

Sensory axons were characterized as either muscle spindle or golgi tendon organ based on response during the rising phase of twitch contractions of the triceps surae muscles; muscle spindles paused, while golgi tendon organ afferents accelerated during twitch contraction. Further study of afferent firing was based responses to muscle stretch including ramp-hold-release (3mm, 20mm/s ramp, 1 sec hold), three successive triangular stretches (4mm/s, 3mm), vibration (50-250 Hz, 80µm), and ramp-hold-release (3mm, 20mm/s ramp, 1 sec hold) with superimposed 100 Hz vibration (80µm) during the 1 sec hold phase. Muscle spindle afferents were characterized as primary Ia when they displayed an initial high frequency burst at the beginning of the ramp or triangular stretch, 1:1 firing entrainment in response to ≥ 100Hz vibration. Group II muscle spindle afferents paused firing during twitch contraction but failed to show initial bursting, or 1:1 entrainment to ≥ 100Hz vibration. All stretches were performed at resting length (L_r) of 10g ± 3g, L_r+ 10g, and L_r+ 20g. Intra-axonal records of action potentials and muscle length and forced changes were digitized (20 kHz), and stored on a computer for later analysis using Spike2 software.
Chapter IV

Specific Aim 1: To test the hypothesis that OX treatment causes proprioceptive deficits by reducing ionic currents that support sustained repetitive firing necessary for sensory encoding

Introduction

Previous study of muscle proprioceptors following OX demonstrated an inability to sustain repetitive firing during static muscle stretch (Bullinger et al., 2011b). It was determined that the afferents were capable of firing during the time of static muscle by superimposing high frequency vibration, thus muscle proprioceptors were capable of firing but just failed to do so in response to static muscle stretch. These results suggested that OX was impacting the encoding process (Bullinger et al., 2011b). The loss of sustained firing by muscle proprioceptors is equivalent to a loss of static muscle length signal to the central nervous system. We predict that this loss of signal will result in behavioral deficits. One of the primary goals of this proposal is to corroborate the effect of OX on muscle proprioceptor encoding and to determine how it impacts with behavior.

The other primary goal of this study is to determine the mechanism(s) underlying OX disruption of muscle proprioceptor encoding. The effects of OX on hold phase repetitive firing suggest that OX may be disrupting ionic currents that support repetitive firing. An inability to repetitively fire suggested a problem with persistent inward Na⁺ current (NaPIC) (Lee and Heckman, 2001; Bullinger et al., 2011b; Xie et al., 2011). NaPIC is essential for repetitive firing in many different groups of neurons:
motoneurons, cortical neur

ganglion neurons (Lee and
Heckman, 2001; Ptak et al.,
2005; Harvey et al., 2006a;
Xie et al., 2011). These currents activate subthreshold causing a subthreshold plateau in
membrane potential that helps limit Na⁺ channel inactivation during the slow
depolarization at the end of the afterhyperpolarization (Harvey et al., 2006a). If OX is
disrupting NaPIC necessary for sustained repetitive firing then additional signs of
impaired repetitive firing are expected from muscle proprioceptors. We know for
instance, in motoneurons NaPIC role in supporting repetitive firing is dependent on the
speed of depolarization (Harvey et al., 2006a). During slow depolarization, without
NaPIC inactivation of the transient Na⁺ channel prevails, preventing action potential
formation (Harvey et al., 2006a). Therefore, OX should disrupt repetitive firing during
slow muscle stretch, supporting its role in disrupting NaPIC.

If OX is disrupting a NaPIC necessary for repetitive firing, then pharmacological
agents that block NaPIC injected acutely should reproduce the firing deficits observed
following OX. Riluzole and phenytoin are two drugs that have been shown to block
NaPIC (Zeng et al., 2005; Theiss et al., 2007; Bellingham, 2011; Xie et al., 2011). If OX
is disrupting NaPIC, then riluzole and phenytoin should similarly reduce repetitive firing
in muscle proprioceptors and will provide the first insight into the underlying mechanism
of deficits following OX.
Methods

Chronic OX Studies

These studies were designed to test effects of chronic OX treatment. Oxaliplatin (BIOTANG, Lexington, MA) dissolved in 5% dextrose was administered via intraperitoneal (i.p.) injection once per week (except week 4 when OX was unexpectedly unavailable) in single doses of 10 mg/kg to reach a cumulative dose of 70 mg/kg over 8 weeks. Ten female rats received these injections concurrently with five female rats that served as sham controls receiving only vehicle (5% dextrose i.p.) on the same schedule. OX effects were assessed by studying rats over a period of 14 weeks spanning periods before, during and after injections. Throughout this period, each rat was closely and frequently monitored for pain or distress. No individual rat reached set criteria established for early removal from the study, e.g. 20% weight loss, vocalization, failure to drink or groom, severe lethargy, self-mutilation, uncontrollable infection.

Repeated measures of body weight, motor task performance, and tail nerve conduction were obtained from all rats before electrophysiological recordings were made in terminal experiments, all as described below.

Behavioral Testing and Other Repeated Measures

Over the course of the study all 15 animals were handled by two individuals who were responsible for all behavioral testing, weight measurements, SNAP recordings, and OX treatment. The ladder rung walking task was chosen for its validated use to detect and describe sensorimotor deficits (Metz and Whishaw, 2002, 2009; Akay et al., 2014). All 15 rats were introduced and trained on the horizontal ladder run apparatus in a single session lasting 4 hours one week prior to the start of the study. Rats were trained to traverse the apparatus in the same direction in order to reach their home cage.
Behavioral testing was performed once per week at the same time of day on different days each week, and lasted approximately 4 h. During a test session, each rat traversed the apparatus 5 times. On separate days animal weight and SNAPs were recorded (see below). Behavioral sessions were video recorded using a high definition camera (Samsung F90 HD Camcorder) aimed toward the left side of the animal; a mirror was placed on the right side of the apparatus to allow clear visibility of the right hind limb (Fig. 1B). All videos were imported onto a laptop computer and exported as QuickTime videos that were stored for analysis. The person performing all analysis was blinded to which rats received treatment. All results were compiled and were subsequently decoded for statistical analyses.

*Ladder rung: Horizontal Apparatus*

The ladder rung apparatus was built as previously described (Metz and Whishaw, 2002), consisting of two Plexiglas walls, 1 m × 0.3 m, connected at the bottom with 1/8 in (~3 mm) diameter steel rods (rungs). Rungs were set at irregular intervals, with a maximum of 5 cm and a minimum of 1 cm distance between rungs. Holes drilled for rungs were 1 cm apart, across the entirety of the Plexiglas, allowing for different iterations (6 iterations were used in total) of rung patterns, preventing animals from learning any single pattern.

*Analysis of Hind Foot Placement*

Two measures of hind foot placement: secure placement, and correct placement were analyzed in control and OX treated animals. All steps taken during ladder walking, from first to last were included in the analysis (Antonow-Schlorke et al., 2013). Once analyzed, the data were averaged and are presented as percent of change, week 0 scores were analyzed, averaged and are represented as zero.
Analysis of Secure Placement

Secure hind foot placement was assessed using the seven-category scale scoring system introduced by Metz and Whishaw (Metz and Whishaw, 2002, 2009). The scoring system, which distinguishes misses, slips, and placement errors in rung contact, was slightly modified by assigning a score of 0 whenever the foot entirely missed a rung, whether or not it was associated with a deep fall. Based on the scoring system above, error was defined as a score of 0–2. The total number of errors in hind foot placement was calculated with no distinction between right and left, and the mean error/step ratio was calculated for all five trails and represented as percent of error. This number was multiplied by −1 in order to convert the values into a measure of the number of secure placements rather than the number of errors per steps. Thus an increase in the number of errors/steps corresponds to a decrease in secure hind foot placement of the hind foot.

Analysis of Correct Placement

During ladder rung walking, normal rats tended to place their trailing hind foot on the exact same rung as the ipsilateral front foot, defined here as correct placement of the hind foot; sequential placement of the ipsilateral hind foot on a different rung was defined as an error in placement and supposed to reflect impaired proprioceptive ability. Placing the hind foot on an incorrect rung, and then replacing to the correct rung, was still considered an error in placement. Correct hind foot placement was defined as the number of errors/steps multiplied by −1. The total number of errors was counted and expressed as an average of error/step ratio for both hind limbs. Multiplying this number by −1 gives a value for correct placement. Thus as the number of errors/ steps increases, the value for correct hind foot placement decreases.
Sensory Nerve Action Potentials (SNAPs)

At the end of each behavioral test session, rats were temporarily anesthetized with isoflurane inhalation via nose cone (2–4% in 100% O2) in order to record SNAPs as described in our earlier reports (Novak et al., 2009; Bullinger et al., 2011b). Two pairs of needle electrodes were inserted sub-dermally in the tail: one pair near the base of the tail for recording SNAPs (compound action potentials) evoked by electrical stimulation through the other pair located 2 cm distally. A ground electrode was inserted near the recording electrodes. An electrical stimulus of 10 mA was applied to elicit and record the SNAP using a Nicolette Viking Quest nerve conduction machine (Natus, Pleasanton, CA). Before removal of electrodes, a permanent marker was used to re-record the position of the electrodes, enabling exact placement of the electrodes for the duration of the study.

Terminal Experiments

Electromechanical responses of individual proprioceptors were examined electrophysiologically in terminal experiments performed on all rats, including those used to study the behavioral effects of OX treatment and those dedicated solely for studying the acute effects of riluzole and phenytoin on sensory signaling.

Acute Pharmacological Studies

Twenty-two rats having received no prior treatment or study were used to measure proprioceptor firing responses in terminal electrophysiological study exactly as described above. Individual muscle proprioceptors were randomly sampled both before and after i.p. injection of riluzole (16 rats), phenytoin (4 rats), or vehicle alone (DMSO in saline). Riluzole was given in doses of 4 mg/kg (4 rats), 6 mg/kg (8 rats), and 10 mg/kg
(4 rats), and phenytoin was given in a single dose of 20 mg/kg dose. Vehicle consisted of 1 mL DMSO combined with 4 mL 0.9% saline.

Data Collection from Muscle Proprioceptors

Intra-axonal records from individual muscle proprioceptors were obtained as described in the general methods section. The parameters recorded from individual muscle proprioceptors are described next. Dynamic and static responsiveness of proprioceptors were measured as different parameters of firing occurring, respectively during changing and constant muscle length. Dynamic response parameters included rate of initial burst firing at the onset of fast ramp stretches, the rate of firing rates at the peak of ramp stretch (peak firing rate). Dynamic thresholds were recorded as the length change necessary to generate sustained repetitive firing during fast and slow ramps. Measures of static responsiveness were measured during the hold phase of ramp-hold-release stretches as the duration and average rate of repetitive firing.

Results

Proprioceptive Movement Deficits in OX-treated Rats

In previous study of chronic OX-treatment in rats, we demonstrated defects in sensory signaling by muscle proprioceptors (Bullinger et al., 2011b). Because these signals are critical for detecting and guiding limb position and movement, we predicted deficits in movements that rely on sensory feedback about limb position (see Introduction). In order to assess sensorimotor function in rats, we tested their performance in walking over horizontally arranged ladder rungs (Metz and Whishaw, 2002, 2009). As rats move with vision directed forward, success in walking relies on proprioceptive function that assists with foot placement on rungs that pass out of sight.
Performance measures in ladder rung walking are illustrated in Fig. 3. All values are expressed as percent change per rat from scores obtained in early task sessions (time 0). Plots for secure (Fig. 3A) and correct hind foot placement (Fig. 3C) illustrate sensorimotor function for each OX-treated rat over time in relation to the mean and 95% confidence intervals computed from values pooled from all five sham control rats. A general pattern emerged over time with values for secure and correct hind foot placement falling below control confidence intervals, despite some tendency for both OX and control rats to improve performance with time. Divergence from control values appeared as early as one week after the first OX dose and continued through the last measurements made 3 weeks after OX-treatment was discontinued.

Closer inspection of Fig. 3 shows the following: First, OX-rats exhibited considerable variability both among individuals and within individuals across time. Compare, for example, secure hind foot placement (Fig. 3A) that was poor throughout and after treatment for OX-rat #3, not distinguishable from normal for OX-rat #5 until treatment was complete, and never abnormal for OX-rat #2. In this respect, our rat model reproduces the high degree of variability also observed for the OX patient population, in which subjective assessment of motor ability varies between and within individuals (Bennett et al., 2012). Second, Fig. 3A shows that values for secure hind foot placement in OX-rats rarely exceeded control means and were never higher than the control 95% confidence intervals. This contrasts with values for correct hind foot placement (Fig. 3C), which for some OX rats (#4 and #8) were better than normal. Thus, rats capable of correct placement were unable to make secure contact, e.g. slips occurred as shown in Fig. 3B. Although these differences between performance in secure vs. correct hind foot placement may prove to be important if verified in a larger study, there was nonetheless qualitative agreement between these measures in 7/10 OX
rats at the time of the last performance measurement (Fig. 3D).

OX-rats were routinely observed for a number of additional factors that might have contributed to poor sensorimotor performance. Although OX-rats did not increase body weight as seen in the sham control rats over the study period, both sham control and OX-rats gained weight. Mean body weight increased 20% for sham controls compared to 10.5% for OX-rats. Following each treatment, OX rats were also less active and had lower appetites. Otherwise, OX-rats exhibited no deterioration in general condition, e.g. severe lethargy, piloerection or failure to groom. During behavioral test sessions, OX animals exhibited similar climbing and gripping capabilities as control rats. From the latter observations, we rule out weakness as the basis for poorer than normal motor performance.

Severe neuropathy, involving dying-back degeneration extending into the peripheral nerve was assessed by measuring SNAPs from the rats’ most distal extremity, the tail, at the end of each behavioral test session. We found no significant difference (p > 0.05) between OX and control rats over time consistent with our earlier study (Bullinger et al., 2011b). Even by the end of behavioral testing, OX-rats were not different than control, respectively, in either SNAP amplitude (mean ± SD; 0.30 ± 0.04 mV, 0.33 ± 0.08 mV) or latency (mean ± SD; 0.80 ± 0.08 ms, 0.82 ± 0.08 ms). These findings rule out the occurrence of neuropathy severe enough to cause axon degeneration in parent sensory nerves, as it does in some though not all OX patients (Burakgazi et al., 2011). However, this measure does not assess degeneration of the distal most endings of sensory neurons (see Discussion).

Proprioceptor Impairment Occurred Together with Proprioceptive Movement Deficits

As a test of our hypothesis that proprioceptive encoding deficits contribute to poor motor performance, we performed in vivo electrophysiological studies on all OX
treated rats at the conclusion of behavioral evaluation in weeks 12 and 13 (cf Fig. 3A,C time scale). Analyses focused on Ia muscle spindle afferents, for which per-animal sample sizes were largest. Fig. 4 compares firing behavior of Ia muscle spindle afferents sampled from sham control and OX-rats. A conspicuous group difference was found in static responsiveness, i.e. the firing that occurs during the static (hold) phase of muscle stretch. Records taken from a control rat (Fig. 4A) show that repetitive firing was sustained throughout static stretch, which is typical for normal Ia muscle spindle afferents and which classifies them as slowly adapting sensory neurons (Lewin and McMahon, 1991). In sharp contrast, records from an OX-rat (Fig. 4B) show that firing was absent during most of static stretch. The reduction in static responsiveness for OX rats as a group averaged >50% (Fig. 4D, Table 1). These findings corroborate the abnormally low static responsiveness that we reported earlier for OX treated rats (Bullinger et al., 2011b).

The average magnitudes of impairment in proprioceptor signaling and behavioral performance were compared among individual OX rats. Fig. 4E shows a significant positive correlation between how securely the hind foot was placed on the rungs and the duration of Ia muscle spindle afferent static responsiveness ($r^2 = 0.76$, $p < 0.01$). Static responsiveness was not significantly related to correct hind foot placement.

**Mechanisms of Impaired Proprioceptor Signaling in OX-treated Rats**

At the time during static muscle stretch when Ia muscle spindle afferents failed to fire, superimposition of mechanical vibration, a fast-dynamic muscle stretch was perfectly effective in eliciting firing (Fig. 4C). Thus, the loss of static responsiveness was not attributable to inability either to generate or conduct spikes centrally, since the Ia muscle spindle afferent remained responsive to fast dynamic mechanical stimulation produced by muscle vibration. Instead we speculated that reduced static responsiveness
originated from impairment of underlying persistent inward current mechanisms. If this were the case, then additional signs of firing impairment would be expected, as they are with suppression of persistent inward currents in other neuronal systems (Harvey et al., 2006a). Specifically, we expected that the threshold for repetitive firing in OX rats would remain unchanged during fast ramp stretch (20 mm/s), but would increase during for slow ramp stretch (4 mm/s). Results illustrated in Fig. 5 confirmed our expectation. Threshold for repetitive firing increased by about 60% with slow but not fast dynamic stretch in OX-rats (Fig. 5C,F, Table 1). Comparison of Fig 5A and B provides insight into the change in threshold. In control rats, repetitive firing is immediately preceded by an initial burst of rapid firing, which occurs at the onset of fast dynamic stretch and in the first of three sequential slow dynamic triangular muscle stretches (Fig. 5A; Haftel et al., 2004). That initial burst was not lost in OX-rats (Fig. 5B), and it occurred at the same threshold for control and OX-rats (see Table 1). These findings indicate that in OX rats, increased threshold was specific to repetitive firing and was not an expression of general insensitivity to mechanical stimulation.

Pharmacological Block of NaPIC Mimics Proprioceptor Impairment with OX

Repetitive firing in response to a static stimulus relies in many neurons on persistent inward current mediated by Na channels (Crill, 1996; Lee and Heckman, 1998, 2001; Harvey et al., 2006a). If OX treatment altered proprioceptor signaling through this mechanism, then we expected to mimic its effect by reducing sodium persistent inward current (NaPIC) in normal rats. Riluzole was selected for its known effects in blocking NaPIC and repetitive firing in the large diameter class of DRG neurons that include muscle proprioceptors (Xie et al., 2011).

Fig. 6 illustrates the effect of acute riluzole injection on the static responsiveness of 1a muscle spindle afferents in normal rats For 1a muscle spindle afferents sampled
from a single rat, comparison of Fig. 6A with 6B demonstrates a reduction in static responsiveness similar to that shown for OX treatment (Fig. 4). Fig. 6D demonstrates that the reduction in static responsiveness was dose dependent. By comparison, OX reduced static responsiveness by about 60% (Fig. 4; Table 1), an amount found for riluzole concentrations between 6 and 10 mg/Kg. If we speculate that OX acts through the same mechanism as riluzole, possibly block of NaPIC, then we would suggest that NaPIC is only partially impaired by OX treatment.

Additional effects of acute riluzole were studied as they were for OX treatment. Fig. 6C shows that la muscle spindle afferents remained sensitive to fast dynamic mechanical stimulation produced by muscle vibration, just as in OX-rats (cf. Fig. 4C). Fig. 7 shows that riluzole increased the threshold for repetitive firing in response to slow stretches, and affected fast stretches only at the highest dose (cf. Fig. 5 for OX rats). Table 1 compares riluzole (at the intermediate dose of 6 mg/kg) and OX treatment for their effects on additional la firing parameters. The match is nearly identical between those parameters that either were or were not significantly different relative to control. The one exception was peak firing rate which was nominally reduced by riluzole, but not significantly so as it was with OX treatment. Collectively, these findings are consistent with the possibility that OX treatment impairs proprioceptor signaling by reducing NaPIC. Table 1 revealed differences between the control groups for riluzole vs. OX that bear consideration. Riluzole control values were taken from la muscle spindle afferents sampled from single rats before riluzole injection, and those values are similar to control values recorded from untreated rats in an earlier study (Bullinger et al., 2011b). However, the OX controls were different from riluzole controls for nearly every parameter. We can only speculate that the OX controls were influenced by the behavioral study protocol or by vehicle injection, nonetheless, these data provide the appropriate controls for OX treated rats, which underwent the same protocol.
Riluzole might have reduced la static responsiveness through its known action of blocking NaPIC in motoneurons (Harvey et al., 2006a; Theiss et al., 2007). Through this action, riluzole might decrease activity of gamma motoneurons, which play a critical role in setting la sensitivity to muscle stretch (Crowe and Matthews, 1964; Matthews, 1972). To determine whether a loss of gamma modulation of muscle spindle gain might underlie OX-induced encoding defects, we sectioned ventral roots (where gamma motoneuron axons exit the spinal cord) and recorded spindle encoding in both controls and riluzole injected rats. Ventral root section had no significant effect (p > 0.05) on la muscle spindle afferent static responsiveness. In control rats, there was no significant difference in the duration of static responsiveness with ventral roots intact (746 ± 315 ms) or ventral roots cut (790 ± 269 ms). Neither did the effect of riluzole depend on gamma motoneuron input, since riluzole reduced the duration of static responsiveness to levels that were not significantly different (p > 0.05) with ventral roots intact (423 ± 438 ms) or cut (518 ± 404 ms).

Mechanistic interpretation of the riluzole effect is confounded by its multiple drug actions. In addition to blocking NaPIC, riluzole activates calcium dependent potassium channels (BK/SK) (Grunnet et al., 2001; Wang et al., 2008), inhibits voltage-gated Ca\(^{2+}\) channels (Huang et al., 1997; Stefani et al., 1997), and modulates glutamate signaling and release (Doble, 1996; Lamanauskas and Nistri, 2008; Bellingham, 2011, 2013), all of which are present in muscle spindles (Bewick and Banks, 2015). In attempt to narrow the set of possible explanations for the riluzole effect, we tested acute injection of phenytoin, which also blocks NaPIC (Lampl et al., 1998; Zeng et al., 2005), but does not share the other actions with riluzole. The effects of phenytoin on la muscle spindle afferent firing were similar to those of riluzole and OX treatment. Fig. 8 shows the shortening of firing duration during static muscle stretch described above for OX treatment and matched to the effect of riluzole at its intermediate dose (6 mg/Kg). The
reproduction of impaired Ia firing in OX-rats by two drugs having predominantly only 
NaPIC blockade in common leads us to the parsimonious though provisional conclusion 
that OX reduces static and slow dynamic responsiveness of Ia muscle spindle afferents 
by reducing NaPIC in the proprioceptor's peripheral nerve terminals.

*Encoding Deficits are Present in Other Muscle Proprioceptors*

The large diameter group II muscle spindle afferents and group Ib tendon organ 
afferents are also muscle proprioceptors that provide essential feedback regarding 
muscle length and force (Matthews, 1972; Jami, 1992). Fig. 9 illustrates the effects of 
OX treatment and riluzole on static responsiveness of these two proprioceptors. For 
group II proprioceptors, static responsiveness shifted, but only slightly and insignificantly 
toward lower than control values in OX rats, although riluzole in the highest dose studied 
here produced a substantial and significant decrease. For Ib golgi tendon organ 
afferents, there was a significant reduction in responsiveness with OX treatment and 
with acute injection of riluzole. This finding had unique value in establishing that impaired 
sensory firing did not require the functional and structural specializations that are present 
in muscle spindles but absent in tendon organs (Hunt, 1974). We suggest, therefore, 
that these findings localize impairment of static responsiveness to the nerve terminals of 
proprioceptors.

*Discussion*

Recently our laboratory discovered that chronic treatment with oxaliplatin in 
rodents produces a selective deficit in sensory encoding by muscle proprioceptors 
(Bullinger et al., 2011b). In particular, sensory neurons supplying muscle spindle 
receptors lose their ability to signal stationary muscle position. Given the primacy of
proprioceptor signals for informing the central nervous system about limb position (Prochazka and Ellaway, 2012; Proske and Gandevia, 2012), we predicted, and in the present study we demonstrated that movements relying on proprioceptive feedback were impaired in OX-treated rats. This finding motivated us to further explore the cellular mechanisms underlying impaired sensory signaling. Our findings suggested that OX treatment chronically impairs proprioceptors through ion-channel mechanisms, which present possible therapeutic targets for relieving some of the disabling effects of OX chemotherapy.

Movement Disability with Proprioceptor Impairment

Sensory information provided by muscle proprioceptors is indispensable to the production of normal movements and postures of body and limbs (Prochazka and Ellaway, 2012; Proske and Gandevia, 2012). These receptors are distributed in nearly all mammalian skeletal muscles where they encode dynamic and static parameters of muscle length and force. Muscle proprioceptors are the major source of sensory feedback used to control movements that are unassisted by visual input or vestibular sense, e.g. reaching for an object outside the visual field. Sensory feedback from muscle proprioceptors is critical not only for making rapid adjustments to unexpected movement conditions (Nichols et al., 1999; Shemmell et al., 2010), but also for maintaining body schema, i.e. a sensorimotor representation of the spatial relations between body parts that is used in guiding voluntary movement (Ivanenko et al., 2011). It follows then that any conditions which interrupt sensory detection, encoding or signal transmission to the central nervous system have the potential to result in postural instability, clumsiness, and ataxia as has been found under a variety of conditions in rodents, cats, and humans (Krinke et al., 1985; Abelew et al., 2000; Sghirlanzoni et al., 2005).
In rats receiving chronic OX treatment, we observed abnormal motor behavior characterized as difficulty with achieving proper limb placement. The following observations suggest a link to altered proprioceptor signaling. First, the behavioral task that OX-treated rats struggled with was one that accentuated reliance on proprioceptor signaling. Specifically, rats walked on ladder rungs, a task that required them to position hind feet on unevenly spaced rungs that passed outside their visual field as they stepped forward; rung positions were randomly changed between sessions to prevent advanced knowledge of their position (Metz and Whishaw, 2002, 2009). This task exposed substantial errors in placing the foot securely and correctly beginning within the first two weeks of OX treatment and extending until the last measurements taken at three weeks after OX treatment. Disability was not attributable to muscle weakness, for which we found no evidence in the rats’ general behavior, e.g. climbing onto objects, or recovering from missteps or falls on the ladder rung apparatus. Second, the same OX rats that performed poorly in the proprioceptive movement task also exhibited impaired sensory signaling in terminal electrophysiological study of muscle proprioceptors. In fact, we found a significant correlation in the magnitude of abnormalities measured in animal and proprioceptor behavior. Third, difficulties with limb placement tested in the ladder rung test are also found in mice in which muscle spindles are selectively eliminated by experimental genetic mutation (Akay et al., 2014). These observations support a possible causal relationship between movement disorders and modified proprioceptor firing observed here for OX treated rats.

Assessing Patient Relevance

With OX chemotherapy, patients report difficulties often characterized as proprioceptive disability, including instability when walking and clumsiness with
manipulating objects ((Bennett et al., 2012). There are good reasons to propose that these movement deficits originate from impaired signaling by muscle proprioceptors. As muscle proprioceptors are the sole source for detecting and signaling of muscle position and force in rats and all other mammalian species studied thus far (Matthews, 1972; Zelena and Soukup, 1983; Jami, 1992), so too are they the origin of this information in humans. Muscle spindles and tendon organs in humans resemble those in rats and other animals in their structure and neural innervation (Swash and Fox, 1972; Nitatori, 1988; Eriksson et al., 1994; Liu et al., 2003) and in their encoding of dynamic and static parameters of muscle conditions, including their sustained (slowly adapting) firing during fixed muscle length or force (Edin and Vallbo, 1990a, b, c; Macefield, 2005).

Experimental manipulations of proprioceptor signaling in humans, e.g. temporarily silencing muscle proprioceptors by nerve compression (Walsh et al., 2010; Inui et al., 2011) demonstrate their necessity for normal movement. Additionally, destruction of muscle proprioceptors in human victims of large fiber neuropathy leads to clumsiness, ataxia and inability to properly position limbs outside visual field (Rothwell et al., 1982). These observations establish strong parallels between humans and another animal species with respect to proprioceptor signaling and its requirement in producing normal movement, and they support our assertion that the effects of OX on proprioceptive signaling and behavior that we observe in rats are likely to have relevance for patients.

Role of Neuropathy

Signaling by primary sensory afferents might be obstructed through any one of several processes and locations, ranging from sensory detection or encoding at afferent endings to conduction and transmission by afferent axons and central synapses. The best documented cause of chronic sensory impediment by OX chemotherapy is
neuropathy, occurring as a length-dependent dying back from the distal ends of the longest sensory axons supplying distal limbs, e.g. feet and hands (Avan et al., 2015). The possibility that neuropathy contributed to present results cannot be definitively discounted. Although we found no evidence of neuropathy in SNAPs measured from the long sensory axons in the rat tail, we did not directly test for histological signs of degeneration in sensory nerve terminals. While acknowledging uncertainty, the following several observations suggested to us that neuropathy was probably not a major contributor to our results. First, none of the proprioceptors sampled from OX rats generated action potentials in response to electrical stimulation of nerve (1 cm proximal to muscle entry) without also firing in response to muscle stretch, thereby giving no evidence that sensory terminals had degenerated in advance of parent axons. Second, direct histological examination in our earlier study showed that the sensory innervation of muscle spindles was not retracted and was grossly normal in appearance (Bullinger et al., 2011b). It is important to note, however, that the cumulative dose of OX was raised from 40 mg in that previous study to 70 mg in the present study, in order to better match the dosage in humans. A cumulative dose of 70 mg/kg in rats is equivalent to approximately 415 mg/m² in humans (Freireich et al., 1966; Reagan-Shaw et al., 2008), a dose that is at just threshold for the earliest detection of neuropathy measured from changes in SNAP amplitude (Grothey, 2003) and well below levels, >700 mg/m², that produce robust neuropathy (Argyriou et al., 2008; Avan et al., 2015). Thus we may not expect the higher dose used in the present study to produce substantially more neuropathy in our OX treated rats, and this supposition is supported by the similarity in OX effects on the mechano-responsiveness of proprioceptors sampled in this and our previous study (cf Table 1 from each paper). Third, the chronic effects of OX were stereotypical in affecting static firing exclusively and did not express widely varying changes in firing behavior that one would expect from individual proprioceptors in
different stages of sensory terminal degeneration. None of these observations are easily reconciled as evidence for neuropathy.

While evidence for neuropathy in patients is in many cases incontrovertible and undoubtedly a significant contributor to disability (Argyriou et al., 2014; Avan et al., 2015), additional chronic effects and their importance cannot be dismissed. For example, pain and alldynia observed following OX (Kiernan, 2007; Fehrenbacher, 2015) are suspected to result, not from neuropathy, but instead from sensory axon hyper-excitability (Park et al., 2009a, 2011b). Neither does neuropathy explain disability reported by patients for whom there is no evidence for a decrease either in SNAPs or in the density of epidermal nerve terminals (Burakgazi et al., 2011). When significant neuropathy does occur, it may not act alone in causing disability. Considering the possibility that OX affects proprioceptor signaling in patients, as we show here for rodents, we expect involvement of proprioceptors distributed throughout the body. Proprioceptors reside in trunk and proximal limbs muscles (Banks, 2006), and the sensory feedback they supply supports sundry movements and postures (Proske and Gandevia, 2009; Roden-Reynolds et al., 2015). Normal walking, for example, relies heavily on sensory feedback from proprioceptors in hip muscles (Roden-Reynolds et al., 2015). Impaired signaling from those sites would be readily capable of contributing to imbalance, difficulty walking, and clumsiness variously described by OX patients. These proximal origins of movement disability originating proximally would be masked, however, and mistakenly assigned to proprioceptors in distal extremities denervated by length-dependent neuropathy, because we commonly interact with the environment through hands and feet. Sensation from the feet is critical for weight bearing activities (Meyer et al., 2004) and from hands for skilled grasping (Witney et al., 2004). All considered, it seems reasonable, and not disproven that factors in addition to distal
neuropathy contribute significantly to chronic disability. This notion could be critically assessed by discriminating tests for proprioceptive disorders in the trunk, shoulders and hips.

**A Mechanism for OX Modification of Proprioceptor Encoding**

Several mechanisms apart from neuropathy might have resulted in selective modification of static firing by proprioceptors following OX treatment. Reduced static firing might have derived from one or another of the numerous specializations of muscle spindle receptors. For example, OX might have impaired static firing by reducing gamma motor drive to the spindle's intrafusal muscle fibers (Matthews, 1962; Boyd and Ward, 1975; Banks et al., 1997). This possibility seemed unlikely, however, given our demonstration that complete experimental elimination of gamma motor input was not capable of significantly reducing proprioceptor responsiveness. More generally, we were able to rule out the necessity of specializations unique to muscle spindles by demonstrating that static responsiveness was equally impaired in Ib proprioceptors that provide the sole neural innervation to relatively non-specialized tendon organ receptors (Hunt, 1974; Jami, 1992; Zelena, 1994). The proprioceptors' parent axons, spanning the distance from muscle to dorsal roots, were fully competent to generate and conduct action potentials from nerve terminals to the spinal cord, even at sustained, high frequency in response to rapid oscillations in muscle length, i.e. vibrations. Finally, the generation of action potentials with high fidelity in response to each length oscillation throughout bouts of muscle vibration demonstrated that the underlying ionic currents generated either in mechano-transduction or in action potential encoding were not chronically disabled by OX (Bewick and Banks, 2015). Elimination of these mechanisms promoted the idea that abbreviated static responsiveness and increased threshold for
repetitive firing involved sustained ionic currents and channels normally generated
during static and dynamic muscle stretch. The suspect channels are ones that contribute
to currents underlying the receptor potential produced through mechano-transduction
(Hunt et al., 1978) and/or currents required to sustain repetitive firing of action potentials
for encoding steady depolarization.

The ion channels that support sustained repetitive firing during steady
depolarization in muscle proprioceptors have not been identified. In numerous other
neurons, repetitive firing to sustained and slow depolarization rests on properties of Na⁺
channels that inactivate slowly and thus generate persistent inward currents (NaPIC)
(Crill, 1996; Lee and Heckman, 1998, 2001; Harvey et al., 2006a). One of these
channels, NaV1.6 is expressed by large diameter DRG neurons (Black et al., 1996;
Rush et al., 2007; Chung et al., 2015), which include muscle proprioceptors as well as
primary sensory neurons supplying skin. Expression of NaV1.6 by the cell body is
matched by expression in the nerve terminals of Merkel cells (Lesniak et al., 2014),
which like proprioceptors are slowly adapting (Iggo and Muir, 1969; Zimmerman et al.,
2014). Furthermore, both NaV1.6 and NaPIC respond acutely to OX (Sittl et al., 2012).
Collectively, these findings led us to predict that OX might abbreviate static
responsiveness and increase the dynamic threshold of for repetitive firing by
compromising NaPIC in the nerve terminals of proprioceptors.

We assessed the importance of NaPIC by testing whether a known
pharmacologic antagonist would mimic the OX effect on static responsiveness and the
dynamic threshold for repetitive firing. We selected riluzole for its action as a blocker of
NaPIC (Benoit and Escande, 1991; Urbani and Belluzzi, 2000; Lamanauskas and Nistri,
2008; Bellingham, 2011) in DRG neurons (Xie et al., 2011). Acute i.p. injection of riluzole
in normal rats reproduced essentially all of the effects that chronic OX had or did not
have on proprioceptor encoding. While having no effect on the normal generation or conduction of action potentials evoked by muscle vibration, riluzole exhibited a dose dependent reduction in static responsiveness. Riluzole also increased repetitive firing threshold during slow ramps as it did in OX treated rats, and at higher doses increased repetitive firing threshold during fast ramps. These findings supported our hypothesis, however, riluzole's additional drug actions, e.g. activation of K\(^+\) current via BK channels (Grunnet et al., 2001; Wang et al., 2008) confound interpretation. Although there is as yet no more specific NaV1.6 antagonist, there is anti-epileptic drug, phenytoin, which shares only riluzole's block of NaPIC, but none of its other drug actions (Lampl et al., 1998; Zeng et al., 2005). Previous study has established that in cats, phenytoin reduced the static responsiveness of muscle spindles (Anderson and Raines, 1974). Our finding that acute administration of phenytoin to normal rats also abbreviated static responsiveness bolsters the plausibility that NaPIC supports static firing in proprioceptors and that it may be the target of chronic OX.

The route by which chronic OX treatment might compromise NaPIC remains to be determined. Some studies (Sittl et al., 2012) though not all (Wu et al., 2009) demonstrate that OX acutely increases and prolongs NaPIC in DRG neurons. In addition, NaV1.6 provides a route through which mechanical allodynia develops, presumably from increased excitability of large diameter sensory neurons, within 24 h of intra-plantar injection of OX in mice (Deuis et al., 2014). These acute effects of OX are opposite to the decreased excitability we observe in muscle proprioceptors. We did find, however, that acute administration of high doses of OX produced spontaneous firing in normal rats, and we are currently testing whether this acute increase in excitability might trigger a compensatory and chronic decrease in excitability. If this is the case then
blocking acute hyper-excitability is a potential treatment that may prevent chronic effects on proprioception.

**Conclusion**

Our findings demonstrate that oxaliplatin treatment has the potential to cause chronic movement disorders by a novel mechanism. In our rodent model of OX neurotoxicity, movement deficits in tasks relying on proprioception co-existed with impaired sensory encoding that selectively diminished the signal for static or slowly changing muscle position. We suggest that proprioceptor signaling is diminished by a chronic effect of OX that impairs NaPIC. Identification of this independent target of OX neurotoxicity offers a novel target for therapeutic intervention.
Fig. 3. Movement disability in OX-treated rats. Rats were scored for errors in hind foot placement while walking on unevenly spaced ladder rungs in sessions spanning 11 weeks. (A, C) For untreated control rats (n=5) data were pooled and plotted as percent change in order to illustrate improvement (training effect) relative to initial task sessions (time 0); mean values (open black circles joined by lines) are bracketed for 95% confidence interval (CI). For each one of 10 OX-treated rats (identified by red number) mean percent change is plotted per task session, and the schedule for OX injection is noted by blue dots. (A) Secure hind foot placement was scored by errors/steps in rung contact multiplied by -1 and computed as percent change from the pre-treatment value for each OX treated rat. (B) Photograph shows left hind limb slip in double image (simultaneous side and underneath views) of OX-treated rat walking on ladder rungs. (C) Correct hind foot placement was scored by errors/steps in correctly placing the trailing hind foot on the same rung as the ipsilateral forelimb multiplied by -1 and computed as percent change from the pre-treatment value for each OX treated rat. (D) Plot of secure vs. correct hind foot placement (unlike Figs. 1A and 1C, values were calculated as percent change from untreated control mean value at week 11); box at origin encompasses variation pooled for untreated control rats.
Fig. 4. OX treatment shortens firing duration during static muscle stretch.

Representative records of Ia muscle spindle afferents firing (grey traces show intra-axonal records of action potentials, black dots indicate instantaneous firing rate) in response to ramp-hold-release muscle stretch (B bottom trace; 3 mm, 20 mm/s, 1 s hold phase). (A) In an untreated control rat, Ia fired during fast-dynamic (ramp) phase and throughout static (hold) phase of stretch. (B) In a rat 4 weeks after 8-week treatment course of OX, Ia fired similar to normal during fast-dynamic phase but stopped firing during the static phase of muscle stretch. (C) For the same Ia muscle spindle afferent as in B, vibration superimposed on muscle stretch (bottom trace; 100 Hz, 80 µm vibration during stretch hold phase) restored firing. (D) Mean± SE for duration of static-phase firing expressed as percent change for pooled samples of Ia muscle spindle afferents in OX-treated rats (71 Ia's in 10 rats) relative to control rats (52 Ia's in 5 rats). (E) Comparison of mean static-phase firing (n > 4 Ia muscle spindle afferents per animal) and secure hind foot placement for individual control (C) and OX (OX) animals expressed as a percent change from mean control values. Secure hind foot placement during horizontal ladder rung task is positively correlated with the duration of static responsiveness ($r^2 = 0.76$, $p < 0.01$, dashed line).
Fig. 5. OX-treatment increases repetitive firing threshold during slow- but not fast-
dynamic muscle stretch. Threshold for repetitive firing was measured from Ia muscle
spindle afferents during fast ramps (20 mm/s; left column) and slow ramps (4 mm/s in 3
successive triangular stretches; right column). Action potential firing and firing rate in
response to muscle stretch (traces described in Fig. 4) were taken from one Ia muscle
spindle afferent in a control rat (A) and one in an OX-treated rat (B). Repetitive firing
threshold (arrow heads) after OX-treatment was similar to control for fast ramps, but
higher than control for slow ramps. Note that threshold of the transient initial burst was
unchanged. (C) Mean ± SE for repetitive firing threshold for fast (left) and slow (right)
ramps expressed as percent change for pooled samples of Ia muscle spindle afferents in
OX- treated rats (58 Ia's in 10 rats) relative to control rats (51 Ia's in 5 rats).
Fig. 6. Riluzole mimics OX-treatment in shortening firing duration during static muscle stretch. Representative records (as described in Fig. 4) of Ia muscle spindle afferent responses to muscle stretch in one rat studied (A) before and (B) after Riluzole injection (6 mg/kg i.p.). Similar to the effect of OX-treatment (Fig. 4), acute Riluzole injection shortened static responsiveness, but had no apparent effect on dynamic responsiveness exhibited during fast-ramp stretch or vibration (C). (D) Reduction in duration of static responsiveness (expressed as percent change from control) for Ia muscle spindle afferents was dose dependent.
<table>
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<tr>
<th>Reaction</th>
<th>Peak Time (sec)</th>
<th>SD</th>
<th>Initial Burst, Pulse/sec</th>
<th>Fast Lamp</th>
<th>Dynamic Limb Threshold, mm</th>
<th>Slow Lamp</th>
<th>Dynamic Limb Threshold, mm</th>
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<td>Glucose</td>
<td>1.5 (g/dL)</td>
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<td>0.15 ± 0.2 ± 3.6</td>
<td>0.71 ± 0.2 ± 3.4</td>
<td>0.15 ± 0.2 ± 3.6</td>
</tr>
<tr>
<td>Ox (mg/dL)</td>
<td>Means ± SD, ox</td>
<td></td>
<td>1.4 ± 0.6 ± 3.4</td>
<td>2.2 ± 0.6 ± 3.4</td>
<td>1.6 ± 0.8 ± 3.4</td>
<td>2.2 ± 0.6 ± 3.4</td>
<td>1.6 ± 0.8 ± 3.4</td>
</tr>
</tbody>
</table>

Static Responsiveness

Dynamic Responsiveness

Duration Stated Limb Threshold, min
Average Limb Rate, Pulse/sec

Hold
Table 1. Muscle length encoding by Ia muscle spindle afferents. Rats in oxaliplatin (OX) study were all subject to the same procedures, i.e. chronic measurement of motor performance and terminal measurement of proprioceptor encoding; 10 received repeated injections of vehicle + OX and 5 received injections of vehicle alone. All rats in riluzole (Ril) study, were examined only in terminal experiments to obtain measurement of proprioceptor encoding before and after Ril injection. n, number of afferents pooled within groups. Nested ANOVA and Tukey’s post hoc tests were used to test for the significance of group differences. * P < 0.05
A Fast Ramp

B Riluzole treated

C

Fast Dynamic Threshold (% change)

Slow Dynamic Threshold (% change)

Riluzole (mg/kg)
Fig. 7. Riluzole mimics the OX-treatment increase in repetitive-firing threshold during slow-dynamic muscle stretch. Representative records of Ia muscle spindle afferent firing in response to muscle stretch (cf. Fig. 3) in a rat studied (A) before and (B) after riluzole injection (6 mg/kg i.p.). Repetitive firing threshold (arrow heads) was similar before and after acute Ril injection for fast ramps, but was substantially higher for slow ramps. Note that threshold of the transient initial burst was unchanged. (C) Mean ± SE repetitive firing threshold for fast (left) and slow (right) ramps expressed as percent change for pooled samples of Ia muscle spindle afferents after relative to before Ril-injection.
Fig. 8. Phenytoin shortens firing duration during static muscle stretch. (A)
Representative records of a la muscle spindle afferent firing (top grey trace action potentials with instantaneous firing rate indicated by superimposed black dots) in response to ramp-hold-release muscle stretch (bottom trace; 3 mm, 20 mm/s, 1 s hold phase) following phenytoin injection (20 mg/kg i.p.). Similar to the acute effect of riluzole (cf Fig. 4), acute phenytoin injection shortened static responsiveness, but had little effect on dynamic responsiveness exhibited during fast-ramp stretch or vibration (data not shown). (B) Duration of static responsiveness (expressed as percent change from control) significantly reduced for 25 la muscle spindle afferents measured in rats injected with phenytoin compared with 21 la muscle spindle afferents in control rats.
Fig. 9. Effects of OX treatment and riluzole on group II spindle and Ib tendon-organ proprioceptors. (A) OX treatment shortened firing duration during static muscle stretch nominally for II's (p = 0.077) and significantly for Ib's. (B) Acute riluzole injection also tended to shorten firing at the highest doses.
Chapter V

Specific Aim 2: To test the hypothesis that 5-HT2 agonists, that increase persistent inward currents, can restore sensory encoding by muscle proprioceptors following OX.

Introduction

Aim one establishes that chronic OX impairs a NaPIC necessary for sustained repetitive firing during normal proprioceptor encoding. This current localizes to the receptor terminal and offers a novel therapeutic target to restore proprioceptor encoding. Recent work has identified the Na\(^+\) channel subtypes present at the sensory nerve terminal. Consistent with NaPIC playing a role in encoding, NaV 1.6 has been identified at the sensory nerve terminal of muscle proprioceptors (Carrasco et al., 2017). NaV 1.6 has been shown in large diameter dorsal root ganglion neurons to mediate a large NaPIC, suggesting that it may be performing a similar task at the sensory nerve terminal (Rush et al., 2007). Targeting this current might restore repetitive firing lost in muscle proprioceptors following OX.

NaPIC’s role in repetitive firing has been well established in numerous types of neurons (Crill, 1996; Lee and Heckman, 2001; Harvey et al., 2006a; Perrier et al., 2013). The subthreshold NaPIC is thought to arise from the same transient Na\(^+\) channels that underlie action potentials (Alzheimer et al., 1993). The majority of voltage-gated Na\(^+\) channels carrying the transient Na\(^+\) current underlying action potentials rapidly inactivate
after an opening period. A small number of these channels likely 1-3% enter a persistent state, giving rise to NaPIC (Alzheimer et al., 1993).

In motoneurons, 5-HT and serotonergic agonists increase the amplitude of NaPIC (Harvey et al., 2006b; Murray et al., 2010; Murray et al., 2011). 5-HT2 receptor activation in motoneurons that are unable to repetitively fire during slow current injection led to robust repetitive firing that correlated with large increases in NaPIC (Harvey et al., 2006b). 5-HT2 receptors are coupled to the Gαq-protein, which through activation of phospholipase C, increases DAG, and IP3 (Harvey et al., 2006b; Murray et al., 2011). PKC is known to phosphorylate Na\(^+\) channels altering their activation and inactivation. Furthermore, PKC has been show to facilitate NaPIC ((Franceschetti et al., 2000).

In order to determine the feasibility of therapeutic restoration of proprioceptor encoding, a non-specific 5HT-2 receptor agonist quipazine was chosen (Chopek et al., 2013). Quipazine was given in terminal experiments following injection with 6 mg/kg riluzole. In the acute setting quipazine improved proprioceptor encoding, this suggested that encoding following OX could be restored. Following chronic OX treatment, a FDA approved 5-HT2C receptor agonist (Witkamp, 2011), lorcaserin was tested. The switch to lorcaserin was necessary to establish the potential for translation to human studies with an FDA approved drug.

**Methods**

All surgical procedures and data collection in this aim are the same as the studies outlined above in general methods and specific aim 1 methods.
Acute Pharmacological Studies

Rats previously untreated were used to measure proprioceptor responses in terminal experiments as described in general methods. Ten animals were used to assess the effects of quipazine on muscle proprioceptors impaired by riluzole. In these animals muscle proprioceptors were sampled before and after i.p. injection of riluzole (6 mg/kg), followed by sampling after i.p. injection of quipazine (10 mg/kg). In five of these animals ventral roots were sectioned prior to any recording to remove any influence of gamma motoneurons on muscle spindle function. Riluzole injections were handled as described in aim 1. Quipazine, 10mg, was dissolved in 1 ml DMSO and 4 ml 0.9% saline and dose by animal body weight (mg/kg).

Chronic OX Studies

In the same animals that underwent chronic OX treatment in specific aim 1, three animals were selected for further study with lorcaserin. Muscle proprioceptors were sampled prior to i.p. injection with lorcaserin, 2 mg/kg. Lorcaserin, 10 mg, was dissolved in 0.9% normal saline and was dosed based on animal body weight (mg/kg). Following collection of muscle proprioceptor responses following lorcaserin, ventral roots were sectioned and sampling continued.

Results

Chronic OX impairs muscle proprioceptor encoding by disrupting a novel NaPIC, leading to behavioral deficits. Identification of this underlying mechanism allows for application of a targeted therapeutic to potentially restore muscle proprioceptor encoding and behavior. If successful in the rodent, this therapeutic would have the potential to translate to human studies and significantly improve patient lives.
Quipazine restores Ia muscle spindle afferent responsiveness after riluzole

In other neuronal subtypes, NaPIC can be increased by activation of 5-HT2 receptors. We applied two different 5-HT2 receptor agonists after either acute riluzole or chronic OX. In the acute study, a dose of 6mg/kg riluzole was chosen because it most closely matches the effects of chronic OX on muscle proprioceptor encoding, specifically Ia muscle spindle afferents. Following acute injection of 6 mg/kg riluzole 68% of sampled 1a muscle spindle afferents fail to fire at least 800 ms of a 1000 ms hold phase, compared to only 25% in control conditions. This drastic loss of sustained repetitive firing represents a significant loss of muscle length and force feedback to the central nervous system.

Within individual animals treated with riluzole, quipazine dramatically improved repetitive firing in Ia muscle spindle afferents. Fig. 9 compares static responsiveness duration for control, 6 mg/kg riluzole, and 10 mg/kg quipazine. Riluzole (6 mg/kg) significantly reduces sustained firing, and following i.p. injection of quipazine there is a significant (p<0.05) increase in static responsiveness. After quipazine, only 33% of Ia muscle spindle afferents failed to fire at least 80% through the hold phase, compared to 68% in riluzole 6 mg/kg. This marks a significant restoration of sustained repetitive firing during static muscle stretch and therefore fixed muscle position and force feedback to the central nervous system. This effect was rapid and occurred within 45 min of injection and lasted for hours following injection.

Quipazine’s ability to increase the duration of static responsiveness restores part of the signal lost following treatment with riluzole. Characteristic of disrupting NaPIC, riluzole also selectively disrupts repetitive firing during slow dynamic stretch, measured by an increase in the repetitive firing threshold (slow dynamic threshold). Similar to impaired repetitive firing during sustained muscle stretch, the loss of repetitive firing
during muscle stretch represents a significant loss of feedback regarding changing muscle length and force to the central nervous system. Fig. 10 compares slow dynamic threshold in control rats, and those treated with riluzole and quipazine. The increase in slow dynamic threshold by riluzole was corrected by quipazine. In fact quipazine reduced slow dynamic threshold to less than that observed in controls. Thus, quipazine increased repetitive firing deficits during slow muscle stretch, restoring the lost feedback to the central nervous system.

**Lorcaserin Restores Ia Muscle Spindle Afferent Responsiveness after OX**

Similar to riluzole (6 mg/kg), following chronic OX 73% of Ia muscle spindle afferents sampled failed to fire at least 800 ms during a 1000 ms hold phase. The similar effects of riluzole and chronic OX on static responsiveness suggest that quipazine or other 5-HT2 agonists should restore firing following chronic OX. Due to the translational implications of restoring proprioceptor encoding following chronic OX, the FDA approved 5-HT2C receptor agonist lorcaserin was chosen (Witkamp, 2011). Fig. 11 shows the effects of lorcaserin on the duration of static responsiveness. Similar to quipazine, lorcaserin (2 mg/kg) significantly increased the duration of static responsiveness. After lorcaserin only 40% of Ia muscle spindle afferents failed to fire at least 800 ms during a 1000 ms hold phase. The effect was not as robust as the 5-HT2 nonspecific agonist quipazine given in the acute setting with riluzole, but was still capable of restoring a significant amount of encoding and therefore muscle length and force feedback after chronic OX.

In order for lorcaserin to have potential as a target treatment following OX it should also significantly reduce the slow dynamic threshold. Fig. 12 compares the slow dynamic threshold in control, OX, and following lorcaserin. Fig. 12A is a representative record following chronic OX increasing the slow dynamic threshold. Fig. 12 B is from the
same animal following injection with lorcaserin (2 mg/kg), and shows the reduction in slow dynamic threshold. Again, the results with lorcaserin were not as robust as those with riluzole, but clearly showed a significant effect on slow dynamic threshold following OX.

Our conclusions from aim 1 suggest that chronic OX is most likely disrupting a NaPIC necessary for muscle proprioceptor encoding. The presence of NaPIC in muscle proprioceptors is novel and any regulation at this level is unknown. NaPIC in other neuronal systems is regulated by Gq-coupled 5-HT2 receptors (Harvey et al., 2006b; Murray et al., 2011). The next step is to determine how quipazine and lorcaserin are working to restore encoding specifically in la muscle spindle afferents. There are two potential locations where these drugs could be working, either peripherally at the spindle terminal, or centrally likely by increasing output from gamma motoneurons. As mentioned in the background section, muscle spindles receive both sensory and motor innervation. The motor innervation arises from gamma motoneurons, which can be further broken down into static and dynamic, based on their effects on either static or dynamic responsiveness (Boyd and Ward, 1975; Banks et al., 1997).

In order to determine where quipazine and lorcaserin were acting to restore encoding, ventral roots were sectioned either prior to any recording in the case of riluzole or following injection of the 5-HT2 agonists in the case of chronic OX. If the selected 5-HT2 agonists are working peripherally, then ventral root section should have no effect on their ability to restore encoding. If they are working centrally, ventral root section should eliminate their effects.

Fig. 13 shows the effects of ventral root section on the effectiveness of quipazine and lorcaserin to increase the duration of static responsiveness. Note, that in the case of quipazine, ventral roots were sectioned prior to any recording and riluzole injection. As discussed in aim 1 ventral root section had no effect on encoding in control or riluzole
treated afferents. In OX animals ventral roots were sectioned following injection and data collection with lorcaserin. In the case of riluzole and quipazine (Fig. 13A) ventral root section eliminates the efficacy of quipazine to restore static encoding. With lorcaserin (Fig. 13B) the trend is similar, but did not reach significance. This is likely because the effectiveness of lorcaserin to restore static responsiveness was less than that of quipazine, however a peripheral effect of lorcaserin cannot be ruled out.

Ventral root section showed similar effects on slow dynamic threshold following 5-HT2 agonist injection. Acutely, eliminating motor influence significantly reversed the effectiveness of quipazine to decrease the slow dynamic threshold. The same results were observed with ventral root section after lorcaserin. Unlike the effects of ventral root section on static responsiveness, ventral root section after lorcaserin significantly increased slow dynamic threshold. This difference most likely arises from the fact that lorcaserin had a larger effect on slow dynamic threshold than the duration of static responsiveness.

Discussion

Disruption of NaPIC by chronic OX significantly impairs muscle proprioceptor encoding resulting in behavioral deficits. The largest effect was on Ia muscle spindle afferents, and so in aim two, known 5-HT2 receptor activators were applied. In the acute setting following riluzole and after chronic OX 5-HT2 agonists significantly increased repetitive firing and were able to restore encoding in Ia muscle spindle afferents. Overall lorcaserin was less effective in restoring proprioceptor function. This may result from lorcaserin being a specific activator of 5-HT2C receptors, whereas quipazine is non-specific. Ventral root section eliminated the effects of 5-HT2 receptor agonists on impaired muscle proprioceptors encoding, suggesting that their actions were central.
Localizing the Actions of Quipazine and Lorcaserin

Results following ventral root section point to a central mechanism for quipazine and lorcaserin. The most likely explanation is that quipazine and lorcaserin increased output from gamma motoneurons, which extrinsically modulate muscle spindle afferent discharge (see section II). The effectiveness of both drugs to restore dynamic and static responsiveness suggests that they increased output from both static and dynamic gamma motoneurons.

Gamma motoneurons are smaller than alpha motorneurons, which in part explains their different membrane properties (Jankowska et al., 1998; Enjin et al., 2012). Despite these differences gamma motoneurons show similar firing frequencies when compared to alpha motoneurons (Jankowska et al., 1998; Enjin et al., 2012). Given that gamma motoneurons must generate repetitive trains of action potentials like alpha motoneurons, it is likely that they express voltage-gated Na$^+$ channels that carry a NaPIC. It is likely that NaPIC in gamma motoneurons is also modulated by 5-HT2 receptors.

5-HT1D has been identified as a novel marker for gamma motorneurons (Enjin et al., 2012), but there is no available data on the expression of 5-HT2 receptors. Previous studies have established that serotonin precursors (5-HTP) increase firing in gamma motoneurons (Ellaway and Trott, 1975; Myslinski and Anderson, 1978; Djupsjobacka et al., 1995; Jankowska et al., 1998), suggesting that serotonin receptors increase their output. This occurs in two different ways, by directly activating 5-HT receptors on gamma motoneurons and/or by reflex activation of gamma motoneurons mediated most likely by chemosensitive group III and IV muscle afferents (Djupsjobacka et al., 1995). Based on the similarities of alpha and gamma motoneurons 5-HT2 agonists likely
increase output by increasing PIC. Although the exact mechanism of gamma motoneuron activation by quipazine and lorcaserin is not discernable based on our results, they significantly improve muscle proprioceptor encoding impaired by riluzole and OX.

**Functional Considerations**

Gamma motoneuron input modulates muscle proprioceptor output in a task dependent manner (Prochazka and Ellaway, 2012). There are several different supported theories of how and when gamma motoneurons fire during movement: alpha-gamma co-activation, tonic- phasic gamma activation, fusimotor set, and forward sensory models (Taylor et al., 2006; Prochazka and Ellaway, 2012; Ellaway et al., 2015). Evidence for all of these theories have been identified in both animal and human studies. As described by its name alpha-gamma co-activation occurs when gamma motoneurons are activated in sync with alpha motoneurons. In this model gamma firing keeps the muscle spindles in a sensitive state to detect unexpected length changes, evidence for which has been found using microneurography (Burke et al., 1978; Aniss et al., 1990; Murphy and Martin, 1993; Kakuda et al., 1996). Other studies in cats favored a tonic phasic activation of gamma motoneurons. In this model static gamma motoneuron discharge is relatively tonic suggesting that group II afferent firing was a temporal template of the intended movement, while dynamic gamma discharge is phasic and occurs just prior to the transition from muscle shortening to lengthening (Taylor et al., 2000b; Taylor et al., 2000a; Taylor et al., 2006). This would sensitize Ia muscle spindle afferents to the beginning of muscle stretch. Similar to tonic- phasic gamma activation, fusimotor set suggests that there is tonic firing from static gamma motoneuron and that dynamic gamma activity was modulated in an activity dependent manner (Prochazka et al., 1985). Lastly forward sensory models are a recent development based on
microneurography in humans that showed that muscle spindle firing reflected muscle length changes, muscle contraction, and was predictive of future movements (Dimitriou and Edin, 2010). The wide variety of evidence for gamma motoneuron function suggests that their output to muscle spindles is linked with activation of alpha motoneurons, and dependent on the motor task (Prochazka and Ellaway, 2012).

Given the large functional impact on muscle spindle output, gamma motoneuron discharge may be able to compensate for impaired encoding following OX. This idea is supported by our results above, which suggest that increased gamma motoneuron output compensates for the encoding deficit following OX. However, this result is complicated by the fact that in our in vivo preparation, gamma motoneuron output is suppressed by anesthesia (Matthews, 1963; Haftel et al., 2004). It is evident from aim 1 that suppressed or eliminated gamma motoneuron input is not responsible for the impaired encoding deficit following OX. It may be possible however that the encoding deficit we observe would not be present in awake freely moving animals because of intact and physiologic gamma motoneuron output. Following chronic OX animals showed significant behavioral deficit and the severity of behavioral deficit was highly correlated with the amount of impaired encoding. This suggests that either normal gamma motoneuron output is not able to compensate for impaired encoding, or chronic OX also impairs repetitive firing in motoneurons. Regardless of which of these is the case, both would be significantly improved by increasing NaPIC and therefore repetitive firing.

While direct study of motorneurons following OX has not been done, recent clinical studies have highlighted motor deficits in patients following chronic OX. For example, following OX patients describe that their “legs feel heavy, like pulling a couple of logs around” (Bennett et al., 2012). These testimonials are corroborated by other studies describing fatigue and increased fall risk in patients following OX (Burakgazi et
al., 2011; Tofthagen et al., 2012; Kneis et al., 2015; Taleb et al., 2017). This may result from impaired motoneuron firing, potentially due to a decrease in NaPIC. Reduced NaPIC in motoneurons has been shown to be an important contributor to weakness and fatigue following sepsis (Nardelli et al., 2016). If alpha motoneuron repetitive firing is disrupted following chronic OX, then it is possible that gamma motoneurons may be similarly affected. An effect on gamma motoneurons would compound muscle spindle dysfunction. This suggests that lorcaserin could potentially restore muscle spindle feedback and alleviate weakness and fatigue following chronic OX.

**Optimal Therapeutic Target**

Results from aim 1 identified an encoding problem intrinsic to muscle proprioceptors that as shown above, can be fixed by extrinsically likely by increasing gamma motoneuron discharge. As discussed above patients also have motor symptoms following OX, suggesting that there might be a problem intrinsic to the motoneurons. Preliminary studies from our laboratory support the idea that following chronic OX motoneurons also have trouble repetitively firing (data not shown). This finding is similar to that in motorneurons following sepsis, which is thought to result from decreased NaPIC (Nardelli et al., 2016). While 5-HT2 agonists would likely correct this issue, which then as suggested by results in this aim would restore encoding in muscle proprioceptors, a more effective treatment would be to target NaPIC in each location. As mentioned regulation of NaPIC within muscle proprioceptors is unknown, but previous studies have shown the 5-HTP increases the output from muscle spindles that have been de-efferented by ventral root section (Ellaway and Trott, 1975). This suggests that serotonin through an unknown receptor can modulate muscle spindle output peripherally. Future studies are needed to identify and characterize modulation of NaPIC in muscle proprioceptors.
Conclusion

Results with riluzole in aim one establishes that impaired encoding intrinsic to muscle proprioceptors following chronic OX likely results from disrupted NaPIC. Application of 5-HT2 receptor agonists, quipazine and lorcaserin, which are known to increase NaPIC, significantly restored muscle proprioceptor encoding following riluzole and chronic OX respectively. Ventral root section determined that these 5-HT2 agonist likely restored encoding by increasing gamma motoneuron output.

While the restoration of la muscle spindle encoding occurred indirectly by gamma motoneuron activation, there is evidence to suggest that motoneuron function may also be disrupted following chronic OX (Burakgazi et al., 2011; Tofthagen et al., 2012; Kneis et al., 2015; Taleb et al., 2017). This suggests that disruption of NaPIC following chronic OX may extend beyond muscle proprioceptors and may help account for fatigue and weakness experienced by patients. Regardless of the exact mechanism, the effectiveness of 5-HT2 agonists at restoring encoding suggest that they could significantly improve behavioral deficits in animal and alleviate proprioceptive deficits in patients.
Fig. 10. 5-HT2 receptor agonist, quipazine significantly increase the duration of static responsiveness following 6 mg/kg riluzole. (A,B) Representative traces of action potential firing (gray lines) with instantaneous firing rates (black dots) in response to muscle stretch (A,B bottom trace; 3mm 20 mm/s). (A) Acute injection of riluzole (6 mg/kg) reduced the duration of static responsiveness in Ia muscle spindle afferents. (B) In the same animal, injection of quipazine (10 mg/kg) restored static responsiveness in Ia muscle spindle afferents. (C) Mean ±SE for duration for static responsiveness in Ia muscle spindle afferents in various treatment conditions.
Fig. 11. Fig. 10. 5-HT2 receptor agonist, quipazine significantly decreased slow dynamic threshold following riluzole 6 mg/kg. (A,B) Representative records of action potential firing (gray lines) with instantaneous firing rates (black dots) in response to ramp-hold-release muscle stretch (A,B bottom trace; 3mm 4 mm/s, 1 s hold phase). (A) Injection of riluzole (6 mg/kg) increased the slow dynamic threshold (arrowheads) in Ia muscle spindle afferents. (B) In the same animal, injection of quipazine (10 mg/kg) reduced the slow dynamic threshold (arrowheads) in Ia muscle spindle afferents. (C) Mean ± SE for duration for slow dynamic threshold in Ia muscle spindle afferents in various treatment conditions.
Fig. 12. 5-HT2C receptor agonist, lorcaserin significantly increase the duration of static responsiveness following chronic OX. (A,B) Representative traces of action potential firing (gray lines) with instantaneous firing rates (black dots) in response to muscle stretch (A,B bottom trace; 3mm 20 mm/s). (A) Chronic OX reduced the duration of static responsiveness in Ia muscle spindle afferents. (B) In the same animal, injection of lorcaserin (2 mg/kg) restored static responsiveness in Ia muscle spindle afferents. (C) Mean ±SE for duration for static responsiveness in Ia muscle spindle afferents in various treatment conditions.
Fig. 13. 5-HT2C receptor agonist, lorcaserin significantly decreased slow dynamic threshold following chronic OX. (A,B) Representative records of action potential firing (gray lines) with instantaneous firing rates (black dots) in response to ramp-hold-release muscle stretch (A,B bottom trace; 3mm 4 mm/s, 1 s hold phase). (A) Chronic OX increased the slow dynamic threshold (arrowheads) in Ia muscle spindle afferents. (B) In the same animal, injection of lorcaserin (2 mg/kg) reduced the slow dynamic threshold (arrowheads) in Ia muscle spindle afferents. (C) Mean ±SE for duration for slow dynamic threshold in Ia muscle spindle afferents in various treatment conditions.
Fig. 14. Ventral root section eliminated the effects of quipazine or lorcaserin on static responsiveness (A, B) and slow dynamic threshold (C,D). (A-D) Mean ±SE for duration for static responsiveness and slow dynamic threshold in Ia muscle spindle afferents following quipazine (A,C) or lorcaserin (B,D) before and after ventral section.
Chapter VI

Specific Aim 3: To determine if spontaneous activity following acute OX in muscle proprioceptors arises from sensory nerve terminals

Introduction

Neurotoxic side effects of the highly effective chemotherapy agent, oxaliplatin (OX) limit its clinical efficacy. OX is commonly used as an adjuvant to surgical tumor removal, and significantly improves five-year survival rates (Andre et al., 2009). However during and following cessation of treatment patients are burdened with debilitating neurotoxicity, which significantly diminishes quality of life. Within hours and for days following OX infusion, greater than 90% of patients experience abnormal sensations (paresthesia), pain (dysesthesia), muscle spasm and fasciculation, and cold induced allodynia. (Argyriou et al., 2008; Argyriou et al., 2014). These positive symptoms typically last less than one week, but re-occur and often worsen following every infusion (Park et al., 2013). The common mechanism underlying these symptoms are changes in the excitability of sensory and motor axons (Adelsberger et al., 2000; Wilson et al., 2002; Lehky et al., 2004; Krishnan et al., 2005; Benoit et al., 2006; Krishnan et al., 2006; Park et al., 2011b; Park et al., 2012a; Argyriou et al., 2013a). Conversely, accumulating doses of OX result in chronic negative symptoms including numbness and tingling in the toes and the feet, as well as sensory ataxia associated with poor balance and increased risk for falls (Beijers et al., 2014; Briani et al., 2014; Cavaletti, 2014; Avan et al., 2015).
These symptoms are thought to arise from a length dependent neuropathy (Beijers et al., 2014; Briani et al., 2014; Cavaletti, 2014; Avan et al., 2015), however recent studies have identified deficits in patients with no signs of neuropathy (Burakgazi et al., 2011). Unlike the transient effects of acute OX, these symptoms persist with little improvement years after drug cessation (Park et al., 2011a). It is unknown if there is a causal link between the acute and chronic syndromes associated with OX chemotherapy, but evidence shows a correlation between the severity of acute symptoms and the development of chronic impairment (Park 2009, Krishnan et al. 2006; Park et al. 2009b).

The positive symptoms following acute OX administration are commonly attributed increased excitability in sensory and motor axons, caused by changes in voltage-gated ion channels. For example, in vitro and in vivo studies of acute OX have shown changes in voltage-gated Na\(^+\) channels, including hyperpolarized shifts in their activation and inactivation (Adelsberger et al., 2000; Grolleau et al., 2001; Benoit et al., 2006). Studies in patients have used threshold tracking techniques to suggest changes in voltage-gated Na\(^+\) channels (Krishnan et al., 2006; Park et al., 2011b). The converging conclusion from these studies is that OX is affecting the transient voltage gated Na\(^+\) channels distributed throughout sensory and motor axons, resulting in the positive symptoms experienced by patients.

Recent studies provide insight into the proposed link between acute and chronic OX neurotoxicity. For example, genetic studies in patients have identified specific polymorphisms in the voltage-gated Na\(^+\) channel gene correlated with the severity of the acute and chronic syndrome (Argyriou et al., 2013b). Aim 1 of this dissertation identified that OX disrupts specific voltage-gated Na\(^+\) channels in muscle proprioceptors leading to impaired sensory encoding and behavior. That aim demonstrated that following a clinically relevant dosage regime of OX muscle proprioceptors displayed impaired sensory encoding; specifically they were unable to maintain repetitive firing. Further
investigation of these changes showed that this encoding deficit resulted from disruption of a subtype of voltage-gated Na\(^+\) current (persistent inward Na current) most likely located in the sensory nerve terminal. NaV 1.6, which is known to mediate a NaPIC localizes to the sensory terminal in muscle proprioceptors (Carrasco et al., 2017). This finding suggests that in addition to the dorsal root ganglion, OX may cause changes in the distal endings of sensory nerves. If acute hyper-excitability following OX is linked to the chronic loss of excitability, then there may be changes within the sensory nerve terminal following OX infusion.

The purpose of this study was to localize the changes following acute OX that are responsible for the positive symptoms experienced by patients. We applied in vivo electrophysiological techniques to localize changes in excitability following acute OX administration. Our in vivo rat model shows all of the signs of acute OX neurotoxicity including spontaneous activity in sensory axons, and muscle fasciculation with corresponding repetitive EMG bursting similar to that observed in patients. Following acute OX injection we tested the excitability of individual sensory axons and applied novel spike triggered averaging techniques to localize the origin of spontaneous firing in sensory axons and EMG bursting.

**Methods**

*Acute OX Studies*

These studies were designed to test effects of acute OX treatment on sensory and motoneurons. Oxaliplatin, (BIOTANG, Lexington, MA) dissolved in 5% dextrose was administered via intraperitoneal (IP) injection one day prior to terminal experiments. Two different dosages were used in this study 20, or 30 mg/kg. The higher dose increased the proportion of spontaneous activity available for STA analysis. Spontaneous sensory
and motor activity was observed, and STA analysis produced the same result at the 20 mg/kg dose.

**Recording Preparation/ Data Collection**

In order to sample muscle proprioceptors, dorsal roots were isolated and placed on bipolar recording hook electrodes. Rootlets were tested for orthodromic action potential volley, and stretch sensitive activity. Within dorsal rootlets individual sensory axons were penetrated with sharp glass microelectrodes filled with 2M K⁺ Acetate⁻. Sensory axons were randomly sampled and selected for recording when they displayed orthodromic action potentials with a conduction delay of < 3 ms following peripheral electrical stimulation of triceps surae nerves. During spike triggered averaging experiments ventral roots were placed in continuity with bipolar electrodes for recording or stimulation to obtain the latency of the compound muscle action potential (CMAP).

**Measurements of Axonal Excitability**

As described above, individual sensory axons were impaled and identified based on orthodromic action potentials in response to stimulation of the Mg/LgS nerves. Techniques described below are the same as those previously used to measure axon parameters (Novak et al. 2009). For the purposes of this study excitability measures were only assess from group Ia and Ib muscle proprioceptors. When membrane potential was ≤-40 mV or less and action potential amplitude was ≥ 40 mV measurements of axon properties were recorded. Depolarizing, 1 ms current pulses injected every second at increments of 0.1 nA were used to determine rheobase current (the current that triggered action potentials). Hyperpolarizing of the same duration and frequency at 1-3 nA was used to determine input resistance and to evoke action potentials at anode break. Intra-axonal records of action potentials, membrane potential, current injection,
and muscle length and force changes were digitized (20 kHz), and stored on a computer for later analysis using Spike2 software.

Measurements of threshold for action potentials and amplitude were made by manually placing cursors in Spike2 at point of inflection during the rising phase of the action potential, and at its peak. The amplitude of the action potential was calculated as the different between the membrane potential and the potential at the peak of the action potential. In order to determine the maximal rate of rise during the action potential (max $\frac{dV}{dt}$), the first derivative of the action potential was taken within Spike2. Lastly, threshold was determined as the difference between the potential at which $\frac{dV}{dt}$ was equal to 100 and the resting membrane potential.

**Spike Triggered Averaging**

Spike triggered averaging techniques (STA) were used to assess the origin of spontaneous firing from muscle proprioceptors following OX. These techniques have been previously described for use in motoneurons, but the principles used here are identical (Bullinger et al., 2011a). Intra-axonal records of spontaneous and mechanically evoked action potentials from sensory axons was used to trigger an average from a bipolar extracellular electrode located peripherally at the entry zone of the Mg/LgS. In other instances, spontaneous EMG bursting was used to trigger an average from ventral roots just distal to their entry into the spinal cord. Fig. 15 shows the general experimental setup and placement of intracellular and extracellular recording electrodes.

**Results**

Intra-axonal recordings were obtained from 15 rats for analysis of axonal excitability and STA techniques. Specifically, axonal excitability was studied in 4 control
and 7 OX (20 mg/kg) rats 24 hours post injection. Five animals were used for sensory STA analysis at doses of 20 (2 rats), and 30 mg/kg (3 rats) at 24 to 96 hours post injection. Motor STA analysis was done in 5 animals 24 hours after injection of 30 mg/kg OX.

Validation of the Rodent Model

Acute neurotoxicity following OX infusion is characterized by symptoms of increased excitability in both sensory and motor systems. For days following infusion of OX, patients describe both sensory and motor symptoms including: paresthesia, cold induced pain, and muscle cramps/fasciculation (Argyriou et al., 2008; Park et al., 2009b; Park et al., 2012a).

In our rodent model we observe sensory and motor dysfunction that persists for days following acute OX injection. Fig. 16 shows examples of sensory dysfunction following OX treatment. In Fig. 16A spontaneous activity recorded from a muscle proprioceptor is shown, notice that firing continues despite shortening the muscle and removing all force. Unprovoked firing in muscle proprioceptors is the proprioceptor correlate of paresthesia arising from cutaneous afferents in patients (Argyriou et al., 2013a). A correlate to cold induced symptoms is shown in Fig. 16B application of room temperature mineral oil to the triceps surae muscles resulted in burst of activity recorded intra-axonally from a la muscle spindle afferent. Lastly, Fig. 16C shows spontaneous muscle activity similar to that observed in patients. Similar to the motor signs of hyper-excitability in patients, EMG showed repetitive (Fig. 16C, top trace) discharge with corresponding force production (Fig. 16C, bottom trace). These electrophysiological measures fit well with patient described muscle fasciculation and cramping following OX infusion (Wilson et al., 2002; Lehky et al., 2004). Therefore our model of acute OX
mimics the clinical presentation following OX infusion and suggest that findings in our rodent model will be highly relevant to patients.

**Additional Signs of Hyperexcitability**

In addition to the signs of hyperexcitability shown above, following OX treatment bursting activity was observed in numerous cells. Similar to Fig. 16 above, Fig. 17A and B show bursting activity in muscle proprioceptors without stimulation. In Fig. 17A the brief burst of repetitive firing persist during muscle shortening (removal of stimulus). Fig. 17B shows a robust increase in repetitive firing for roughly 20 sec before returning to a baseline despite no stimulation (muscle stretch). Bursts of activity can also be induced by muscle stretch. Fig. 17C and D show bursts of activity elicited by either ramp-hold-release or triangular muscle stretch that persist after after the muscle stretch paradigm is removed. In fact, during these burst of activity the axons become unresponsive to additional muscle stretch stimuli suggesting that their origin may be from the sensory terminal responsible for generating action potentials.

**Axonal Excitability**

Hyperexcitability in muscle proprioceptors qualitatively mimics changes in excitability that result in the positive symptoms in patients. The converging hypothesis from other studies is that acute OX alters axonal voltage-gated Na\(^+\) channels. In order to assess changes in Na\(^+\) channels we tested the excitability of individual sensory axons in vivo. Table 2 compares axonal parameters in control and acute OX animals. Previous studies have demonstrated that OX shifts the activation and inactivation of voltage-gated Na\(^+\) channels to more negative potentials. This shift would move threshold closer to resting membrane potentials and would reduce the overall amplitude of Na\(^+\) current. Fig. 18 looks at the relationship between resting membrane potential and
the potential at which threshold is reached. As membrane potential becomes more negative there is less build up of voltage-gated Na\(^+\) channel inactivation which results in a lower threshold (Novak et al. 2009). Fig. 18B also shows that at a given membrane potential cells recorded from OX animals may have slightly lower thresholds than controls at a similar membrane potential. If this were the case then the millivolts needed to reach threshold should be reduced in OX cells. Fig. 18D shows the mean millivolts to threshold is slightly reduced in OX, but this did not reach significance. Additionally, consistent with previous study we found that overall the amplitude of Na\(^+\) current was reduced in muscle proprioceptors following OX. Fig. 18A shows the effects of resting membrane potential on the maximal rate of rise (max DV/DT). At similar membrane potentials, axons following acute OX showed lower max DV/DT suggesting that decreased number of voltage-gated Na\(^+\) channels were participating in the action potential. Reduced max DV/DT also accounts for the reduction in action potential amplitude in table 2. These changes may result from either decreased total numbers of channels or by a hyperpolarized shift in the inactivation when compared to control (Novak et al., 2009), which has been shown previously in acute OX (Adelsberger et al., 2000; Grolleau et al., 2001; Benoit et al., 2006). A comparison of means shows that while max DV/DT was reduced following OX, this effect did not reach significance.

Localizing the Acute Effects of OX

Our assessment of individual axon excitability following acute OX showed similar changes to those found in previous studies using in vitro or threshold tracking techniques. While these changes are consistent with the previous literature, their magnitude seemed insufficient to account for the robust spontaneous activity in muscle proprioceptors. If changes in axon excitability seem insufficient to cause spontaneous activity, then where does spontaneous activity originate?
In order to answer this question we applied novel spike triggered averaging (STA) techniques to localize the origin of spontaneous sensory and motor activity. If spontaneous sensory and motor activity arise from a single epicenter, then we should obtain a single average using STA. Conversely, if spontaneous activity arises from numerous epicenters, as suggested by an increase in axonal exitability, then we should either obtain multiple averages with different latencies, or no clear average at all.

**Origin of Spontaneous Sensory Activity**

In 14 identified muscle proprioceptors from 7 rats spontaneous firing recorded intra-axonally was used to trigger an average from the peripheral recording electrode. In 8 of these 14, stretch evoked activity was used to trigger and average from the peripheral recording electrode. The latencies of spontaneously, and stretch evoked, and electrically evoked (conduction delay) events were compared to determine the the origin of spontaneous activity following acute OX. Fig. 19A and B shows two muscle proprioceptors in which STA on spontaneous and stretch evoked activity was obtained. In both cases spontaneously evoked STA latency occurs in the negative time window, meaning that the spontaneous action potentials crossed the peripheral recording electrode prior to being recorded intra-axonally in the dorsal roots. Comparison of these latencies in each muscle proprioceptor with the latency of stretch evoked activity and the electrically evoked delay confirms that spontaneous activity arose from a peripheral origin, most likely the sensory nerve terminal. Fig. 20 compares the mean latencies for spontaneously and stretch evoked STA, and the electrically evoked delay. Electrically evoked delays were consistently the shortest, followed by spontaneous and then stretch evoked. The slightly longer latency in stretch evoked may result from the relatively higher firing rates achieved in response to stretching the muscle as compared to spontaneous activity. Also, stretch evoked delays may have been effected by the act of
stretching which moves the peripheral electrodes. Regardless, in no case was the spontaneously evoked average faster than the electrically evoked delay for the same muscle proprioceptor and there was no significant differences between groups.

STA analysis suggests that spontaneous activity in muscle proprioceptors is arising from a peripheral epicenter, most likely the sensory nerve terminal. In order to validate the STA method we applied the potent Na$^+$ channel blocker bupivacaine locally at the entry zone of the triceps surae nerves. If activity is arising from a peripheral epicenter, than blocking action potential initiation peripherally should eliminate activity recorded from the dorsal roots. Fig. 21A and B shows two examples of the effects of locally applied bupivacaine on spontaneous activity in a muscle proprioceptors. In both cases spontaneous firing ceases within approximately 30 seconds. Conduction blockade was confirmed in the second case (Fig. 21C) by failure to record action potentials in response to muscle stretch. Results with bupivacaine confirm those obtained by STA, that spontaneous activity in muscle proprioceptors arises from a peripheral epicenter.

**Origin of Spontaneous Motor Activity**

In order to determine the origin of spontaneous muscle activity, we used spontaneous EMG activity to trigger an average into ventral roots. If spontaneous muscle activity results from aberrant motoneuron firing, then we should detect an average in the negative time window at a latency consistent with the delay of the compound muscle action potential. In 5 animals, spontaneous EMG activity was used to obtain 8 averages from from ventral roots. Fig. 22 shows two of these examples with their corresponding EMG and force traces. In Fig. 22A an average from that contained two peaks with latencies of 3.2 and 3.5 ms. Fig. 21B shows another example of an average containing two peaks with latencies of 2.9 and 3.3 ms respectively. The two
different peaks in each of these examples likely arise from two different motor units with slightly different conduction velocities.

The total range of STA latencies observed was from 2.02 to 4.1 ms, which is consistent with the range of delays observed in the CMAP. All of these averages were in the negative time window, meaning that the activity crossed the ventral root electrode prior to being recorded by the EMG electrodes. Thus following acute OX spontaneous motor activity arises from a spinal cord epicenter, most likely the motoneuron.

STA analysis demonstrates that spontaneous EMG activity results from motor unit firing evident by the averages obtained within ventral roots. In order to validate the STA technique we paired down the ventral root until discriminable activity was evident. Fig. 23A shows the resulting ventral root record with clear activity (arrowheads). This activity then traveled to the periphery and activates the muscle, resulting in the EMG trace shown in Fig. 23B (3.8 ms delay). Activation of this motor unit causes a small increase in muscle force shown in Fig. 23C.

If spontaneous muscle activity arises from motor unit firing, then blocking the acetylcholine receptor with pancuronium bromide should block neuromuscular transmission, eliminating spontaneous EMG activity. Fig. 24 shows the effect of pancuronium bromide on spontaneous EMG activity. In the first instance all spontaneous EMG activity ceases and there is no change in force (Fig. 24A). Paralysis was confirmed by lack of force generation following supramaximal stimulation of the triceps surae nerves (Fig. 24B). In a second animal pancuronium bromide was given and paralysis was confirmed by lack of force production following nerve stimulation, however spontaneous EMG activity was still present (Fig. 24C, top trace). Note that despite the spontaneous EMG activity there is no increase in muscle force (Fig. 24C, bottom trace). This result was observed in a third animal following pancuronium bromide and suggests that in addition to spontaneous motor unit activity originating in the spinal cord there is
spontaneous activity intrinsic to the muscle fiber(s), similar to myotonia (Lehky et al., 2004; Novak et al., 2009)

**Disconnecting Sensory from Motor**

The normal function of muscle proprioceptors is to provide feedback about muscle length and force to the central nervous system. In the case of the Ia muscle spindle afferent this occurs through a monosynaptic connection with alpha motoneurons. Therefore, it is possible that spontaneous activity in muscle proprioceptors is contributing to the spontaneous muscle activity observed with EMG. The converse is also true, that spontaneous motor activity could be driving the firing from muscle proprioceptors. This could occur either from the changes in muscle length and force associated with motor unit firing, or in the case of muscle spindle direct input to intrafusal muscle fibers from gamma motoneurons.

The only way to be sure that spontaneous activity in muscle proprioceptors is a result of changes within the sensory cell it to eliminate the influence of alpha and gamma motoneurons on extrafusal and intrafusal muscle fibers. This can be accomplished by either i.p. injection of pancuronium bromide, a potent inhibitor of nicotinic acetylcholine receptors, or by ventral root section. Fig. 25 shows the effects of both of these procedures on spontaneous firing following OX. Clearly, spontaneous activity persists after removal of motor influence by ventral root section (Fig. 25A) or pancuronium bromide injection (Fig. 25B). In the same cell as Fig. 25B, STA analysis shows a latency consistent with a peripheral epicenter (Fig. 25C). These results eliminate the possibility that spontaneous motor activity is driving the spontaneous sensory activity.

Conversely, in order to eliminate any effect of spontaneous sensory inputs to motoneurons we section the dorsal roots just prior to entering the spinal cord. Fig. 26 shows that even after eliminating sensory input, we still observed spontaneous EMG
activity and increases in muscle force. Just as before, that activity triggered an average in the ventral root in the negative time window. Thus, increased spontaneous activity from muscle proprioceptors is not necessary for spontaneous motor unit discharge, suggesting the OX is increasing the intrinsic excitability of motoneurons.

Discussion

Following clinically relevant doses of OX, our rodent model showed signs of sensory and motor hyperexcitability which are qualitatively similar to the symptoms experienced by patients. Our model showed spontaneous activity in muscle proprioceptors which correlates with paresthesia experienced by patients. We also observed spontaneous increases in muscle force with corresponding repetitive EMG bursting. Further investigation showed that this activity resulted from motor unit discharge and from myotonic like activity intrinsic to the muscle, both of which have been observed in patients (Wilson et al., 2002; Lehky et al., 2004). Using novel in vivo intraxonal recording techniques to assess the previously reported increase in axonal excitability (Adelsberger et al., 2000; Grolleau et al., 2001; Benoit et al., 2006; Park et al., 2011b) we found changes that seemed unlikely to account for the large amount of spontaneous activity. We then used STA techniques to determine the origin of spontaneous sensory and motor activity, and found that in both instances an epicenter consistent with the normal sites of action potential initiation and not in axons or dorsal root ganglion.

Origin of Spontaneous Sensory and Motor Activity

STA analysis demonstrates that spontaneous sensory and motor activity following acute OX localizes to a single peripheral and central epicenter, respectively. This must be the case for two reasons: 1. If a single average was obtained, then there is
a single focal point generating activity, and 2. If the latency of these averages is consistent with the normal electrically evoked delay then they must have originated at or distal the recording electrode in the case of sensory activity and at or proximal to the recording electrode in the case of motor activity. These two points eliminate the possibility of multiple epicenters along the length of the motor and sensory neuron, including the sensory neuron cell body in the dorsal root ganglion.

The origins of spontaneous sensory and motor activity were validated by further experimental manipulation. Application of bupivacaine to the nerve entry zone caused conduction block and stopped spontaneous sensory activity. This clearly demonstrated the peripheral origin of spontaneous sensory activity. Spontaneous motor activity was confirmed by fine dissection of ventral roots which allowed for single motor units to be discriminated. This activity resulted in EMG activity with normal delay and force production. Spontaneous motor unit activity was also confirmed by blocking acetylcholine receptors with pancuronium bromide. Pancuronium bromide stopped motor unit EMG bursting and revealed spontaneous EMG activity intrinsic to the muscle. This activity likely results from the firing of random muscle fibers similar to myotonia congenita (Novak et al., 2015). This finding is not surprising, as acute OX patients show signs of myotonia like symptoms, such as percussion myotonia and EMG studies have shown signs of myotonia (Wilson et al., 2002; Lehky et al., 2004). This myotonia like activity was present in 2 out of 3 rats tested, however unlike the motor unit activity it produced no detectable changes in force. This suggests that while there is likely an increase in the intrinsic excitability of muscle fibers, the spontaneous firing in motor units may have a larger role in the symptoms experienced by patients.

It is clear that both spontaneous sensory and motor activity originate from sites of normal action potential initiation. With respect to the motor side this is both the motoneuron and individual muscle fibers. This demonstrates that in addition to a
peripheral effect, OX has a significant effect within the central nervous system. Localization of spontaneous activity to these sites provides insight into the underlying mechanism(s).

**Mechanism of Spontaneous Activity**

The ability to generate trains of repetitive action potentials requires voltage-gated Na\(^+\) channels. Voltage-gated Na\(^+\) channels conduct multiple types of Na current. For instance, the transient Na\(^+\) current is responsible for the generation of action potentials, and persistent inward Na currents (NaPICs) are essential for generating trains of action potentials (Crill, 1996; Lee and Heckman, 1998, 2001; Harvey et al., 2006a). Transient Na currents are necessarily present at action potential initiation sites, the distribution of NaPIC is less obvious. However, given its essential role in repetitive firing, it or other mechanisms supporting repetitive firing must be present at every site that trains of action potentials are initiated (Lee and Heckman, 2001). The role of voltage-gated Na\(^+\) channel dysfunction following acute OX has been studied in numerous species and preparations, including human axons by threshold tracking. The prevailing conclusion from these studies is that OX effects voltage-gated Na channels leading to an increase in axonal excitability (Adelsberger et al., 2000; Grolleau et al., 2001; Benoit et al., 2006; Krishnan et al., 2006; Park et al., 2011b).

In the motoneuron, NaPIC has been shown to be essential for the initiation and maintenance of repetitive firing (Lee and Heckman, 1998, 2001; Harvey et al., 2006a). Using pharmacologic blockade, NaPIC has also been shown to be essential for muscle proprioceptor encoding (Vincent et al., 2015). Study of Na\(^+\) currents in muscle have identified a NaPIC that is present in individual muscle fibers (Gage et al., 1989). Additionally, channels like NaV 1.6, which have been shown to carry a NaPIC (Rush et al., 2007), in addition to a transient Na current, localize to the motoneuron axon initial
segment (Kole and Stuart, 2012), sensory nerve terminal of muscle proprioceptors, and cutaneous receptors (Lesniak et al., 2014; Carrasco et al., 2017). The majority of studies of acute OX neurotoxicity have identified changes in the transient Na\(^+\) current. Specifically, this is thought to occur by a shift in the activation and inactivation of the voltage-gated Na\(^+\) current, which narrows the difference between resting membrane potential and action potential threshold (Adelsberger et al., 2000; Benoit et al., 2006). In a more recent study acute OX has been shown to increase NaPIC in sensory neurons resulting in bursting (Sittl et al., 2012).

Insight into the relative contribution of transient Na\(^+\) current versus NaPIC to the hyperexcitability following acute OX comes from the types of spontaneous activity. Both sensory and motor spontaneous activity consists of bursts of repetitive firing. Fig. 17C&D shows bursts of activity set off by muscle stretch, suggesting that the depolarization resulting from muscle stretch activated a current leading to a burst of repetitive firing. Similarly, Fig. 16C shows a burst of repetitive EMG activity. As discussed above, repetitive firing relies on NaPIC, suggesting that our findings may result from changes in NaPIC.

Further support for a change in NaPIC comes from our assessment of axonal excitability. While we observed changes similar to previous studies in transient Na\(^+\) current present in axons, the magnitude of these changes seem unlikely to be the main contributor to the large amount of spontaneous activity in our model. Additionally, if changes in transient Na\(^+\) current were the sole mechanism for spontaneous activity, then we might have expected our STA results to be different. Transient Na\(^+\) current is present at every node of ranvier along the sensory and motor axon. If changes in this current would result in multiple epicenters that would either give no clear average with STA analysis, or the localization of the epicenter would be at different points along the axon. Our results with STA consistently localize to a single peripheral or central
epicenter. Therefore, while changes in transient Na\(^+\) current cannot be ruled out as a contributor to spontaneous activity, our results suggest that changes in NaPIC are the major contributor. Said in another way, spontaneous activity is present without significant changes in the transient Na\(^+\) current.

Based on previous studies and our results it is likely that acute OX causes an increase in a NaPIC present at action potential initiation sites, where it increases excitability resulting in spontaneous sensory and motor activity. With respect to spontaneous motor unit discharge this increase likely occurs at the axon initial segment. Whereas dendrites, which also have NaPIC (Prather et al., 2001; Heckman et al., 2003; Heckmann et al., 2005), may contribute to spontaneous activity initiated by synaptic drive, our results demonstrate that dendritic NaPIC activated by synaptic input is not necessary given that motor unit bursting persists after removing a large amount of synaptic input by dorsal root section.

Spontaneous, myotonia like activity in muscle fibers likely arises from an increase in the NaPIC normally found in skeletal muscle (Gage et al., 1989). Given that muscle spindles are comprised of a sensory nerve terminal that innervates intrafusal muscle fibers it is possible that some sensory activity could arise from spontaneous discharge and contraction of the intrafusal fibers they innervate. While this could contribute, the majority of activity likely arises from increased NaPIC that is present and essential for normal encoding in the sensory nerve terminal.

**Conclusion**

OX infusion results in debilitating sensory and motor symptoms. We have demonstrated that our rodent muscle proprioceptor model shows qualitatively similar types of spontaneous activity in sensory and motor systems. Following acute OX, we observed spontaneous activity in muscle proprioceptors, EMG bursting, and
temperature dependent increases in activity. Previous studies have suggested that these symptoms arise from changes in voltage-gated Na$^+$ channels present in sensory and motor axons (Adelsberger et al., 2000; Grolleau et al., 2001; Wilson et al., 2002; Benoit et al., 2006). We tested this hypothesis by assessing the excitability of individual axons using in vivo intra-axonal recordings. The changes, while similar to previous studies were very small and seemed unlikely to account for the amount of spontaneous activity we observed. Further analysis using novel STA techniques localized the origin of spontaneous sensory and motor activity to the normal site of action potential initiation, ie sensory nerve terminal, motoneuron, and muscle fiber. This suggests that OX targets specific mechanisms at these locations. Given that spontaneous activity consisted of bursts of repetitive firing our results suggest that acute OX may increase NaPIC present at action potential initiation sites. This finding fits well with results from aim 1, which suggests that chronic OX effects NaPIC present at the sensory nerve terminal. Overall our report provides insight into the effects of acute OX and suggest that changes in NaPIC may link acute and chronic changes following OX.
Fig. 15 Experimental Setup. Evoked and spontaneous action potentials were recorded intra-axonally within dorsal roots. These action potentials were used to trigger an average from the peripheral bipolar electrode. The latencies of electrically, stretch evoked and spontaneous averages will be compared. Spontaneous EMG spikes recorded from the triceps surae were used to trigger averages from ventral roots. The latency of these averages were compared with the compound muscle action potential latency elicited by ventral root stimulation.
Fig. 16. Validation of acute OX neurotoxicity in our rodent model. Representative traces, A,B gray lines are action potentials, black dots show instantaneous firing rates. (A) Spontaneous activity from a muscle proprioceptor following OX injection. Note that the muscle proprioceptor keeps firing despite decreasing muscle length and force. (B) Burst of activity from a muscle proprioceptor following application of room temperature mineral oil to the body of the triceps surae muscle. Force trace below shows the moment when oil was applied. (C) EMG trace (top) showing repetitive motor unit discharge and corresponding force production (bottom trace).
Fig. 17. Representative examples of bursting activity following OX. Representative traces of spontaneous activity (A-D), gray lines are action potentials, black dots show instantaneous firing rates. (A) Brief bursts of activity from a muscle proprioceptor that persist despite muscle shortening. (B) Extended period of unprovoked repetitive firing. (C,D) Bursting activity in muscle proprioceptors can be initiated by muscle stretch (C) ramp hold release, 3 mm, 4 mm/s, 1 sec hold phase, (D) triangular stretch, 3 mm 4 mm/s).
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Fig. 18. Measures of axonal excitability in control and OX treated rats. (A) Plot of max DV/DT (rate of rise of the action potential) versus membrane potential. Compared to control, OX axons have lower max DV/DT suggesting lower less Na\(^+\) channels are participating in action potential formation. (B) Changes in threshold are largely attributed to changes in membrane potential. There is some tendency for OX axons to fall below the line, suggesting that they rest closer to threshold. (C) Mean ± SE comparison of max DV/DT in control and OX axons. (D) Mean ± SE comparison of millivolts to threshold in control and OX axons.
Millivolts to threshold (mV)

<table>
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<tbody>
<tr>
<td>5.3 ± 2.0</td>
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<td>6.7 ± 1.6</td>
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<td>5.5 ± 1.4</td>
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<tr>
<td>4.0 ± 1.0</td>
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<tr>
<td>5.0 ± 0.8</td>
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<tr>
<td>6.7 ± 1.7</td>
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<tr>
<td>7.0 ± 1.9</td>
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<tr>
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<tr>
<td>8.2 ± 2.1</td>
</tr>
<tr>
<td>8.9 ± 2.3</td>
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<tr>
<td>9.4 ± 2.5</td>
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Input resistance (MΩ)

Max O/D

Mho (ohm)

Anode break ap size (MΩ)

AP amplitude (MΩ)

Membrane potential (Mv)

<table>
<thead>
<tr>
<th>Means ± SD</th>
<th>Min</th>
<th>Max</th>
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<tbody>
<tr>
<td>2.2 ± 1.9</td>
<td>0.6</td>
<td>7.4</td>
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<tr>
<td>3.3 ± 1.5</td>
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<td>7.0</td>
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<tr>
<td>4.2 ± 1.0</td>
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</tr>
<tr>
<td>10.0 ± 2.1</td>
<td>4.0</td>
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Control
Table 2. Assessment of excitability parameters in axons. In control (4 rats) and 24 hours after acute oxaliplatin (20 mg/kg, 7 rats) axonal parameters were recorded intraaxonally in terminal experiments. n, number of afferents pooled within groups. Nested ANOVA and Tukey’s post hoc tests were used to test for the significance of group differences. * P < 0.05
Fig. 19. Two representative examples of spike triggered averaging in muscle proprioceptors showing spontaneous activity. Dashed line denotes time zero (time of action potential recorded intra-axonally). (A,B) Averages obtained using STA from stretch (top) and spontaneous (bottom) activity in muscle proprioceptors following OX. (C) Mean ± 95% confidence interval comparison of latencies from spontaneous, stretch evoked averages with the electrically evoked delay.
Fig. 20. Comparison of electrically evoked delay with STA latencies from spontaneous and stretch evoked activity. Mean \pm 95\% confidence interval comparison of latencies from spontaneous, stretch evoked averages with the electrically evoked delay.
**Fig. 21. Effects of bupivacaine applied locally to the nerve entry zone.** A-C gray lines are action potentials, black dots show instantaneous firing rates. (A,B) Arrowheads show when bupivacaine was locally applied. Within 30 sec spontaneous activity ceased. (C) Conduction block was confirmed in the cell shown in B, by an absence of stretch responses.
Fig. 22. STA analysis of spontaneous EMG activity. Dashed lines denote time zero (time at which the EMG spike occurred). (A,B) Two examples of averages (bottom trace) in ventral roots obtained from spontaneous EMG activity (top trace) with corresponding changes in force (middle trace).
Fig. 23. Spontaneous motor unit firing results in EMG activity and corresponding increase in muscle force. (A) Ventral root record from bipolar recording electrode shows two bursts of activity (arrowheads). (B) EMG record with activity arising 3.8 ms after ventral root activity. (C) Muscle force trace showing increases in muscle force following EMG activity.
**Fig. 24. Spontaneous muscle activity results from motor unit and muscle fiber firing.** (A) EMG record (top trace) showing spontaneous activity with corresponding changes in force (bottom trace). Following injection of pancuronium bromide EMG activity disappears. (B) Following pancuronium bromide injection supramaximal stimulation of the peripheryal nerve (arrowheads) results in no increase in muscle force. (C) Following paralyzation with pancuronium bromide, EMG record (top trace) showing spontaneous muscle fiber activity with no change in muscle force (bottom trace).
Fig. 25. Elimination of motor influence had no effect on spontaneous activity in muscle proprioceptors. (A) Ventral root section prior to recording did not prevent spontaneous firing. (B) Similarly, paralyzation with pancuronium bromide had no effect on spontaneous activity. (C) In the same cell as (C), pancuronium injection had no effect on the STA latency.
Fig. 26. Elimination of sensory input had no effect on spontaneous muscle activity. Cutting dorsal roots did not eliminate spontaneous EMG bursting (top trace) or force production (middle trace). STA analysis of EMG record produced an average (bottom trace) from ventral roots.
Chapter VII

Conclusions

Adjuvant treatment with OX chemotherapy results in neutotoxic side effects which greatly impact the lives of patients during and long after cessation of treatment. Given the relatively high disease free survival rate following colorectal cancer, these neurotoxic side effects force patients to endure a lower quality of life. Aims in this dissertation specifically target acute and chronic OX neurotoxicity. We have identified novel mechanisms of proprioceptive deficits following chronic OX that may account for the disparity in the severity of patient reported symptoms and those predicted by physicians. Our study of acute OX has led to novel conclusions about where activity that underlies symptoms originates and how this might shed light on the underlying mechanisms.

Chronic OX Neurotoxicity

Our animal model of chronic OX replicates the clinical scenario in which patients without neuropathy develop debilitating proprioceptive deficits. Through our use of in vivo electrophysiology we identified a deficit in encoding which correlated with the severity of behavioral impairment. Through pharmacologic investigation we discovered that OX most likely disrupted a NaPIC necessary for normal proprioceptor function. This finding is novel and provides for the first time an explanation of deficits without neuropathy. Importantly this finding may also help explain the disparity in severity of symptoms described above and provides a potential therapeutic target (Fig. 27).
Targeting NaPIC was capable of restoring encoding, and because of the correlation between the encoding deficit and behavioral impairment, suggests that this treatment may alleviate proprioceptive deficits following chronic OX.

**Acute Neurotoxicity**

Our acute OX model shows qualitatively similar changes to those that underlie positive symptoms in patients. It is evident from our results and previous studies that these symptoms arise from an increase in excitability. Unlike previous studies, significant changes in axonal excitability as assessed by measurements of transient Na$^+$ current were not present. Further investigation with STA techniques localizes these changes to the normal action potential initiation sites. This and the types of activity present suggested that acute OX may be altering excitability, potentially by increasing NaPIC.

**Connecting Acute and Chronic OX Neurotoxicity**

A major goal of OX research has been to identify a causal link between acute and chronic forms of OX neurotoxicity. Previous studies have identified that the development and severity of acute symptoms correlates with the development and severity of chronic symptoms (Krishnan et al., 2006; Park et al., 2009a). Specific polymorphisms in voltage-gated Na$^+$ channels have been shown to correlate with the severity of acute and chronic OX neurotoxicity (Argyriou et al., 2013b). The studies have provided support for a link between acute and chronic OX neurotoxicity, but have failed to identify a causal link or to develop a preventive treatment.

This results presented above provide for the first time, insight into a potential unified mechanism for acute and chronic OX neurotoxicity. Results in this dissertation demonstrate two main points: 1. Acute and chronic OX neurotoxicity localize to the
sensory nerve terminal, and 2. Acute and chronic OX neurotoxicity may have opposite
effects on NaPIC. Acute OX likely increases NaPIC in muscle proprioceptors leading to
spontaneous activity for days following injection. After several injections of OX and after
treatment cessation, NaPIC is decreased in muscle proprioceptors leading to impaired
encoding.

These results suggest that NaPIC activity is being homeostatically controlled.
Regulation of NaPIC based on cellular excitability has been shown in other clinical
scenarios. For instance, following chronic spinal cord injury, in order to compensate for
loss of descending excitatory inputs and lower motoneuron excitability, there is a
clinically significant increase in PICs within motoneurons leading to spasticity (Gorassini
et al., 2004; Li et al., 2004; Murray et al., 2010; Murray et al., 2011).

We propose that during OX treatment the increased excitability and positive
symptoms experienced by patients result from increased NaPIC in sensory and
motoneurons. After multiple infusions of OX, hyperexcitability stresses sensory and
motoneurons. In an attempt to compensate the neurons reduce their excitability by
decreasing the amount of NaPIC, contributing to the negative symptoms experienced by
patients. Although not studied here, a failure to reduce excitability may result in
excitotoxicity and cell death consistent with the other proposed cause of chronic OX
neurotoxicity (Carozzi et al., 2015). Acute hyperexcitability is a potential therapeutic
target aimed at alleviating the positive symptoms experienced by patients and
preventing the development of chronic persistent negative symptoms (Fig. 27).
OX Infusion → Acute Symptoms → Chronic Symptoms

Preventative Therapy

Targeted Therapeutics

Multiple Infusions
Fig. 27. Clinical progression of OX neurotoxicity. In the majority of patients acute neurotoxicity occurs with every OX infusion. Accumulating doses of OX result in chronic neurotoxicity, which persists after treatment cessation. Aim 2 established a potential therapeutic target to alleviate chronic neurotoxic symptoms. If acute and chronic neurotoxicity are casually linked, then blocking acute changes will prevent the development of chronic symptoms.
Chapter VIII

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### Appendix A: Commonly used Abbreviations

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<th>Abbreviation</th>
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<tbody>
<tr>
<td>OX</td>
<td>Oxaliplatin</td>
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<tr>
<td>CIPN</td>
<td>Chemotherapy induced peripheral neuropathy</td>
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<tr>
<td>PIC</td>
<td>Persistent inward current</td>
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<tr>
<td>NaPIC</td>
<td>Sodium persistent inward current</td>
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<td>SNAP</td>
<td>Sensory nerve action potential</td>
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<td>STA</td>
<td>Spike triggered averaging</td>
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<tr>
<td>EMG</td>
<td>Electromyograph</td>
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<tr>
<td>I.P.</td>
<td>Intraperitoneal</td>
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<tr>
<td>Mg/LgS</td>
<td>Medial gastrocnemius/Lateral gastrocnemius and Soleus muscles</td>
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<tr>
<td>DV/DT</td>
<td>Rate of rise of action potential</td>
</tr>
<tr>
<td>DEG/ENaC</td>
<td>Mechanically gated Na⁺ channel</td>
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<tr>
<td>Peizo 2</td>
<td>Mechanically gated ion channel</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>nA</td>
<td>Nanoamps</td>
</tr>
<tr>
<td>ms</td>
<td>Millisecond</td>
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<tr>
<td>mg/kg</td>
<td>Milligrams/kilogram</td>
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<tr>
<td>NaV</td>
<td>Voltage-gated Na⁺ channel</td>
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<tr>
<td>PCO₂</td>
<td>Partial pressure expired carbon dioxide</td>
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<tr>
<td>µm</td>
<td>Micrometers</td>
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<td>Ril</td>
<td>Riluzole</td>
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<td>OX</td>
<td>Oxaliplatin</td>
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<tr>
<td>Ia</td>
<td>primary muscle spindle afferent</td>
</tr>
<tr>
<td>Group II</td>
<td>Secondary muscle spindle afferent</td>
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<tr>
<td>Ib</td>
<td>Primary golgi tendon organ afferent</td>
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