

University of Alberta

Neuronal Mechanisms of Hyperexcitability in Individuals with Spasticity after
Spinal Cord Injury and Individuals with Bruxism

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Centre for Neuroscience

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Fall 2013

Edmonton, Alberta

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ABSTRACT

Motoneuron hyperexcitability is a characteristic of several different motor disorders. We examined neuronal mechanisms of hyperexcitability in two of these disorders: spasticity after spinal cord injury (SCI) and bruxism. Involuntary muscle spasms after SCI occur as a result of uncontrolled increases in motoneuron excitability. Brainstem-derived serotonin (5HT) and noradrenaline (NA) normally facilitate motoneuron excitability and inhibit sensory transmission to motoneurons. After SCI, monoamine levels are drastically reduced below the lesion. In this thesis we first examined the role of various monoamine receptors in modulating motoneuron excitability after SCI in humans. In individuals with incomplete SCI we showed that ligand-activated 5HT₂ and NA- α 1 receptors on motoneurons activate large persistent inward currents (PICs) which drive long-lasting involuntary muscle spasms after injury. These results indicate that the residual levels of monoamines below an incomplete lesion can affect motoneuron function. In contrast, PIC activation after motor complete SCI is *solely* mediated by constitutive or "spontaneously" active 5HT₂/NA- α 1 receptors. The emergence of constitutively-active receptors after motor complete SCI appears to be an adaptive mechanism to recovery motoneuron excitability in response to a severe loss of 5HT and NA. Although the emergence of constitutively-active receptors is beneficial in restoring lost motoneuron function after injury, it also contributes to spasticity when combined with the disinhibition of sensory afferent transmission. Sensory afferent transmission is normally inhibited by the activation of 5HT₁ receptors located on excitatory interneurons and sensory afferent terminals. We examined in motor complete SCI participants whether application of a 5HT₁ agonist, zolmitriptan, could restore lost inhibition after injury. Zolmitriptan effectively restored inhibition of sensory transmission to the motoneurons and reduced the triggering of PIC-mediated spasms. Lastly, we

examined whether enhanced PIC activation contributed to sustained masseter muscle activity in persons suffering from bruxism. Periods of involuntary masseter activity occur during sleep microarousals where monoaminergic drive to motoneurons is increased. Despite the increase in PIC-activating monoamines during periods of bruxism, we did not find enhanced PIC activation in individuals with bruxism when compared to age-matched controls. Further work is needed to elucidate the mechanisms behind this disorder.

ACKNOWLEDGEMENTS

First and foremost I would like to thank Monica Gorassini for being a wonderful supervisor, but above all, an amazing mentor. Without Monica's guidance I would not have been able to produce the body of work that is presented in this thesis. She is not only an extremely talented researcher but she is also a very generous, understanding and down to earth person. She has shown me that it is possible to be successful at what you do while also enjoying life to the fullest and that lesson is something I plan to live by! I can only hope that I can follow in her footsteps as I move forward in both my professional and personal life. Grazie tantissimo Monica!

A special thank-you goes out to Jennifer Nevett-Duchcherer as well. It takes an extraordinary individual to constantly deal with the administrative headaches-and yes I caused some over the last 5 years!-so thank you Jenn! It didn't go unnoticed. In addition to your assistance in the lab I also enjoyed our daily "girl chat". I will miss our morning chats when I move on! To the Gorassini lab members, Ephrem Zewdie and Elizabeth Condliffe, I thank you for making the lab environment so enjoyable to work in. You are an awesome bunch of people who were always willing to help, to share ideas and to simply have fun.

I would like to thank my supervisory committee members: Dave Collins, Karim Fouad, Kelvin Jones and Christine Thomas. Dave and Karim, you were there with me from the beginning and I am so thankful for all your feedback and support throughout the last 5 years. Dave, I will never forget my time with you, Flo and your lab members (Austin, Alyssa, Matheus and Yoshi) in Australia! It was a wonderful experience with wonderful people that I will definitely always remember.

Last but not least I would like to thank my family and close friends for constantly being there for me when I needed them. I am extremely lucky to have a such a supportive network behind me-you all know who you are. I want you to know that your constant support and love truly made a difference. I love you all.

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CHAPTER 1: INTRODUCTION

A version of this chapter is in preparation for publication.
D'Amico JM and Gorassini MA. *Frontiers in Neuroscience* : Review article

1.1 Foreword

Neuronal hyperexcitability can be implicated in various pathological states. This thesis examines the mechanisms behind the development of motoneuron hyperexcitability in individuals suffering from spasticity after spinal cord injury and in individuals presenting with involuntary chewing and teeth grinding in bruxism. In both circumstances individuals exhibit sustained, involuntary muscle activity which can be debilitating to everyday life and in some cases, leads to chronic pain conditions. Shedding insight onto the mechanisms behind these involuntary muscle contractions may allow for the development of more effective therapeutic agents with limited unwanted side effects.

1.2 Spasticity after Spinal Cord Injury

In the months following a spinal cord injury (SCI), patients develop spasticity which is characterized by involuntary muscle spasms and muscle contractures, both of which can interfere with residual motor function and produce pain. Although spasticity can be quite debilitating, muscle spasms can have a positive impact in daily life (Satkunam 2003; Adams and Hicks 2005). Involuntary activation of paralysed muscles can allow individuals to stand when their weakness may not permit it, facilitate daily activities such as transfers and dressing and prevent pressure ulcers which occur on bony prominences as a result of immobility (Satkunam 2003; Adams and Hicks 2005). Thus, treatment for spasticity should only be considered if it interferes with residual function, produces pain or puts the individual at risk of hurting themselves (Satkunam 2003).

Spasticity is quite common after SCI, with slightly over 80% of all people sustaining a SCI developing spasticity (Maynard et al. 1990; Skold et al. 1999). Clinically, spasticity can present with muscle hypertonus, hyperreflexia, clonus and long-lasting cutaneous reflexes in response to non-noxious stimuli (Nielsen et al. 2007; Woolacott and Burne 2006; Adams and Hicks 2005; Pandyan et al. 2005; Ivanhoe and Reistetter 2004). The classical definition of spasticity by

Lance (1980) as a “velocity-dependent increase in tonic stretch reflexes to phasic stretch, in the absence of voluntary activity” (Lance 1980, 1990) has been broadened to include the various presentations of the disorder. The more updated and accepted definition by Pandyan (2005) describes spasticity as a “disordered sensorimotor control, resulting from an upper motoneuron lesion, presenting as intermittent or sustained involuntary activation of muscles” (Pandyan et al. 2005). Several changes occur after a spinal cord injury that lead to and drive the development of spasticity, ranging from general decreased inhibition to unchecked, increased excitation, which produces an environment that is ripe for the triggering and maintenance of involuntary spasms.

1.3 Current Treatment Options

1.3.1 Physical Therapy

Currently, spasticity can be treated with both physical therapy and pharmacological interventions. Physical therapy can successfully reduce spasticity and avoids the negative side effects associated with pharmacological treatments. Three approaches to physical therapy interventions include passive, active and electrical stimulation strategies. Passive interventions such as stretching (Merritt 1981), range of motion exercises (Harvey et al. 2009), prolonged standing in a frame (Odeen and Knutsson 1981) and passive cycling (Rayegani et al. 2011) have all produced significant reductions in clinical measurements of spasticity, including the Ashworth score, pendulum test and Penn spasm frequency scores. Active strategies include gait re-training (Manella and Field-Fote 2013; Dietz et al. 1994) and hydrotherapy where exercises are performed in a therapeutic pool (Kesiktas et al. 2004). Twelve weeks of body-weight supported locomotor training in participants with incomplete SCI significantly reduced ankle extensor spasticity as measured by reflex thresholds, ankle clonus and quadriceps spasm duration (Manella and Field-Fote 2013). Additionally, hydrotherapy that included 20 minutes of active exercises, in addition to typical rehabilitative interventions that included passive range of

motion exercises, significantly decreased the Ashworth and Penn spasm frequency scores, and resulted in a significant decrease in the dosage of baclofen prescribed to individuals when compared to typical rehabilitative therapy alone (Kesiktas et al. 2004).

In combination with the active strategies mentioned above, functional electrical stimulation can be used to further reduce spasticity. Functional electrical stimulation (FES) is electrical stimulation of the muscle or nerve that results in the production of functionally relevant movements (Moe and Post 1962) and is commonly used in conjunction with active cycling or rowing exercises. FES-assisted cycling produced significant reductions in the Ashworth score when compared to passive cycling (Krause et al. 2008), highlighting the increased effectiveness of active physical therapy strategies

Although FES treatment in some studies reduced clinical measurements of spasticity (Krause et al. 2008; Granat et al. 1993; Carty et al. 2013; Mirbagheri et al. 2002), FES has also been shown to increase spasticity when applied over a period of 4-8 weeks (Robinson et al. 1988; Douglas et al. 1991). The initial decrease in spasticity that occurs after a single bout of FES is thought to result from muscle fatigue given that the fatigue typically lasts for a couple of hours, occurs more rapidly during repetitive electrical stimulation than during volitional contractions (Marsolais and Edwards 1988; Riener 1999) and is more severe in paralyzed muscles (Gerrits et al. 1999, 2003). However, over time FES produces significant increases in muscle strength and volume (Granat et al. 1993; Skold et al. 2002), which in turn may produce stronger muscle spasms to worsen spasticity (Robinson et al. 1988; Skold et al. 2002).

Localized electrical stimulation, which differs from FES because it does not result in the production of functional movements, can also be used to reduce spasticity of affected muscles (Robinson et al. 1988b, Aydin et al. 2005; van der Salm et al. 2006). These electrical stimulation paradigms include direct muscle stimulation, transcutaneous electrical nerve stimulation (TENS) and epidural

spinal stimulation. Direct muscle stimulation over the belly of the triceps surae for a single 45 minute session (pulse width 0.3ms, frequency 30Hz, burst duration 4s every 4s) significantly reduced modified Ashworth scores measured in plantar flexors but the effects were short-lasting (van der Salm et al. 2006). In contrast, long-term direct muscle stimulation over the quadriceps muscle produced either increases or no changes in spasticity at 4 and 8 weeks post-intervention (Robinson et al. 1988b). More promising results have been obtained with TENS protocols which involve stimulating the nerve that innervates the target muscle (Aydin et al. 2005; Possover et al. 2010). When comparing the effects of TENS to baclofen, 15 days of TENS over both tibial nerves (15 minute sessions, pulse frequency 100Hz) was as effective in reducing lower limb Ashworth and spasm frequency scores when compared to baclofen alone (Aydin et al. 2005). Here, the reduction of spasticity was long-lasting (24hours) and long-term treatment was more substantial compared to a single session (Aydin et al. 2005), indicating the feasibility of TENS as a long-term treatment for spasticity. Epidural spinal cord stimulation, which involves implanting flexible electrodes above the dura to electrically stimulate spinal roots, has also been tested for the treatment of spasticity (Pinter et al. 2000; Barolat 1995). When proper placement of the stimulator (over L1, L2 and L3 roots) and optimized stimulus parameters are employed (frequency 50-100Hz, amplitude 2-7V, stimulus width 0.21ms), epidural spinal cord stimulation effectively reduces Ashworth and pendulum test measures and EMG activity recorded during passive flexion and extension of the knee (Pinter et al. 2000). Additionally, epidural spinal cord stimulation produced reductions in spasticity lasting up to 2 years in a larger study (Barolat 1995). However, some negative effects reported include stimulator failures and loss of effectiveness over a ~10 year period (Midha and Schmitt 1998).

Surprisingly, despite the numerous and effective physical therapy strategies to treat spasticity, individuals with SCI are typically prescribed pharmacological interventions as the first line of treatment (Rekand et al. 2012). Further studies comparing the effectiveness of physical therapy and

pharmacological treatments are needed to determine the best approach for treating and managing spasticity after SCI.

1.3.2 Pharmacological Treatments

Current pharmacological treatments for spasticity in SCI include oral administration of baclofen, tizanidine, clonidine and sodium dantrolene (Gracies et al. 1997; Nance 2001; Elbasiouny et al. 2010; Meleger 2006). The most commonly prescribed anti-spastic, baclofen, a GABA-B agonist, acts presynaptically to reduce the amount of excitatory neurotransmitter released into the synapses (Curtis et al. 1997). Baclofen can be administered orally and although it is quite effective in reducing muscle spasms, it can have detrimental side effects such as drowsiness, and can impair residual motor function such as walking in patients with incomplete SCI (Norman et al. 1998; Nance 1994). Additionally, in cases of severe spasticity, baclofen has been administered via an intrathecal pump (Stempien and Tsai 2000; Lazorthes et al. 1990; Krach 2001a). However, when baclofen is suddenly withdrawn this can result in deleterious effects such as increased spasticity, fever, altered mental state, seizures, hepatic and renal failure, brain injury and death (Awaad et al. 2012; Mohammed and Hussain 2004; Meythaler et al. 2003; Nielsen et al. 2002). Similar to baclofen, tizanidine and clonidine act to enhance presynaptic inhibition via activation of noradrenaline- α_2 receptors (Gracies et al. 1997; Nance 1994; Krach 2001b; Davidoff 1985), and similarly, negative side effects such as drowsiness, overall weakness and cardiovascular side effects have been shown (Nance 1994; Gracies et al. 1997; Krach 2001a). Site-specific relief of spasticity can be obtained through botulinum toxin injections into specific muscles. Botulinum toxin acts peripherally at the neuromuscular junction and results in decreased activation of nicotinic acetylcholine receptors (Simpson 2004; Das and Park 1989; Burgen et al. 1949). This allows a more localized reduction in spasticity, but this comes at the cost of muscle strength (Das and Park 1989). Recent research in stroke has also suggested that one month after botulinum toxin injections, there is depression

of recurrent inhibition in the motoneurons (Marchand-Pauvert et al. 2013), which ironically is counterproductive in treating spasticity. Therefore, currently there is no *ideal* pharmacological treatment available for alleviation of spasticity after SCI. Further examination of the mechanisms that lead to involuntary motoneuron activity in spasticity will provide knowledge of more suitable drug targets and allow for the development of specific anti-spastic medications with less side effects, while also providing the opportunity to specifically cater treatment to individuals based on their primary desires which may differ depending on their level of residual motor function.

Mechanisms of Spasticity after SCI

1.4 Changes in Muscle Properties

Chronically after a SCI, changes occur in the skeletal muscles below the lesion as a result of muscle disuse. These changes can produce increased passive tension of the muscles which contributes to muscle contractures (Dietz and Sinkjaer 2007). With disuse after SCI, muscle atrophy first occurs as measured by decreased fibre diameter, cross-sectional area and volume (Biering-Sorensen et al. 2009; Lotta et al. 1991; Malisoux et al. 2007; Scelsi et al. 1982). In addition to muscle atrophy, changes in the length of the muscle occur which affect the length-tension relationship of the muscle (McDonald et al. 2005; Pelletier and Hicks 2010). In individuals with contractures, as measured by decreased passive range of motion during dorsiflexion, there was an accompanying shift in the force-angle curves for the plantarflexors suggesting that muscle shortening of the plantarflexors contributes to contractures after SCI (McDonald et al. 2005; Pelletier and Hicks 2010). Additionally, it was suggested that rehabilitation programs should focus on maintaining plantarflexor muscle length to reduce contractures after SCI (Pelletier and Hicks 2010). Changes in muscle fiber composition can occur after spinal cord injury as well although different results have been obtained when examining non-spastic versus spastic muscles. Several animal and human studies have found a general transition toward myosin heavy

chain (MHC) type II fibres, specifically, a decrease in the more fatigue-resistant type I and IIa fibres with a significant increase in type II fatigable fibres (Scelsi et al. 1982; Lotta et al. 1991; Malisoux et al. 2007; Roy et al. 1992, 1984). When comparing fibre types in humans, there was a 6-fold increase in type IIX fibres in spastic muscles compared to control muscles (Malisoux et al. 2007). Additionally, after SCI there is a decrease in the oxidative capacity of muscles with a shift in metabolic profile towards the fast, glycolytic, fatigable fibres (Biering-Sorensen et al. 2009; Scelsi et al. 1982). However, in the studies described above, it is not known if the muscles studied were flaccid or if they displayed frequent muscle spasms. It is reasonable to assume that the disuse associated with flaccid paralysis and the level of muscle activity present in spastic muscles would have differential effects on skeletal muscle properties. In fact, when directly examining spastic muscles after SCI the results are contrary to those described above. Muscle fiber composition in the spastic tail muscle at different stages post-SCI in the rat confirm this notion by showing that *acutely* after injury when the tail is flaccid and spasticity has not yet developed, there is a transition from Type I to predominantly Type II fibres when compared to control animals (Harris et al. 2005). However, as spasticity develops in the tail 2 or more months after injury, the tail muscle recovers from the fibre type composition changes with a partial return of normal muscle fiber types and morphology (Harris et al. 2005, 2007). These results indicate that, similar to exercise which has also been shown to prevent the muscle fibre changes that occur after SCI (Roy et al. 1999), the muscle activity produced in spastic muscles after SCI allows retention of normal muscle fiber type composition. Therefore it is likely that muscle-fiber composition does not play a major role in spasticity after SCI in muscles that are frequently activated by involuntary spasms (Harris et al. 2007) but likely plays a larger role in more flaccid muscles.

Given the changes in muscle composition that can occur after SCI, the mechanical properties of muscle fibres, such as contractile and twitch forces (Rochester et al. 1995; Scott et al. 2006; Gaviria et al. 1999; Gordon et al. 1993; Butler et al. 2003), half-relaxation time (Hager-Ross et al. 2006; Shields 1995;

Shields et al. 1997 Rochester et al. 1995), time to peak tension (Shields et al. 1997), shortening velocity (Gaviria et al. 1999; Shields et al. 1997), force-frequency relationship (Gerrits et al. 1999, 2001, 2005 ; Thomas 1997; Hager-Ross et al. 2006) and fatigue-related properties (Scott et al. 2006; Gerrits et al. 2000; Gaviria et al. 1999) have also been reported to change. To briefly summarize these results, after SCI, muscles display *faster* contractile properties, decreased force production and less fatigue-resistance. Again, neither of the studies reported above described in detail the presence or degree of spasticity in the muscles examined. When examining flaccid tail muscle in rats after acute SCI, these muscles were similarly more fatiguable than tail muscles from normal rats. However, the spastic tail muscles displayed *slower* contractile properties than normal muscles, including longer twitch contraction and relaxation times and slower relaxation from tetanus (Harris et al. 2007). Given the absence of change in myofiber type composition in spastic rats (Harris et al. 2005), the authors attribute the change in contractile properties to impaired myoplasmic calcium buffering and/or reduced calcium sequestering into the sarcoplasmic reticulum (Harris et al. 2006). Coinciding with the rat results, large subject to subject variability in muscle contractile properties (as measured by frequency response characteristics of the ankle muscles) was found in a group of chronic SCI participants consisting of ten ASIA A participants ranging from 2-18 years post-injury (Hidler et al. 2002). When subdividing the SCI group into individuals with various degrees of spasticity, as determined by Ashworth scores, tendon tap reflexes, Penns spasm frequency scores and ankle clonus, individuals with high measures of spasticity exhibited frequency response characteristics indicative of *slow* contractile properties that were similar to those of the control group, whereas individuals with low levels of spasticity displayed faster contractile properties (in agreement with previous studies) when compared to the control group (Hidler et al. 2002). Therefore, it seems that in individuals with high spasticity, the involuntary muscle spasms help to preserve contractile properties of the muscle fibres (Harris et al. 2006; Hager-Ross et al. 2006; Hidler et al. 2002; Thomas 1997), and because there is no significant difference in contractile properties

between the high spasticity SCI group and the control group, it is likely that changes in these muscle properties do not greatly contribute to the development of spasticity after SCI (Hidler et al. 2002).

In addition to changes in muscle tissue that may occur after SCI (especially in flaccid muscles), changes in non-muscle tissue also occur when atrophic myofibers become replaced by adipocytes, collagen and amorphous substances (Olsson et al. 2006; Scelsi et al. 1982) which can increase the intrinsic stiffness of the muscle (Schleip et al. 2006; Mirbagheri et al. 2001). In support of this finding, when specifically examining the passive tension properties of spastic muscles after SCI compared to muscles from control individuals at three different levels: the whole-muscle (vastus lateralis) *in vivo*, the single muscle fibre and the isolated myofibril *in vitro* (Olsson et al. 2006), it was found that despite increases in passive tension at the whole muscle and muscle fibre level (specifically Type IIX fibres only) there were *no* changes in passive tension at the myofibril level (Malisoux et al. 2007; Olsson et al. 2006). In accordance with the myofibril findings, the muscle protein titin, which is a main contributor to passive tension (Labeit and Kolmerer 1995; Horowitz et al. 1986), remained unchanged in spastic SCI muscles when compared to control muscles, as did the properties of the intermediate filaments (Olsson et al. 2006). Given the above findings, it has been hypothesized that the increased passive tension evident at the individual muscle fibre and whole muscle levels is most likely due to the replacement of myofibrils by amorphous substances (e.g. collagen, connective tissue: Olsson et al. 2006; Katz and Rymer 1989; Scelsi et al. 1982) as muscle atrophy occurs rather than due to changes at the myofibril level (e.g sarcomere length unchanged) (Olsson et al. 2006).

Several studies have focused on the changes in muscle properties that occur after injury but how this relates to the development of spasticity after SCI is unclear. Although the contribution of muscle and other tissue properties to spasticity is undoubtedly important, in this thesis I will mainly focus on the

neuronal mechanisms behind the development of involuntary muscle spasms after SCI.

1.5 Neuronal Mechanisms

1.5.1 Spinal Shock Acutely after Injury

Acutely following a SCI, the spinal cord enters a quiescent, areflexic state termed "spinal shock" (Dietz 2010; Ditunno et al. 2004; Hiersemenzel et al. 2000; Ashby et al. 1974). In the weeks and months that follow, the spinal cord transitions through 4 phases: an areflexic state, reflex return and presentation of abnormal reflexes, early hyper-reflexia and late hyper-reflexia (Ditunno et al. 2004). During the areflexic state there is complete muscle paralysis, flaccid muscle tone, loss of tendon reflexes and loss of sensation caudal to the lesion (Bastian 1890). This areflexic period is physiologically characterized by motoneuron hyperpolarization (Schadt and Barnes 1980) due to significant, abrupt disruption in descending background activity to the motoneurons (Ashby et al. 1974). Specifically, the disappearance of dendritic, voltage-activated sodium and calcium persistent inward currents (PICs) in the motoneurons greatly reduces motoneuron excitability.

PICs are comprised equally of a TTX-sensitive persistent Na current, and a low-voltage activated (LVA), slowly inactivating nimodipine-sensitive L-type Ca current (CaV1.3) (Li and Bennett 2003). Due to their low threshold, PICs can be activated sub-threshold to cell firing and dramatically amplify synaptic input to the motoneuron at recruitment (Lee and Heckman 2000). PICs produce a sustained depolarization, or plateau potential, which can last for many seconds to produce self-sustained firing, i.e., firing that occurs with synaptic inputs that are lower than that needed to initially recruit the motoneuron (Bennett et al. 2004; Heckman et al. 2005; Hounsgaard et al. 1988; Hultborn et al. 2003; Lee and Heckman 2000). Self-sustained firing is mainly fueled by the calcium PIC (CaPIC) since it inactivates more slowly than the NaPIC, the latter normally

inactivating over the first few seconds in a plateau potential (Li and Bennett 2003). However, due to its quick re-activation properties, the sodium PIC (NaPIC) can contribute to slow, steady rhythmic firing as it regeneratively helps to accelerate the rate of rise of the membrane potential on the upswing of the afterhyperpolarization (AHP) to trigger cell firing (Harvey et al. 2006a; Lee and Heckman 2001; Miles et al. 2005). Because PICs provide substantial amounts of depolarization to the motoneuron, their immediate loss after injury significantly reduces spinal excitability as can be seen in acutely-spinalized rats that are areflexic in response to brief dorsal root stimulation, despite the activation of long excitatory postsynaptic potentials (EPSPs) which appear acutely after injury (Fig. 1-1: Li et al. 2004a).

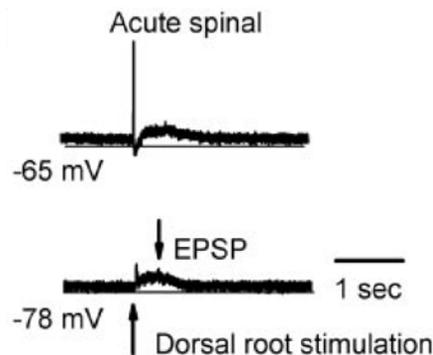


Figure 1-1 Spinal shock in acutely-spinalized rat

Motoneuron recording in response to dorsal root stimulation in an acutely-injured rat. Note the absence of a reflex response even at resting membrane potential (top panel) due to elimination of PICs acutely after injury. The long-duration motoneuron EPSP (~1s) appears acutely after injury. From Li et al. 2004.

1.5.2 Re-emergence of PICs in Chronic SCI

In the days and weeks that follow a SCI, corresponding to phases 3 and 4 of Ditunno's model, the spinal cord regains excitability, demonstrated by increased H-reflexes, increased F-wave persistence and the return of flexor reflex responses (Hiersemenzel et al. 2000; Leis et al. 1996). Interestingly PICs also re-emerge during these phases and are most likely the major contributor to the overall increase in motoneuron excitability that occurs chronically after injury. In

a chronically injured rat, the same brief sensory stimulation that was applied to the dorsal roots of the acutely-injured rat in Figure 1-1 now evokes long-lasting (>5s) reflex responses or spasms (Fig. 1-2: Li et al. 2004a). The spasm is mediated by the underlying plateau potential provided by activation of the PICs because when the PICs are deactivated by hyperpolarizing the motoneuron, the self-sustained firing disappears and all that remains is the long (200-900ms) polysynaptic EPSP component that was similarly present in the motoneuron from an acutely-injured rat. Elimination of the plateau-mediated spasm can also be produced by blocking the Na and Ca PIC components via TTX and nimodipine respectively (Li et al. 2004a). It is worth noting that increased self-sustained firing produced by the re-emergence of PICs coincides with the development of spasms in chronically-injured animals since acutely-spinalized animals with no PICs do not yet display prolonged muscle spasms. Based on the above animal studies, the reemergence of PICs seems *essential* to the production of involuntary, sustained muscle spasms in response to brief innocuous stimulation, a clinical symptom that can also be seen in people suffering a SCI.

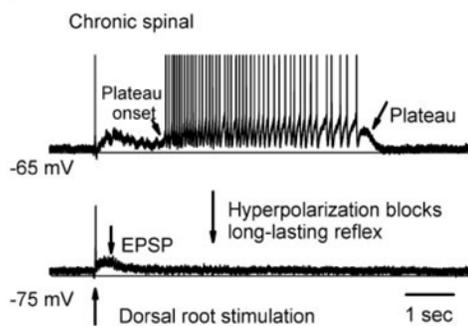


Figure 1-2 Re-emergence of PICs after chronic SCI in the rat

Dorsal root stimulation applied to a motoneuron from a chronic spinal rat. At resting membrane potential, PIC activation produces a plateau potential and self-sustained firing in response to stim.

Bottom panel: Hyperpolarization of the motoneuron deactivates the PICs eliminating the spasm. Note the similar long EPSP. From Li et al. 2004.

1.5.2.1 Estimating Persistent Inward Currents in Humans

The use of paired motor unit recordings in humans has allowed for indirect evidence that PICs contribute to the involuntary activation of motoneurons during muscle spasms in chronic SCI. The basis of estimating PIC activation using the firing profiles of two motor units (motoneurons) is shown from direct intracellular

recordings in the rat (Fig. 1-3, Gorassini et al. 2004). The amplitude of the PIC can be estimated indirectly during current clamp when the motoneuron is activated by a triangular current profile (Bennett et al. 2001) as follows. During the ascending phase of the injected current, there is an acceleration in the membrane potential of the test motoneuron just prior to the initial spike (arrow: Fig. 1-3A), demonstrating that the PIC is activated just before or near the time of motoneuron recruitment (at a level of current marked by the first dashed line: Fig. 1-3A). Once activated, the added depolarization provided by the PIC allows the motoneuron to fire at levels of injected current (input) below that needed to recruit the motoneuron (below top horizontal line: Fig 1-3A), with cell firing stopping only when the injected current is substantially reduced (at second dashed line). The difference in injected current between derecruitment and recruitment of the motoneuron (ΔI) is then used as a measure of the PIC amplitude because it is the amount of current that needs to be removed to counteract the added depolarization provided by the PIC and stop the motoneuron from firing.

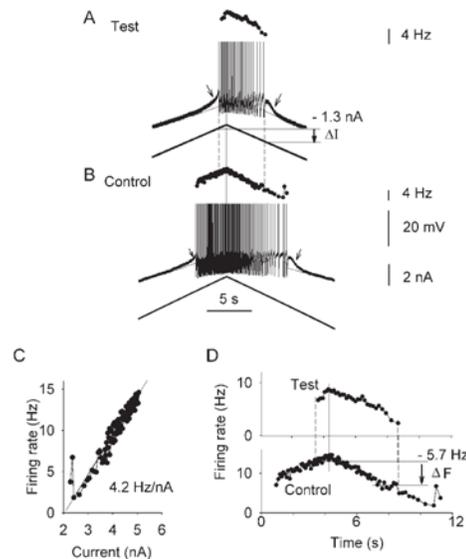


Figure 1-3 Estimating PICs in humans

A&B) Firing rate profiles of a control and test motoneuron in response to the same intracellular current ramp injection. Measurement of PIC as the difference in current input at recruitment and derecruitment of the test motoneuron (dashed lines): ΔI value. **C)** Linear relationship between the firing rate and injected current in the control motoneuron. **D)** The control motoneuron is serving as a measure of input to the test motoneuron. Measurement of the PIC as the difference in firing rate of the control motoneuron at recruitment and derecruitment (dashed lines) of the test motoneuron: ΔF value. (Gorassini et al. 2004).

In addition to using the amount of current injection as a measure of input to the motoneuron, one can also use the firing rate of a second motoneuron (control motoneuron in Fig. 1-3B) that receives a similar input as the later recruited test motoneuron. This is possible because the firing rate of a motoneuron is linearly related to the amount of current injection (or synaptic input) it receives once the PIC is fully activated, as shown in Fig. 1-3C, (see also Bennett et al. 2001; Hsiao et al. 1997, 1998; Binder et al. 1996).

Thus, when using the firing rate (F) of the control motoneuron as a measure of input to the test motoneuron, rather than the injected current (Fig. 1-3D), the difference in firing rate of the control motoneuron at derecruitment and recruitment of the test motoneuron (dashed lines) corresponds to the reduction in input that is required to counteract the added depolarization from the PIC ($F_{\text{derecruitment}} - F_{\text{recruitment}} = \Delta F$). Dividing the ΔF value (5.7 Hz) by the slope of the F-I relationship measured in Fig. 1-3C (4.2 Hz/nA) results in an estimation of current (1.4 nA) that is close to the amplitude of the PIC measured from the injected current profile (1.3nA in Fig. 1-3A).

1.5.2.2 Paired Motor Unit Analysis (ΔF Measure) in Human SCI

By using the firing rate profiles of two motoneurons (motor units) as described above, the contribution of PICs to the activation of motoneurons during involuntary muscles spasms has been estimated in participants with SCI (Fig. 1-4: Gorassini et al. 2004). Here the firing rate of a lower-threshold control motor unit from the tibialis anterior (TA) muscle that is activated by a weak voluntary contraction (Fig. 1-4 bottom trace) is used as a measure of input to the TA motoneuron pool and to a higher threshold test motor unit. As evidence of this, vibration of the TA tendon (hatched squares) produced a transient increase in the firing rate of the control unit and a transient recruitment of the higher-threshold test unit (Fig. 1-4: middle trace). Vibration of the tendon did not produce a strong

enough afferent input to evoke a muscle spasm. However, vibration of the medial arch of the foot (Fig. 1-4: black square) produced a greater increase in the firing rate of the tonically-active control unit, signifying greater synaptic input to the motor units, and consequently resulted in stable recruitment of the higher-threshold test unit. In fact, the newly recruited test unit continued to fire well after the removal of the vibratory input, and exhibited sustained firing even at levels of synaptic input (as measured by the firing rate of the control unit) well below the level needed to recruit the test unit (Fig. 1-4: difference between horizontal lines, arrow indicates ΔF ; Gorassini et al. 2004). By comparing the estimated PIC amplitude ($\Delta F = 5\text{Hz}$) to the total amount of rate modulation in the test unit during a contraction or involuntary spasm (12Hz), it is estimated that the underlying PIC contributes $\sim 40\%$ to the self-sustained firing of motor units in SCI participants (Gorassini et al. 2004) and therefore plays a major role in driving involuntary muscle spasms after injury in humans.

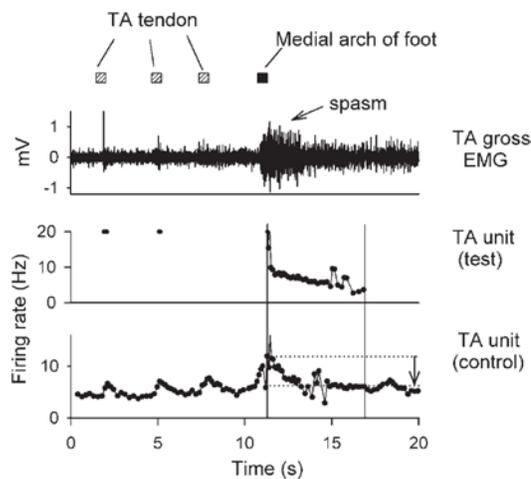


Figure 1-4: ΔF during spasms in SCI Spinal-cord injured participant: *Top trace:* tibialis anterior gross surface EMG during sustained dorsiflexion and application of transient tendon vibrations (grey squares) and vibration of the medial arch of the foot (black square). *Middle trace:* Firing rate of higher-threshold test unit in tibialis anterior in response to vibrations. *Bottom trace:* Firing rate profile of tonically active lower-threshold control motor unit in tibialis anterior. From Gorassini et al. 2004.

Using the paired motor unit technique to estimate PIC amplitude in human participants relies on the assumptions that: 1) the firing rate of the control unit is an accurate representation of input to the test unit and 2) the pair of motor units receive a common synaptic drive (Gorassini et al. 2004). To ensure that the above assumptions hold true, it is essential to pick activation trials where the onset of

discharge of the control and test motor units is separated by at least 3 seconds to ensure that the PICs are fully or nearly fully activated in the control unit when the test unit is recruited, given that it can take ~500 ms for the slow calcium component of the PIC to activate (Li et al. 2004a; Murray et al. 2011b). This ensures that any subsequent changes in the firing rate of the control unit during a spasm or contraction are indeed representative of the changes in synaptic input rather than from the rapid acceleration in the firing rate that occurs during activation of the PIC (Fig. 1-3A). Similarly, the test unit needs to be activated for a minimum of 3 seconds to ensure full PIC activation, and therefore eliminate the chance of underestimating the PICs in the test unit. Thirdly, the correlation between the smoothed firing rate of a control and test motor unit (Fig. 1-5) is measured to ensure that both units are being modulated in the same way in response to changes in synaptic input, therefore ensuring that both units are receiving a common synaptic drive. Only trials with r^2 values greater than 0.8 (e.g. Fig. 1-5) are included for analysis to ensure that the majority of the modulation in the firing rate of the test unit can be accounted for by the modulation in the firing rate of the control unit. In summary, the ΔF measures can provide a reasonable estimate of PIC amplitude from human motor unit recordings when the above assumptions are accounted for.

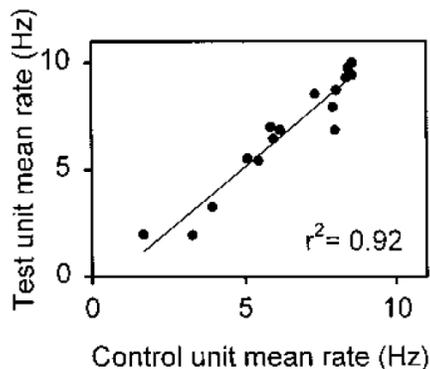


Figure 1-5 Firing rate correlation between test and control motor unit
Linear regression of the mean firing rate of the test motor unit plotted against the mean firing rate of the control motor unit (500ms bins). From Gorassini et al. 2002

1.5.3 Modulation of PICs by Monoamine Receptors After Injury

1.5.3.1 PICs Require Activation of Monoamine Receptors

In addition to being voltage-sensitive, PICs require the concomitant activation of serotonergic and noradrenergic receptors located on the motoneurons (Harvey et al. 2006a; Harvey et al. 2006b). Initial studies in the decerebrate cat illuminated the role of monoamines in PIC activation. The large PICs and sustained reflex responses present in decerebrate cats were blocked by methysergide; a serotonin (5HT) and noradrenaline (NA) receptor blocker (Hounsgaard et al. 1986; Hounsgaard et al. 1988). The 5HT and NA in the decerebrate cat likely came from axons whose cell bodies originated in the brainstem given that acutely after a complete transection of the spinal cord, in which all descending 5HT and NA input to the motoneurons is eliminated, the PICs were drastically reduced or eliminated. However, PICs could be restored via application of the 5HT precursor, 5HTP (Hounsgaard et al. 1988). Similarly, intracellular studies in turtle, guinea pig and rat motoneurons have demonstrated a role for 5HT and NA in the facilitation of motoneuron PICs (Hsiao et al. 1998; Harvey et al. 2006a; Harvey et al. 2006b; Hounsgaard and Kiehn 1989; Hsiao et al. 2005; Li et al. 2007; Conway et al. 1988).

1.5.3.2 Descending Monoamines are Reduced after Injury

As described above, monoamine receptor activation is essential for PIC activation; therefore, the immediate disappearance of PICs acutely after injury can be attributed to the sudden disruption in descending monoaminergic tracts. After a complete transection, there is a significant disappearance of 5HT and NE fibres (Carlsson et al. 1963) and within 7 and 9 days after injury, the levels of 5HT and NE are reduced to insignificant levels below the transection (Anden et al. 1964). Carlsson and colleagues were the first to identify monoaminergic tracts in the

spinal cord whose cell bodies originated in the brainstem (Carlsson et al. 1963).

Serotonin is synthesized from the amino acid L-tryptophan via the enzymes L-tryptophan hydroxylase and amino acid decarboxylase (AADC) and then stored in three different places in the body: in neurons of the CNS and intestinal myenteric plexus, in enterochromaffin cells in the mucosa of the GI tract, and in blood platelets (Feldman et al. 1997). For the purpose of this thesis we will only be focusing on neuronal, descending sources of serotonin. Serotonin exerts its actions on neuronal excitability via seven types of receptors: 5HT₁, 5HT₂, 5HT₃, 5HT₄, 5HT₅, 5HT₆, 5HT₇, with all of them being G-protein coupled receptors (GPCRs) except for the 5HT₃ receptor which is a ligand activated channel (Hoyer et al. 2002; Nichols and Nichols 2008; Boess and Martin 1994). This thesis focuses specifically on the 5HT₁ and 5HT₂ receptors. The 5HT₁ receptors are further subtyped into 5HT_{1A}, 5HT_{1B}, 5HT_{1D} and 5HT_{1F}, all coupled to the adenylyl cyclase inhibiting G-i pathway (Hoyer et al. 2002; Boess and Martin 1994). Similarly, 5HT₂ receptors are subtyped into 5HT_{2A}, 5HT_{2B} and 5HT_{2C} receptors which are all G-q coupled receptors (Hoyer et al. 2002; Boess and Martin 1994).

Noradrenaline, along with dopamine and adrenaline, is a catecholamine synthesized in the CNS, adrenal chromaffin cells and sympathetic nerves. It is converted from the amino acid L-tyrosine into L-Dopa via tyrosine hydroxylase (Kopin 1968). L-Dopa is then converted into dopamine via AADC, and subsequently into noradrenaline via the enzyme dopamine beta hydroxylase (Kopin 1968). Similar to serotonin, noradrenaline exerts its actions via three different types of GPCRs. Initially receptors were divided solely into α and β subtypes (Ahlquist 1980); however recently the α -receptors have been further subdivided into α_1 and α_2 (Bylund 1992) receptors with α_1 receptors coupled to downstream G-q pathways, similar to 5HT₂ receptors (Gershengorn 1989; Minneman and Esbenshade 1994; Ruffolo et al. 1991), and the α_2 receptors coupled to downstream G-i pathways, similar to 5HT₁ receptors (Ruffolo et al. 1991).

Given the abrupt disruption in descending monoaminergic sources and the *essential* role they play in PIC activation, it was necessary to elucidate the mechanisms behind the re-emergence of PICs chronically after injury in the absence of monoamines. 5HT and NA receptors are indeed active below a complete transection since blocking these receptors, specifically the 5HT_{2b,c} and NA α 1 receptors, with specific receptor antagonists (e.g, cyproheptadine) eliminates PIC activation in chronically spinalized rats (Fig. 1-6A: Harvey et al. 2006b; Murray et al. 2011a). This is quite interesting given the significant reduction in serotonin immunolabeling below the level of a complete transection (Fig. 1-6B: Murray et al. 2011a). Similarly there is a dramatic reduction in dopamine- β -hydroxylase labeling below the transection, indicating a dramatic reduction in noradrenaline-synthesizing neurons (Fig. 1-6B: Rank et al. 2011).

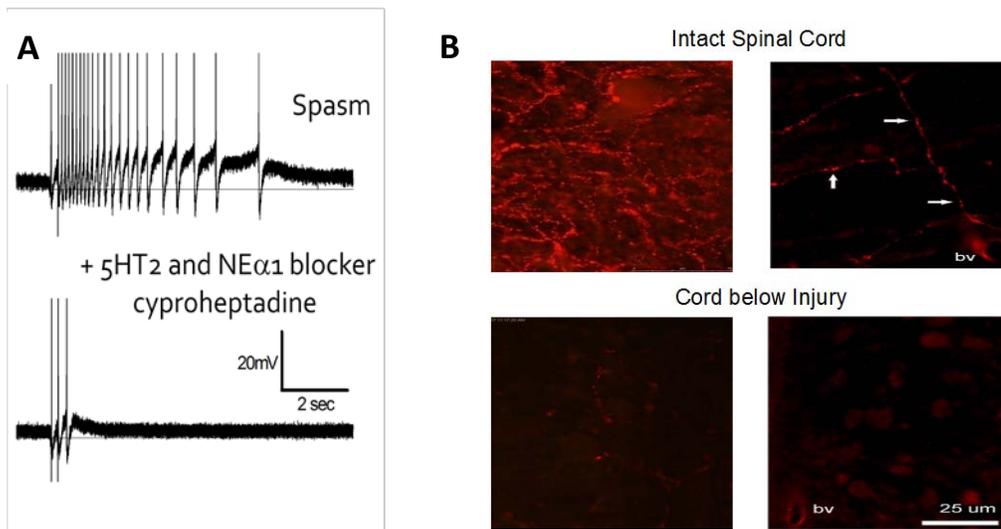


Figure 1-6 Activation of 5HT₂/NA- α 1 receptors after injury despite reduction in monoamines below injury level

A) Blocking of 5HT₂ and NA- α 1 receptors eliminates PIC-mediated spasm in a chronically-injured rat (Harvey et al. 2006) B) *Left panel:* Serotonin immunolabeling above and below a complete spinal transection in a rat (Murray et al. 2010). *Right panel:* Dopamine beta hydroxylase (D β H) staining above and below a complete transection in a rat (Rank 2011).

Thus, the next set of experiments described below examines how the recovery of motoneuron PICs in the months following a complete SCI can occur in the absence of monoamines below the injury.

1.5.3.3 Constitutively-active 5HT_{2b,c} and NA- α 1 Receptors

PIC-modulating 5HT_{2b,c} and NA- α 1 receptors are GPCRs coupled to a hetero-trimeric G protein which consists of an α , β and γ subunit (Heckman et al. 2003; Raymond et al. 2001). Activation of GPCRs induces the release of GDP and GTP that are bound to the α -subunits. The α -subunit in Gq coupled receptors, such as the 5HT₂ and NA- α 1 receptors, acts to stimulate the β isoform of the enzyme phospholipase C (PLC). This results in the hydrolysis of phosphatidylinositol bisphosphate into the second messengers inositol trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ increases calcium mobilization via release of intracellular IP₃-regulated calcium stores and DAG activates the downstream protein kinase C (PKC) (Mizuno and Itoh 2009) which subsequently phosphorylates and results in activation of channels such as the Na and Ca channels mediating the PICs. An interesting characteristic of GPCRs, like the 5HT₂ and NA α 1 receptors, is their ability to display constitutive receptor activity (Navailles et al. 2006; Berg et al. 2005; Seifert and Wenzel-Seifert 2002; Gether et al. 1997). GPCRs exist in equilibrium between inactive (R) and active (R*) receptor states. In the active state, the receptor is coupled to its G-protein and can activate downstream signaling pathways, and subsequently motoneuron PICs for example. Receptors can spontaneously enter their active state without activation by a ligand and this is termed "constitutive receptor activity". On the other hand, ligand-activated receptors can only enter their active state when the appropriate ligand binds to the inactive receptor state (Fig. 1-7).

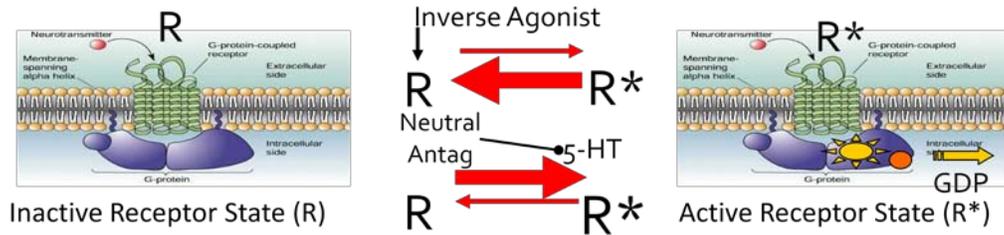


Figure 1-7: Receptor state equilibrium

Receptor state equilibrium: inactive receptor state (R), and active receptor state (R*) where the receptor is coupled to its G-protein and can activate downstream pathways. Mechanisms of action of inverse agonists and neutral antagonists Adapted from <http://nhsjs.com/wp-content/uploads/2010/02/gprotein.jpg>

5HT_{2C} and NA- α 1 receptors demonstrate a degree of constitutive receptor activity, which has been measured by basal IP₃ levels, as well as through pharmacological methods in HEK-293 cells, Cos-7 cells and in vitro preparations (Navailles et al. 2006; Berg et al. 2005; Chanrion et al. 2008; Herrick-Davis et al. 2000; Rauser et al. 2001). To examine whether constitutive activity in 5HT₂ and α 1 receptors arises after spinal cord injury, and therefore explains the re-emergence of PICs, the effects of two types of antagonists on PIC activation was examined in completely transected rats (Murray et al. 2010): an inverse agonist and a neutral antagonist (Figs. 1-7 and 1-8).

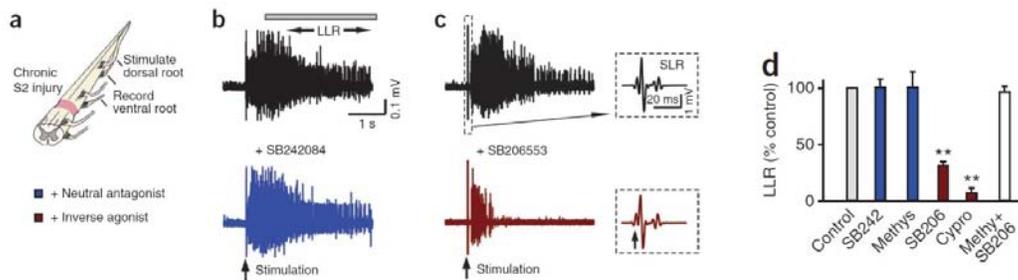


Figure 1-8: Constitutive receptor activity in chronic SCI rat

A) Experimental set-up: In-vitro ventral root recordings in response to brief dorsal root stimulation in chronically spinalized (S2) rats. **B)** PIC-mediated long-lasting reflex (LLR) before (black trace) and after SB242084 (blue trace: neutral antagonist). **C)** Evoked LLR before (black trace) and after SB206553 (red trace: inverse agonist). **D)** Group means of the LLR (normalized to predrug levels % control) after application of the neutral antagonists (blue), inverse agonists (red) and both (white). From Murray et al. 2010.

An *inverse* agonist stabilizes the resting state of a receptor (R), thereby altering the equilibrium between receptor states and subsequently reducing *both* constitutive receptor activity *and* conventional ligand-activation of the receptor (Chanrion et al. 2008; Herrick-Davis et al. 2000; Westphal and Sanders-Bush 1994) (Figs. 1-7 & 1-8). The 5HT₂ and NA- α 1 inverse agonists cyproheptadine and SB206 effectively reduced PIC-mediated, long-lasting reflexes in chronically-spinalized rats (Murray et al. 2010; Fig. 1-8C&D: red traces/bars). In contrast, the 5HT₂ and NA- α 1 *neutral antagonists* methysergide and SB242, which do not affect the equilibrium between receptor states and only block the ligands/agonists from activating the receptor, had *no effect* on PIC-mediated responses (Fig. 1-8B&D: blue traces/bars). By utilizing both types of antagonists it was possible to determine the presence of constitutive receptors as follows. Since the *inverse agonist* reduced 5HT₂ and NA- α 1 receptor activity and its downstream effects (e.g., reduction of PIC-mediated long-lasting reflexes) and the *neutral antagonist* had no effect, then the effects of the inverse agonists can be solely attributed to blocking constitutively-active receptors below the lesion. Additionally, similar results were obtained when examining more specifically the constitutive receptor activity of NA- α 1 receptors after injury: the inverse agonists prazosin (NA- α 1) and WB4101 (NA- α 1) significantly reduced PIC-mediated responses in chronic spinal rats whereas the neutral antagonists methysergide (5HT₂/NA α 1), and REC (NA α 1) had no effect (Appendix: Murray et al. 2010; Rank et al. 2011). Additionally, mRNA analysis revealed an upregulation in the isoform of the 5HT_{2C} receptor that displays the highest degree of constitutive receptor activity (Murray et al. 2010). In **Chapter 2** we examine whether constitutively-active receptors mediate PIC activation and the production of muscle spasms after *incomplete* and *complete* spinal cord injuries in humans. We expect that the mechanisms behind the facilitation of PICs and subsequent spasticity will be different in these two populations due to varying injury severities and subsequently the amounts of residual monoamines available below the injury.

1.5.4 Changes in Sensory Transmission to Motoneurons after SCI: Emergence of Long-duration EPSPs

As demonstrated in Figures 1-1 and 1-2, activation of afferents evokes prolonged NMDA-mediated EPSPs in motoneurons immediately following a spinal cord transection, (Bennett et al. 2001a) that can trigger the recovered PICs in chronic animals and produce involuntary muscle spasms. The duration of these long EPSPs in response to single-shock stimulation of cutaneous afferents is approximately 740 ms in acutely-injured and 960 ms in chronically-injured animals (Li et al. 2004a). Similar results have been obtained in response to sural nerve stimulation in motoneurons of chronically spinalized cats (Baker and Chandler 1987). As mentioned earlier, the emergence of long EPSPs is not sufficient to mediate prolonged involuntary muscle spasms; however, they are essential to trigger the slow-activating calcium PIC which can take up to 500ms to fully activate (Li et al. 2004a; Moritz et al. 2007).

Similar to the above results in spinalized animals, brief sensory activation evokes long EPSPs in individuals with SCI. Two methods exist to measure postsynaptic potentials (PSPs) from motor unit recordings in human participants. The post-stimulus time histogram (PSTH) plots the probability of a motor unit firing and is time-locked to the sensory stimulus. Because this technique only measures probability, it is limited by count and synchronization errors since the probability of a motoneuron firing is not only dependent on the underlying membrane potential but also on the firing history of the motoneuron, which can be affected by refractory periods in the AHP (Turker and Cheng 1994; Turker et al. 1997). A second method, which has been demonstrated to more accurately represent the underlying PSP in motoneurons is the peristimulus frequencygram (PSF) technique (Turker and Powers 1999; Turker and Powers 2003). With PSFs, the amplitude, shape and duration of motoneuron PSPs are estimated by recording changes in motor unit firing rates in response to brief afferent stimulation while participants perform a steady background contraction. The instantaneous firing rates of tonically active single motor units are then plotted time-locked to a

sensory stimulus (Fig. 1-9). Because the firing rate of a motoneuron, for the most part, reflects the net current reaching the soma (Baldissera et al. 1982; Powers et al. 1992), any subsequent change in firing rate in response to the stimulus should reflect the underlying shape of the PSP, especially for PSPs lasting >300ms. PSFs therefore avoid the synchronization and count errors that can occur with PSTHs. PSFs in response to brief (<20ms) stimulation of the medial arch of the foot have been obtained in both non-injured control and SCI participants (Fig. 1-9, Norton et al. 2008). In an SCI participant, the firing rate of a tonically active motor unit increased above baseline for a duration of ~1s in response to stimulation of the medial arch (Fig. 1-9A Norton et al. 2008). This is most likely due to an EPSP lasting for ~1s, similar to that measured in motoneurons in chronically spinalized rats (Fig. 1-9B above). In comparison, in a control participant (Fig. 1-9B), the PSF displayed an overall increase in the firing rate of the motor unit above the pre-stimulus rate that lasted for only ~300ms with a pause in the firing rate after the initial acceleration and an interposed cluster of action potentials at firing rates near/or below the mean background rate (red circle: Fig. 1-9B Norton et al. 2008). This profile was predicted to result from a 300ms EPSP with a fast intervening IPSP. To confirm the above hypothesis, a current profile was injected into a rat motoneuron (inset Fig. 1-9C) to produce the hypothesized PSP (grey trace: Fig. 1-9C) as determined when cell firing was prevented by hyperpolarization. When the membrane potential was returned to rest and the cell was allowed to fire, the firing rate of the motoneuron produced a PSF profile (Fig. 1-9C) similar to that observed in the control participant in Fig. 1-9B confirming the above prediction. In summary, the PSF profiles of SCI participants differed significantly from uninjured participants in two aspects: the duration of the EPSP was prolonged by ~700ms and an intervening IPSP was absent in SCI participants. These results suggest an overall increase in the excitability of excitatory networks and/or a general reduction in the excitability of inhibitory networks after SCI in humans.

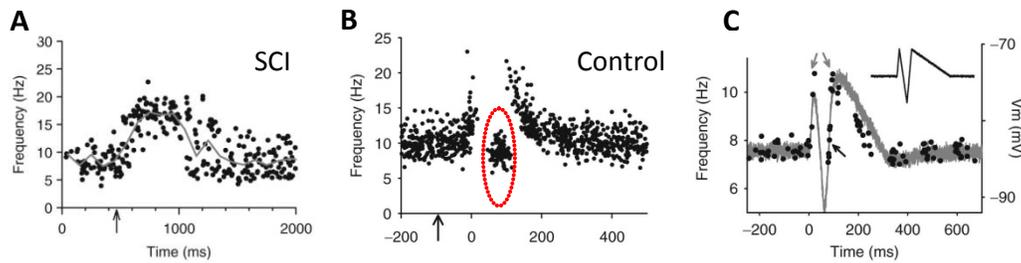


Figure 1-9: Peristimulus frequencygrams in SCI and control participants
 PSF profiles obtained from **A)** an incomplete SCI participant **B)** an uninjured control participant **C)** a rat motoneuron that mimics the PSF profile obtained in the uninjured control participant. Time of stimulation marked by arrow in A and B. The intracellular current injection required to obtain this profile is shown as an inset and the response to this profile in a hyperpolarized motoneuron is shown by the grey trace. From Norton et al. 2008.

1.5.5 Spinal Inhibitory Pathways after SCI

Although the animal and human studies described above mainly examined changes in transmission of cutaneous afferent pathways to motoneurons after injury, transmission to motoneurons from other sensory afferent pathways is also altered after SCI (Fig. 1-10). Generally, there is an overall increase in transmission of excitatory pathways and a reduction in the transmission of inhibitory pathways to the motoneurons.

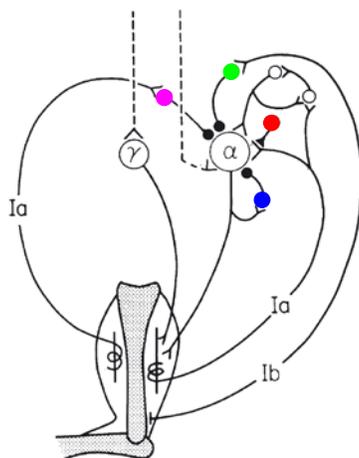


Figure 1-10: Spinal inhibitory circuitry
 Diagram of various spinal pathways to the motoneurons with different interneurons denoted by coloured circles. Renshaw cell (blue circle), Ia interneuron (pink circle), Ib interneuron (green circle) and PAD interneuron (red circle). Adapted from Nielsen et al. 2007.

1.5.5.1 Presynaptic Inhibition

Presynaptic inhibition effectively modulates the efficacy of afferent transmission to the motoneurons via primary afferent depolarizing interneurons (PAD INs, red circle, Fig. 1-10). Although presynaptic inhibition can occur at all afferent terminals, studies in humans have mainly focused on presynaptic inhibition of Ia afferents. The mechanisms of presynaptic inhibition involve the activation of fast, ionotropic GABA_A receptors and slow, metabotropic GABA_B receptors. Activation of GABA_A receptors on primary afferent terminals produces an efflux of Cl⁻, resulting in primary afferent depolarization and subsequently, reducing the size of the propagating action potential. This ultimately results in less calcium influx and less neurotransmitter release (Rudomin and Schmidt 1999; Seki et al. 2003; Rudomin 2009). Activation of the G-protein coupled GABA_B receptors results in modulation of the calcium channels in afferent terminals, similarly resulting in less calcium influx and less neurotransmitter release at the afferent terminals (Rudomin and Schmidt 1999; Soto et al. 2006; Castro et al. 2006).

Mailis and Ashby examined transmission from Ia afferents to motoneurons after SCI by recording motor unit action potentials in the soleus muscle in response to low-intensity (~0.8xMT) posterior tibial nerve stimulation (Mailis and Ashby 1990). In only the most spastic SCI participants, there was an increase in the monosynaptic Ia EPSP peak of the PSTH as well as the emergence of a late peak 11-15ms later which was not seen in uninjured control and non-spastic participants, suggesting the emergence of an oligosynaptic, group I excitatory pathway after injury. This enhanced transmission in Ia afferent pathways after injury may be due to a reduction in presynaptic inhibition on Ia afferent terminals. Paired electrical stimulation of the homonymous afferents to a muscle can be used to measure the level of presynaptic inhibition at the Ia-motoneuron synapse. For example, a prior conditioning stimulus at sub-threshold intensities applied to the posterior tibial nerve activates PAD INs which in turn presynaptically inhibits Ia afferents from the homonymous posterior tibial nerve

(Fig. 1-11A: red pathway ; Lev-Tov et al. 1983; Birnbaum and Ashby 1982). Examining the effects of paired homonymous electrical volleys on motor unit firing at various interstimulus intervals (ISIs) (posterior tibial nerve at $\sim 0.8 \times \text{MT}$ for conditioning and test volleys, ISIs: 5, 10, 30, 50, 100ms) produced two peaks of increased firing above baseline in the PSTHs of both uninjured (top trace, Fig. 1-11B) and SCI participants (bottom trace Fig. 1.11B, Mailis and Ashby 1990). The first peak is produced by the arrival of the Ia inputs from the first conditioning volley and similarly the second peak results from the test volley input. Typically, the second peak in the PSTH is reduced in comparison to the first peak at ISIs of 20 and 50ms due to presynaptic inhibition of Ia afferents evoked from the first conditioning volley. When plotting the logarithm of the area of the second peak expressed as a percentage of the area of the first peak, the ratio was always larger in SCI participants when compared to control participants at all ISIs between 5-30ms (dashed line Fig. 1-11C) and specifically at the 30ms ISI the depression of the second peak seen in uninjured control participants was absent in SCI participants.

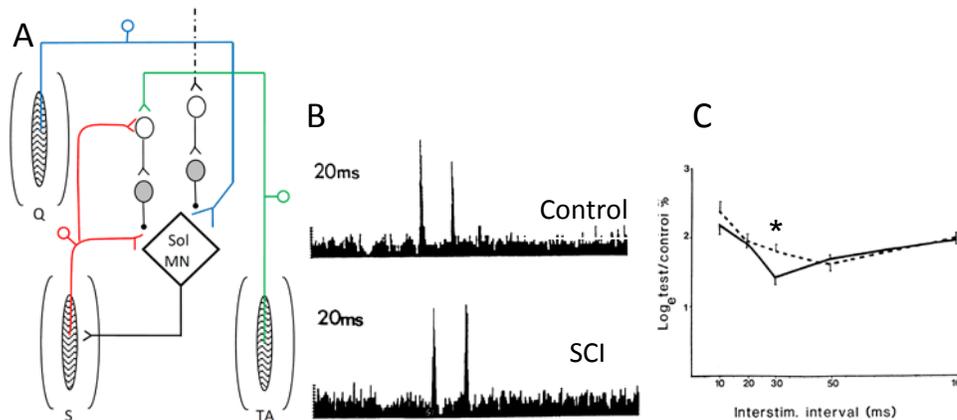


Figure 1-11 Measuring presynaptic inhibition in humans

A) Methods to measure presynaptic inhibition and the spinal circuitry involved. Red trace: paired homonymous electrical volleys, Blue trace: Heteronymous Ia facilitation of soleus motoneurons, Green trace: D1/D2 electrically-evoked presynaptic inhibition. *S* soleus muscle, *Q* quadriceps muscle, *TA* tibialis anterior muscle, *Sol MN* soleus motoneuron. Adapted from *Circuitry of the Human Spinal Cord: Pierrot-Deseilligny*. **B)** PSTH obtained from an uninjured control (top trace) and an SCI participant (bottom trace) in response to paired homonymous electrical stimulation of the PT nerve. Taken from Mailis and Ashby 1990. **C)** Ratio of the second peak measured as a % of the first peak in the PSTHs obtained from uninjured control (solid line) and SCI participants (dashed line) at the various interstimulus intervals. Taken from Mailis and Ashby 1990.

These findings indicated decreased presynaptic inhibition in SCI participants; however, these results are not exclusive to presynaptic inhibition since prior activation of the same afferents can also produce postactivation depression (also termed PAD) at the Ia-motoneuron synapse (Curtis and Eccles 1960; Katz et al. 1977; Hultborn et al. 1996). To control for this, paired heteronymous electrical volleys can also be used to assess the level of tonic background presynaptic inhibition at Ia-motoneurons synapses (Hultborn et al. 1987; Meunier and Pierrot-Deseilligny 1989). Specifically, the size of the monosynaptic, heteronymous facilitation of the soleus H-reflex from a conditioning supramaximal stimulation of the femoral nerve (4-5xMT) is modulated by the level of background presynaptic inhibition onto femoral Ia afferents (Fig. 1-11A: blue trace) This heteronymous facilitation of the soleus H-reflex was greater in SCI participants when compared to control participants, indicating reduced levels of tonic background presynaptic inhibition after SCI (Faist et al. 1994). Lastly, conditioning stimuli (3-5 shocks at 300Hz; 0.95xMT) applied to the afferents in the nerve supplying the antagonist muscle (e.g. TA) inhibits afferent transmission from Ia afferents in the agonist (e.g. soleus) test volley through activation of PAD interneurons (Fig. 1-11A: Green trace). The conditioning antagonist volley produces two phases of inhibition: an early D1 inhibition at ISIs of 10-25ms and a late, longer-lasting D2 inhibition at ISIs of 70-200ms in uninjured participants (Mizuno et al. 1971). These two phases of inhibition reflect the strength of presynaptic inhibition on afferent terminals because these same conditioning volleys do not affect cortically-evoked MEP responses at similar ISIs, therefore excluding postsynaptic inhibitory mechanisms (Faist et al. 1996; Capaday et al. 1995). When an H-reflex in the flexor carpi radialis (FCR) muscle was conditioned by a prior stimulation of the antagonist radial nerve, there was a complete disappearance of the expected early D1 inhibition in the FCR H-reflex in tetraplegic participants, indicating reduced presynaptic inhibition of Ia afferents in the median nerve (test volley) (Aymard et al. 2000). In conclusion, presynaptic inhibition appears to be significantly reduced in individuals with SCI when compared to healthy control participants;

however, there is no correlation between the reduction in presynaptic inhibition and the Ashworth measure of spasticity in these individuals (Faist et al. 1994). The reduction in presynaptic inhibition after SCI most likely results from decreased descending facilitation of PAD interneurons after injury (Fig. 1-11A) which alters the tonic level of presynaptic inhibition at the Ia-motoneuron synapse.

1.5.5.2 Reciprocal Ia Inhibition

Reciprocal Ia inhibition is a disynaptic pathway through Ia interneurons (pink circle: Fig. 1-10) which are activated by Ia afferents of the agonist muscle that subsequently inhibit motoneurons of the antagonist muscle. Reciprocal inhibition therefore ensures that antagonist muscles remain relaxed during contraction of the agonist muscle, preventing undesired co-contractions. Reciprocal Ia inhibition can be measured by examining the effects of a conditioning volley (low threshold 0.8-1.0 x MT, ISI=2-4ms) in the nerve supplying the agonist muscle on the test H-reflex response in the antagonist muscle (Mizuno et al. 1971; Kots and Zhukov 1971; Crone et al. 1987). In SCI participants, suppression of the soleus H-reflex by a prior conditioning stimulation of the common peroneal nerve supplying the tibialis anterior muscle was greatly reduced compared to control participants (Crone et al. 2003). In fact, reciprocal inhibition was replaced by reciprocal facilitation, which was most likely due to unmasking of this reciprocal facilitatory pathway from the removal of descending inputs after injury (Yanagisawa 1980). In contrast, there appears to be an increase in reciprocal inhibition from soleus Ia afferents to tibialis anterior motoneurons (Ashby and Wiens 1989). The discrepancies between the two studies can be explained by the differences in cortical descending facilitation of Ia interneurons. In non-injured individuals, the dominant corticospinal facilitatory effect is directed towards flexor motoneurons and their associated Ia interneurons (Brouwer and Ashby 1991). Since Ia interneurons mutually inhibit each other (Hultborn et al. 1976), the facilitation of tibialis anterior Ia interneurons results in inhibition of soleus Ia interneurons. The results of the above interaction is

tonically stronger reciprocal inhibition from tibialis anterior to soleus and weaker reciprocal inhibition from soleus to tibialis anterior muscles in non-injured individuals. However, after SCI, there is a significant loss in tonic, descending facilitation of flexor Ia interneurons which may account for the observed decrease in reciprocal inhibition to soleus motoneurons as well as the increase in reciprocal inhibition to tibialis anterior motoneurons. Although it is apparent that reduced reciprocal inhibition may contribute to increased motoneuron excitability after injury there is no correlation between the reduction in reciprocal inhibition and the degree of spasticity, as measured by the Ashworth scale, in individuals with SCI (Crone et al. 1994; Nielsen et al. 2007).

1.5.5.3 Post-Activation Depression or Rate-Dependent Depression

Post activation depression (PAD₂) is a form of inhibition that results in decreases of the H-reflex in response to repetitive nerve stimulation (Crone and Nielsen 1989). PAD₂ produces a dramatic, long-lasting (~10-15s) inhibition of H-reflexes and motoneuron activity (Crone and Nielsen 1989; Magladery et al. 1952; Rothwell et al. 1986; Hultborn et al. 1996). Proposed mechanisms behind PAD₂ have included presynaptic inhibition (Schieppati et al. 1985), although this mechanism has been disproven (Hultborn et al. 1996), changes in intrinsic motoneuron excitability (Olsen and Diamantopoulos 1967), also disproven (Hultborn et al. 1996), activity in spinal and supraspinal interneuronal circuits (Rossi et al. 1988) and decreases in neurotransmitter release at the Ia-motoneuron synapse (Katz et al. 1977; Mark et al. 1968). The level of PAD₂ can be measured by examining rate-dependent depression of the H-reflex which is the gradual decrease in H-reflex size that occurs when trains of reflexes are elicited at frequencies between 1 and 10 Hz (Lloyd and Wilson 1957). Rate-dependent depression is decreased in individuals after SCI (Schindler-Ivens and Shields 2000) and can be increased by a single bout of treadmill training (Trimble et al. 1998). Additionally, PAD₂ can be measured by examining the effects of tendon vibration on H-reflex responses because vibration results in repeated activation of Ia afferents. Tendon vibration failed to attenuate H-reflexes in participants with

chronic SCI when compared to uninjured individuals, once again indicating reduced PAD₂ after SCI (Calancie et al. 1993).

1.5.5.4 Recurrent Inhibition

Recurrent inhibition involves the transmission from motor axon collaterals to Renshaw cells (blue circles: Fig. 1-10), which in turn inhibit the same motoneurons that activate them, subsequently providing a negative feedback loop (Eccles et al. 1954). Recurrent inhibition was increased in participants with SCI compared to healthy individuals (Shefner et al. 1992) when using the homonymous, paired H-reflex technique (Pierrot-Deseilligny and Bussel 1975). Therefore, recurrent inhibition most likely does not play a major role in the development of hyperexcitability after injury.

1.5.5.5 Flexor Reflex Pathways

Flexor reflex afferent pathways include the group II, joint and cutaneous afferents that activate ipsilateral flexors and contralateral extensors with inhibition of ipsilateral extensors (Fig 1-12A). In uninjured, control participants noxious cutaneous stimulation normally results in a short-latency (~50-70ms) flexor withdrawal response with a duration of ~50ms (Hugon 1967; Pedersen 1954; Shahani and Young 1971). Interestingly, in complete SCI participants, stimulation of the purely cutaneous sural nerve, as well as the mixed tibial nerve, typically only results in long-latency (>100ms) excitation of flexors (TA muscle) and an absence of the short-latency FRA response exhibited in uninjured controls (Roby-Brami and Bussel 1987). A long-latency FRA response can also be obtained in uninjured control participants, however, unlike the SCI participants, it's threshold for activation is much higher than that for the early FRA response (Shahani and Young 1971). It has been demonstrated in spinalized cats that the early and late FRA are distinct pathways involving separate interneurons whereby the early FRA pathway tonically inhibits the late FRA pathway (Fig. 1-12B: Anden et al. 1964, 1966a,b,c; Jankowska et al. 1967b; Lundberg 1979). However, this inhibition can be released by application of L-DOPA/NA (Fig. 1-

12B) resulting in the emergence of long FRA responses in these animals (Anden et al. 1966a,b,c; Jankowska et al. 1967b; Lundberg 1979), which is similar to those present in individuals with complete SCI.

The above organization has been suggested to be present in humans based on the findings that the latency of the late FRA response in SCI participants is increased as the duration and intensity of the FRA stimulation is increased (Roby-Brami and Bussel 1987; Carlsson et al. 1963; Anden et al. 1964; Rank 2011). It is interesting to note however, that the late FRA response in humans can be elicited by afferents with activation thresholds of $\sim 0.75 \times \text{MT}$ corresponding to low-threshold cutaneous afferents (Roby-Brami and Bussell 1987). Additionally, the increase in the latency of the late FRA response is elicited by stimulation of afferents with thresholds of $\sim 3 \times \text{MT}$ indicating activation of high-threshold cutaneous afferents and group II/III muscle afferents (Roby-Brami and Bussell, 1987). Therefore, the increase in the latency of the late FRA response resulting from increased stimulation intensity and duration is most likely due to activation of a separate higher-threshold inhibitory pathway to the motoneurons via this second group of afferents rather than the organization proposed in the acute spinalized cat with L-DOPA.

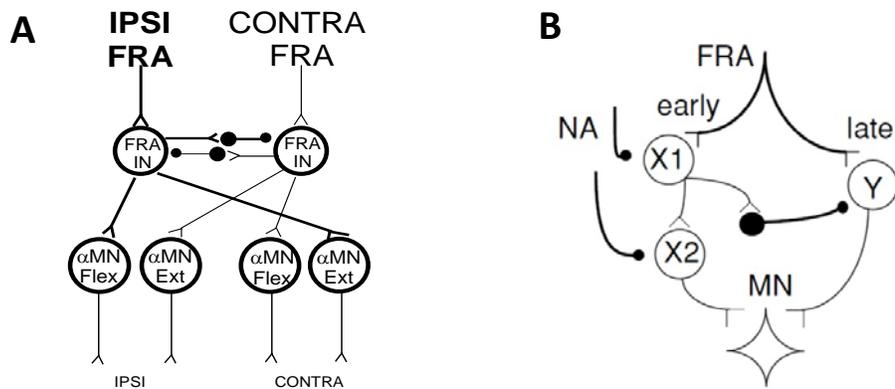


Figure 1-12: Flexor reflex afferent pathways

A) Half-centre organization of long-latency flexor-reflex afferent circuitry. FRA interneurons activated by ipsilateral FRAs facilitate ipsilateral flexion and contralateral extension. Ipsilateral FRA interneuron inhibits contralateral FRA interneuron. **B)** Early FRA pathway inhibits late FRA pathway. Taken from *Circuitry of the Human Spinal Cord*: Pierrot-Deseilligny.

1.5.6 Mechanisms for Reduced Spinal Inhibition after Injury

The level of inhibition in the spinal cord is influenced by several factors. Decreased inhibition in the spinal cord can result from direct decreases in facilitation of inhibitory interneurons from damaged descending pathways. Additionally, changes in the intrinsic excitability of the various interneurons interposed in the inhibitory pathways can contribute to decreased inhibition after injury due to changes in the levels of various neuromodulators after injury. Two proposed mechanisms are illustrated in the next two paragraphs.

1.5.6.1 Reversal of Chloride Potential

Downregulation and decreased membrane insertion of the specific KCC2 chloride transporter has been shown to occur after SCI (Boulenguez et al. 2010). The KCC2 pump functions to remove chloride from the neuron in exchange for potassium in order to maintain the chloride equilibrium potential at $\sim 70\text{mV}$. The decrease in KCC2 activity results in an increase in intracellular chloride concentration and a depolarization ($\sim 10\text{mV}$) of the reversal potential for chloride (Fig. 1-13).



Figure 1-13 KCC2 pump model in normal and SCI conditions

A) KCC2 function in normal neuron removes Cl from the neuron therefore allowing Cl influx during activation of GABA and Gly ligand-activated receptors. **B)** Downregulated KCC2 expression in SCI neurons increases intracellular Cl therefore resulting in efflux of Cl during activation of GABA and Gly receptors. (Adapted from Price et al. 2005)

This change in chloride reversal potential subsequently results in a switch from GABA_A and glycine-mediated inhibition to excitation because chloride moves out of the cell rather than into the cell during activation of the above receptors. Interestingly, inhibition can be restored via activation of TrkB receptors with BDNF, which results in upregulation of the KCC2 pump (Bouleguez et al. 2010). More recently, activation of 5HT_{2A} receptors via a calcium-independent PKC pathway has also been shown to restore KCC2 expression after injury and therefore restore lost inhibition (Bos et al. 2013). These results may aid the development of a 5HT_{2A} receptor agonist to restore inhibition after SCI.

1.5.6.2 Disinhibition as a Result of Decreased Monoamines

Monoamines modulate sensory activation of motoneurons mainly by regulating overall inhibition of sensory transmission. Therefore, it is no surprise that after SCI, when the majority of descending monoamines have been eliminated, disinhibition of inputs to the motoneurons occurs. Monoamines exert their actions on sensory transmission both pre and post-synaptically via activation of G-q and G-i coupled receptors. Both serotonin and noradrenaline facilitate Ia and Ib inhibitory interneurons via activation of G-q-coupled 5HT₂ and NA- α 1 receptors, subsequently resulting in increased Ia reciprocal inhibition and Ia/Ib non-reciprocal inhibition (Hammar and Jankowska 2003; Jankowska et al. 2000). Additionally, serotonin and noradrenaline have been shown to reduce pain transmission in animal models (Yoshimura and Furue 2006; Millan 2002) and similarly application of monoamine reuptake inhibitors effectively reduces pain in humans (Marks et al. 2009).

Activation of G-i-coupled receptors, such as the 5HT₁ and NA- α 2 receptors, modulates excitability via different downstream pathways. In brief, G-i-coupled receptors facilitate their downstream actions via both the G- α and G- $\beta\gamma$ subunits. The G- α subunit inhibits adenylyl cyclase (AC), results in a subsequent decrease in cAMP and decreased phosphorylation by protein kinase A (PKA) (Hein 2006; Boess and Martin 1994; Hoyer et al. 2002). The G- $\beta\gamma$ subunit directly inhibits neuronal calcium channels while increasing conductance of

inwardly-rectifying potassium channels and MAP kinases ERK 1/2 (Hein 2006; Cooper 2003; Boess and Martin 1994; Hoyer et al. 2002). Overall, the G-i coupled receptors (such as 5HT1 and NA α 2) have inhibitory actions in the CNS. Activation of 5HT1 and NA- α 2 receptors on the terminals of group I/II muscle, skin and high threshold pain afferents results in decreased transmission in these pathways (Yoshimura and Furue 2006; Jordan et al. 2008; Rekling et al. 2000; Jankowska et al. 1994; Millan 2002). Additionally, activation of these receptors on excitatory interneurons such as propriospinal neurons and group II interneurons, directly reduces excitatory transmission to motoneurons (Jankowska et al. 1993, 1994; Rekling et al. 2000; Jordan et al. 2008).

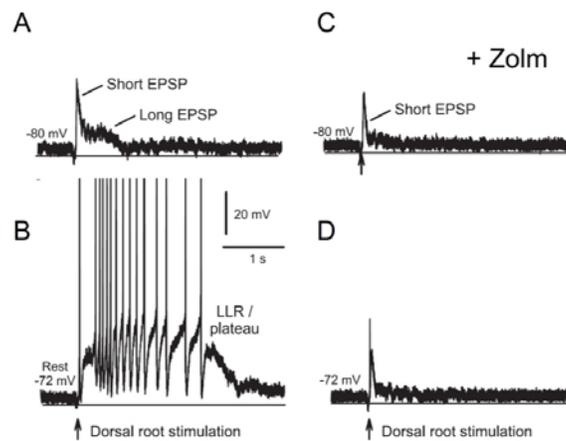


Figure 1-14 Effect of zolmitriptan on sensory transmission in SCI rat

A) Recording from a hyperpolarized rat motoneuron after chronic SCI in response to dorsal root stimulation. Note short and long-duration EPSP components. **B)** Recording from the same motoneuron in A at resting membrane potential triggers PIC-mediated long-lasting reflex. **C)** Recording from same motoneuron in A at hyperpolarized membrane potential after application of 5HT1 agonist zolmitriptan. Note elimination of long-duration EPSP. **D)** Recording from same motoneuron in C at resting membrane potential. Elimination of long EPSP no longer triggers PIC-mediated long-lasting reflex. (Murray et al. 2011)

Given the significant role that G-i coupled 5HT1 and NA- α 2 receptors play in modulating sensory transmission to the motoneurons, it was important to determine whether application of specific agonists to these receptors could restore lost inhibition in completely-transected rats. As mentioned in previous paragraphs and shown in Fig. 1-14A, after a complete spinal transection rat

motoneurons held at a hyperpolarized potential to prevent cell discharge display both a short and long EPSP component in response to brief sensory afferent stimulation. At resting membrane potential where the motoneuron can then fire, the long EPSP is sufficient to trigger the slow-activating PICs and subsequently self-sustained discharge during a muscle spasm (Fig. 1-14B).

Interestingly, when the specific 5HT1 agonist zolmitriptan is applied, the long (but not short) EPSP is eliminated (Fig. 1-14C: Murray et al. 2011). Additionally, with the elimination of the long EPSP, the PICs are no longer activated at resting membrane potential (Fig. 1-14D: Murray et al. 2011) and self-sustained firing is not triggered. Similar results have been demonstrated through the use of the NA- α 2 agonist, clonidine (Rank et al. 2011) indicating that application of these agonists can restore lost inhibition of sensory transmission after injury. It is interesting to note that, unlike the 5HT2 and NA- α 1 receptors, the G-i coupled 5HT1 and NA- α 2 receptors do not adapt to the absence of monoamines after injury and do not become constitutively-active to preserve inhibition of sensory transmission. In *Chapter 3* we examine whether transmission of sensory inputs to motoneurons can be reduced in individuals suffering a *motor complete* spinal cord injury through the use of the 5HT1 receptor agonist, zolmitriptan, and whether or not this agonist indirectly decreases the triggering of PICs and muscle spasms by sensory inputs.

1.6 Hyperexcitability in the Trigeminal Motor System

Moving from spinal motoneuronal hyperexcitability, the third project in this thesis examines mechanisms behind the development of hyperexcitability in the trigeminal motor system, namely in individuals suffering from the temporomandibular disorder sleep bruxism, which is characterized by sustained masseter EMG activity and teeth clenching and grinding during sleep.

1.7 Basic Organization of the Trigeminal System

The trigeminal nerve (V) is the fifth cranial nerve and contains three branches: the ophthalmic (O), maxillary (Mx) and mandibular (Mn) nerves (Fig. 1-15). Of the three, the mandibular branch is the only one that has both a sensory and a motor component which innervates the jaw-opening and jaw-closing muscles. Cell bodies of the afferents entering via the trigeminal nerve are located in either the trigeminal ganglion or the trigeminal mesencephalic nucleus (NVmes). The sensory component of the trigeminal system consists of a main principal sensory nucleus (NVsnpr) that receives primary afferent input from all three branches of the trigeminal nerve. Caudal to the primary sensory nucleus lie the nuclei of the spinal trigeminal tract. These nuclei consist of the nucleus oralis (NVspo) and nucleus caudalis (NVspc), the latter being further subdivided into the α , β and γ components. The trigeminal motor nucleus (NVmot) itself lies medially to the sensory complex at the same level as the primary sensory nucleus. The motor nucleus contains mainly motoneurons innervating masticatory muscles, as well as commissural interneurons which connect the two motor nuclei to allow bilateral masticatory movements (Westberg and Kolta 2011; McDavid et al. 2008; McDavid et al. 2006). Motoneurons innervating the jaw-closing muscles are located dorsally and laterally in the rostral and middle-thirds of the nucleus, whereas motoneurons innervating jaw-opening muscles are located ventromedially in the caudal part of the nucleus. The jaw-closing muscles are the masseter, temporalis, and medial pterygoid. The jaw-opening muscles are the digastric, lateral pterygoid, geniohyoid, mylohyoid and stylohyoid muscles. In *Chapter 4* we focus on the jaw-closing masseter muscle because it displays sustained involuntary muscle contractions in patients suffering from bruxism.

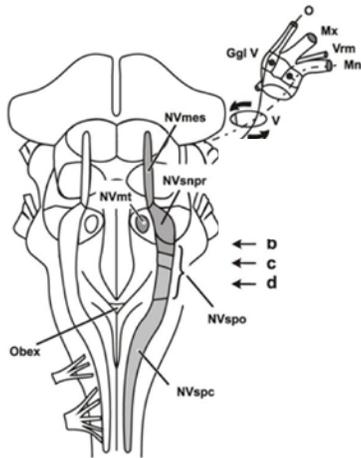


Figure 1-15: Basic organization of the trigeminal system

Organization of brainstem nuclei thought to be involved in mastication. *Mn* mandibular nerve; *V* trigeminal root; *NVmes* mesencephalic nucleus; *NVsnpr* principal sensory nucleus; *NVmt* trigeminal motor nucleus; *NVspo* nucleus oralis of spinal trigeminal tract; *NVspc* nucleus caudalis of spinal trigeminal tract. (Taken from Westberg and Kolta, 2011)

1.8 Components in the Control of Mastication

1.8.1 Descending Cortical Input

Rhythmic jaw movements in animals can be induced by repetitive electrical stimulation to the face area of the somatosensory cortex, basal ganglia, lateral hypothalamus, amygdala and midbrain reticular formation, resulting in a pattern of jaw-opening and jaw-closing muscle activity resembling natural chewing (Kawamura and Tsukamoto 1960; Hashimoto et al. 1989). In humans, EEG activity reaches a maximum level at the central position of the skull during voluntary chewing. This area corresponds to the central masticatory area in monkeys (Yoshida et al. 2000). Additionally, brain positron emission tomography and fMRI show that, similar to animal studies, the primary somatosensory and supplementary motor areas, insula, cerebellum and striatum are active during gum chewing (Momose et al. 1997; Onozuka et al. 2002). Projections from these areas to the brainstem result in activation of the masticatory CPG circuitry responsible for generating rhythmic masticatory activity.

1.8.2 Masticatory Central Pattern Generator (CPG)

In the isolated brainstem preparation, where the spinal cord is removed from a decerebrate animal and the cervical nerves are severed to remove all

afferent inputs, rhythmical masticatory motor patterns (fictive movements) can be elicited, indicating the presence of a masticatory central pattern generator (CPG) (Dellow and Lund 1971; Lund 1991; Kogo et al. 1996). The masticatory CPG receives cortical input arising mainly from the contralateral cortex (Dellow and Lund 1971; Tal 1987; Nozaki et al. 1986b). Additionally, although afferent input is not required to produce rhythmical chewing it is essential in modulating chewing; therefore components of the CPG receive various types of afferent input which are described in section iii) *Afferent Inputs*, below.

The masticatory CPG consists of two components: a rhythm generator and a burst generator. Rhythm generation is mediated by cells located in the medial brainstem reticular formation between the trigeminal motor nucleus and the inferior olive (Nozaki et al. 1986a&b; Yasui et al. 1985). Lesioning of this region in animals abolished mastication (Nozaki et al. 1986a; Chandler and Tal 1986) and intracellular recordings from neurons in this region show that these neurons are indeed rhythmically active during fictive mastication (Nozaki et al. 1986a). Two rhythm generators exist in the masticatory system (Fig. 1-16) and can operate independently as demonstrated by a cut along the midline of the caudal medulla which eliminated rhythm generation on the side ipsilateral to stimulation, while rhythm generation on the contralateral side remained intact (Chandler and Tal 1986). Corticobulbar stimulation produces excitation of the dorsal part of the nucleus reticularis paragigantocellularis (PGC) (Ermirio et al. 1989) (Fig. 1-16); however, intracellular recordings from these neurons have revealed that they do not fire rhythmically, but rather induce rhythmic firing in the downstream gigantocellularis (GC) neurons (Nozaki et al. 1986a; Ermirio et al. 1989). These neurons do not have direct projections to masticatory motoneurons and therefore affect masticatory activity mainly via projections to premotor interneurons located adjacent to the trigeminal motor nucleus (Lund 1991).

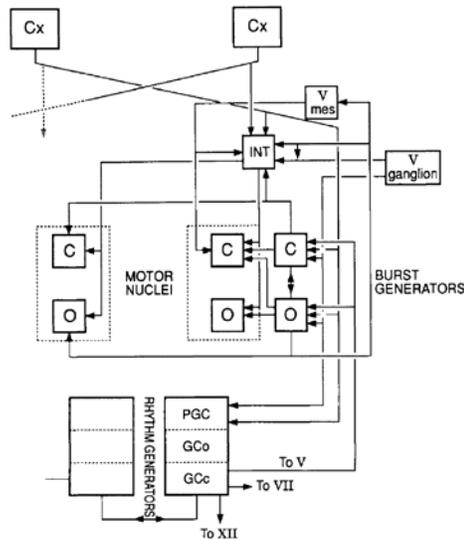


Figure 1-16 Proposed CPG circuitry
PGC paragigantocellularis; *GC* gigantocellularis; *C* jaw-closing; *O* jaw-opening; *V* ganglion trigeminal ganglion; *Vmes* mesencephalic nucleus; *INT* interneurons; *Cx* descending cortical input. (Taken from Lund 1991)

Last order premotor interneurons have direct excitatory monosynaptic glutamatergic and GABAergic/ glycinergic inhibitory connections to masseter motoneurons and receive input both from descending sources such as the somatosensory cortex and the various afferents in the masticatory system. As such, they are in an ideal situation to integrate masticatory information. Horseradish peroxidase (HRP) injections into the trigeminal motor nucleus made it possible to identify all last order interneurons that projected to masseter motoneurons. HRP is retrogradely transported via axonal uptake; thus any neurons that accumulate the tracer are last-order premotor interneurons. These neurons were found to be located bilaterally in the supratrigeminal, intertrigeminal and juxtatrigenial areas of 'regio h' which lies adjacent to the trigeminal motor nucleus and the principal sensory nucleus (Kolta et al. 2000: Fig. 1-17A). Additionally, labeling was found ipsilaterally to the injection site in the dorsal part of the nucleus oralis and nucleus caudalis (Fig. 1-17A). Lastly, within the trigeminal motor nucleus, commissural interneurons projecting to contralateral motoneurons were labeled (McDavid et al. 2006, 2008) with these interneurons most likely serving the role of coordinating bilateral jaw movements (Westberg and Kolta 2011).

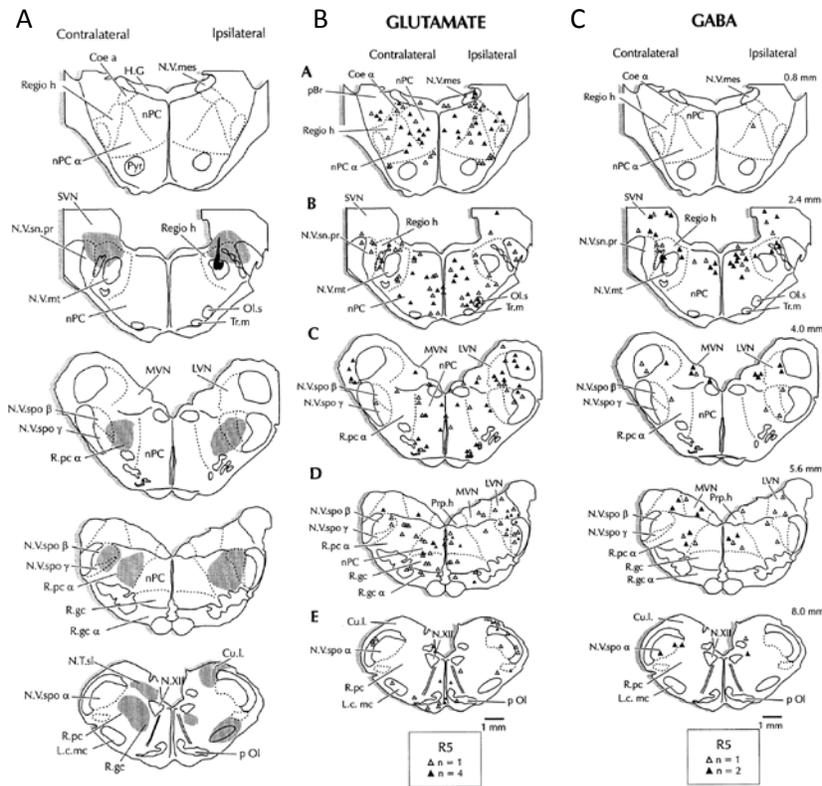


Fig 1-17 Last-order premotor interneurons projecting to trigeminal motor nucleus **A)** Retrogradely-labelled premotor neurons with monosynaptic connections to masseter motoneurons in the rat. **B)** Glutamate immunoreactive premotor neurons. **C)** GABA immunoreactive premotor neurons. Taken from Kolta et al. 2000.

In the isolated brainstem preparation, cuts just behind the trigeminal motor nucleus did not affect mastication (Dellow and Lund 1971; Kogo et al. 1996); therefore the second component of the CPG, the burst generators, are most likely located in areas proximal to the trigeminal motor nucleus such as the premotor interneurons in 'regio h' (Fig. 1-17). It is fitting that this area also receives input from the rhythm generators (Fig. 1-16; Lund 1991) and has direct monosynaptic glutamatergic and GABAergic/glycinergic projections to jaw-opening (O: Fig. 1-16) and jaw-closing (C: Fig. 1-16) motoneurons (Westberg and Kolta 2011; Kolta et al. 2000; Kolta 1997)(Fig. 1-16 CPG circuitry & Fig. 1-17B,C immunolabeling). These same neurons may also play a role in pattern generation because recordings from these neurons in anesthetized and paralyzed rabbits demonstrated that they exhibit rhythmical bursts in firing which occur in phase with fictive rhythmic mastication (Donga et al. 1990). Despite the ability of the

masticatory CPG to produce rhythmical masticatory activity in isolated preparations, input from the various afferents greatly modulates motor output during natural chewing.

1.8.3 Afferent Inputs

Trigeminal motoneurons, CPG components and higher centres receive afferent input conveying different types of information that shapes the masticatory motor output (Fig. 1-18). The importance of peripheral feedback was demonstrated in lightly anesthetized rabbits where removal of sensory feedback from periodontal receptors reduced facilitation of the jaw-closing motoneurons when a test strip was placed between the teeth (Lavigne et al. 1987; Morimoto et al. 1989). Additionally, destruction of the muscle spindle cell bodies eliminated facilitation of the jaw-closer motoneurons (Morimoto et al. 1999). In humans, individuals with dental bridges are unable to display bite-to-bite variations in force when compared to control individuals (Haraldson 1983). Additionally, individuals who have lost their teeth display a reduction of 20-40% in the bite force when compared to controls (Haraldson et al. 1979; Slagter et al. 1993). It is apparent that the peripheral input provided by the various afferents in the masticatory system (Fig. 1-18) can shape the overall motor activation patterns.

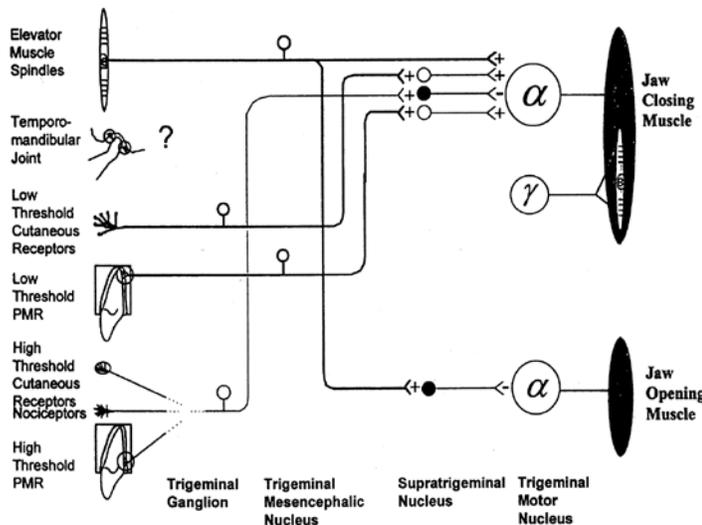


Figure 1-18
Afferent inputs to jaw motoneurons
Organization of different afferent inputs to jaw-closing masseter motoneurons (Turker et al. 2002)

1.8.3.1 Cutaneous Afferents

Afferent inputs from receptors located in the skin and oral mucosa have been recorded in the mandibular division of the trigeminal nucleus in rabbits (Appenteng et al. 1982) and in humans (Barlow 1987; Johansson et al. 1988). These receptors consist of slow-adapting receptors with both small and large receptive areas (SAI and SAI) as well as rapidly-adapting receptors with small and large receptive areas (RAI and RAI) (Johansson et al. 1988). In humans, electrical stimulation of the peri-oral skin or mucosa results in excitation of the jaw closers when the stimuli is just above perception threshold, and inhibition of the jaw closers with stronger stimuli (Brodin and Turker 1994). It is unknown, however, whether these afferents play a major role in modulating mastication.

1.8.3.2 Temporomandibular Joint (TMJ) Afferents

Information regarding joint angle during mastication is provided by temporomandibular joint afferents which are sensitive to displacement velocity. Innervation of the human TMJ capsule has been studied with receptor types including: free nerve endings which convey pain information, Ruffini endings and Golgi organs which acts as static mechanoreceptors and Vater-Pacini corpuscles which act as dynamic mechanoreceptors (Thilander 1961; Storey 1976). During unloading of the jaw, which occurs once the teeth have fractured a brittle object, a short-latency (10-20ms) reduction in EMG activity of the masseter muscle occurs (Turker 2002). This unloading activates TMJ receptors as well as muscle spindles and periodontal receptors as described below. Interestingly, in humans jaw unloading without movement of the jaw seems to be solely mediated by periodontal receptors (Turker and Jenkins 2000); therefore, TMJ receptors most likely contribute only when there is a change in jaw position during unloading.

1.8.3.3 Muscle Spindles

Masseter motoneurons also receive information from muscle spindle afferents. The spindle afferent cell bodies are located in the mesencephalic nucleus and therefore it is possible to record their activity with microelectrodes in

awake animals (Matthews 1976). Recordings from awake and anaesthetized cats have helped demonstrate that velocity sensitivity to stretch was more predominant for primary muscle afferents, with firing rates of up to 600Hz recorded at the start of jaw opening (Turker 2002). On the other hand, activity in the secondary muscle afferents was proportional to the degree of opening (80-200Hz) (Turker 2002). In humans, only the jaw-closing muscles contain muscle spindles (Lennartsson 1979; Kubota and Masegi 1977). Brief rapid stretch of the masseter muscle by a downward tap to the chin with a tendon hammer results in a profound excitatory response in the masseter with a monosynaptic latency of ~8ms (Turker 2002). The hammer tap producing the brief muscle stretch most likely results in activation of cutaneous and TMJ receptors as well, therefore further studies have utilized slow, controlled stretches of the masseter to try to isolate muscle spindle afferent effects and similarly, during these controlled stretches a powerful monosynaptic response was present in the masseter (Turker 2002).

1.8.3.4 Periodontal Mechanoreceptors (PMRs)

PMRs are located near the apex of the tooth root (Ness 1954). Mechanical and electrical stimulation of these receptors in cats was used to determine their firing properties in response to stimulation of the teeth (Linden 1990). PMRs convey information regarding the amount of tension that is applied to the tooth and are sensitive either to the force applied to the tooth or the rate of the force change (Turker 2002). Direct stimulation of the receptors revealed that there are two groups of PMRs: in the first group, cell bodies are located in the trigeminal ganglion and these afferents are rapidly adapting and fire in short bursts at the start of jaw closure during mastication (Linden 1990). The second group of periodontal afferents has cell bodies located in the mesencephalic nucleus (Linden 1990). These afferents are slowly adapting and their firing rates increase proportionally to bite force (Linden 1990). In humans, microneurography results have generally agreed with the animal findings (Trulsson and Johansson 1994; Trulsson et al. 1992). The major role of the PMRs is to convey information

regarding unloading and loading of the teeth during chewing. During mechanical unloading of the tooth, inhibition of the sEMG in both the ipsilateral and contralateral masseter muscles occurs (Turker and Jenkins 2000). This inhibition is abolished by local anaesthesia of the teeth (Turker and Jenkins 2000). This reflex likely serves as a protective measure to inhibit jaw closing forces once a piece of food has been fractured by the teeth, thereby inhibiting an excessive closing force that may result in tooth damage. During rapid loading of the tooth (for example chewing a hard object like a stone) activation of the rapidly-adapting PMRs results in a short latency (~12ms) inhibition of the jaw-closing muscles (Sessle and Schmitt 1972; van der Glas et al. 1985; Brodin et al. 1993). In contrast, during slow loading of the teeth (for example chewing your food), activation of the slow-adapting PMRs induces a mainly excitatory response in jaw-closing motoneurons (Lavigne et al. 1987; Lund and Lamarre 1973; Turker et al. 1994). In summary, the slow-adapting PMRs act to reinforce jaw-closure during chewing, however if a hard object (like a stone) is present in your food activation of the rapidly-adapting PMRs produces strong inhibition of jaw-closing muscles to prevent tooth damage.

1.9 Normal Rhythmic Masticatory Muscle Activity during Sleep

Rhythmic masticatory muscle activity (RRMS) occurs during sleep in healthy individuals. Moving from wakefulness, individuals cycle through different sleep stages that can be divided into either non-REM and REM sleep with non-REM sleep further subdivided into light sleep (stages 1 and 2) and deep sleep (stages 3 and 4) based on EEG recordings. These stages occur in 3-6 cycles throughout the night and are separated by an interval of approximately 60-90 minutes. As the individual shifts through the various sleep stages, the release of substances such as ACh, histamine, dopamine, orexin, NA and 5HT, which normally facilitate excitability of cortical and thalamic arousal neurons, is decreased (Kilduff and Peyron 2000; Fung et al. 2001; Mignot et al. 2002; Rye

2003; Lavigne et al. 2003) (Fig. 1-19). As a result, during REM sleep, limb and jaw muscle tone decreases to a state of complete muscle atonia. One mechanism responsible for the decreased excitability of jaw motoneurons is a reduction in the descending cortical drive during sleep (Evarts 1963). Additionally, increases in the firing of the nuclei pontis caudalis (NVspc) activates two separate pathways that subsequently results in decreased excitatory drive to jaw motoneurons during sleep. The first pathway involves activation of pontine cholinergic neurons which in turn facilitate glutamatergic neurons in the medial medullary reticular formation. The glutamatergic neurons facilitate glycinergic neurons which have direct inhibitory projections to jaw motoneurons (Lavigne 2003). The second pathway results in the facilitation of GABAergic neurons that directly inhibit 5HT and NA neurons that maintain an excitatory drive on motoneurons (Rechtschaffen and Siegel 2000). In addition to changes in the inputs to the motoneurons, intrinsic motoneuron excitability is also decreased during sleep muscle atonia. By recording the membrane potential from jaw-closing motoneurons in guinea pigs during the different sleep states it was determined that as the animal transitions from wakefulness through to REM sleep, the membrane potential becomes progressively hyperpolarized (wakefulness: -55mV , NREM sleep: $<-60\text{mV}$, REM sleep: $<-75\text{mV}$) as a result of postsynaptic glycine-mediated inhibition of the motoneurons (Chase et al. 1980, 1989; Chandler et al. 1980; Chase 1983). All of these mechanisms should contribute to overall muscle atonia during sleep, yet periods of RMMA are frequently present during sleep.

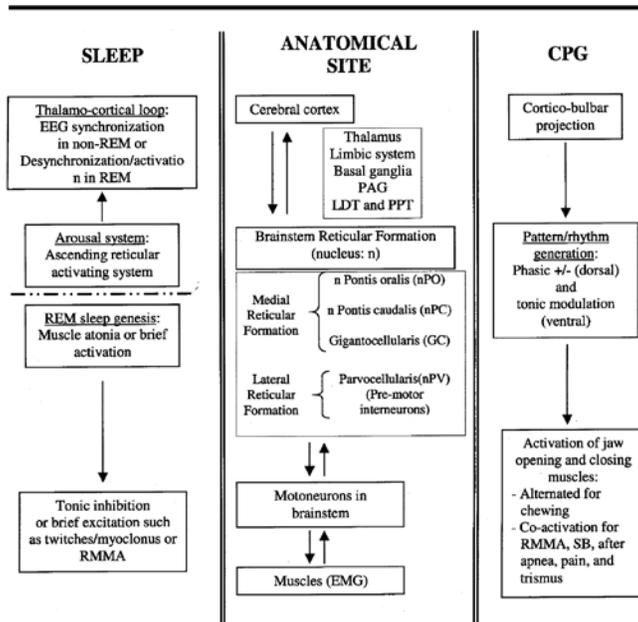


Figure 1-19: Role of the brainstem reticular formation in sleep

Role of brainstem reticular formation in sleep (REM and NREM sleep stages) and rhythmic masticatory muscle activity (CPG) and the corresponding anatomical sites responsible. (Taken from Lavigne et al. 2003)

Periods of RMMA during sleep are characterized by phasic and tonic bursts in masseter EMG activity (Lu et al. 2005; Kato et al. 2011; Kato et al. 2007; Anacleto et al. 2010; Inoue et al. 1999). RMMA is similar to chewing during wakefulness and occurs in ~60% of people without detrimental tooth grinding and destruction (Lavigne et al. 2001). Periods of RMMA coincide with the incidence of microarousals which are associated with a shift of EEG activity lasting 10-15s, and occurring mainly during the early NREM sleep stages (Quattrochi et al. 2000; Halasz 1998; Halasz et al. 2004). Additionally, the intensity of masseter EMG activation during RMMA is correlated to the intensity of the microarousals measured by the degree of desynchronized cortical activity in animals (Kato et al. 2007; Kato et al. 2003). Coincidentally, during microarousals there is an increase in the discharge of neurons located in the raphe nuclei, locus coeruleus, subcoeruleus and A5/A7 cells, which all release excitatory 5HT and NA to the trigeminal motoneuron pool (Leung and Mason 1999; Sakai and Crochet 2001; Takahashi et al. 2010; Crochet and Sakai 1999). The increase in the release of excitatory neuromodulators most likely contributes significantly to the increase in motoneuron excitability during microarousals.

1.10 Masticatory Muscle Disorder During Sleep: Bruxism

Bruxism is a movement disorder of the masticatory system that is characterized by teeth grinding and clenching during sleep due to sustained masseter activity during periods of RMMA (Lobbezoo and Naeije 2001; Lobbezoo et al. 2006). Sleep bruxism is reported by 8% of the population (Lavigne et al. 2003) and can result in damaging tooth wear and destruction, breakage of dental restoration, temporal tension headaches and disruption of sleep, and hence, is of great concern to dentists (Lavigne et al. 2008). Bruxism can result in acute and chronic myofascial pain that can be quite debilitating. Possible underlying causes include dental occlusions, genetic factors, stress and anxiety, high levels of oromotor activity, and most recently, the enhanced activation of arousal and cardiac autonomic pathways during sleep have been implicated (Lavigne et al. 2007). Currently, morphological factors and dental occlusions are thought to play a small role since there is a poor relationship between self-reported and/or clinically established bruxism and occlusal factors (Manfredini et al. 2004; Demir et al. 2004; Cheng et al. 2004). As such, the causes of bruxism are currently thought to be mediated by central, rather than peripheral factors (Lobbezoo and Naeije 2001), although the mechanisms have yet to be fully understood.

1.11 Possible Mechanism: Abnormal RMMA in Sleep Bruxism

Individuals suffering from sleep bruxism experience increased microarousals during sleep when compared to non-symptomatic age and sex-matched participants, with bruxer participants experiencing 9.5 microarousals/hour compared to 5.7 microarousals/hour in controls (Lavigne et al. 2001). In line with these results, individuals with bruxism display increased episodes of RMMA (5.8 episodes/hour) compared to control participants (1.8 episodes/hour) that are of higher masseter EMG frequency and amplitude (Lavigne et al. 2001). In

studies where microarousals were evoked experimentally by sensory stimulation in both healthy and bruxer individuals, RMMA was triggered seven times more frequently during microarousals in sleep in bruxer participants and 86% of induced periods of RMMA were associated with pathological teeth grinding (Kato et al. 2011; Kato et al. 2003; Kato et al. 2001a). These results imply that periods of RMMA are more easily triggered in bruxer participants and result in greater sustained masseter activity when compared to healthy individuals.

As mentioned earlier, during microarousals there is an overall removal of inhibition to masseter motoneurons and an increase in the monoaminergic drive to these same motoneurons. Given the enhanced activation of PICs in models of decreased inhibition (after SCI) and the role that monoamines play in the facilitation of PICs, in *Chapter 4* we examined whether participants with bruxism have greater masseter PIC activation than in non-bruxer control participants. Increased PICs in the masseter motoneurons of individuals with bruxism may essentially provide the added depolarization to masseter motoneurons which allows the masseter muscle to display pathological levels of sustained activity during periods of RMMA which otherwise do not normally result in teeth clenching and grinding in healthy individuals.

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CHAPTER 2:
CONSTITUTIVELY-ACTIVE 5HT2/ α 1
RECEPTORS FACILITATE MUSCLE
SPASMS AFTER HUMAN SPINAL
CORD INJURY

A version of this chapter has been published.
D'Amico JM, Murray KC, Li Y, Chan KM, Finlay MG, Bennet DJ and Gorassini MA.
Journal of Neurophysiology 109(6): 1473-1484, 2013.

2.1 Introduction

In rats, the recovery of motoneuron excitability in the months following a complete spinal cord injury is mediated, in part, by increases in constitutively active 5HT₂ and α ₁ receptors on the motoneuron (Murray et al. 2010; Rank et al. 2011). Continual activation of these monoaminergic receptors in the absence of the ligands serotonin and noradrenaline below the injury allows for continual facilitation of voltage-activated, persistent inward calcium currents (CaPICs) via downstream Gq-coupled pathways (Heckman et al. 2003; Hounsgaard et al. 1988; Mizuno and Itoh 2009; Perrier et al. 2002). Combined with the presence of prolonged, sensory-evoked excitatory postsynaptic potentials (EPSP's; Baker and Chandler 1987; Li et al. 2004a) and reduced inhibitory postsynaptic potentials (IPSP's; Boulenguez et al. 2010; Norton et al. 2008), the recovered CaPICs are more readily triggered and depolarize the motoneuron to produce unchecked and prolonged involuntary muscle spasms after spinal cord injury.

Evidence for constitutive receptor activation in animal models of complete spinal cord injury was provided by the combined finding that neutral antagonists, which only block ligand activation of the 5HT₂ (e.g., SB242084/methysergide) and α ₁ (REC15/2739) receptors were ineffective in reducing the CaPIC whereas inverse agonists (e.g., 5HT₂: SB206553/cyproheptadine; α ₁: WB4010/prazosin), which block both ligand *and* constitutive activation of these receptors, effectively reduced the CaPIC (Murray et al. 2010; Rank et al. 2011). In addition, mRNA analysis revealed that increases in the unedited and constitutively active isoform of the 5HT_{2C} receptor (INI) occurred below the injury site where levels of serotonin were greatly diminished (Murray et al. 2010). In this same study, we also provided evidence in human participants with spinal cord injury that cyproheptadine, an inverse agonist to 5HT₂ and α ₁ receptors, also decreased long-lasting reflexes (spasms), which are thought to be mediated by CaPIC activation (Gorassini et al. 2004). However, as these participants had preserved motor function, there existed the possibility that residual, descending sources of serotonin and noradrenaline were still present below the injury to provide ligand

activation of the 5HT2/ α 1 receptors. Therefore, this leaves the possibility that cyproheptadine could have acted solely by blocking the ligand activation of the 5HT2/ α 1 receptors on the motoneuron, requiring further experiments to prove that constitutive monoaminergic receptor activity plays a role in the facilitation of involuntary motoneuron activity (muscle spasms) in humans with chronic spinal cord injury.

To this end, in this study we first tested if the participants with motor incomplete injuries from the Murray et al. (2010) study indeed had residual sources of serotonin that facilitate CaPIC mediated long-lasting reflexes by examining if a serotonin reuptake inhibitor (i.e., citalopram) could facilitate such reflexes. Because we observed evidence for such facilitation, we then compared the effects of the inverse agonist cyproheptadine and the neutral antagonist chlorpromazine to 5HT2 and α 1 receptors in participants with motor or motor/sensory complete injuries where descending sources of serotonin and noradrenaline are likely greatly reduced or absent. Similar to the animal studies, we examined if only the inverse agonist, and not the neutral antagonist, was effective in reducing CaPIC-mediated reflexes to provide more substantive evidence for the presence of constitutive monoaminergic receptor activation in humans after spinal cord injury. We also used paired motor unit recordings to provide a more direct estimate of the amplitude of CaPIC compared to the long-lasting reflexes (as per Udina et al. 2010), and explored the role of constitutive 5HT2/ α 1 receptor activity on this CaPIC estimate.

2.2 Methods

All experiments were approved by the Health Research Ethics Board at the University of Alberta and conformed to the Declaration of Helsinki with off-label drug use approved by Health Canada Clinical Trials. All participants gave written, informed consent prior to participating in the study. In total, 6 uninjured control participants with no known neurological injury or impairment and 9 *incomplete* spinal cord injured (iSCI) and 9 motor or motor/sensory *complete* SCI

(cSCI) participants with traumatic injuries took part in this study (see Table 2-1 for details). Additionally, drug screens were carried out and verified by authors JDA, KMC and MF to rule out contraindications for the various drugs. One individual with SCI was excluded from the study because of warfarin use, which interacts with cyproheptadine.

2.2.1 Drug Administration

All 5HT₂/α₁-receptor drugs were administered orally and were housed in a two-part telescoping capsule to blind participants as to which drug they were taking. For example, iSCI participants took either cyproheptadine (8 mg), a serotonin 5HT₂/α₁-receptor inverse agonist in spinal motoneurons (Murray et al. 2010) or citalopram (20 mg), a selective serotonin reuptake inhibitor or SSRI (Hyttel 1982). Increases in PIC-mediated responses (described in Reflex analysis and Estimation of PIC amplitude below) by citalopram in iSCI participants indicated the presence of spared, functional sources of serotonin. We were only able to test citalopram in 4 of the 9 iSCI participants (2M-5M, * in Table 2-1) because 4 of the others were already taking an SSRI and one out-of-town subject was not able to return for the second experiment. cSCI participants took either cyproheptadine (12 mg) or an equivalent dose of chlorpromazine (12.5 mg), a 5HT₂/α₁-receptor neutral antagonist (see Discussion and Herrick-Davis et al. 2000; Rauser et al. 2001; Richelson and Nelson 1984). Evidence to suggest the presence of constitutive 5HT₂/α₁ receptor activity was considered if only the inverse agonist (cyproheptadine), which blocks both constitutive and ligand activation of the receptor, and not the neutral antagonist (chlorpromazine), which only blocks ligand activation of the receptor, was effective in reducing the PIC-mediated responses. If SCI participants were on oral baclofen, they were asked to skip their morning pill before the experiment. Non-injured control participants were given the same oral dose of citalopram and chlorpromazine. JDA, who performed the data analysis, was also blinded to the drug given. Heart rate and blood pressure were measured before and every 30 minutes after drug intake.

Participants were also asked to report any changes in physiological sensations from the drug.

2.2.2 Long-duration Reflexes

Reflex recordings were done in SCI participants only, seated in their wheelchairs with limbs unconstrained. Two surface electrodes (2.2 x 3.3 cm, Kendall Soft-E, Chicopee, MA, USA), were placed over the tibialis anterior and soleus muscles to record electromyographic (EMG) signals. The surface EMG was amplified 1000 times, filtered using a band-pass of 10-1000 Hz (Octopus, Bortec Technologies, Calgary Canada) or 20-2500 Hz (Model 2024F Intronix Technologies, Bolton, ON, Canada). The EMG signals were digitized using Axoscope hardware and software at a rate of 5kHz (Digidata 1440 Series, Molecular Devices, Sunnyvale, USA) and stored on a personal computer for off-line analysis. To evoke long duration (> 1 s) reflex responses in the tibialis anterior, which we have previously demonstrated to be largely mediated by Ca⁺⁺-dependent PICs (Gorassini et al. 2004; Li et al. 2004a), we stimulated cutaneomuscular afferents supplying the side and sole of the foot with long pulse trains. These many second-long reflexes (or spasms) were evoked at rest by electrical stimulation to the medial arch of the foot (300Hz, 14 pulses, 0.5ms pulse width) using a DS7A constant current stimulator (NL703, Digitimer, Hertfordshire, UK). The intensity of stimulation was chosen to maximize the duration of the evoked reflex without being too painful for the subject. Higher stimulation intensities were needed in the motor complete SCI participants (75.0 ± 22.0 mA) compared to the incomplete SCI participants (27.6 ± 11.8 mA, $p < 0.001$ Mann-Whitney). Stimulation was repeated 6 times at 5-10s intervals for each trial. Two to three trials were taken before drug administration to establish a stable baseline. Reflex measures were repeated every 30 minutes after drug intake for 2 hours. Although peak plasma levels of the various drugs occur ≈ 2-4 hours after oral intake (Bezchlibnyk-Butler et al. 2000; Mendes et al. 2012; Yeung et al. 1993), we only tested motoneuron excitability until 2 hours post-drug because in pilot studies, maintaining a similar seated posture for more than 3 hours (+ 1 hour

pre-drug measures) was too fatiguing for the non-injured control participants who are not accustomed to sitting for long, uninterrupted periods. As with other drug studies (Ziemann 2004), we were able to observe effects of the various drugs on neuronal pathways within 30 to 120 minutes after drug intake, well before their estimated peak plasma times.

2.2.3 Reflex Analysis

Three components of the long-duration reflex response were measured using custom-written Matlab software (The MathWorks, Natick, MA). The early-latency (onset at 50-70 ms after first stimulation pulse), short-lasting polysynaptic reflex (SPR) was defined as a distinct, large amplitude response that occurred in the first 50 ms of the reflex (as per Murray et al. 2010, 2011a; Rank et al. 2011). The SPR, which was only evoked in the iSCI participants (see also Roby-Brami and Bussel 1987), is useful to examine sensory-evoked EPSP's in isolation because they are not inhibited by a block of CaPICs with isradipine (Murray et al. 2011a). The more polyphasic and longer-lasting polysynaptic reflex (LPR), which included the start of the reflex response up until 300 ms after the first stimulation pulse, contains a mixture of both sensory-evoked EPSP's and CaPIC activation because its amplitude is reduced to around 50% by isradipine (Li et al. 2004a; Murray et al. 2011a). The later, long-lasting reflex component (LLR) was defined as the time window from 500ms after the first stimulation pulse to the end of the reflex response in the pre-drug trial, as per Murray et al. 2010, 2011a,b and Rank et al. 2011 (LLR duration: 1445 ± 704 ms in iSCI and 645 ± 454 ms in cSCI participants). Thus, the LLR represents a period where most of the sensory synaptic drive to the motoneuron (EPSP) has subsided and is produced mainly by a depolarization from the CaPIC (Li et al. 2004a; Norton et al. 2008).

To measure the amplitude of the various reflex components using Matlab software, each EMG trace was first rectified and the mean EMG was calculated for the SPR, LPR and LLR time windows defined above. The mean rectified background noise, measured from 100 ms before the stimulation, was subtracted

from the data. The mean EMG for each of the 6 stimulation trials were averaged together to obtain SPR, LPR and LLR values for each time point. All SPR, LPR and LLR values post drug (at 30, 60, 90 and 120 minutes) were then expressed as a percentage of the pre drug value (post-drug/pre-drug x 100%). Because there was no statistical difference between the two SPR, LPR or LLR values measured immediately before the drug intake, the two baseline values were averaged together for each reflex component. Percent values at each time point were averaged across participants for each of the different drug experiments.

2.2.4 Motor Unit Recordings

Motor unit recordings in iSCI and non-injured control participants were used to examine the effects of the various monoamine drugs on the estimation of PIC amplitude using paired motor unit analysis (as per Udina et al. 2010). Motor unit recordings were taken during the same experimental session as the reflex recordings in the iSCI participants. Participants were seated in their wheelchairs or in a comfortable chair with the foot strapped onto a metal plate that was coupled to a force transducer. Participants were tasked with tracking a triangle drawn on a transparency placed over the computer screen using either the dorsiflexion torque or integrated surface EMG signal, whichever they preferred. The y-axis of the torque or EMG signal was adjusted so that only 2-3 motor units were recruited during the ascending phase of the contraction and the x-axis was adjusted so the total contraction lasted at least 15 s. Intramuscular electrodes used for recording single motor unit action potentials were inserted into the tibialis anterior muscle using a 25 gauge needle (for electrode details see Udina et al. 2010). Intramuscular EMG was amplified 5000 times, high-pass filtered at 200 Hz using an isolated, high impedance amplifier (Model 2024F Intronix Technologies, Bolton, ON, Canada), with a DC frequency response to 10 kHz, containing an imbedded Butterworth filter with a 12 dB/octave cut-off. All data were digitized using a Power 1401 A/D converter and Spike2 (Version 6) software (Cambridge Electronics Design, Cambridge, UK) using a sampling rate

of 25 kHz for the intramuscular EMG, 5kHz for surface EMG and 100 Hz for the torque signal.

2.2.5 Estimation of PIC amplitude

Data were analyzed off-line using commercial spike discrimination software (Spike2, Cambridge Electronic Design). Single motor unit action potentials were first selected by setting a horizontal threshold, and inspecting each waveform visually to identify a given motor unit based on waveform shape. When possible, the same motor-unit pair (control and test) was followed both before and after drug administration. However, due to electrode movement during the long experiment (typically 3-4 h), one or both of the motor-unit pairs could be lost, and new unit pairs were sampled after drug intake. In the cyproheptadine experiments, 11 of the 15 motor unit pairs recorded in the iSCI participants were the same both pre and post-drug whereas in the chlorpromazine experiments, 5 of the 7 motor unit pairs recorded in the non-injured control participants were the same both pre and post-drug.

The amplitude of PIC activation was estimated utilizing the paired motor unit analysis technique (Bennett et al. 2001; Gorassini et al. 2002, 2004). The times of occurrences for the spikes obtained in Spike 2 were exported via a text file to a custom-written Matlab program. The instantaneous firing rates of the units were then calculated as the reciprocal of each interspike interval. The firing rate profile of a lower-threshold control motor unit was used as a measure of the synaptic input to the motoneuron pool and specifically, to a relatively higher-threshold motor unit, termed the test unit. To calculate the firing rate of the control unit at recruitment and de-recruitment of the test unit, a fifth-order polynomial was used to smooth the firing rate profiles. The smoothed firing rate of the control unit at recruitment and de-recruitment of the test unit was determined automatically, and the difference in smoothed firing rate of the control unit when the test unit was de-recruited compared to when it was recruited was computed: $\Delta F = F_{\text{derecruitment}} - F_{\text{recruitment}}$. This ΔF , therefore, corresponds to

the reduction in synaptic input needed to counteract the depolarization from the PIC and provides an indirect estimate of PIC amplitude (Bennett et al. 2001; Gorassini et al. 2004). The accuracy of the ΔF measure relies on how well the firing rate of the control unit reflects the synaptic drive to the test unit, which requires that both units receive a common synaptic drive. To estimate this, the smoothed firing rate of the control unit was plotted against the smoothed firing rate of the test unit and the Coefficient of Variation (r^2) was calculated (as per Gorassini et al. 2002; Udina et al. 2010).

Five of a possible 8 to 10 contraction trials were chosen to calculate an average ΔF for each subject at each time point measured (pre drug, 30, 60, 90 and 120 minutes post drug). Contractions were chosen based on the recruitment profiles of the motor units, i.e. if the recruitment of the control and test motor units were separated by more than 2 s, if the test unit was activated for 2 s or more during the ascending phase of the contraction to ensure full CaPIC recruitment, and if the rate of increase in firing rate of the control unit during the ascending phase of the contraction was similar to the rate of decrease in firing rate during the descending phase of the contraction (Udina et al. 2010). All participants, including the iSCI, were able to produce triangular firing rate profiles of the control motor unit that lasted for at least 10 to 15s. These profiles were produced to best emulate the firing rate profiles used to calculate PIC amplitude from intracellular recordings in rat motoneurons (Gorassini et al. 2004). Post-drug ΔF values were expressed as a percentage of the pre-drug value, and averaged across participants.

2.2.6 In vitro ventral root recordings of long duration reflexes in rats

To verify that the human-approved drug chlorpromazine works as a neutral antagonist on 5HT₂/ α ₁-receptors in spinal motoneurons, i.e., only blocking ligand activation of the receptor, we used the in vitro sacral spinal cord preparation, a rat model which has been used to distinguish inverse agonists from neutral antagonists (Murray et al. 2010). Under urethane anesthesia (1.8 g·kg⁻¹)

the whole spinal cord caudal to the S2 injury was removed from chronic (>2 months) spinal rats and immersed in oxygenated artificial cerebrospinal fluid (ACSF; flowing 8 ml·min⁻¹); recordings were made starting 2.5 hr later, as detailed previously (Bennett et al. 2001; Li and Bennett 2003). Ventral (S4 and Co1) and dorsal (Co1, coccygeal) roots were mounted on silver wires above the ACSF and covered with Vaseline. The dorsal root was stimulated with a single pulse (0.1 ms, 0.02 mA, 3x's reflex threshold; repeated 5 times at 10s intervals for one trial, trials were repeated every 12 minutes), and the long-lasting reflex (LLR) response was recorded on the ventral roots, and then analyzed as for the surface EMG in humans. The LLR was quantified by averaging the rectified ventral root activity over a time-window 500-4000 ms post stimulus, a period previously shown to reflect the motoneuron Ca PIC activity in isolation (Murray et al. 2010; Rank et al. 2011). A 5 μ M dose of chlorpromazine (from Sigma-Aldrich) was used, a dose in the whole sacral spinal cord that is known to be effective for other neutral antagonists to the 5HT_{2C} receptor, such as SB242084, that have similar binding affinities as chlorpromazine (K_i < 20nM, see Table 4 in Murray et al. 2011b). The LLR was quantified at 30 minutes post-drug application because the effect of 5HT₂/ α 1 receptor antagonists applied in vitro peak at this time (Murray et al. 2010). A lack of effect of chlorpromazine on the LLR was taken as evidence that it acted as a neutral antagonist given that in chronically injured animals, there are little to no functional sources of serotonin for it to interfere with (Murray et al. 2010). Further, to verify that the 5 μ M dose of chlorpromazine was effective, the EC₅₀ of the 5HT₂ receptor agonist, α -methyl-5HT (Murray et al. 2011b), was measured in response to a prior application of 5 μ M of chlorpromazine. At 15 minute intervals 10, 100, 1000 and 10,000 nM of α -methyl-5HT was applied cumulatively starting at 15 minutes after chlorpromazine administration. An increase in the EC₅₀, i.e., the concentration of α -methyl-5HT needed to produce 50% of its maximal facilitation of the LLR, would indicate that a 5 μ M dose of chlorpromazine was effective in blocking a ligand if present and thus, acts as a neutral antagonist to the 5HT₂ receptor at this dose.

2.2.7 Statistics

All statistics were performed using SigmaPlot 11 software (Systat Software, Inc). All values presented in the text are listed as means \pm standard deviation and data in the figures are presented as means \pm standard error. Normality was tested with the Shapiro-Wilk test for SPR, LPR, LLR and ΔF values. One factor repeated-measures ANOVA for normally distributed data was used to determine the effect of the various drugs over time on the SPR, LPR, LLR and ΔF values and repeated-measures ANOVA on ranks was used for non-normally distributed data. Post-hoc Bonferroni corrected t-tests were used to isolate which post-drug response at the 30, 60, 90 or 120 minute time point differed from the pre-drug response. Significance was set to $p \leq 0.05$.

2.3 Results

2.3.1 Cyproheptadine: Incomplete SCI

Involuntary muscle spasms (S) in the tibialis anterior muscle were sometimes triggered after a voluntary dorsiflexion contraction (C) as shown in Figure 2-1A (top trace) for a participant with a T2-4 incomplete SCI (3F in Table 2-1). The incidence of these contraction-induced spasms decreased 60 minutes following the oral administration of the inverse agonist cyproheptadine (8 mg, Fig. 2-1A bottom trace). In this same participant, cyproheptadine also reduced the long-duration reflexes (i.e., muscle spasms) in the tibialis anterior triggered by electrical stimulation to the medial arch of the foot (Fig. 2-1B). Both the EPSP/PIC-mediated long polysynaptic reflex (LPR) and the PIC-mediated long-lasting reflex (LLR: see Methods for definitions) were reduced. In the 8 incomplete SCI (iSCI) participants tested (1M-8M, Table 2-1), cyproheptadine decreased both the LPR and LLR components of the long-duration reflex (Figs. 2-1D and 2-1E; one-way ANOVA, LPR: $F=3.66$, $p < 0.05$; LLR: $F=7.73$, $p < 0.001$). Post-hoc testing revealed a significant decrease in both the LPR and LLR at 60, 90 and 120 minutes compared to the pre-drug value (Bonferroni paired t-test; all p

<0.05). In contrast, the pure EPSP-mediated component of the reflex, the short lasting polysynaptic reflex (SPR, see inset in Fig. 2-1B), was not affected by cyproheptadine (Fig. 2-1C; $F=0.27$, $p = 0.89$).

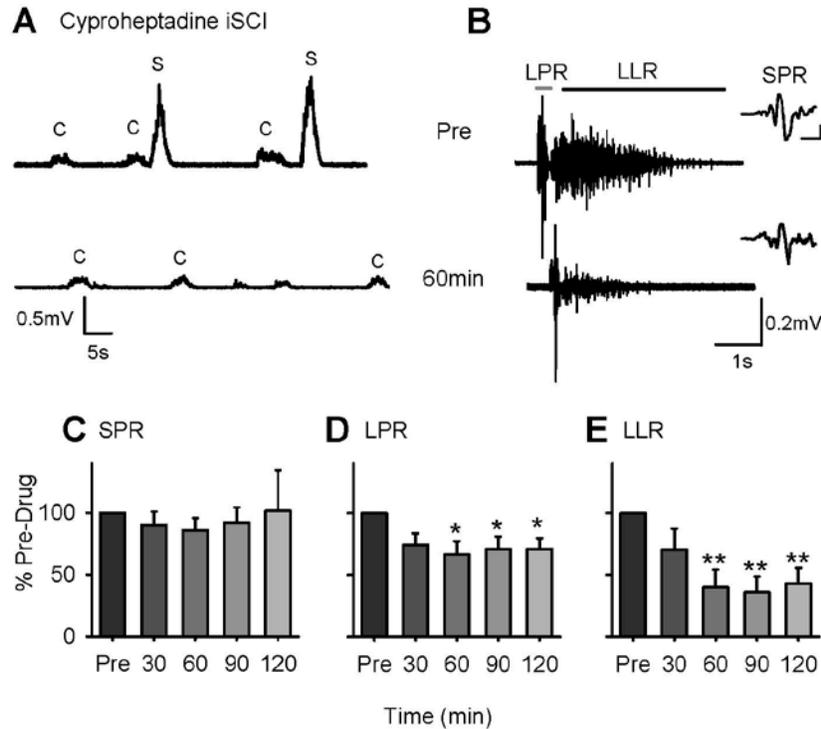


Figure 2-1 Cyproheptadine in incomplete SCI

A) Rectified surface EMG from tibialis anterior (TA) during dorsiflexion contractions (C) that trigger involuntary muscle spasms (S) in a T2-4 incomplete SCI participant before (Pre) and 60 minutes after (60 min) an 8mg dose of cyproheptadine (3F in Table 2-1). **B)** Same participant in A, unrectified surface EMG during long-duration reflex evoked in the TA from medial arch stimulation (22mA, 300 Hz, 14 pulses, 0.5 ms wide) before (top) and ≈60 minutes after (bottom) cyproheptadine. The long polysynaptic reflex (LPR: from start of reflex to 300ms post stimulation) is marked by the grey bar and the long-lasting reflex (LLR: from 500ms after stimulation to 3500 ms) is indicated by the black bar. Insets to the right show short polysynaptic reflex (SPR) on expanded time scale: scale bars 20ms/0.2mV. Bottom trace shifted to the right to reveal SPR. **C)** Average of SPR, LPR (**D**) and LLR (**E**) expressed as a percentage of Pre-drug values at different time points across the 8 iSCI participants tested (1M-8M, Table 2-1). Parts of the data from 6 of the 8 iSCI participants have been published previously (Murray et al. 2010). Error bars in this and subsequent figures represent mean±standard error. * $p < 0.05$, ** $p < 0.005$.

In 6 of the 8 iSCI participants (# in Table 2-1), we were able examine the effect of cyproheptadine on the estimated amplitude of the motoneuron PIC using paired motor unit analysis (see “Estimation of PIC amplitude” in Methods).

Briefly, the firing rate of a lower-threshold, control motor unit recorded from the tibialis anterior (bottom graphs) was used as a measure of synaptic drive to a higher-threshold, test motor unit (top graphs) during a triangular voluntary dorsiflexion (Fig. 2-2A). The difference in the smoothed firing rate of the control motor unit when the test motor unit was de-recruited, compared to when it was recruited (ΔF), was used as a measure of the reduction in synaptic input needed to counteract the depolarization from the PIC and provides an indirect estimate of PIC amplitude. As shown for the single subject in Figure 2-2A, the higher-threshold test motor unit was de-recruited at a higher firing rate of the control motor unit at 60 minutes after cyproheptadine (right panels) compared to before (left panels). This produced a decrease in the ΔF measurement (arrows in Fig. 2-2A) to signify a reduction in the estimated PIC. In the 6 iSCI participants where 2 motor units of different recruitment thresholds could be distinguished for the 2 hours after drug intake, there was a significant decrease in the ΔF from cyproheptadine (Fig. 2-2B; $F = 4.85$, $p = 0.007$), with the ΔF significantly smaller by 29% (on average) at 30 and 60 minutes post-drug ($p < 0.05$).

Although the test unit was de-recruited earlier, the firing rate profile of the control motor unit remained similar after cyproheptadine with a mean firing rate throughout the contraction of 8.51 ± 0.35 Hz post-drug compared to 8.8 ± 1.1 Hz before drug intake ($p = 0.63$), signifying that the voluntary synaptic drive was similar before and after drug intake. Likewise, the test motor units were activated for a similar duration of time during the ascending phase of the contraction before cyproheptadine intake (median 3.53s) compared to after (median 3.21s, $p = 0.62$), ensuring that enough time was given for full activation of the CaPIC. Lastly, a large percentage of the firing rate profile of the test unit could be accounted for by the firing rate profile of the control unit, both before and after cyproheptadine administration (median $r^2 = 0.80$ pre-drug, 0.73 post-drug at 60 minutes), indicating that the control unit provided similar information about the synaptic drive to the test unit both before and after drug intake.

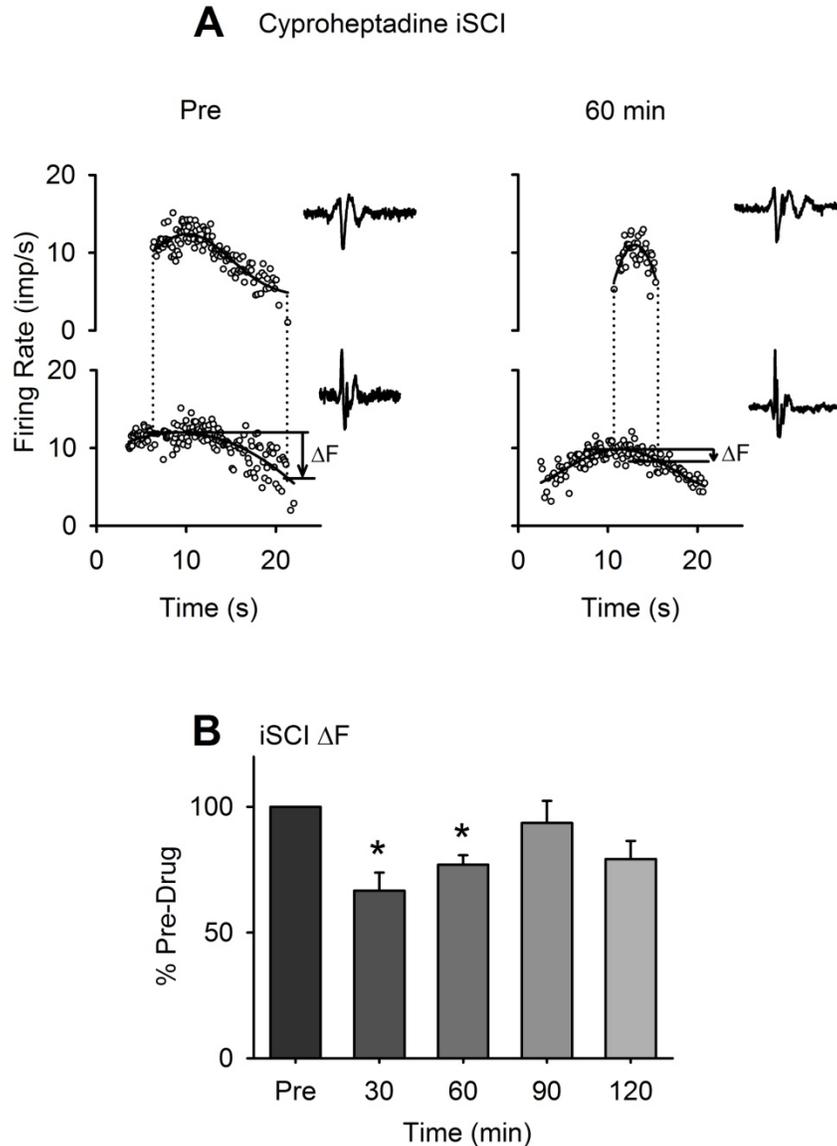


Figure 2-2 Effects of cyproheptadine on estimated PIC

A) Instantaneous firing rate of lower-threshold control (bottom panels) and higher-threshold test (top panels) motor units during isometric dorsi-flexion before (left graphs) and 60 minutes after (right graphs) oral intake of 8 mg cyproheptadine in iSCI participant. The thick black line represents a 5th order polynomial fit through the firing rate points. Dashed vertical lines mark the time of occurrence of recruitment and de-recruitment of the higher-threshold test unit. Solid horizontal lines indicate the firing frequency of the control unit when the test unit was recruited and de-recruited, with the magnitude of difference in the firing rates between the two time points (ΔF) marked by the arrow. Insets are overlays of motor unit action potentials. The change in shape of the motor units over time was gradual, allowing for their continual identification. **B)** Average ΔF expressed as a percentage of the Pre-drug values at different time points across the 6 iSCI participants tested (3F-7M, 9M, # in Table 2-1). * $p < 0.05$.

2.3.2 Citalopram: Incomplete SCI

Due to the incompleteness of the injury, the iSCI participants examined above could have had residual sources of serotonin or noradrenaline below the injury from spared descending axons. Thus, the observed decreases in the PIC-mediated responses (LLR's and ΔF 's) from the inverse agonist cyproheptadine could have arisen not only from suppression of constitutive 5HT₂/ α ₁ receptor activation but also by blocking ligand-activation of the receptor. To test for this possibility, in 4 of the iSCI participants tested, a 20mg dose of citalopram, a selective serotonin reuptake inhibitor (SSRI), was also given in a separate experiment to examine if the PIC-mediated LLR could be facilitated by increases in the release of serotonin from spared descending axons. We first examined in non-injured (NI) control participants, who have intact descending 5HT axons, if a 20mg dose of citalopram was effective in increasing the PIC-mediated ΔF . As shown for a single control subject in Figure 2-3A, at 90 minutes post drug, the test motor unit continued to fire at much lower rates of the control motor unit just before de-recruitment compared to before the drug, thereby producing a larger ΔF (see arrow). An average increase in the ΔF of ~80% was obtained in 3 uninjured control participants ($79 \pm 11\%$ increase averaged across all time points; data not shown), verifying that a 20 mg dose of citalopram should be effective in increasing the PIC-mediated LLR if iSCI participants have some residual descending monoaminergic axons. As shown for the same iSCI participant in Figure 2-1, a 20mg dose of citalopram prolonged the long-duration reflex evoked from stimulation of the medial arch of the foot (Fig. 2-3B). The average LLR increased by $152 \pm 67\%$ in the 4 iSCI participants tested with citalopram (Fig. 2-3D; $F = 3.56$, $p = 0.04$); however, none of the individual values post-drug reached statistical significance compared to pre-drug ($p > 0.05$). In contrast, the mixed sensory/PIC mediated component of the response (LPR) remained unchanged over time ($F = 0.56$, $p = 0.70$).

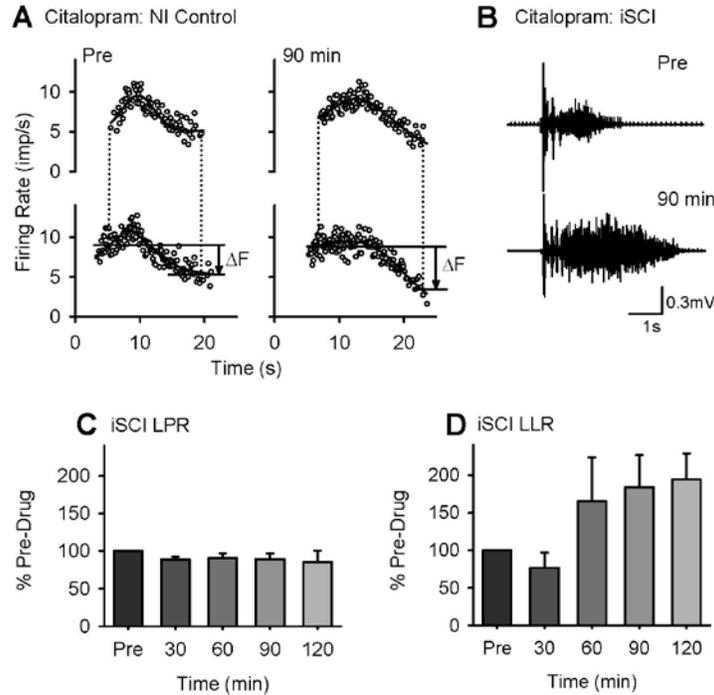


Figure 2-3 Citalopram in non-injured controls and incomplete SCI

A) Paired motor unit recordings as in Fig. 2-2A before (left graph) and 90 minutes after 20 mg citalopram administration (right graph) in a single non-injured (NI) control participant. **B**) Long-duration reflex recorded in TA (medial arch stimulation: 15.7mA, 300 Hz, 14 pulses, 0.5 ms pulse width) in a single iSCI participant (3F, Table 2-1) before and \approx 90 minutes after 20 mg citalopram. **C**) Average of LPR and LLR **(D)** expressed as a percentage of Pre-drug at different time points across the 4 iSCI participants tested (2M-5M, * in Table 2-1).

Based on these results, it is likely that many of the iSCI participants tested had spared, descending sources of monoamines. Thus, we cannot determine how much the reduction of PIC-mediated responses by the inverse agonist cyproheptadine was due to a suppression of constitutive 5HT2/ α 1 receptor activity or due to a reduction of ligand-activation of the receptor. As a result, we repeated these experiments in SCI participants with motor or motor/sensory complete injuries (cSCI) where there should be little to no residual sources of serotonin and noradrenaline below the lesion (Murray et al. 2010; Rank et al. 2011).

2.3.3 Cyproheptadine: Motor and Motor/Sensory Complete SCI (cSCI)

Figure 2-4A shows the gradual decrease in the long-duration reflex, especially the LLR component, from a motor/sensory complete SCI participant (16M, Table 2-1) after 12 mg dose of cyproheptadine. The dose of cyproheptadine was increased to 12 mg because an 8 mg dose was minimally effective in two pilot participants with motor complete SCI (data not shown). The data from 9 cSCI participants (10M-18M, Table 2-1) showed similar effects with a $48 \pm 12\%$ reduction in the LLR averaged over 30 to 120 minutes after drug intake (Fig. 2-4C; $F=13.93$, $p < 0.001$) and a smaller $22 \pm 10\%$ reduction in the LPR over the same time period (Fig. 2-4B; $F=4.75$, $p=0.005$). Post-hoc analysis revealed that many time points were significantly smaller post-drug (see Figs. 2-4C and B; all $p < 0.05$). A distinct, early-latency SPR was recorded in only 2 of the 9 cSCI participants and thus, was not analyzed further.

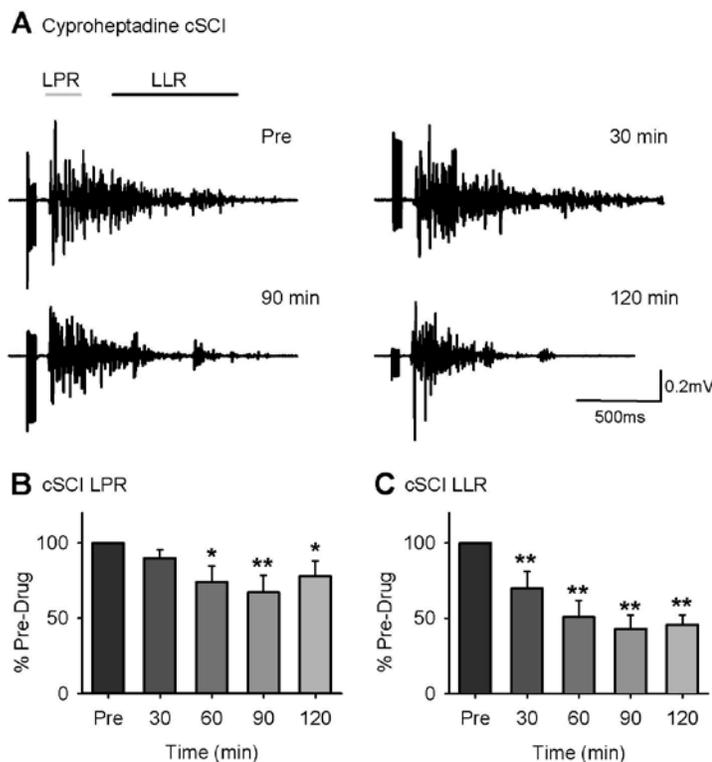


Figure 2-4
Cyproheptadine in complete SCI
A) Long-duration reflexes recorded in TA (medial arch stimulation: 60mA, 300Hz, 14pulses, 0.5ms pulse width) in single cSCI participant (subject 16M, Table 2-1) before, 30, 90 and 120 minutes after 12 mg cyproheptadine. **B)** Average of LPR and LLR **(C)** expressed as a percentage of Pre-drug at different time points across the 9 cSCI participants tested (10M-18M, Table 2-1). * $p < 0.05$, ** $p < 0.005$.

2.3.4 Chlorpromazine: Motor and Motor/Sensory Complete SCI

To further exclude the possibility that the effects of cyproheptadine on the cSCI long-duration reflex was due to a block of endogenous serotonin or noradrenaline activating the receptors (and therefore could only result from a decrease in constitutive receptor activity), we also examined if the neutral antagonist chlorpromazine, which only blocks ligand-activation of the 5HT₂/α₁ receptor in transfected cells (Herrick-Davis et al. 2000; Rauser et al. 2001), had no effect on the LPR and LLR. We first needed to determine if chlorpromazine acted as a neutral antagonist on 5HT₂/α₁ receptors in spinal motoneurons. In chronically spinalized rats with no functional sources of serotonin or noradrenaline (Murray et al. 2010; Rank et al. 2011), chlorpromazine was ineffective in reducing the PIC-mediated LLR (Fig. 2-5A, top graph) at a dose (5μM) comparable to that used for other 5HT₂/α₁ receptor neutral antagonists. The average amplitude of the LLR was 109.1±12.7% of the pre-drug value at 30 minutes post drug application (Fig. 2-5A, bottom graph; p = 0.14), confirming that it was not acting as an inverse agonist in spinal motoneurons but only as a neutral antagonist. Moreover, a prior application of 5μM chlorpromazine increased the EC₅₀ of α-methyl-5HT, a 5HT₂ receptor agonist, to 2030±1200 nM (n = 6) compared to an EC₅₀ of 62.84±26.51 nM (n = 14, Murray et al. 2011b) when α-methyl-5HT is applied alone (data not shown, p < 0.001). The 30-fold increase in the EC₅₀ of α-methyl-5HT by chlorpromazine demonstrated that a 5μM dose was effective in blocking a ligand if one was present and thus, chlorpromazine acted as a neutral antagonist at this dose.

When we examined the effects of chlorpromazine on the ΔF in non-injured (NI) control participants having full sources of serotonin/noradrenaline, chlorpromazine was effective in reducing the ΔF at 90 and 120 minutes post-drug intake (F = 6.72, p = 0.011; 90 and 120 min p < 0.05). Contractions were similar before and after drug intake as reflected in similar mean firing rates of the control motor units (pre-drug: 10.76±1.27 Hz vs. post-drug: 10.35±1.94 Hz, p = 0.50) and

median duration of test motor unit activation during the ascending phase of the contraction (pre-drug: 5.5s vs. post-drug: 5.1s, $p = 0.26$). Similar to the cyproheptadine data, a large percentage of the firing rate profile of the test unit could be accounted for by the firing rate profile of the control unit, both before and after chlorpromazine (median $r^2 = 0.78$ pre-drug, 0.82 post-drug at 90 minutes).

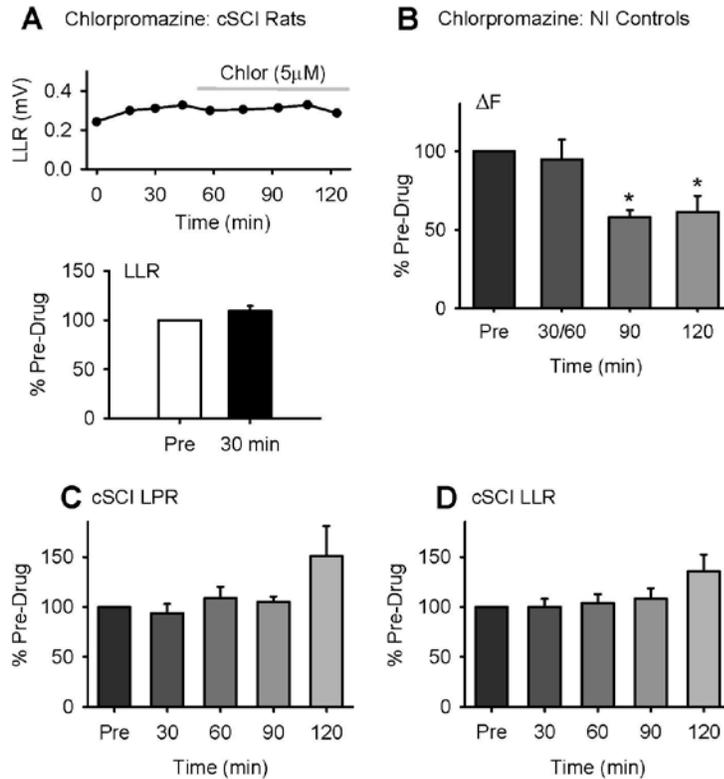


Figure 2-5 Chlorpromazine in rat complete SCI, human non-injured controls and complete SCI

A) Top graph: amplitude of LLR measured in ventral root evoked from single-pulse, dorsal root stimulation (0.1 ms, 0.02 mA, 3x's reflex threshold) from a single, completely spinalized rat before and after 5 μ M of chlorpromazine (time of drug application marked by grey line). Bottom graph: average of LLR expressed as percentage of Pre-drug at 30 minutes after (black bar) application of chlorpromazine to bath solution ($n = 6$ roots). **B)** Average ΔF expressed as percentage of Pre-drug at different time points across 4 non-injured (NI) control participants. **C)** Average of LPR and LLR **(D)** expressed as percentage of Pre-drug at different time points across 7 of the 9 cSCI participants tested (10M-16M, ^ in Table 2-1). * $p < 0.05$.

In contrast to the non-injured controls, chlorpromazine had no effect on either the LPR (Fig. 2-5C) or LLR (Fig. 2-5D) in the cSCI participants with likely little to no residual serotonin or noradrenaline below the injury (LPR: Chi-square = 1.44, $p = 0.84$; LLR: $F = 1.16$, $p = 0.36$). When analyzing separately the effects of chlorpromazine in the 3 SCI participants with some sensory preservation (10-12M, Table 2-1), the LPR and LLR across all post-drug time points were still unchanged ($5 \pm 4\%$ and $0 \pm 10\%$ change, respectively), suggesting that although there was some sparing in ascending sensory pathways, there was unlikely any functional descending sources of the ligand serotonin or noradrenaline to the motoneuron in these participants. Taken together, these results suggest that chlorpromazine acts as a neutral antagonist on spinal motoneurons, decreasing 5HT2/ α 1 receptor activation only if there are residual sources of the ligand present (i.e., as in non-injured controls) and does not reduce constitutive 5HT2/ α 1 receptor activity (i.e., in chronic spinal rats and cSCI participants) that is reduced by the inverse agonist cyproheptadine.

2.4 Discussion

Motoneuron PICs are facilitated by the activation of 5HT2 and α 1 receptors (Harvey et al. 2006a, 2006b; Hounsgaard et al. 1988; Hsiao et al. 2005; Perrier and Cotel 2008), specifically the 5HT2B/C (Murray et al. 2011b) and α 1A (Rank et al. 2011) receptor subtypes. In this study cyproheptadine, an inverse agonist which reduces both constitutive and ligand activation of 5HT2/ α 1 receptors, reduced the PIC-mediated LLR in iSCI participants. However, citalopram, a selective serotonin reuptake inhibitor, also increased the LLR in all of the iSCI participants tested, indicating that functional sources of serotonin are likely present in incomplete spinal cord injury (see also Thompson et al. 2011). Thus, we cannot determine if the reduction of the LLR by cyproheptadine in the iSCI group was produced *solely* by a reduction in constitutive 5HT2/ α 1 receptor activity given that there was likely ligand activation of the receptor as well (cf Murray et al. 2010). When tested in motor or motor/sensory complete SCI participants with likely little to no functional sources of descending monoamines

from spared or sprouted axons (see “Spared descending monoaminergic tracts...” below), cyproheptadine still reduced the LLR substantially. Moreover, the neutral antagonist chlorpromazine, which only blocks ligand-activation of the 5HT2/ α 1 receptor, had no effect on the LLR in the cSCI participants at oral doses that were very effective in reducing ΔF (PIC) measures in uninjured controls having descending sources of 5HT (as shown by citalopram). The combined results showing that the inverse agonist reduced the LLR (which blocks both ligand and constitutive receptor activation), but the neutral antagonist had no effect (which only blocks ligand activation of the receptor), suggests that after motor complete SCI, PIC-mediated long-lasting reflexes (i.e., spasms) are facilitated solely by the presence of constitutive 5HT2/ α 1 receptor activation on motoneurons, as previously shown in animal models of complete SCI (Murray et al. 2010; Rank et al. 2011).

2.4.1 Selectivity of cyproheptadine in reducing the motoneuron PIC

Reduction of the LLR by cyproheptadine in SCI participants could have arisen by reducing the transmission of sensory afferent inputs onto the motoneuron, which then subsequently activate the motoneuron PIC to trigger a LLR or spasm (Li et al. 2004a; Norton et al. 2008). However, as shown for the iSCI participants the SPR, occurring in the first 50 ms of the reflex, was not affected by cyproheptadine. This short-latency, short-lasting polysynaptic response has been shown in rats to be solely mediated by a short-lasting, sensory-evoked EPSP because it is not reduced by blocking the CaPIC with isradipine (Murray et al. 2011a) nor is it affected by blocking of 5HT2/ α 1 receptors (Murray et al. 2010). Moreover, because the CaPIC takes 50 ms or longer to activate (Li et al. 2004a; Murray et al. 2011a), it is unlikely that it contributes substantially to motoneuron activation during this early time. The longer-latency and longer-lasting (\approx 200 ms) LPR on the other hand is mediated by both a long EPSP and the CaPIC, as revealed by blocking PICs with either cell hyperpolarization or isradipine (Murray et al. 2011a). In contrast, the majority of the LLR (85-90%) is

mediated by CaPIC activation. It is interesting that in the SCI participants, cyproheptadine was proportionally more effective in reducing reflex components that contained proportionally larger amounts of CaPIC activation as determined from the rat studies. For example in iSCI participants, the SPR containing little to no CaPIC was reduced by 5% on average, the LPR that is partly mediated by the CaPIC was reduced by 30%, and the LLR which is mainly mediated by the CaPIC was reduced by 60%. This suggests that cyproheptadine selectively reduced the CaPIC via its action on the 5HT2/ α 1 receptors.

In agreement with the PIC-mediated LLR, the more direct estimation of PIC amplitude via the ΔF measures was also reduced by cyproheptadine. Thus, the ΔF measurement is sensitive to drug effects as was also shown for the chlorpromazine data in this paper and previously for amphetamine administration in non-injured control participants (Udina et al. 2010). We were able to follow the same units during voluntary contractions both before and after drug intake for the majority of the motor unit pairs (see “Estimation of PIC amplitude” in Methods), which helped to reduce the influence of sampling bias on the ΔF measurements. Moreover, the firing rate relationship between the control and test motor units and the mean rates of the control units were not affected by the drugs, suggesting that the synaptic drive to the TA motoneuron pool did not change after drug intake to potentially influence the ΔF measurements.

2.4.2 Selectivity of cyproheptadine in blocking 5HT2/ α 1 receptors

Cyproheptadine is a broad spectrum antagonist, binding with high affinity (K_i 's < 30nM) to 5HT2 (A, B and C: Boess and Martin 1994; Bonhaus et al. 1997; Martin 1997), noradrenergic (α 1: Yoshio et al. 2001), histamine (H1: Moguilevsky et al. 1994), cholinergic (M1-5: Stanton et al. 1993) and dopamine (D3:Toll et al. 1998) receptors. However, the reduction of the motoneuron PIC by cyproheptadine is mainly mediated by blocking the 5HT2B/C and α 1 receptors because specific antagonists to these receptors completely mimic the action of cyproheptadine on the CaPIC (Harvey et al. 2006a, 2006b). Moreover, blocking

the other receptors that cyproheptadine binds to, such as H1 (Harvey and Bennett unpublished data), D3 (Dai et al. 2009) and M2 (Miles et al. 2007), does not affect the motoneuron PIC. Therefore, although cyproheptadine can strongly affect dopamine, muscarine and histamine receptors, its effects on the motoneuron, as measured by the LLR and ΔF , are likely restricted to affecting the 5HT2 and $\alpha 1$ receptors.

2.4.3 Chlorpromazine as neutral antagonist on 5HT2/ $\alpha 1$ receptors

Similar to cyproheptadine, chlorpromazine also has high affinity (K_i 's < 20 nM) to 5HT2 (A and C; Boess and Martin 1994; Herrick-Davis et al. 2000; Rauser et al. 2001; Toll et al. 1998), 5HT6 (Kroeze et al. 2003; Roth et al. 1994), noradrenergic ($\alpha 1$: Kroeze et al. 2003; Richelson 1988; Richelson and Nelson 1984), dopamine (D2, D3, D4: Kroeze et al. 2003; Richelson 1988; Seeman et al. 1997; Stormann et al. 1990; Toll et al. 1998) and histamine (H1: Kroeze et al. 2003; Richelson 1988) receptors. However, as stated above, dopamine, histamine and 5HT6 receptors do not modulate motoneuron PIC activation (Murray et al. 2011b) so chlorpromazine's actions on these receptors would likely not affect the LLR and ΔF measurements in this study.

There is strong supportive evidence that chlorpromazine, at the doses used in this study, acted as a neutral antagonist to 5HT2/ $\alpha 1$ receptors on motoneurons. For example, 5 μ M of chlorpromazine was ineffective in suppressing the 5HT2/ $\alpha 1$ receptor-mediated facilitation of the LLR in chronically injured animals where there are little to no functional sources of the ligand 5HT (Murray et al. 2010). However, this same dose was effective in decreasing the efficacy of the 5HT2 receptor agonist, α -methyl-5HT, in facilitating the LLR (by 30-fold), providing strong evidence that the 5 μ M dose used was effective in blocking ligand activation of the 5HT2 receptor when present. In support of this, in COS-7 cells transfected with the highly constitutive isoform of the human 5HT2C (INI) receptor, chlorpromazine does not modulate 5HT2C receptor activity when applied in isolation but only suppresses the facilitation of 5HT2C receptors after

the application of the ligand 5HT (Herrick-Davis et al. 2000). In the lumbar spinal cord, Barbeau and colleagues also demonstrated in chronically spinalized rats that spontaneous leg muscle activity induced by 5HTP, which is known to be mediated by 5HT₂/ α 1 receptor facilitation of the motoneuron PIC, is suppressed by chlorpromazine (Barbeau et al. 1981). Likewise, suppression of PIC-mediated polysynaptic reflexes by chlorpromazine only occurs in acutely injured animals (Ito et al. 1982), where there are abundant stores of 5HT present (Murray et al. 2010), but not in chronically injured animals where levels of the ligand 5HT are nearly abolished. The high affinity of chlorpromazine to 5HT_{2C} (6.1 nM) and α 1 (2.6 nM) receptors and its strong suppression of motoneuron PIC activation only when a ligand is present, strongly supports our claim that chlorpromazine is acting as a neutral antagonist, failing to reduce the LLR in rat spinal cords devoid of 5HT but reducing the ΔF in non-injured controls with descending sources of 5HT.

2.4.4 Constitutive 5HT₂/ α 1 receptor activity in iSCI

Although we cannot prove the existence of constitutive 5HT₂/ α 1 receptor activity in the motor incomplete spinal cord participants using pharmacology, this does not mean that constitutive receptor activity is absent in these participants or in the non-injured control participants for that matter. For instance, in unlesioned control spinal cords approximately 10% of the 5HT_{2C} receptors in the sacral spinal cord are of the unedited INI and weakly edited VSI isoforms (Murray et al. 2010) that display higher degrees of constitutive receptor activity compared to the more edited isoforms of the receptor (e.g., VSV). The presence of some degree of constitutive activity likely ensures a basal level of motoneuron PIC-facilitation from 5HT₂/ α 1 receptors given that the number of brainstem serotonergic neurons with descending projections to the spinal cord is relatively low (\approx 3000) in rodents compared to other descending pathways (Jacobs et al. 2002). Thus, with incomplete SCI there may be an increase in the proportion of monoaminergic receptors with constitutive activity to compensate for the partial loss of serotonin and noradrenaline below the injury.

2.4.5 Spared descending monoaminergic tracts after motor complete SCI

Persons with motor/sensory (AIS A) or motor (AIS B) complete injuries typically have little white matter sparing based on tensor diffusion imaging studies (Ellingson et al. 2008; Petersen et al. 2012). In addition, patients with bilateral surgical lesions with sparse ($\approx < 10\%$) white matter sparing to the posterior half of the spinal cord, where descending monoaminergic fibres to the ventral horn are located (Holstege and Kuypers 1987; Martin et al. 1978), demonstrate remarkable recovery of leg movements and even walking function in the months following surgery (Nathan 1994). Thus, persons with permanent motor or motor/sensory complete deficits, like the participants studied here, likely have extensive white matter damage in the posterior half of the spinal cord that contain descending monoaminergic axons. The lack of effect of the ligand-blocking neutral antagonist chlorpromazine in completely injured rats and motor complete SCI participants also supports this assumption. Future experiments demonstrating a lack of effect of serotonin/noradrenaline reuptake inhibitors on PIC-mediated reflexes evoked from spinal segments below the injury would also support this claim.

2.4.6 Clinical implications

Because functional sources of monoamines are present below the level of injury following *incomplete* spinal cord injury, as shown from the citalopram results, motoneuron excitability is likely facilitated by both ligand and constitutive activation of monoaminergic receptors. A contribution from ligand activation of monoaminergic receptors to spasticity in incomplete spinal cord injury is evidenced by the findings that oral administration of the neutral antagonist chlorpromazine, in a controlled study, reduced muscle tone in patients with multiple sclerosis where spasticity was primarily the result of spinal cord involvement (Cohan et al. 1980, see also Basmajian and Szatmari 1955). In contrast, in *motor complete* SCI, it appears that motoneuron excitability is solely

maintained via the activation of constitutive monoaminergic receptor activation because we found that blocking ligand activation of monoaminergic receptors with neutral antagonists was ineffective. The slow recovery of motoneuron excitability in the first few months after SCI, especially in severe injuries, is likely due to the slow increase of constitutive 5HT₂/α₁-receptors, which then reactivate the motoneuron PIC. Moreover, inhibition of excitatory sensory inputs onto the motoneuron is also reduced after SCI (Boulenguez et al. 2010; Murray et al. 2011a; Norton et al. 2008), creating a perfect storm to trigger the unchecked activation of PICs and associated involuntary muscle spasms.

The findings in this study have potentially important therapeutic implications as they point to a new target to pharmacologically reduce the unwanted activation of motoneurons producing involuntary muscle spasms. In contrast to the most commonly prescribed anti-spastic medications, baclofen (Lioresal®) and tizanidine (Zanaflex®), which act to decrease sensory afferent transmission (see Fig. 2-5 in Discussion of accompanying D'Amico et al. 2012), inverse agonists to 5HT₂/α₁ receptors (cyproheptadine, SB206553) exert their effects directly on the motoneuron by suppressing the activity of both ligand and constitutively active receptors to reduce the facilitation of voltage-dependent CaPICs via Gq-coupled pathways. Although severely reducing PIC activation would not be desirable in patients with incomplete SCI because it would also weaken activation of the motoneuron from spared descending inputs, this is not a concern in motor complete SCI.

Cyproheptadine has been shown to be as effective in reducing excessive muscle activation compared to clonidine and baclofen in spastic, spinal cord injured patients (Nance 1994) and at a dose of 12mg/day, it was well tolerated during a 2-month trial (Wainberg et al. 1990). Associated with cyproheptadine's anti-histamine and anti-cholinergic properties were side effects such as drowsiness, dry mouth, appetite stimulation and thus, the potential for weight gain although the latter has not been quantified in a closed, placebo controlled trial in patients with spinal cord injury (see Gracies et al. 1997 for review, Toth and

Szonyi 1976). The development of more specific inverse agonists to 5HT₂/α₁-receptors, with less side effects than cyproheptadine or SB206553 (carcinogenic by-products), may lead to better oral control of involuntary muscle spasms in SCI, especially in motor complete patients where suppression of unwanted muscle activity is the primary goal. However, moderate suppression of motoneuron excitability leading to a decrease in clonus and involuntary spasms by cyproheptadine can produce improvements in residual walking function in participants with incomplete spinal cord injury (Wainberg et al., 1990). Based on our better understanding of the mechanism of action of cyproheptadine, perhaps closed placebo controlled trials and long-term studies are warranted to determine the potential cost/benefits of cyproheptadine in both complete and incomplete SCI. Additionally, in severe cases of spasticity where intrathecal delivery of baclofen is used (Lazorthes et al. 1990; Nielsen et al. 2002; Stempien and Tsai 2000), it may be worthwhile to test if cyproheptadine, which acts directly on motoneurons and thus can suppress all spared aberrant inputs (descending and peripheral), is more effective compared to baclofen which acts to only suppress sensory transmission to the motoneuron (Curtis et al. 1997; Li et al. 2004b).

Subject Code	Age, yr	Age of Injury, yr	Injury Level	AIS	MAS	Penn Spasm Scale	Preserved Sensation	
							Light touch	Pinprick
1M	61	6	C3, C6	C	0	2	✓	✓
2M†	54	12	C5-C6	C	3	3	✓	✓
3F*†	49	6	T2-T4	C	0	3	✓	✓
4F*†	23	2	C6-C7	C	1	3	✓	✓
5M*†	50	12	C6-C7	D	3	2	✓	✓
6M*	51	31	C3-C4	D	1	2	✓	✓
7M*	53	3	C1-C3	D	1	2	✓	✓
8M	61	2	C3-C5	C	NA	1	✓	✓
9M*	45	3	C4-C5	D	1	3	✓	✓
10M‡	33	13	C5-C6	B	1	3	Left: ✓ Right: ✓	Left: ✓ Right: ✓
11M‡	29	7	C4-C5	B	1	3	Left: none Right: upper shin and foot	Left: outer shin Right: ✓
12M‡	31	1.5	T3-T4	B	0	3-4	Left: ✓ except front of shin Right: none	Left: ✓ Right: inner side of leg
13M‡	45	3	T4-T5	A	1	2	Left: none Right: none	Left: none Right: none
14M‡	21	11	C6-C7	A	1	2	Left: none Right: none	Left: none Right: none
15M‡	24	6	C5-C6	A	2	1	Left: none Right: none	Left: none Right: none
16M‡	22	2	T4	A	2	3	Left: none Right: none	Left: none Right: none
17M	53	8	C7-T1	B	NA	NA	Left: none Right: none	Left: none Right: none
18M	23	2	T4-T5	A	2	3	Left: none Right: none	Left: none Right: none

Table 2-1 Demographic and clinical description of SCI participants

Description of participants including age of the participant and their injury at the time of recording, level of injury and ASIA International Score (AIS), modified Ashworth (0-4) and Penn Spasm Frequency Scale (Part 1: 0-4). The last two columns characterize the preservation of light touch and pin prick sensation from the knee downwards: ✓ indicates preserved sensation. All participants were tested with cyproheptadine; # indicates participants in whom paired motor unit analysis was performed, * indicates participants tested with citalopram, ^ indicates participants tested with chlorpromazine. Shaded area indicates participants with preserved motor function in legs.

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CHAPTER 3:

***REDUCTION OF SPINAL SENSORY
TRANSMISSION BY FACILITATION OF
5HT1B/D RECEPTORS IN NONINJURED
AND SPINAL CORD-INJURED HUMANS***

A version of this chapter has been published.
D'Amico JM, Li Y, Bennet DJ and Gorassini MA.
Journal of Neurophysiology 109(6): 1485-1493, 2013.

3.1 Introduction

After a complete spinal cord injury (SCI), levels of serotonin and noradrenaline below the lesion decrease greatly because the major supply of these neuromodulators comes from descending pathways originating in the brainstem (Anden et al. 1964; Carlsson et al. 1963; Jacobs et al. 2002; Jordan et al. 2008; Rekling et al. 2000). Serotonin and noradrenaline normally inhibit transmission of ascending and segmental sensory pathways via the activation of Gi- coupled serotonin (5HT1) and noradrenaline ($\alpha 2$) receptors located on sensory afferent terminals and excitatory interneurons (Clarke et al. 2002; Di Pasquale et al. 1997; Engberg et al. 1968; Jankowska et al. 1993; Jordan et al. 2008; Manuel et al. 1995; Millan 2002; Rekling et al. 2000; Singer et al. 1996; Yoshimura and Furue 2006). The reduction of 5HT1 and $\alpha 2$ receptor activation (Murray et al. 2011; Rank et al. 2011) results in the enhanced transmission of low-threshold, cutaneomuscular afferent pathways to produce abnormally long (≈ 1 s), excitatory postsynaptic potentials (EPSPs) in response to brief sensory stimulation in both rat and human motoneurons (Li et al. 2004a; Norton et al. 2008). These long polysynaptic EPSPs contribute to spasticity after SCI by triggering slowly activating, calcium persistent inward currents (CaPICs) that drive self-sustained motoneuron firing and involuntary muscle spasms (Bennett et al. 2001b; Gorassini et al. 2004; Heckman et al. 2008; Li et al. 2004a)

One strategy to reduce muscle spasticity after SCI has been to facilitate the activation of Gi-coupled pathways to reduce sensory transmission to motoneurons. The common anti-spastic medication baclofen, a GABA_B-receptor agonist, and clonidine/tizanidine, $\alpha 2$ -receptor agonists, activate Gi-coupled pathways to reduce cAMP and Ca⁺⁺ entry into synaptic terminals (Curtis et al. 1997). This ultimately results in reducing the duration of polysynaptic EPSPs from about 1s to less than 50 ms and as a consequence, the CaPIC is not activated and muscle spasms are reduced (Li et al. 2004b; Murray et al. 2011; Rank et al. 2011). However, both baclofen and tizanidine produce undesirable side effects such as tolerance, sedation and hypotension (Davidoff 1985; Gracies et al. 1997;

Krach 2001; Meleger 2006; Rosche 2002) and thus, are not optimal oral anti-spastics. Recently, another strategy to activate Gi-coupled pathways via 5HT1 receptors has been tried where both short- and long-latency polysynaptic EPSPs were reduced by either 5HT1B or 5HT1F receptor facilitation (Murray et al. 2011), leading to a reduction in CaPIC activation and muscle spasms. Application of the various 5HT1B/F receptor agonists did not reduce the CaPIC itself, demonstrating that their anti-spastic effects were mediated by reducing the duration of sensory, synaptic activation of the motoneuron so that the slowly activating CaPICs were not recruited.

One of the 5HT1 receptor agonists tested in the Murray et al. 2011 study was zolmitriptan, a 5HT1B/D receptor agonist that is approved for human use to treat migraines (Martin 1997; Martin et al. 1997; Peterlin and Rapoport 2007). Although zolmitriptan cannot be used on a daily basis due to adverse side effects, and thus cannot be used as an anti-spastic, we tested as a proof of principle if facilitation of 5HT1B/D receptors in humans also reduces segmental sensory transmission to motoneurons in both non-injured control and SCI participants. To examine the activation of the motoneuron by sensory inputs in isolation without CaPIC activation, we examined the effect of zolmitriptan on the soleus H-reflex. The EPSP produced during a monosynaptic reflex is very short, $\approx 5-10$ ms in rat and cat motoneurons (Baker and Chandler 1987; Edwards et al. 1989; Jimenez et al. 1991; Li et al. 2004b) and estimated to be ≈ 30 ms in humans based on motor unit recordings (Miles et al. 1989). Thus, depolarization of the motoneuron during an H-reflex is too brief (< 50 ms) to activate a CaPIC and any reduction in motoneuron output from zolmitriptan would likely be due to a reduction in its sensory synaptic activation. We also examined in SCI participants if long-lasting, polysynaptic reflexes activated by cutaneous stimulation to the medial arch of the foot, and the subsequent long-lasting reflexes (spasms) they trigger, were also reduced by zolmitriptan to determine if facilitation of 5HT1B/D receptors is a potential strategy to reduce spasticity after SCI. Parts of this data have been published in abstract form (D'Amico and Gorassini 2012).

3.2 Methods

Experiments were approved by the Health Research Ethics Board at the University of Alberta and conformed to the Declaration of Helsinki. The off-label use of the anti-migraine drug zolmitriptan, in non-injured and SCI participants, was approved by Health Canada-Clinical Trials. All participants gave written, informed consent prior to participating in the study. In total, 6 non-injured control (35 ± 13 yrs, 2 female) and 7 SCI participants with motor complete injuries (35 ± 10 yrs, 2 female) took part in the study (Table 3-1). Three of the 7 SCI participants (1-3M, Table 3-1) also took part in the 5HT₂ receptor study of D'Amico et al. 2012. Drug screens were performed to rule out contraindications for zolmitriptan and to ensure participant safety. Five other SCI participants were excluded from the study due to drug contraindications from antidepressants and one subject was excluded due to a blood clotting disorder that made it unsafe to administer zolmitriptan. A further 2 SCI participants were excluded from the study (both motor complete, T11/12 and T6/7) because appreciable H-reflexes could not be evoked from either leg.

3.2.1 Drug Administration

All participants were required to come to the lab on two separate occasions (separated by at least 1 week) to receive placebo or the drug zolmitriptan in random order. Drug and placebo were housed in a two-part telescoping capsule to conceal the identity of the drug. Non-injured control and SCI participants received a 10mg dose of the 5HT_{B/D} agonist zolmitriptan (Proietti-Cecchini et al. 1997; Visser et al. 1996; Werhahn et al. 1998). Placebo was a sugar pill with similar weight to the zolmitriptan tablets. JDA, who performed the data analysis, was also blinded until data analysis was completed. Heart rate and blood pressure were measured before and every 60 minutes after drug intake. Participants were asked to report any physiological sensations after taking zolmitriptan or placebo. Because plasma concentrations of zolmitriptan are detectable at 15 minutes after oral intake with peak concentrations occurring

at 2-4 hours (Peterlin and Rapoport 2007), reflex recordings were taken every 30 min for 2 hours after drug intake. This allowed us to examine the onset of the drug affect and make measurements near the time of peak plasma concentrations. In pilot experiments, taking reflex recordings beyond 2 hours after drug intake was too fatiguing for the participants.

3.2.2 H-reflex Recordings

H-reflex recordings were obtained in both non-injured and SCI participants. H-reflexes were evoked in the soleus muscle because they are readily elicited at rest, which was important for the SCI participants as they could not produce voluntary contractions. All non-injured participants, and SCI participants who were able to transfer safely, were placed in a supine position on a padded table. Two SCI participants were examined in a reclined position in their powered wheelchair (1M and 4F in Table 3-1). Two surface electrodes (2.2 x 3.3 cm, Kendall Soft-E, Chicopee, MA, USA) were placed over the right soleus muscle to record EMG signals. The soleus H-reflex was evoked by stimulating the tibial nerve (DS7A constant current stimulator NL703, Digitimer, Hertfordshire, UK) through a monopolar electrode once the best position was found with a probe electrode (1ms pulse width, return electrode placed over patella). The surface EMG signal was amplified 200 or 1000 times (depending on the size of the response) and filtered using a bandpass of 20-2500Hz (Model 2024F Intronix Technologies, Bolton, ON, Canada). All signals were digitized at a rate of 5kHz using Axoscope hardware and software (Digidata 1440 Series, Molecular Devices, Sunnyvale, USA) and stored on a personal computer for offline analysis.

3.2.3 H-reflex recruitment curves

H-reflex responses were evoked at incrementing stimulus intensities to produce a recruitment curve before (2 baseline curves) and 30, 60, 90 and 120 minutes after drug/placebo intake. Prior to each H-reflex recruitment curve, the motor threshold (MT) was determined online as the stimulation intensity required

to elicit an M-wave of approximately 100 μ V. The stimulation intensity was expressed as a multiple of motor threshold (xMT) and was set from below H-reflex threshold (ranging from 0.5 to 0.7 xMT) to when the H-reflex decreased after its peak (ranging from 1.2 to 1.6 xMT) in steps of 0.05 xMT. This ensured that a minimum of 8-9 points were collected along the steep portion of the H-reflex recruitment curve. Five reflexes were evoked at each stimulation intensity. Stimuli were delivered every 3s which allowed enough time to manually increase the stimulation intensity after every 5th trial. The maximal motor response (Mmax) was measured after each recruitment curve.

H- and M-wave amplitudes were measured as peak-to-peak using custom-written software in Matlab (The MathWorks, Natick, MA). The five H and M-wave amplitudes, evoked at each stimulation intensity, were averaged together and normalized to Mmax. The amplitude of the normalized H and M-waves were plotted at each stimulation intensity, then expressed as a function of motor threshold (MT), to produce H and M-wave recruitment curves. To standardize the measurement of MT across the different time points and participants, MT was recalculated off-line using the x-intercept method (as per Kerr and Vujnovich 2002; Lundbye-Jensen and Nielsen 2008). Briefly, the steep portion of the M-wave recruitment curve was fitted with a straight line and its x-intercept was calculated as the new MT, producing better alignment of the M-wave recruitment curves. The H-reflex recruitment curve (up to its peak) was fitted with a 3-parameter sigmoid function [$H(s) = H_{max}/(1+e^{m(S50-s)})$] (Klimstra and Zehr 2008). The peak H-reflex (Hmax) and the stimulation intensity producing 50% of the Hmax (S50) were measured off the fitted curve. The slope parameter “m” was too variable because it depended on the number of points along the recruitment curve and therefore, was not analyzed (see also Klimstra and Zehr 2008). Typically 98% of the variance in the H-reflex recruitment curve was accounted for by the sigmoidal fit with r^2 values ranging from 0.92 to 0.99 (median = 0.99) in non-injured controls and from 0.87 to 0.99 (median = 0.98) in SCI participants. The threshold to evoke an H-reflex (Hthresh) was measured as the stimulus intensity required to elicit an H-reflex that was 5% of Hmax. Because there was large variability in the

size of H-reflexes between SCI participants, the 3 parameters of the H-reflex recruitment curve (Hmax, S50 and Hthresh) at the 30, 60, 90 and 120 minute time points were expressed as a percentage of the pre-drug value and averaged across subjects.

3.2.4 Cutaneomuscular Reflex Recordings

Cutaneomuscular reflexes were recorded in SCI participants only because long-lasting responses (> 1s) cannot be evoked in non-injured control participants. Cutaneomuscular reflexes were evoked in the tibialis anterior (TA) muscle because it has previously been shown that long-lasting responses, likely mediated by CaPIC activation, are readily produced in the TA after SCI (Norton et al. 2008). Cutaneomuscular afferents supplying the side and sole of the foot were stimulated with long pulse trains applied to the medial arch of the foot (300Hz, 14 pulses, 0.5ms pulse width: DS7A constant current stimulator) at an intensity that was just below pain threshold (40 ± 15 mA on average). Surface EMG signals from the TA were amplified 1000 times and filtered using a band-pass of 20-2500Hz (Model 2024F Intronix Technologies, Bolton, ON, Canada). Both limbs were tested and the TA muscle that exhibited the longest reflex response pre-drug was used. In most SCI participants, the right TA had the longest responses except for participants 2M and 5M. Stimulation was repeated 6 times every 6 seconds for each trial. Three to four pre-drug reflex responses were recorded until two consecutive responses fell within 10% of each other. These last two pre-drug reflex responses were averaged together to form the baseline reflex response. Cutaneomuscular reflex recordings were repeated at 30, 60, 90 and 120 minutes after drug intake and were performed immediately after each H/M recruitment curve.

3.2.5 Cutaneomuscular Reflex Analysis

The cutaneomuscular reflex was divided into two components: a long-latency polysynaptic reflex (LPR) and a long-lasting reflex (LLR) as per Murray et al. 2010, 2011 and Rank et al. 2011. The LPR, which includes the start of the

reflex response up until 300 ms after the first stimulation pulse, contains a mixture of both sensory-evoked EPSPs and CaPIC activation because its amplitude is reduced to $\approx 50\%$ by the Ca^{++} -channel blocker isradipine (Li et al. 2004a; Murray et al. 2011). The average latency of the LPR was 84 ± 14 ms, with an average duration of 215 ± 15 ms. The later, long-lasting reflex component (LLR) was defined as the time window from 500ms after the first stimulation pulse to the end of the reflex response in the pre-drug trial, as per Murray et al. 2010, 2011 and Rank et al. 2011 (LLR duration: 400 ± 158 ms). Thus, the LLR represents a period where most of the sensory synaptic drive to the motoneuron (i.e., EPSP) has subsided and is produced mainly by a depolarization from the CaPIC (Li et al. 2004a; Norton et al. 2008).

In Matlab, each EMG trace was first rectified and the mean EMG was calculated for the time windows of the two reflex components (LPR: start of reflex to 300ms post stimulation, LLR: 500ms post-stimulation to end of pre-drug response). The mean rectified background noise, measured from 100ms before the stimulation, was subtracted from the data. The mean EMG for each of the 6 sweeps were averaged together to obtain LPR and LLR values for each time point. All values were expressed as a percentage of the pre-drug value and then averaged across subjects for each experimental session (zolmitriptan or placebo).

3.2.6 In vitro monosynaptic and polysynaptic reflex recordings

To examine the effects of zolmitriptan applied directly to the spinal cord on monosynaptic reflexes that are similar to the H-reflexes recorded in our human participants, we used the *in vitro* sacral spinal cord preparation (Bennett et al. 2001a; Li and Bennett 2003). Under urethane anesthesia ($1.8 \text{ g} \cdot \text{kg}^{-1}$), the whole spinal cord caudal to S2 (sacral) was removed from chronic spinal rats and immersed in oxygenated artificial cerebrospinal fluid (ACSF; flowing $8 \text{ ml} \cdot \text{min}^{-1}$); recordings were made starting 2.5 hr later, as detailed previously (Bennett et al. 2001a; Li and Bennett 2003). Ventral (S4 and Co1, coccygeal) and dorsal (Co1) roots were mounted on silver wires above the ACSF and covered with Vaseline.

The dorsal root was stimulated with a single pulse (0.1 ms, 0.02 mA: $\approx 3x$'s sensory afferent threshold; repeated $5x$'s every 10s for one trial, trials were repeated every 12 minutes). With this stimulation, a mono-synaptic reflex with a latency of 2 ms and lasting for ≈ 4 ms was evoked in the ventral roots. A 300nM dose of zolmitriptan (AstraZeneca, Mississauga, ON, Canada) was used; a dose in the whole sacral spinal cord that is known to reduce polysynaptic EPSPs (Murray et. al. 2011).

3.2.7 Statistical Analysis

All statistical analysis was performed using Sigmaplot 11 software. Values in the text are expressed as mean \pm standard deviation and in the graphs as mean \pm standard error. Normality for the parameters of the H-reflex recruitment curve (Hmax, S50 and Hthresh) and for the LPR and LLR components of the cutaneomuscular reflexes was first tested with the Shapiro-Wilk test. For each separate experiment (placebo or zolmitriptan), a one-way repeated measures ANOVA for normally distributed data and a one-way repeated measures ANOVA on ranks (Chi Square test) for non-normally distributed data was used to determine if there was an effect of the drug on the reflex parameters over the 30, 60, 90 and 120 minute time points. To compare between experiments and to determine whether placebo and zolmitriptan had different effects on the reflex parameters, a two-way repeated measures ANOVA was used with the within subject factors "drug" and "time". A post-hoc Holm-Sidak test, which corrects for multiple comparisons, was used to determine at which time points the zolmitriptan data differed from the placebo data. Significance was set to $p < 0.05$ in all cases.

3.3 Results

3.3.1 Effects of zolmitriptan on the H-reflex recruitment curve

A 10 mg oral dose of the 5-HT_{1B/D} receptor agonist zolmitriptan reduced the amplitude of the maximum H-reflex (H_{max}) in both non-injured and spinal cord injured participants. The peak-to-peak amplitude of H_{max} was reduced 120 minutes after zolmitriptan intake at similar stimulation intensities to pre-drug as reflected in the matched M-wave before (black trace) and after (gray trace) drug intake for both non-injured control (Fig. 3-1A) and SCI (Fig. 3-1C) participants. As shown for the participants in Fig. 3-1, the average unnormalized H_{max} measured before zolmitriptan intake was significantly larger in controls ($2.95 \pm 1.36 \text{ mV}$) compared to SCI participants ($1.35 \pm 1.31 \text{ mV}$, $p=0.05$). Likewise, M_{max} was larger in controls ($6.00 \pm 2.50 \text{ mV}$) compared to SCI ($3.10 \pm 1.58 \text{ mV}$, $p=0.03$), resulting in H_{max}/M_{max} ratios being similar between the two groups (controls: 0.51 ± 0.17 ; SCI: 0.42 ± 0.25 , $p=0.46$).

As shown from the corresponding H-reflex recruitment curves from the these two participants (Figs. 3-1B&D), zolmitriptan mainly affected the amplitude of the H-reflex and not its overall excitability as there were no lateral shifts in the recruitment curve plotted as a function of motor threshold (MT). The reduction in H-reflex size occurred even though the M-wave recruitment curves remained unchanged, signifying a reduction in the transmission of Ia afferent pathways to the soleus motoneuron pool. As in most subjects, the decrease in H-reflex amplitude was most pronounced at 90 and 120 minutes after drug intake (triangles). In 4/6 non-injured controls and in 5/7 of the SCI participants, the H-reflex was suppressed at all stimulation intensities, as shown for the two participants in Figures 3-1B&D. In the remaining participants, H-reflexes began to decrease near S50, the stimulation intensity producing half of H_{max}. H-reflex recruitment curves before zolmitriptan intake (Pre1&2: solid circles, black lines) and at 30 minutes post-drug (open circles, dark gray line) were reproducible,

suggesting that the H-reflex did not spontaneously decrease over time, similar to the recruitment curves at all time points after placebo intake (data not shown) .

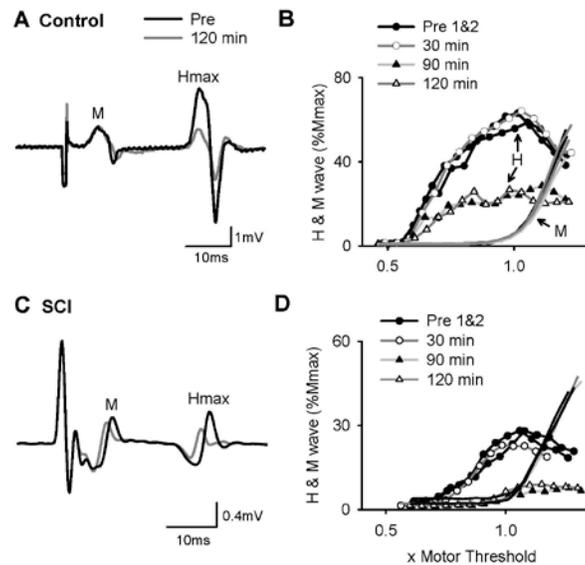


Figure 3-1 H-reflexes after zolmitriptan in uninjured control and SCI participant
A) M-wave (M) and maximum H-reflex (Hmax) recorded in a single uninjured control participant before (black trace) and 120 minutes after (gray trace) 10 mg of zolmitriptan.
B) Corresponding H-reflex and M-wave recruitment curve from same participant in A plotted as peak-to-peak and normalized to Mmax. Stimulation intensity expressed as a multiple of motor threshold. The two pre-drug H-wave recruitment curves (Pre1&2) are represented by black lines and solid circles, the 30 min curve by a dark gray line and open circles, the 90 min curve by a light gray line and solid triangles and the 120 min curve by a dark gray line and open triangles (60 minute data not shown for clarity). M-wave recruitment curves have similar line color schemes with no symbols for clarity. **C)** and **D)** similar to (A) and (B) but for a T3-4 SCI participant (2M in Table 3-1).

3.3.2 Group Data: H-reflex recruitment curve

When plotting the normalized Hmax as a percentage of the pre-drug value across the different time points, Hmax after zolmitriptan intake (solid circles) deviated from placebo values (open circles) at 60 minutes and onwards, with Hmax being reduced to $67.5 \pm 0.27\%$ and $70.6 \pm 0.21\%$ of pre-drug values at 120 minutes in non-injured controls (Fig. 3-2A) and SCI participants (Fig. 3-2B) respectively. There was a significant reduction in Hmax over time after zolmitriptan intake compared to pre-drug (controls: $F= 4.77$, $p= 0.007$; SCI: $F= 3.318$, $p=0.027$) but not after placebo (controls: $F= 0.62$, $p=0.65$; SCI: $F=0.42$,

p=0.79). Two-way ANOVA revealed a significant drug x time interaction (F=3.507, p=0.025), with post-hoc tests showing Hmax after zolmitriptan intake was significantly smaller compared to placebo at the 60, 90 and 120 minutes time points (all p < 0.05). There were no significant increases in Mmax over time (expressed as % of pre-drug, Figs. 3-2A&B bottom graphs) after either placebo or zolmitriptan in both controls and SCI participants (all F and Chi squares > 0.55 and 1.86 respectively, all p > 0.33), indicating that the observed decreases in the normalized Hmax did not result from dividing Hmax by a steadily increasing Mmax.

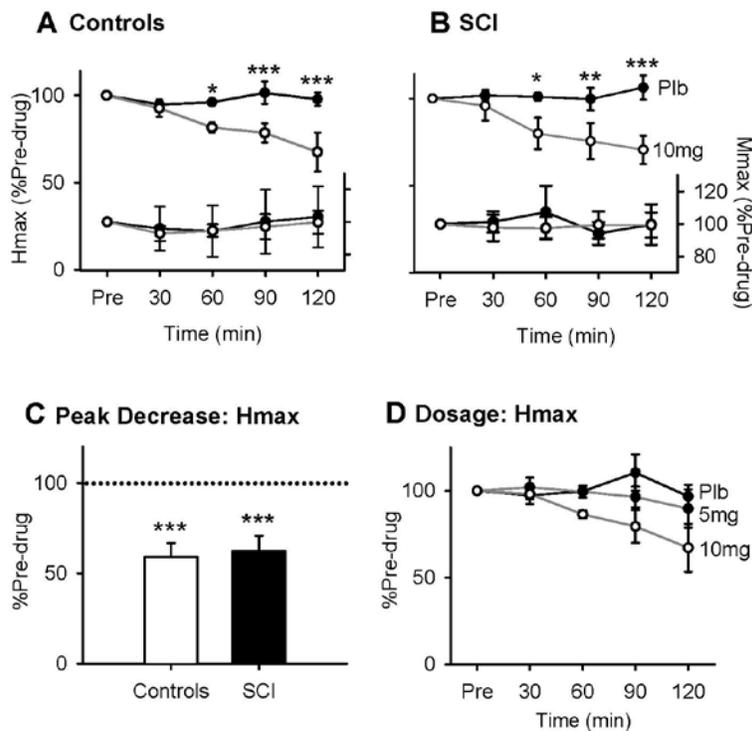


Figure 3-2 Group Data: Hmax and Mmax after zolmitriptan

A) Top panel: Averaged Hmax, expressed as a percentage of pre-drug, at 30, 60, 90, and 120 minutes after zolmitriptan (open circles) and placebo (Plb, solid circles) intake in 6 uninjured control participants. Bottom panel: Average Mmax expressed as a percentage of pre-drug at all time points after zolmitriptan (open circles) and placebo (solid circles). **B)** Same as in (A) but for averaged data across the 7 SCI participants. **C)** Peak decrease in Hmax (expressed as % of Pre-drug) irrespective of time after zolmitriptan intake for both uninjured control (open bar) and SCI (solid bar) participants. **D)** Averaged Hmax (expressed as % of Pre-drug) for 3 uninjured control participants receiving placebo (black line), 5mg (gray line) and 10mg (open circle) of zolmitriptan. Error bars in this and following graphs represent mean \pm standard error. * p < 0.05, ** < 0.01, *** < 0.005.

In some subjects, the peak reduction in Hmax did not occur at 120 minutes after zolmitriptan intake but earlier at 90 minutes (n = 3 control, n = 1 SCI) or 60 minutes (n = 1 control, n = 1 SCI). Thus, when plotting the peak decrease in Hmax occurring at either of these time points (60, 90 or 120 minutes), the Hmax as a percentage of pre-drug was even lower at $58.9 \pm 0.1\%$ for controls and $62.3 \pm 0.23\%$ for SCI participants (Fig. 3-2C), with the peak decrease in Hmax similar between the two groups (p = 0.78). In 3 preliminary control participants, the dosage of zolmitriptan needed to be at least 10 mg to see a reduction in Hmax given that 5mg, like placebo, did not produce a decrease in Hmax (Fig. 3-2D). Finally, as reflected in the recruitment curves of Figure 1, there were no changes in Hthresh or S50 for non-injured control and SCI participants after zolmitriptan or placebo (all F > 0.16, all p > 0.12).

3.3.3 Cutaneomuscular Reflex in SCI

Long-duration reflex responses (spasms) were evoked in the tibialis anterior (TA) muscle in response to a train of pulses (300Hz, 14 pulses, 0.5ms pulse width) applied to the medial arch of the foot as shown for the two participants in Figures 3-3A&B. Both the long polysynaptic component of the reflex (LPR, marked by gray bar), which is mediated by both sensory-evoked EPSP's and PICs, and the long lasting reflex component (LLR, marked by black bar), which is mainly mediated by PICs (see *Cutaneomuscular Reflex Analysis* in Methods for rationale) were reduced by zolmitriptan. It was possible to evoke long-duration reflexes in 6 of the 7 motor complete SCI participants. Similar to the H-reflex, zolmitriptan reduced the size of the LPR over time (Fig. 3-3C), decreasing it to $46.1 \pm 0.32\%$ of pre-drug values at 120 minutes (F= 8.92, p<0.001). In comparison, there was no decrease of the LPR after placebo intake (Chi square=7.07, p=0.13). A two-way ANOVA revealed a significant drug x time interaction (F=5.325, p=0.004), with the LPR after zolmitriptan significantly smaller compared to placebo at the 60, 90 and 120 minute time points (p < 0.05). The reduced LPR consequently resulted in a reduced or nearly abolished LLR (spasm) after zolmitriptan (F= 7.26, p=0.002), but not placebo (Chi square=3.68,

$p=0.45$), with the LLR being reduced to $25.0 \pm 0.39\%$ of its pre-drug value at 120 minutes. Two-way ANOVA revealed a significant drug x time interaction ($F=3.67$, $p=0.026$), with the LLR significantly smaller after zolmitriptan compared to placebo at the 30, 60, 90 and 120 minute time points ($p < 0.05$).

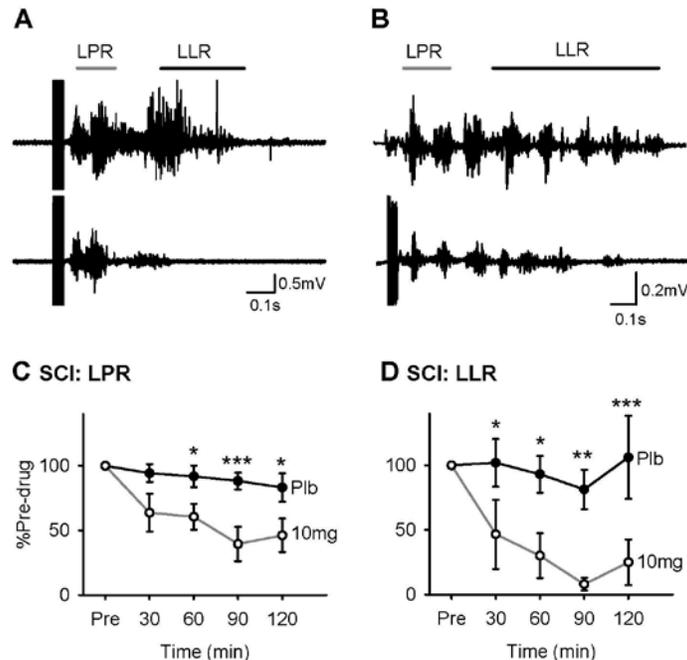


Figure 3-3 Zolmitriptan and CMR in SCI participants

A) Overlay of 6 unrectified TA EMG traces before (upper traces) and after 10mg zolmitriptan (bottom traces) in single C6-7 SCI participant (5M, Table 3-1). Grey bar denotes the window calculated for the long polysynaptic reflex (LPR, start of reflex to 300ms), and the black bar denotes the long-lasting reflex (LLR, 500ms post stimulation to end of reflex). **B)** Similar to **A** but for C6-7/L3 SCI participant (4F, Table 3-1). **C)** Averaged LPR, expressed as a % of Pre-drug at 30, 60, 90 and 120 minutes after 10 mg zolmitriptan (open circles) and placebo (open circles) in 6 of the 7 SCI participants (1M-6M, Table 3-1). **D)** Same as in **C** but for the LLR in 5 of the 7 SCI participants (1M-5M, Table 3-1).

3.3.4 Monosynaptic Reflexes after Direct Application of Zolmitriptan to Rat Spinal Cord

Because zolmitriptan was given orally in the control and SCI participants, this leaves open the possibility that the reduction in H-reflexes could have been due, in part, to systemic actions of the drug on 5HT_{1B/D} receptors located on blood vessels in the spinal cord, a distinct possibility given that the main clinical

use of zolmitriptan is to reduce vasodilatation during migraines (Martin 1997; Peterlin and Rapoport 2007). Therefore, we examined the effects of applying zolmitriptan directly to the spinal cord on monosynaptic reflexes evoked from Co1-dorsal root stimulation in an *in vitro* sacral spinal cord preparation (see Methods). When 300nM of zolmitriptan was applied directly to the spinal cord, the amplitude of the monosynaptic reflex was reduced (Fig. 3-4A), similar to that seen for the H-reflex in human participants (Fig. 3-1). Zolmitriptan reduced the size of the monosynaptic reflex by 40% or more in 5 out of 5 rats tested at 15 minutes after bath application of the drug.

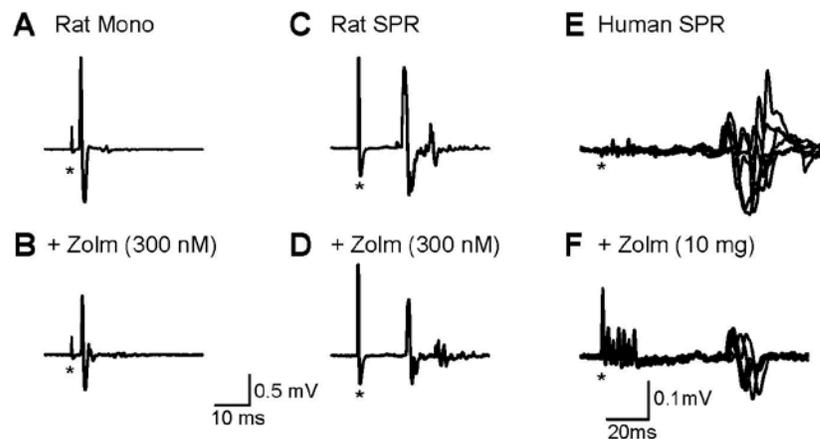


Figure 3-4 Effects of zolmitriptan verified in rat model of SCI

A) Monosynaptic reflex (Mono) recorded from a ventral root of a chronically spinalized rat that did not display a polysynaptic reflex response. **B)** Monosynaptic reflex from A following 300nM bath application of zolmitriptan. **C)** Short-latency polysynaptic reflex (SPR) recorded from a ventral root of a different chronically injured rat (no monosynaptic response was evoked). **D)** Polysynaptic reflex from C after 300nM application of zolmitriptan (modified from Fig. 5 in Murray et al. 2011). **E)** Overlay of 6 SPR's recorded from tibialis anterior in spinal cord injured participant (3M, Table 3-1) before and **F)** 120 minutes after 10mg of oral zolmitriptan. In all figures asterisks mark time of single pulse, or start of multiple pulse, stimulation.

3.3.5 Short-latency polysynaptic reflexes: rat and human

In some rats, rather than a monosynaptic reflex, a short-latency polysynaptic reflex (SPR) was evoked in the ventral root which lasted from 10 to 40ms post-stimulation (Fig. 3-4C) and that was also reduced by zolmitriptan (Fig. 3-4D from Murray et al. 2011). A similar distinct SPR was also evoked in 3 of the

SCI participants during the cutaneomuscular reflex recordings (see also D'Amico et al. 2012). The SPR had a latency of ≈ 70 ms and a duration of 50ms (Fig. 3-4E). In all 3 SCI participants, zolmitriptan reduced the SPR to 56% of its pre-drug value 120 minutes after drug intake, as shown for the SCI participant in Figure 3-4 (6M, Table 3-1).

3.4 Discussion

We demonstrated that facilitation of 5HT_{1B/D} receptors with zolmitriptan, but not placebo, reduced sensory transmission to motoneurons as evidenced by the suppression of H-reflexes in non-injured and spinal cord injured participants. Likewise in participants with SCI, zolmitriptan reduced long-latency polysynaptic reflexes evoked by cutaneomuscular stimulation to ultimately reduce the triggering and overall amplitude of long-lasting reflexes (spasms). Although zolmitriptan cannot be taken orally on a daily basis, these results open the possibility that other 5HT_{1B/D} receptor agonists may be useful to control sensory transmission and reduce the triggering of muscle spasms after spinal cord injury.

3.4.1 Mechanism of action of Zolmitriptan on soleus H-reflexes

In most control and SCI participants (8/13), the H-reflex was reduced at all stimulation intensities indicating that all reflex pathways, including those with the lowest thresholds, were affected by 5HT_{1B/D} receptor facilitation. In the remainder of participants (5/13), only H-reflexes activated at stimulation intensities $>S50$ were reduced by zolmitriptan, indicating that only the higher-threshold sensory pathways were affected in these participants. In all participants, there was a consistent decrease in Hmax at matched amplitudes of Mwave activation, the later an indirect indication that the number of sensory afferents activated pre and post-drug was similar (Misiasek 2003; Zehr 2002). The decrease in Hmax and the absence of any change in Hthresh or S50 suggests that facilitation of 5HT_{1B/D} receptors by zolmitriptan specifically reduced the transmission of sensory-activated inputs in the H-reflex pathway without reducing

the excitability of motoneurons (Misiaszek 2003). This finding in the human is in agreement with animal studies where zolmitriptan specifically reduced sensory-evoked EPSPs but did not influence motoneuron properties such as input resistance, resting membrane potential and spike threshold (Murray et al. 2011). Taken together, this suggests that the reduction in H-reflexes was due to decreases in transmission of sensory pathways to the motoneuron, likely via increases in pre-synaptic inhibition on terminals of sensory afferents or excitatory interneurons or from post-synaptic inhibition of excitatory interneurons, as a result of 5HTB/D receptor facilitation. In addition, because H-reflexes were evoked at a rate of 0.33 Hz, a frequency where “rate-dependent” or “homosynaptic” depression occurs (Crone and Nielsen 1989), zolmitriptan may have reduced the amplitude of the H-reflexes by facilitating a rate-dependent, inhibitory mechanism.

3.4.2 Mechanism of action of Zolmitriptan on cutaneomuscular reflexes

Similar to the Ia-mediated H-reflex pathway, zolmitriptan also reduced short and long-latency polysynaptic reflexes (SPR and LPR) evoked from cutaneomuscular afferent stimulation in participants with spinal cord injury. Reduction of the polysynaptic reflexes was also associated with a reduction in long-lasting reflexes (LLR or spasms). As shown from animal studies with very similar cutaneous reflexes to humans, the LPR can last for 500-1000ms and is a $\approx 50\%$ mixture of EPSP and PIC activation whereas the LLR, which lasts for many seconds, is mainly mediated by PIC activation (Murray et al. 2011). It is the long EPSP during the LPR that provides sufficient depolarization of the motoneuron to activate the CaPIC which then drives self-sustained firing of the motoneuron during a muscle spasm. Because zolmitriptan only reduces sensory activation of the motoneuron and not the PIC (Murray et al. 2011), the reduction in LLR (spasm) activity was likely mediated by the inability of the reduced LPR to trigger a CaPIC and self-sustained firing of the motoneuron. Although we did not estimate the effects of zolmitriptan on PIC activation in this study (e.g., with

paired motor unit analysis), we believe a similar mechanism occurred in the human participants with SCI. For instance, results from the H-reflex experiments suggest that sensory transmission, but not motoneuron excitability, was affected by 5HT1B/D-receptor facilitation.

3.4.3 Effect of Zolmitriptan on sensory transmission likely via 5HT1B/D receptors

Zolmitriptan is a commonly prescribed anti-migraine medication that is able to cross the blood-brain barrier, though only at relatively high doses (Proietti-Cecchini et al. 1997; Visser et al. 1996; Werhahn et al. 1998). It displays high affinity to 5HT1B ($K_i=5.01\text{nM}$) and 5HT1D ($K_i=0.63\text{nM}$) receptors with modest affinity to the 5HT1F receptor ($K_i=63.09\text{nM}$) (Martin et al. 1997). Zolmitriptan likely exerts at least part of its effects on the transmission of cutaneomuscular afferent pathways via activation of the 5HT1B receptor given that in animal studies, the potency of various 5HT1 receptor agonists in reducing cutaneous polysynaptic reflexes and sensory-evoked EPSPs was correlated to the published binding affinity of only 5HT1B and 5HT1F receptor agonists and not to other 5HT receptor agonists (Murray et al. 2011). Likewise, only 5HT1B-receptor antagonists reversed the effects of zolmitriptan on long-latency polysynaptic reflexes in animals. Thus, the activation of 5HT1B, and not 5HT1D receptors, by zolimitriptan likely produced the reduction of cutaneomuscular polysynaptic reflexes evoked in the SCI participants in this study. It remains to be determined in animal studies whether the action of zolmitriptan in reducing the Ia/Ib-mediated H-reflex is also mediated via the 5HT1B receptor or if there is also involvement of the 5HT1D receptor that is also facilitated by zolmitriptan (Honda et al. 2003).

It is interesting that zolmitriptan produced similar decreases in H-reflexes in participants with and without SCI at the 10mg dose, even though reduced levels of endogenous 5HT were likely present below the lesion in the SCI participants (Murray et al. 2010). This suggests that after SCI, 5HT1B/D receptors do not develop supersensitivity to applied agonists, similar to findings in rats where

intravenous administration of sumatriptan, a similar 5HT1B/D receptor agonist, depressed the monosynaptic reflex to the same degree in non-injured and spinal cord injured rats (Honda et al. 2006). It would be interesting to examine if other 5HT receptors, such as 5HT1A, 5HT2A and 5HT7 receptors, which have been shown to help facilitate locomotion after spinal cord injury (Antri et al. 2005; Vinay et al. 2012), develop supersensitivity to 5HT receptor agonists in the presence of reduced levels of endogenous 5HT.

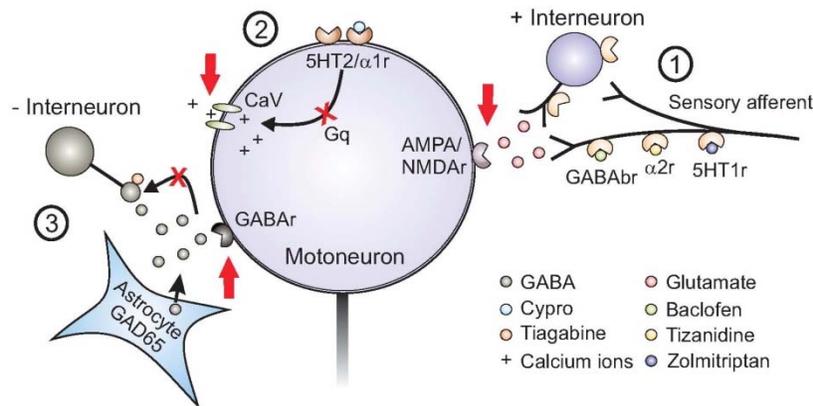


Figure 3-5 Target sites for anti-spastic drugs

Pre-synaptic (1), motoneuron (2) and GABAergic (3) sites of action for anti-spastic drugs. Site 1: Gamma-Aminobutyric Acid (GABA)_b-, α ₂- and 5HT₁- receptors located on presynaptic sensory terminals or on pre- or postsynaptic sites on interposed excitatory interneurons activated by baclofen, tizanidine and zolmitriptan respectively to reduce glutamate release and activation of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors on motoneuron. Site 2: 5HT₂/ α ₁-receptors on motoneuron with constitutive or ligand activation which facilitates downstream voltage-gated calcium channels (CaV) mediating PICs via Gq-protein coupled pathways. Inverse agonists switch the 5HT₂/ α ₁-receptors into their inactive state to reduce activity in the Gq pathway, lessen facilitation of CaV receptors, reduce PICs and consequently, muscle spasms. Site 3: Spinal injection of the HIV1-CMV-GAD65 lentivirus leads to an increase in GAD65 gene expression and GABA release from astrocytes. Combined systemic administration of tiagabine, a GABA reuptake inhibitor, increases levels of GABA to a sufficient level to activate pre and post-synaptic GABA receptors to reduce spasticity.

3.4.4 Clinical Implications

Following spinal cord injury, the activation of Gq-coupled pathways in the motoneuron via constitutive 5HT₂/ α ₁ receptors facilitates the activation of CaPICs

that mediate, in part, the self-sustained activation of motoneurons during involuntary muscle spasms evoked by brief sensory afferent inputs. This is illustrated in the schematic of Figure 3-5 which summarizes the animal and human data from Murray et al. 2010/2011, Rank et al. 2011 and the previous (D'Amico et al. 2013) and current manuscripts. One strategy to reduce muscle spasms is to reduce 5HT2/ α 1 receptor activity via inverse agonists such as cyproheptadine (site 2, Fig. 3-5), resulting in a direct reduction of motoneuron excitability, rather than reducing sensory inputs to the motoneuron like many anti-spastic drugs used currently (discussed below). Thus, the suppression of 5HT2/ α 1 receptors, specifically 5HT2B/C and α 1A receptors, has the potential to reduce excessive muscle activation regardless of the etiology of the spasticity given that the final common pathway, the motoneuron, is directly affected. This strategy would be useful in patients where reducing muscle spasticity is a more important goal than preserving residual motor function, such as for patients with motor complete spinal cord injuries or severe brain damage where functional motor movements are lost and spasticity produces painful contractures and joint deformities. These studies highlight the need to develop inverse agonists to 5HT2B/C and α 1A receptors that are more specific than cyproheptadine, which has undesirable side effects of drowsiness, histamine receptor activation and appetite stimulation (Gracies et al. 1997).

In patients with residual motor function, severely reducing motoneuron excitability to alleviate muscle spasticity may not be the best strategy as this would also reduce activation of the motoneuron by preserved descending inputs. Another strategy to reduce spasticity in this population would be to restore the balance between excitatory and inhibitory activation of the motoneuron by sensory afferent and interneuronal inputs via the activation of Gi-coupled pathways (site 1, Fig. 3-5) with GABA_B- (baclofen, Curtis et al. 1997; Li et al. 2004b), α 2- (tizanidine, Krach 2011; Meleger 2006) and, based on the current study, 5HT1B/D- (zolmitriptan) receptor activation. The main anti-spastic effect of these drugs is to reduce the sensory-evoked EPSP from direct afferent and interposed interneuronal inputs, allowing an unmasking of an IPSP to ultimately

reduce the unchecked activation of CaPICs in the motoneuron (Li et al. 2004b; Murray et al. 2011; Rank et al. 2011). However, all of these Gi-coupled drugs taken orally have unwanted side-effects such as drowsiness and drug tolerance (Krach 2001; Meleger 2006; Nielsen et al. 2002; Rosche 2002). In addition, zolmitriptan cannot be taken daily due to the risk of harmful byproduct production and ironically, induction of headaches (Martin 1997; Peterlin and Rapoport 2007). Again, this study highlights the need to develop better 5HT1B/D, and possibly 5HT1F receptor agonists (Murray et al. 2011), with fewer side effects than baclofen or tizanidine. Alternatively, a moderate suppression of motoneuron activity by cyproheptadine (Wainberg et al. 1990), combined with suppression of sensory inputs, may also strike a proper balance between spasticity control and preservation of residual movements in patients with incomplete injuries.

Although the anti-spastic drugs shown in Figure 3-5 can be problematic when taken orally, results from the studies summarized here open new possibilities for spinally directed approaches in controlling spasticity, such as the use of intrathecal drug delivery. First, cyproheptadine may provide better control of spasticity than baclofen because it works directly on the motoneuron, thereby preventing aberrant descending inputs from the cortex and brainstem from activating the motoneuron that baclofen does not effect. This approach may be useful for many causes of spasticity such as ALS, cerebral palsy and brain trauma/injury in addition to spinal cord injury. Second, intrathecal baclofen can have potentially fatal side effects if suddenly withdrawn, as occurs during sudden blockage of the catheter (Awaad et al. 2012; Lazorthes et al. 1990; Meythaler et al. 2003; Mohammed and Hussain 2004; Stempien and Tsai 2000). Thus, a potential strategy would be to give a combination of GABA_B-, α 2- and 5HT1B/D/F- receptor agonists, which all converge to activate Gi-coupled pathways, at individually lower doses to potentially reduce severe side effects after sudden drug withdrawal.

Another spinally targeted strategy has recently been proposed by Marsala's group (site 3, Fig. 3-5) whereby in a rat model of ischemic spinal cord

injury, increases in GABA release and reduction of spastic stretch reflexes were produced by the combined upregulation of GAD65 gene expression in lumbar astrocytes (from spinal injections of lentivirus) and the systemic administration of tiagabine, a GABA uptake inhibitor (Kakinohana et al. 2012). With spinally targeted interventions (intrathecal or spinal transfections), spasticity may be better controlled without the unwanted side effects of sedation, tolerance and appetite stimulation. The combined use of these strategies, including activating 5HT1B/D/F receptors and suppressing 5HT2/α1 receptor activity, provides new avenues for antispastic treatment.

Subject Code	Age, yr	Age of Injury, yr	Injury Level	AIS	Cause	MAS	Penn Spasm Scale	Preserved Sensation	
								Light touch	Pinprick
<i>1M</i>	29	7	C4-C5	B	Trauma	1	3	Left: none Right: upper shin and foot only	Left: outer shin only Right: ✓
<i>2M</i>	31	1.5	T3-T4	B	Trauma	0	3-4	Left: ✓ except front of shin Right: none	Left: ✓ Right: inner side of leg only
<i>3M</i>	33	13	C5-C6	B	Trauma	1	3	Left: ✓ Right: ✓	Left: ✓ Right: ✓
<i>4F</i>	33	11	C6-C7, L3	B	Trauma	0	3	Left: ✓ Right: ✓	Left: ✓ Right: ✓
<i>5M</i>	58	5	C6-C7	B	Trauma	1+	2	Left: ✓ Right: ✓	Left: ✓ Right: ✓
<i>6M</i>	32	3	C7	A	Trauma	1	2	Left: none Right: none	Left: none Right: none
<i>7F</i>	33	15	T7	B	Trauma	4	2	Left: ✓ Right: ✓	Left: ✓ Right: ✓

Table 3-1 Demographic and clinical description of SCI participants.

Demographics of SCI participants including age of participant and their injury at time of experiment, injury level, ASIA Impairment Scale (AIS), cause of injury, Modified Ashworth Score (MAS) and Penn Spasm Frequency score. Last two columns describe spared light touch and pinprick sensation of lower leg. ✓ indicates preserved sensation from tested area of knee downwards.

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CHAPTER 4:
***ACTIVATION PROPERTIES OF MASSETER
MOTONEURONS IN PARTICIPANTS WITH
AND WITHOUT BRUXISM***

A version of this chapter has been submitted for publication.
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Journal of Neurophysiology, JN-00536-2013, 2013.

4.1 Introduction

Jaw muscles are involved in both simple and complex oral-motor behaviours, such as eating, drinking, talking and breathing, as well as non-functional activities such as tooth grinding and clenching. These muscles can be characterized into either jaw-closing or jaw-opening muscles. In this study we focused on the properties of motoneurons innervating the jaw-closing masseter muscle. The masseter muscle is a multipennate structure with different compartments having various proportions of muscle fibre types and directions, with each compartment activated in different ways depending upon the motor task (Ogawa et al. 2006; Gaudy et al. 2000; Nordstrom and Miles 1991; van Eijden and Turkawski 2001). Motoneurons innervating the masseter muscle are located dorsally and laterally in the rostral and middle thirds of the trigeminal motor nucleus (Westberg and Kolta 2011). These motoneurons receive excitatory glutamatergic and inhibitory glycinergic/GABAergic inputs from premotor neurons located in areas surrounding the principal motor nucleus (Anaclet et al. 2010; Nakamura et al. 2008; Bae et al. 1999). In addition, trigeminal motoneurons receive direct serotonergic inputs from the nuclei raphe obscurus, raphe pallidus and raphe dorsalis (Kolta et al. 1993; Li et al. 1993; Nagase et al. 1997; Schwarz and Peever 2010), as well as norepinephrine inputs from the locus subcoeruleus, A5 and A7 cells and sparse innervation from the locus coeruleus (Schwarz and Peever 2010; Leger et al. 2009; Fort et al. 1990; Schwarz et al. 2008; Fenik et al. 2002).

Similar to motoneurons innervating the limb muscles, trigeminal motoneurons display bistable membrane properties such as plateau potentials and burst oscillations where long-lasting periods of depolarization can occur under low levels of synaptic drive (Hsiao et al. 1998). These properties are mediated by voltage-activated, sodium and calcium persistent inward currents (PICs) that are in turn, facilitated by serotonin and norepinephrine receptors located on the motoneurons (Schwarz et al. 2008). For example, application of serotonin can induce a negative slope region in the current-voltage relationship of trigeminal

motoneurons that is subsequently abolished when the persistent L-type Ca^{2+} and Na^{+} currents are blocked with nimodipine and tetrodotoxin respectively (Hsiao et al. 1998; Hsiao et al. 1997). Given the demonstration of strong PIC activation in animals, we examined if trigeminal motoneurons in the human also exhibit indirect evidence of PIC activation by using a paired motor unit analysis technique developed for limb muscles (Gorassini et al. 2004). Evidence for PIC activation, namely motor unit activity that persists under levels of synaptic drive lower than that needed to initially recruit the motor unit (i.e., self-sustained activity), was examined during isometric, voluntary contractions onto a bite bar (Turker et al. 2004).

Motor units innervated by trigeminal motoneurons are recruited in an orderly fashion, with small motor units having small spike amplitudes and twitch tensions being recruited at lower bite forces than larger motor units (Yemm 1977; Goldberg and Derfler 1977). Once securely recruited, masseter motor units can fire steadily for at least 5 min during a static contraction (Farella et al. 2011). Motor unit firing rates increase linearly with increasing force, with higher threshold units having a larger range of firing rate modulation than lower threshold units and with most units reaching a plateau in firing near 30imp/s (Lund et al. 1979; Derfler and Goldberg 1978). In this study we examined further the relationship between firing rate and recruitment threshold, specifically if masseter motor units display an "onion skin effect", a phenomenon where higher-threshold units fire at lower rates than lower-threshold units (De Luca and Hostage 2010; De Luca and Erim 1994). The onion skin effect has been observed in various motor units from the upper and lower limbs with the parent motoneuron originating in the spinal cord (De Luca and Hostage 2010; Tanji and Kato 1973; Monster and Chan 1977; Kanosue et al. 1979; Rose and McGill 2005; Stashuk and de Bruin 1988). We wanted to examine here if the onion skin effect was present in motor units innervated by motoneurons located in the pons.

Lastly we indirectly examined, via ΔF measurements, whether individuals with bruxism who experience involuntary (self-sustained) teeth grinding and clenching during sleep (Lobbezoo and Naeije 2001; Lobbezoo et al. 2006) display

increased monoaminergic drive to their trigeminal motoneurons under awake conditions. During sleep, muscles are typically atonic; however, there are periods of rhythmic masticatory muscle activity characterized by phasic (teeth grinding) and tonic (clenching) bursts of activity (Lu et al. 2005; Kato et al. 2011; Anacleto et al. 2010; Kato et al. 2007; Inoue et al. 1999) that coincide with the presence of microarousals (Quattrochi et al. 2000; Halasz 1998; Halasz et al. 2004). Interestingly, the discharge of neurons in the raphe nuclei, locus coeruleus, subcoeruleus and A5/A7 cells, which release PIC-facilitating serotonin and norepinephrine to the trigeminal motoneuron pool, increase during microarousals (Leung and Mason 1999; Sakai and Crochet 2001; Takahashi et al. 2010; Crochet and Sakai 1999). Individuals with bruxism experience increased numbers of microarousals during sleep (Kato et al. 2011; Lavigne et al. 2001; Kato et al. 2001a; Kato et al. 2003), and likely increases in monoaminergic drive to trigeminal motoneurons. Thus, we examined with paired motor unit analysis if participants with bruxism display larger estimates of PIC amplitude during voluntary contractions compared to non-bruxing controls to determine if tonically elevated levels of monoaminergic drive to trigeminal motoneurons are present in bruxers, even in the absence of microarousals and rhythmic masticatory muscle activity. Parts of the data from this paper have been published in abstract form (Yavuz et al. 2010).

4.2 Methods

4.2.1 Participants

Protocols were approved by the Human Ethics Committee of Ege University in accordance with the Declaration of Helsinki. All participants provided informed written consent prior to the experiment. Nine non-Bruxer (NBrux) control participants and 13 Bruxer (Brux) participants were examined [NBrux = 26 ± 3.7 years (range 24 to 35), 2 males; Brux = 22 ± 3.1 years (range 19 to 29), 5 males, $p = 0.02$]. Although the Brux group was significantly younger by 4 years compared to the NBrux group, we do not expect this small age

difference to play a large role in our estimates of PIC amplitude and motor unit firing properties. Brux participants were assessed by a clinician (author AS) and their level of bruxism was scaled from 0 to 5 using the Visual Analogue Scale (Scott and Huskisson, 1976) with 0 = no bruxing, 1 = no pain and no tooth abrasion, 2 = light pain and no tooth abrasion, 3 = mild pain and some tooth abrasion, 4 = severe joint pain and tooth abrasion and 5 = continuous bruxing. There were 5 Brux-2, 3 Brux-3 and 5 Brux-4 participants in this group.

4.2.2 Motor Unit Recordings

Each participant sat in a dental chair adjusted for height so that the horizontal plane of his/her upper dental arch was aligned with the upper bite plate of a custom-built mastication apparatus (Turker et al. 2004). Bite plates were coated with a semi-rigid dental impression material (3M Express™, 3M ESPE, St. Paul, MN, USA) that was moulded to each participant's teeth to ensure that contact force and jaw position were similar across participants. The bite bar was coupled to a handmade force transducer [Kyowa (KFG-5-120-C1-11) strain gauge] to monitor the force profiles of the bite. Participants were given a visual display of their exerted bite force on a computer screen. A triangular line was drawn on a transparency and overlain on the computer screen. The participants were instructed to produce a force profile that followed the drawn line with the offset, vertical and horizontal scales of the computer display adjusted to match the initial level, strength and speed of the contraction respectively. The strength and acceleration of the contraction was adjusted to ensure that at least two motor units (a control and test unit, see Estimation of PIC Amplitude below) were recruited during the ascending phase of the contraction. The strength of the contraction was expressed as a percentage of their maximum voluntary contraction (%MVC), which was obtained by averaging the bite force from three maximum contractions. On average, the peak of the contractions were ~15-20% MVC and lasted for ~15-20 s.

Intramuscular electrodes were used to record single motor unit action potentials in the masseter muscle. TeXon® insulated (except for their tips) silver

bipolar wire electrodes (100 μm diameter with insulation; 70 μm core diameter) were inserted into the deep masseter muscle using a sterile 25G needle. The needle was then withdrawn, leaving the fish-hooked wires in the belly of the muscle (Prasartwuth et al. 2008). Surface electromyography (EMG) was recorded from the masseter muscle, amplified by 1000x and bandpass filtered between 20Hz and 500Hz. Intramuscular EMG signals were amplified by 300x and high-pass filtered at 100Hz. EMG and force signals were amplified using a CED1902 Quad-system and digitized using a CED Power1401-8 channel converter and Spike 2 (Version 6.07) software using a sampling rate of 20 kHz for the intramuscular EMG, 2kHz for the surface EMG and 2kHz for the force signal. A lip-clip (see Turker et al. 2004 for details) was used as a ground.

4.2.3 Data Analysis

Data were analyzed offline using spike discrimination software (Spike 2, Cambridge Electronic Design, Cambridge, UK). Single motor unit action potentials were selected by first setting a horizontal threshold that was at least 3 standard deviations above background noise. The selected motor units were then visually sorted based on waveform shape. When possible, the same two units were tracked for every participant (NBrux participants: 39/45 motor unit pairs, Brux participants: 56/65 motor unit pairs).

4.2.4 Estimation of PIC Amplitude (ΔF)

The amplitude of PIC activation was estimated using the paired motor unit analysis technique (Gorassini et al. 2002a; Gorassini et al. 2004) as follows. The times of occurrences for the single motor unit action potentials obtained in Spike 2 were exported via a text file to Matlab for further analysis in a custom-written Matlab program (The MathWorks, Inc, Natick, MA, USA). The instantaneous firing rates of the units were then calculated as the reciprocal of each interspike interval. The firing rate profile of a lower-threshold control motor unit was used as a measure of the synaptic input to the motoneuron pool and specifically, to a

relatively higher-threshold motor unit, termed the test unit. To calculate the firing rate of the control unit at recruitment and derecruitment of the test unit, a fifth-order polynomial was used to smooth the firing rate profile. The smoothed firing rate of the control unit at recruitment and derecruitment of the test unit was determined automatically, and the ΔF measurement was calculated as the difference in smoothed firing rate of the control rate when the test unit was derecruited compared to when it was recruited, i.e. $\Delta F = F_{\text{derecruitment}} - F_{\text{recruitment}}$. The ΔF , therefore, corresponds to the reduction in synaptic input needed to counteract the depolarization from the PIC and provides an indirect estimate of PIC amplitude (Udina et al. 2010).

For each participant, 5 contraction trials were selected to calculate the mean ΔF . Only contraction trials with symmetrical force profiles were included to ensure smooth increases and decreases in synaptic input to the motoneurons. Contractions with abrupt increases or decreases in the force profile, which can effect recruitment and de-recruitment of motor units (Nordstrom and Miles 1991), were omitted. Only trials where the control unit fired for at least 2s before the test unit was recruited were included to ensure that the PIC was fully activated in the control motoneuron given that the calcium component of the PIC can take at least 500ms to activate (Li et al. 2004a; Moritz et al. 2007). This ensured that any changes in firing rate of the control motor unit only reflected changes in synaptic input onto its motoneuron and not from abrupt depolarizations produced during PIC activation. In total, 45 contraction trials (5 x 9 participants) were used to calculate the mean ΔF for the Non Bruxer group and 65 trials (5 x 13 participants) were used to calculate the mean ΔF for the Bruxer group.

4.2.5 Common Synaptic Drive to Control and Test Units

To ensure that the firing rate of the control motor unit approximated the synaptic input to the test motor unit, we needed to ensure that both units were receiving a common synaptic drive (De Luca and Erim 1994) by determining if both units were being modulated in a similar manner. To do this, the smoothed firing rate of the control unit (fit with a 5th order polynomial) was plotted against

the smoothed firing rate of the test unit and the coefficient of determination (r^2) of the rate-rate plot was measured. Only trials where $r^2 \geq 0.7$ were used, ensuring that at least 70% or more of the rate modulation of the test unit could be accounted for by the rate modulation of the control unit. Fifteen of the 110 unit pairs analyzed had r^2 values below 0.7, which may have resulted from recording units from different functional compartments in the masseter muscle (see Introduction).

4.2.6 Onion Skin Effect

To measure the onion skin effect, we compared the relationship between the mean firing rate and recruitment thresholds for the control and test motor units in a pair from the 9 NBrux participants. As mentioned earlier, the higher-threshold test motor units were recruited at least 2s or more after the control units during the ascending phase of the contraction. The recruitment threshold for all motor units was measured as the force at which the motor unit began to fire, expressed as a % of MVC. Mean firing rates were calculated in a time period when both the control and test motor units were active during the contraction. This ensured that firing rates were measured during equivalent levels of synaptic input. In addition, firing rates were only measured after the units were securely recruited. For example, slow start-up firing rates of the test motor unit were excluded. In 10 of the 45 NBrux contractions analyzed, a second test motor unit (test-2) that was recruited after the first test unit (test-1) was also analyzed and compared to the original control and test-1 motor units. One participant was excluded as an outlier because the average force, expressed as a % of MVC, was 2 times higher than the rest of the participants, most likely due to an underestimation of the true MVC in this participant. In total, the mean firing rate and recruitment threshold of 40 control, 40 test-1 and 10 test-2 motor units were measured.

To determine if motor units within a pair that had large differences in recruitment thresholds also had large differences in mean rates (and vice versa for motor units with small differences in recruitment thresholds), the difference in

mean firing rate between the two units in a pair (e.g., test-1 minus control, test-2 minus control and test-2 minus test-1) was plotted against the difference in recruitment threshold between the two units in a pair and a correlation coefficient (r) was calculated (60 motor unit pairs in total). Differences in firing rates and recruitment thresholds were measured between motor unit pairs for each participant, rather than comparing values across all motor units in a group, in order to reduce inter-subject variability that can influence the onion skin effect (De Luca and Hostage 2010).

4.2.7 Statistics

All statistics were performed using SigmaPlot 11 software (Systat Software). Values presented in the text and in Figs. 1C and 1D are means \pm standard deviation (SD) and data in Figs. 2C, D and 4C are presented as means \pm standard error (SE). Normality for the distribution of ΔF , recruitment threshold and mean firing rate values was tested with the Shapiro-Wilk test. One-way ANOVA was used on normally distributed data (e.g., mean firing rates of control, test-1 and test-2 units), whereas a one-way ANOVA on ranks was used for non-normally distributed data (e.g., recruitment thresholds between control and test units). Post hoc Student's t-tests (Bonferroni corrected) and Dunn's test were used to determine if there were differences in the mean ΔF values and motor unit firing properties (e.g., mean rates, difference in recruitment times of control and test units, etc., see Table 1) between the NBruX and BruX groups. Linear regression analysis was used to determine if the differences in recruitment thresholds between control and test units varied linearly with the difference in their mean firing rates and if there was a relationship between ΔF values and the peak %MVC force produced during a contraction for both the NBruX and BruX participants. Significance was set to $p \leq 0.05$.

4.3 Results

4.3.1 ΔF : *Non-Bruxer Control Participants*

In the 9 non-bruxer (NBruX) control participants, estimates of PIC amplitude activated in masseter motoneurons were obtained using the paired motor unit analysis technique. Briefly, the firing rate of a lower-threshold control unit was used as a measure of the synaptic input to a higher-threshold test unit during a triangular voluntary contraction (Fig. 4-1A). As demonstrated for this NBruX participant, the higher-threshold test unit (middle graph) was derecruited at a much lower level of estimated synaptic input (i.e., firing rate of control unit, bottom graph) compared to when it was recruited, to give an estimated PIC amplitude (ΔF) of 3.8 imp/s. That is, to counter-act the added depolarization from the PIC to derecruit the test unit, the synaptic input to the test motoneuron had to be reduced by an amount that produced a decrease in the firing rate of the control unit by 3.8 imp/s. To determine if the firing rate of the test motor unit was modulated in a similar manner as the control motor unit, and thus, receiving the same synaptic drive as the control motor unit, the smoothed firing rate of the test unit was plotted against the smoothed firing rate of the control unit (Fig. 4-1B). The coefficient of determination for the rate-rate plot was high ($r^2=0.96$), indicating that 96% of the modulation of the test unit could be accounted for by the modulation of the control unit, and that the use of the control unit as a measure of synaptic input to the test unit was justified. The rate-rate plot also shows the hysteretic firing pattern of the test motor unit where, during the descending (relaxation) phase of the contraction (white circles), the test motor unit continued to fire at levels of synaptic input well below the level needed to recruit it (at asterisk), indicative of self-sustained firing due to the sustained depolarization provided by the PIC.

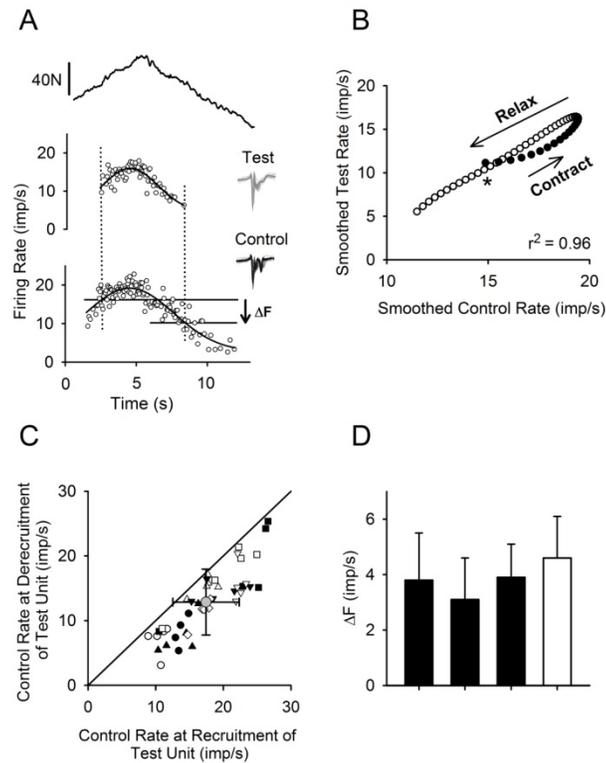


Figure 4-1 ΔF in Non-Bruker participants

A) Instantaneous firing rate of lower-threshold control (bottom) and higher-threshold test (middle) motor unit during isometric contraction (bite force: top trace). Thick black line represents 5th order polynomial (smoothed rate) fit through the firing rates. Dotted vertical lines mark time of recruitment and de-recruitment of the test unit. Solid horizontal lines indicate smoothed firing rate of control unit when test unit was recruited and derecruited, with the difference between the two rates (ΔF) marked by the arrow. **B)** Smoothed mean firing rate of control unit from A plotted against smoothed mean firing rate of test unit during contraction (black circles) and relaxation (open circles) phase of contraction. * marks beginning of test unit firing. **C)** Control unit firing rate at time of recruitment of test unit plotted against control unit rate when test unit was de-recruited for 45 contractions from the 9 NBrux participants (5 contractions per participant, different symbol for each participant). Solid line marks slope of 1 (parity line). Mean of data is shown by the large gray circle and error bars represent SD. **D)** Mean ΔF 's (\pm SD) measured in biceps brachii, soleus and tibialis anterior muscles (black bars) compared to mean ΔF for masseter muscle (white bar).

When plotting the smoothed firing rate of the control unit when the test unit was recruited against the smoothed firing rate of the control unit when the test unit was derecruited for all contraction trials ($n = 45$, Fig. 4-1C, different symbol for each NBrux participant), all data points fell below the parity line indicating that the test units were derecruited at lower levels of synaptic input

than when they were first recruited. The mean ΔF measured for masseter motoneurons was 4.6 ± 1.5 imp/s (SD) (Fig. 4-1D) and is in line with ΔF values reported in different muscles of the upper and lower limbs [tibialis anterior: 3.9 ± 1.2 imp/s, soleus: 3.1 ± 1.5 imp/s (Gorassini et al. 2002a; Udina et al. 2010), biceps brachii: 3.8 ± 1.7 imp/s (Mottram et al. 2009)].

4.3.2 Onion Skin Effect

We also examined in the Non-BruX participants if masseter motor units display an onion skin effect, i.e., if the lower-threshold control motor units had a higher mean firing rate compared to the higher-threshold test (test-1 and test-2 units, see “Onion Skin Effect” in Methods) motor units. When plotting the firing rates of sequentially recruited control (black circles) and test units (test-1: open circles, test-2: grey circles, Figs. 4-2A and B), the lower-threshold control motor units typically had a faster mean firing rate compared to the higher-threshold test-1 or test-2 motor units. A noticeable onion skin effect was observed in 8 of the 9 NBruX participants. When averaged across the NBruX group, the control, test-1 and test-2 motor units had sequentially higher thresholds of recruitment (Fig. 4-2D) and correspondingly, lower mean firing rates (Fig. 4-2C, see values in legend).

To determine if the pairs of motor units with larger differences in recruitment thresholds also had larger differences in mean firing rates, the difference in recruitment threshold (ΔRT) between sequentially recruited units in a pair (e.g., test-1 minus control, test-2 minus control or test-2 minus test-1, Fig. 4-3A) was plotted against the corresponding difference in mean firing rate between the two units in a pair (Fig. 4-3B). There was a significant, linear relationship between the difference in mean rate between units in a pair with an increasing difference in their recruitment thresholds ($r = 0.43$, $p = 0.0007$).

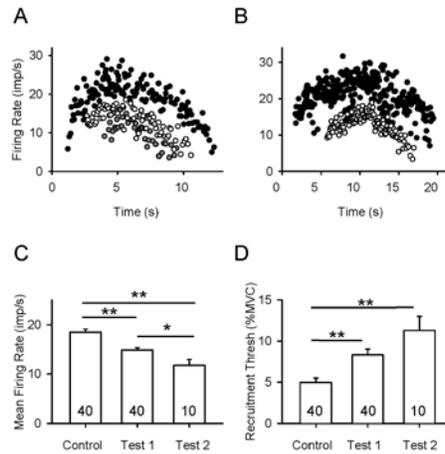


Figure 4-2 Mean Firing Rate and Recruitment Threshold

A) Firing rate profiles of three sequentially recruited motor units during an isometric, triangular contraction in a NBruX participant (control: black circles; test-1: white circles; test-2: gray circles). **B)** Same as in A for a control and test-1 unit pair in another NBruX participant. **C)** Group mean firing rates of early-recruited control units (18.5 ± 3.9 imp/s) and later-recruited test-1 (14.9 ± 3.0 imp/s) and test-2 (11.8 ± 3.7 imp/s) units from NBruX participants. Numbers of units analyzed are indicated in each bar graph and error bars represent SE of the mean. A one-way ANOVA with post-hoc Bonferroni t-tests were used. **D)** Recruitment thresholds, expressed as a %MVC, for control (5.0 ± 3.4 %), test-1 (8.3 ± 4.5 %) and test-2 (11.3 ± 5.4 %) units. A one-way ANOVA on ranks with post-hoc Dunn's test was used. * $p < 0.05$, ** $p < 0.001$.

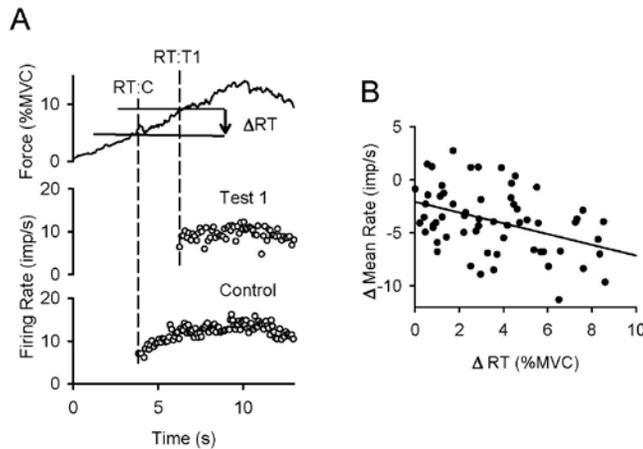


Figure 4-3 Differences in recruitment thresholds and mean firing rates

A) Calculation of recruitment threshold difference between control (bottom) and test-1 (middle) motor units during a voluntary contraction (force expressed as %MVC, top). Dashed vertical lines mark start of firing of control and test-1 units and corresponding recruitment forces for the control (RT:C) and test-1 (RT:T1) units. Arrow marks the difference in recruitment force (ΔRT) between the two units. **B)** Difference in mean rate between a control and test-1, control and test-2 or test-1 and test-2 motor unit pair plotted against the corresponding difference between their recruitment thresholds ($n = 60$ unit pairs). A linear regression is fit through the data points ($r = 0.43$, $p = 0.0007$).

4.3.3 ΔF : Bruxer Participants

ΔF measurements were obtained from bruxer participants (Brux) to determine if the involuntary chewing and teeth clenching present in this group were associated with larger estimates of PICs compared to control participants, even during awake conditions. The ΔF values obtained from the Brux participants were all within the range of values obtained in the control NBrux group (Fig. 4-4C), as shown for the two Brux-2 and Brux-4 participants in Figs. 4-4A and 4B (Brux-2 = 4.1 imp/s; Brux-4 = 5.8 imp/s). The mean ΔF in the Brux group (4.5 ± 1.2 imp/s) was not significantly different than the mean ΔF in the control NBrux group (4.6 ± 1.6 imp/s, $p = 0.83$). However, the Brux-4 group, who have severe joint pain and tooth abrasion, had ΔF values that were all higher than the mean ΔF of the NBrux controls (5.6 ± 0.5 imp/s, marked by gray triangles in Fig. 4-4C), but this difference was not significant ($p = 0.19$), likely owing to a small number of participants in this group ($n = 5$).

In all, the firing rate profiles of the motor units in the Brux and NBrux groups were similar during the isometric contractions with no differences in mean rates of the control and test motor units measured throughout the contraction (Table 4-1). In addition, the control and test motor units were modulated in a similar manner in both groups, with a mean r^2 value of ~ 0.81 in the smoothed rate-rate plots. There was at least 3 seconds of separation between the recruitment time of the control and test motor units in both groups and the test unit was active for at least 3 seconds during the ascending phase of the contraction (Table 4-1), two important requisites when estimating PIC amplitude with paired motor unit analysis, as outlined in the Discussion. On average, the Brux group reached higher peak forces in terms of %MVC during the isometric contraction compared to the NBrux group (Table 4-1), although the difference was not significant. Moreover, when plotting the size of the ΔF against the peak force reached during a contraction (Fig. 4-4 D), there was no relationship between the two for either group (NBrux: $r = 0.22$, $p = 0.15$; Brux: $r = 0.15$, $p = 0.27$).

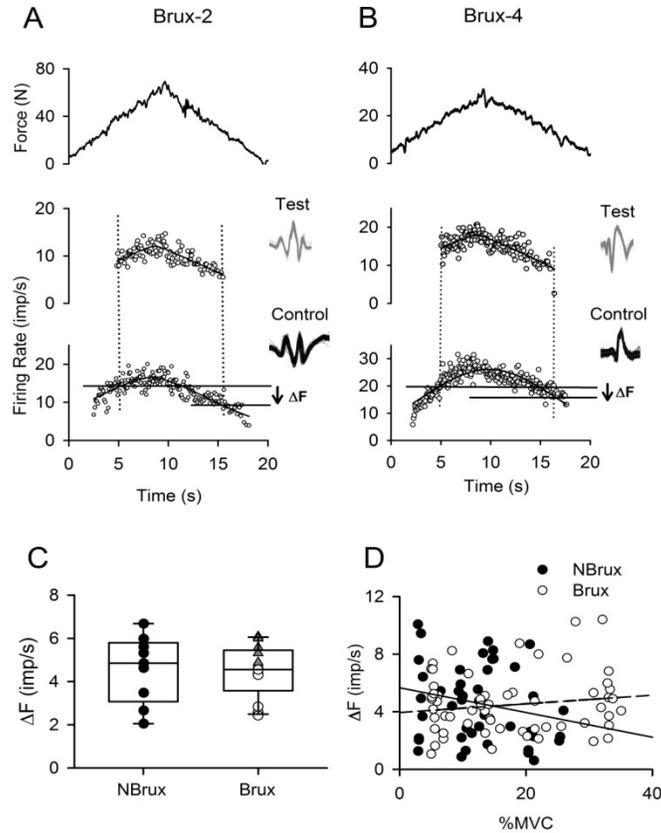


Figure 4-4 ΔF in Bruxer Participants

A) Instantaneous firing rate of a lower-threshold control (bottom) and higher-threshold test (middle) motor units during isometric contraction in Brux-2 (**A**) and Brux-4 (**B**) participants. Same format as Figure 1. Note different scales in A and B. **C)** Group mean ΔF from NBruX (black bar: 4.6 ± 1.6 imp/s) and BruX (white bar: 4.5 ± 1.2 imp/s) participants (Student's t-test, $p = 0.83$). **D)** ΔF plotted against peak force (%MVC) reached during each contraction for the 9 NBruX (black circles, solid line, $n = 45$ contractions) and 13 BruX participants (open circles, dashed line, $n = 65$ contractions).

	Control Mean Rate (imp/s)	Test Mean Rate (imp/s)	Rate-Rate r^2 (range)	Test Activation Time (s)	Control-Test Recruit Diff (s)	Peak Force (%MVC)
NBruX	16.9 ± 3.3	13.5 ± 2.0	0.81 ± 0.1 (0.71-0.95)	3.7 ± 1.2	2.9 ± 1.3	12.6 ± 6.7
BruX	16.0 ± 4.2	11.8 ± 2.3	0.81 ± 0.1 (0.71-0.89)	2.9 ± 0.7	3.2 ± 1.2	17.2 ± 10.5

Table 4-1: Motor unit firing rate and contraction force characteristics

Comparison of: 1) firing rate of control and 2) test motor units during entire contraction; 3) coefficient of variation (r^2) between the smoothed firing rate of the control and test motor unit (range in brackets); 4) duration of time the test unit was active for during the ascending phase of the contraction; 5) time difference between the recruitment of the control and test unit and 6) peak force of the contraction (expressed as %MVC) for both NBruX and BruX groups. Values represent mean \pm SD. For mean r^2 , the median r^2 was calculated for each participant and this value was then averaged across participants in a group. Student's t-test was used to compare values between groups (all $p > 0.05$).

4.4 Discussion

Similar to animal studies following the application of serotonin or serotonin receptor agonists (Hsiao et al. 2005), PICs are activated in human trigeminal motoneurons as estimated by recording pairs of motor units in the masseter muscle. Unlike the animal experiments recorded *in vitro*, there is likely sufficient endogenous levels of serotonin and/or norepinephrine in the awake human to allow for activation of PICs during voluntary contractions. Excessive monoaminergic drive to trigeminal motoneurons was not present in the awake bruxer participants, who present with involuntary chewing and teeth clenching during sleep, as indicated by estimates of PIC amplitudes that were similar to the non-bruxing controls. Lastly, similar to motoneurons in limb muscles, trigeminal motoneurons display a consistent onion effect where, during moderately sized contractions of 20% MVC or less, lower-threshold motor units discharge at higher rates (by 4 to 7 imp/s on average) compared to higher-threshold units.

4.4.1 Validity of ΔF Measurements

Estimating the amplitude of a PIC via paired motor unit analysis relies on how well the control motor unit reflects the level of synaptic input onto the test motor unit since any discharge of the test unit occurring at levels of synaptic input below that needed for recruitment can be attributed to PIC activation. After recruitment of a PIC, the firing rate of a motoneuron is linearly related to the injected or synaptic current it receives (Hsiao et al. 1998; Hsiao et al. 1997; Gorassini et al. 2004; Bennett et al. 2001; Binder et al. 1996). Because of this, the firing rate of one motoneuron (control) that receives the same input as another (test) can be used as a measure of input to both motoneurons. The contractions employed in this study were designed to maximize the possibility that the firing rate of the control motor unit indeed reflected the degree of synaptic input to its motoneuron and to the test motoneuron as well. For example, we only used trials where the control motor unit was active for at least 3 seconds before the test unit

(see Table 4-1) to ensure that the PIC in the control unit was fully or nearly fully activated given that it can take ~500 ms for the slow calcium component of the PIC to activate (Li et al. 2004a; Moritz et al. 2007; Murray et al. 2011). After PIC activation, any changes in the firing rate of the control motor unit (motoneuron) should mainly reflect changes to its synaptic input and not from an added depolarization during PIC activation, which can occur near the time of recruitment (Li et al. 2004, Udina et al. 2010). Likewise, we only chose trials where the test unit was active for at least 3s during the ascending phase of the contraction (Table 4-1), again to ensure that the PIC was fully and securely activated.

As mentioned above, if two motoneurons receive the same synaptic input, then the firing rate of one motoneuron (or motor unit) can be used as a measure of input to the other. One indication that both motoneurons are receiving common inputs is that their firing rates are modulated in a similar manner during a muscle contraction. For this reason, we plotted the relationship between the smoothed firing rates of the control and test motor units and on average, 80% of the modulation in firing of the test motor unit could be accounted for by the modulation in firing rate of the control motor unit ($r^2 = 0.8$ on average, Table 1). The firing rates of the control and test motor units also closely followed the trajectory of the force profiles (see Figs. 4-1, 4-3 and 4-4), indicating that the units were firing within a sensitive range of their input-output properties. In trigeminal motoneurons, the relationship between firing rate and injected current remains linear up to ~50imp/s (Hsiao et al. 1997) when recorded in vitro, whereas the firing rate of trigeminal motoneurons or motor units continue to increase with increasing force up until ~30 imp/s when recorded in vivo (Lund et al. 1979). The peak firing rates of the control units during the $\leq 20\%$ MVC contractions performed in the current study were ≤ 30 imp/s, indicating that the control motoneurons fired in a range that was sensitive to changes in synaptic inputs. In summary, the firing behaviour of the control motor unit in relation to the test motor unit and bite force suggests that it was a good approximation of synaptic input to the test motor unit, enabling a reasonable estimation of PIC amplitude

and its presence in trigeminal motoneurons, similar to that found in animal studies.

4.4.2 Onion Skin Effect

In 8 of the 9 NBrux participants, the firing rates of the lower-threshold control motor units were faster than the firing rates of the higher-threshold test motor units, even near the peak of the ~20% MVC contraction. The discrepancy in firing rates between motor units of different thresholds could be mediated by differences in how a given synaptic input is transduced in the motoneuron. For example, in the higher-threshold motoneurons with lower input resistance (higher conductance), only the top of the synaptic input profile may have reached the axon hillock to produce lower firing rates compared to the lower-threshold motoneurons where a larger portion of the synaptic current reaches the axon hillock to produce faster firing. This of course assumes that the motoneuron pool receives equal amounts of a given synaptic input, which may be the case for descending inputs that drive voluntary contractions but can vary for activation of the motoneuron pool by primary spindle afferents (Binder et al 1996; Heckman and Binder 1993a,b; Powers and Binder 1995). If the synaptic drive to the higher-threshold motor units increased beyond that used for the ~20% MVC contractions, the firing rates of these units would likely increase further, potentially matching or even exceeding that of the lower threshold units, especially at very high levels of contraction effort (Heckman and Binder 1993a,b; Kanosue et al. 1979; Grimby and Hannerz 1976; Tansey and Botterman 1996; Kuo et al. 2006; Manuel et al. 2009; Bigland and Lippold 1954).

The strategy of “onion skin” recruitment has been proposed by De Luca and colleagues as a method to help relieve the CNS of having to modulate the input/output properties of each motoneuron separately (De Luca and Erim 1994; DeLuca 1985). It allows for a common synaptic drive to the recruit the motoneuron pool in an orderly fashion, whereby fatigue-resistant small motoneurons are recruited first and fire at faster rates, and fatigue-prone larger

motoneurons are recruited later and fire at slower rates (De Luca and Hostage 2010). This motor control strategy helps to prevent fatigue, which is quite relevant to the masticatory system as it is involved in sustained motor activities such as chewing and talking.

4.4.3 Amplitude of PICs activated during voluntary contractions is normal in Bruxer participants

The presence of involuntary chewing and teeth clenching that occur during sleep in the Brux participants is not associated with abnormally large PICs activated during voluntary contractions under awake conditions. It may be that large PICs are only present during periods of involuntary chewing and teeth clenching given that these involuntary motor behaviours occur during periods of microarousals when monoaminergic drive to the trigeminal motoneuron pool is high (Leung and Mason 1999; Sakai and Crochet 2001; Takahashi et al. 2010; Crochet and Sakai 1999). In line with this, drugs such as amphetamine and serotonin reuptake inhibitors, which increase levels of norepinephrine and serotonin respectively, increase episodes of involuntary activity in bruxism (Lavigne et al. 2003; See and Tan 2003) and the amplitude of PICs in limb motoneurons (D'Amico et al. 2013; Udina et al. 2010). Thus, the amplitude of PICs should, in future studies, be estimated during sleep when involuntary muscle activity is present. The use of non-invasive surface EMG and motor unit action potential decomposition techniques (De Luca et al. 2006; Farina et al. 2004) could facilitate recordings of motor unit activity during microarousals without disrupting sleeping patterns.

4.4.4 Large PICs in chronic pain?

The Brux-4 group, who are characterized as having chronic pain and tooth abrasion, displayed the highest ΔF values that were consistently above the mean ΔF measured in the other Brux-2 and Brux-3 participants and in the control NBrux group. Although there was only a trend for the ΔF measured in the Brux-4

group to be higher than the ΔF measured in controls ($p = 0.19$, likely due to the small numbers in this group), it does suggest that the presence of chronic pain may increase the excitability of motoneuron PICs. The experimental induction of pain in the masseter muscle can induce changes in the firing behaviour of motor units and increase the number of motor units recruited during a contraction (Minami et al. 2012; Sohn et al. 2004, see also Tucker and Hodges 2009). In addition, there is a reduction in both the duration and amplitude of inhibitory reflex responses evoked in the masseter muscle during tonic painful stimulation (Svensson et al. 1999). These findings, including our own, suggest that chronic pain may increase the excitability of trigeminal motoneurons to maintain muscle force, potentially by increasing the excitability of PICs. Further studies in more Brux-4 participants or during periods of experimentally induced pain are needed to resolve this issue.

4.4.5 Conclusions

Similar to animal studies, PICs are activated in trigeminal motoneurons during voluntary contractions in the human. Both the onion skin effect and the activation of PICs likely facilitate the sustained activation of the masseter muscle which is required during motor activities such as chewing and talking.

4.5 Bibliography for Chapter 4

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CHAPTER 5: DISCUSSION

5.1 Brief General Summary of SCI Findings

This thesis builds on work previously performed in the adult sacral spinal rat model. The translation of this work to humans after SCI is essential for future clinical applications of these findings. To briefly summarize, the role of 5HT₂ and NA- α 1 receptors in motoneuron hyperexcitability and involuntary muscle spasms was examined in humans after SCI. It was determined that in individuals with motor-incomplete SCI, 5HT₂/ α 1 receptors on motoneurons and downstream PICs were activated by residual levels of monoamines below the injury. Although we could not definitively conclude the presence of constitutively-active receptors in these individuals, it is likely that there is a certain degree of constitutively-active receptors below the lesion. However, in individuals with more severe motor-complete SCI, traditional ligand activated monoamine receptors did not play a role in PIC activation, but was driven solely by the presence of constitutively-active monoamine receptors located on the motoneurons. The presence of constitutively-active monoaminergic receptors on motoneurons results in the unchecked activation of large PICs which drive involuntary spasms after injury in humans.

In addition to examining changes at the motoneuron level, we examined the neuromodulation of sensory inputs to the motoneurons, specifically the role of 5HT₁ receptors. Similar to rat studies, the application of a 5HT₁ receptor agonist reduced sensory input to the motoneuron (as measured by the H-reflex and cutaneomuscular reflexes) and as a result, the triggering of PIC-mediated involuntary spasms was also reduced. Interestingly, despite the likely reduction of 5HT levels after injury, the 5HT₁ receptors did not display increased sensitivity when compared to non-injured, control participants. Therefore it seems that, unlike the 5HT₂ and NA- α 1 receptors, the 5HT₁ receptors do not undergo adaptive changes after injury.

5.2 Limitations of SCI Studies

Although we have used the most suitable electrophysiological and pharmacological methods available to examine the proposed questions in this thesis there were still some limitations to the studies performed. In addition to the limitations already mentioned in the discussions of each chapter, additional limitations are examined below.

One such limitation to our pharmacological studies in human participants is determining the exact site of action of our various receptor antagonists and agonists in the spinal cord. Although we know that the various drugs in this study are targeting the appropriate receptors based on their pharmacological profiles obtained in cellular assays and animal models, we cannot be certain where these receptors are located in the human spinal cord, and therefore cannot be certain at which site the drugs are exerting their action with respect to spasm generation. Limited studies have utilized autoradiographic labelling for specific receptors in the postmortem human spinal cord. Postmortem quantitative autoradiographic studies of the human spinal cord have determined the presence of 5HT₁ receptors exclusively in the superficial layer of the spinal dorsal horn (Laporte et al. 1996). These results coincide with the effects of the 5HT₁ agonist, zolmitriptan, on sensory transmission to motoneurons. Given the above labeling results, it is likely that zolmitriptan was not exerting its actions via receptors located on the motoneurons and was indeed acting via modulation of the inputs to the motoneurons since 5HT₁ receptor labeling was not evident in the ventral horn of the human spinal cord. Additionally, zolmitriptan binds to receptors located on blood vessels and can produce vessel constriction, most notably of the cranial arteries (Martin 1997). Although we were able to demonstrate that zolmitriptan similarly reduces the monosynaptic reflex in an *in vitro* rat preparation where changes to blood vessel diameter has a reduced effect on neuronal excitability, we cannot completely rule out the effects of blood vessel constriction on H-reflexes in the human spinal cord.

Similar labelling studies have not yet been performed for the 5HT_{2C} receptors in the human spinal cord, therefore we cannot be certain of the site of action of our 5HT₂ receptor antagonists, cyproheptadine and chlorpromazine. However, we can rely on our indirect EMG results which have demonstrated that blocking the 5HT₂ receptors does not affect the sensory component of the evoked reflex responses and therefore it is likely that these receptors are located on the motoneurons themselves and not on interposed sensory afferents or interneurons in the reflex pathway.

A second limitation in *Chapter 2* was the validity of the paired motor unit analysis technique because the antagonists used in the study may have affected the firing profiles of the earlier-recruited control motor units given their effects on motoneuron excitability. If the antagonists affected the excitability of the control motor units only this would have affected the validity of the control unit as a measure of the synaptic input to the test units. However, it is reasonable to assume that cyproheptadine affected both the control and test motor units in a similar manner. Mean firing rates of both the control and test units did not change after drug intake during voluntary contractions of similar forces. Additionally, the correlation between the firing rate of the control unit and the firing rate of the test unit remained similar after drug intake ($r^2 \sim 0.80$) when compared to pre-drug values. Taken together, these results indicate that the earlier recruited control unit still remained a viable estimate of the synaptic input to the later-recruited test unit post-drug intake and therefore any changes reflected in the ΔF measurements post-drug were most likely due to changes in the PICs themselves rather than due to the effect of cyproheptadine on the excitability of the control motor unit alone. However, the firing rate of the control unit in general may not always completely reflect the synaptic input it and the test unit receives. Compartmentalization of synaptic inputs to the TA motoneuron pool, firing rate adaptation in response to inactivation of ion channels and partial activation of PICs may alter the firing response of motoneurons to synaptic inputs. Thus, interpreting the amplitude of PIC activation from paired motor unit analysis must always be made with caution.

Thirdly, in *Chapter 2*, we were unable to determine if constitutively-active receptors were present and played a role in PIC activation in individuals with motor-incomplete SCI and whether there was a relationship between the degree of constitutive receptor activity and lesion severity. If we were able to utilize an inverse agonist and neutral antagonist approved for human-use that had the identical affinities for each receptor (5HT1 and NA- α 1) then we may have been able to determine whether constitutively-active receptors played a role in PIC activation after incomplete SCI by using the same dosage of both the inverse agonist and neutral antagonist. If the inverse agonist reduced the PICs more than the neutral antagonist then we could attribute some of these actions to the blocking of constitutively-active receptors. Additionally, if two such antagonists were available we could also calculate the ratio of constitutively-active to ligand-activate receptors which contribute to PIC activation and we could have determined whether this ratio was correlated to the severity of the lesion. Although the above findings may have been interesting, there are currently no inverse agonists and neutral antagonists available for human-use that possess identical affinities to the receptors of interest in our study.

In *Chapter 3* we were able to determine that application of the 5HT1 receptor agonist "restored" sensory inhibition to the motoneurons. However, due to the methodology used in the study we were unable to determine whether 5HT1 receptor activation acted to restore inhibition via increased presynaptic inhibition *or* increased post-activation depression. The 4s ISI used to obtain the H-wave recruitment curves was not long enough to exclude increased post-activation depression (Lundbye-Jensen and Nielsen 2008; Crone and Nielsen 1989) as a potential mechanism for the restored inhibition. Although it would have been useful to perform H-wave recruitment curves at intervals where post-activation depression is not present (>10s, Crone and Nielsen 1989) this would have greatly increased the duration of the study and individuals, especially with SCI, would have become quite uncomfortable and this may have resulted in increased spasticity in these individuals. However, it may have been useful to examine H-wave amplitudes at a specific M-wave amplitude pre and post-drug intake using

ISIs of 10-15s to determine whether post-activation depression was indeed affected after zolmitriptan intake.

5.3 Upregulation of Constitutive Receptor Activity: Adaptive Mechanism?

The 5HT_{2C} receptor RNA undergoes post-transcriptional editing at various sites which leads to several different isoforms of the receptor that each display a certain degree of constitutive receptor activity (Niswender et al. 1999; Nakae et al. 2008). The unedited INI isoform shows the highest degree of constitutive activity, while editing reduces this constitutive activity in isoforms such as the VNI. mRNA analysis of the various 5HT_{2C} receptor isoforms has determined that in response to chronic injury there is an upregulation in the INI isoform, which displays the highest degree of constitutive receptor activity, and downregulation in the VNI isoform, which displays the lowest degree of constitutive receptor activity (Murray et al. 2010). Therefore, it seems that the switch in receptor isoforms occurs in response to injury in these animals. Perhaps this switch to constitutive receptor activation is a mechanism to adapt to the significant reduction of serotonin below the lesion. It would be interesting to determine whether supplying 5HT or its receptor agonists to animals with SCI would inhibit this adaptation after injury. It has already been demonstrated that in systems deprived of 5HT the receptors become constitutively-active and that this process can be reversed via chronic application of 5HT agonists (Gurevich et al. 2002). The use of 5HT agonists in humans is not a suitable method to examine this mechanism since most agonists have serious side effects. However, it would be interesting to determine whether a supply of tryptophan (5HTP), which has the ability to cross the blood-brain barrier (BBB), would prevent this type of adaptive change. This method would rely on the assumption that the enzymes necessary for converting 5HTP to 5HT, such as tryptophan hydroxylase and AADC, are present below the lesion site. By further understanding what triggers this adaptive mechanism we can then determine methods to help prevent or modulate the

degree of constitutive receptor activity and this will have implications on motoneuron excitability and spasticity in individuals with SCI.

5.4 PIC Modulation by 5HT and NA: Future Directions

5.4.1 Availability of 5HT Below the Lesion

Unlike studies performed in completely transected rats, injury severity and location is quite heterogeneous in the human SCI population. As such, a limitation to the SCI studies was the inability to anatomically verify the extent of the lesions (through magnetic resonance imaging or diffusion tensor imaging) in our participants. Thus, it is impossible to conclude, especially in our motor-complete SCI participants, the extent of damage to the descending monoaminergic tracts and subsequently, the residual level of monoamines that can be expected below the lesion. In any case, although we cannot be certain of the absence of monoamines below the level of a "motor-complete" injury, we can be certain that the residual amounts that may still be present are not functional since the neutral antagonist, chlorpromazine, had no effect on PIC-mediated responses in motor-completes but did have an effect in uninjured controls.

In completely spinalized rats, the SSRI citalopram had no effect on long-lasting reflexes or PICs, indicating that there were no residual serotonergic neurons below the lesion (Murray et al. 2010). This suggests that residual serotonin from spinal neurons or blood platelets (Biardi et al. 2005) below a complete injury do not have a functional effect on motoneuron PICs and muscle spasms. Thus, a lack of effect from the SSRI citalopram should indicate the absence of residual serotonergic neurons below a complete SCI. In contrast, in individuals with incomplete spinal cord injury who have some residual motor function, citalopram facilitates involuntary long-lasting reflexes (muscle spasms) after injury as demonstrated in *Chapter 2* (D'Amico et al. 2013; Thompson et al. 2011). These results indicate that in individuals with spared motor function,

descending sources of serotonin are able to facilitate the large sodium and calcium PICs in the motoneuron that mediate muscle spasms. Despite preliminary evidence with the neutral antagonist chlorpromazine, the effects of an SSRI remain to be tested in persons with motor-complete SCI to determine if there are functional sources of serotonin below a complete injury. If we do not find evidence for residual, functional sources of serotonin in participants with motor-complete SCI, this will support our results obtained in *Chapter 2* indicating that most likely there are no residual serotonergic neurons below a severe lesion and therefore activation of 5HT2 receptors below a complete spinal cord injury is purely mediated by constitutive 5HT2 receptor activity.

5.4.2 Activation of PICs by Peripheral Noradrenaline

In vivo studies in the completely-transected rat have demonstrated that, unlike results obtained in the *in vitro* model, NA- α 1 receptors are indeed activated by the ligand NA below a complete lesion despite the significant elimination of descending noradrenergic sources (Rank et al. 2011). It has been suggested that the source of NA comes from the periphery since peripheral injection of NA into the peritoneum increase PIC-mediated responses. It appears that peripheral NA is able to cross the blood-brain barrier (BBB) and bind to central NA- α 1 receptors because blocking these central receptors via intrathecal injection of the NA- α 1 receptor antagonist prazosin counteracts the facilitation of PIC-mediated responses when peripheral NA is increased (Rank 2011). The above phenomenon seems to be exclusive for NA since similar findings were not obtained *in vivo* with serotonin antagonists (Murray et al. 2010; Rank 2011).

To determine whether the same phenomenon occurs in humans after SCI, we are currently examining if increasing the release of peripheral noradrenaline affects PIC-mediated spasms. Amphetamine will be used to increase the release of NA as it blocks both monoamine oxidase, which normally breaks down NA in synaptic terminals and the packaging of NA into vesicles. These two actions of amphetamine increase the concentration of NA in synaptic terminals and as a

result, reverses the direction of the noradrenaline transporter (NET) so that it releases NA into the synaptic cleft rather than removing it from the cleft (Fleckenstein et al. 2007). Increases in peripheral NA by amphetamine occurs by increasing the release of NA from kidney adrenal glands (Goldstein et al. 2003), resulting in increased levels of blood NA that subsequently crosses the BBB via NET to activate motoneuron PICs (Rank 2011). In the same complete SCI participants who have no functional descending sources of serotonin, the effects of amphetamine on PIC-mediated spasms will be examined. It is probable that these participants will not have descending sources of NA either since it is likely that 5HT and NA fibres travel together in the spinal cord. If amphetamine produces facilitation of PIC-mediated spasms in the motor-complete SCI participants where citalopram had *no effect*, this will suggest that similar to the *in vivo* rat model, peripheral NA is able to cross the blood brain barrier to activate central $\alpha 1$ receptors on the motoneuron and facilitate muscle spasms in humans. These findings will have clinical implications for the treatment of spasticity. Specifically, the use of a NA- $\alpha 1$ *neutral* antagonist may be a suitable anti-spastic that will act *directly* at the motoneuron to reduce excessive PIC activation, however it will *not completely* eliminate PIC activation since the constitutively-active receptors will still be activated. This may provide the right balance between managing spasticity and preserving a level of motoneuron excitability that does not negatively impact residual motor function.

5.5 Application to Other CNS Disorders

5.5.1 Amyotrophic Lateral Sclerosis (ALS)

It would be interesting to examine whether the development of constitutive receptor activity occurs in other CNS disorders that produce degeneration of serotonergic/noradrenergic neurons. ALS is a neurodegenerative disorder involving both upper and lower motoneurons and prognosis is quite slim once diagnosed. On average, death occurs within 2-5 years of diagnosis, with only 20% of patients living more than 5 years and 10% of patients living beyond

10 years (Zoccolella et al. 2009). One major mechanism implicated in the development of ALS is neuronal excitotoxicity resulting in neuron death (Palecek et al. 1999; Bruijn et al. 2004). Additionally, animal studies have demonstrated that ALS mice display larger NaPICs when compared to normal, control mice (Kuo et al. 2005; Pieri et al. 2009). Interestingly, NaPICs are significantly increased only in low input resistance motoneurons (i.e. large motoneurons) which coincidentally are also the first neurons to degenerate in ALS (Kuo et al. 2005), therefore it seems that abnormal PIC activation most likely contributes to overall excitotoxicity in ALS. This has yet to be examined in humans with ALS.

Given the role that monoamines play in modulating PIC activation, researchers have examined the neuromodulation of PICs in ALS mice. It is interesting to note that in mouse models of ALS there is a significant reduction in spinal levels of serotonin in both symptomatic and non-symptomatic mice (Dentel et al. 2012), mimicking the conditions present after SCI. The reduction of spinal 5HT is due to degeneration of serotonergic neurons in the brainstem. Despite the reduction in serotonin, the 5HT₂ receptors are still active and subsequently the PICs remain active as evidenced by the long-lasting PIC-mediated responses that can be elicited in these ALS mice (Dentel et al. 2012). Mouse ALS studies have implied that the 5HT₂ receptors become *constitutively-active* (Dentel et al. 2012); however, this conclusion is not definitive since only an inverse agonist to 5HT₂ receptors was tested and therefore, the reduction in motoneuron activity may have been due to blocking of an unknown proportion of ligand-activated receptors. It would be interesting to examine in humans with ALS whether PIC activation is enhanced (through paired motor unit recordings) and how PIC activation is modulated by serotonin. It has been demonstrated both through human autopsy results (Dentel et al. 2012) and plasma serotonin levels (Dupuis et al. 2010) that similar to animal studies, humans suffering from ALS also suffer from degeneration of serotonergic neurons and descending tracts and therefore the emergence of constitutively-active 5HT₂ receptors, or alternatively receptor super-sensitivity, is likely. It is well known that the need exists to develop new therapeutic treatments in addition to riluzole, which is currently the only FDA-

approved treatment for ALS (Cifra et al. 2010; Lamanauska and Nistri 2008), and applying the techniques mentioned in this thesis to humans with ALS provides one avenue to do so. Future drug development may result in a conjunctive therapy to be used with riluzole to improve quality of life (decreased spasticity) and hopefully increase survival rate after ALS diagnosis.

5.5.2 Parkinson's Disease (PD)

Parkinson's disease is another neurodegenerative disorder typically characterized by degeneration of dopaminergic neurons in the substantia nigra pars compacta (Damier et al. 1999). However, it has been demonstrated that neurodegeneration extends to other areas such as the brainstem, resulting in progressive degeneration of serotonergic neurons located in the raphe nuclei and noradrenergic neurons located in the locus coeruleus (Zarow et al., 2003; Braak, 2003, 2009; Halliday, 1990). Post-mortem studies in human PD participants have revealed a significant reduction (~70%) of cells in the locus coeruleus (Zarow et al., 2003) as well as significant cell loss in the caudal raphe nuclei when compared to age-matched control participants (Braak & Del Tredici, 2009). Individuals with PD normally present with: bradykinesia, akinesia, rigidity, resting tremor, postural instability and gait dysfunction. It is interesting to note that although dopamine has typically been implicated in the above-listed motor disorders, it is well-known that both 5HT and NA modulate motor output in healthy individuals and therefore the degeneration of monoaminergic neurons in individuals with PD most likely contributes to the presentation of PD-related motor disorders. Specifically, decreased 5HT results in postural instability and tremor (Iacono et al., 1997; Doder et al., 2003), whereas decreased NA can produce difficulties in gait initiation (Barbeau and Rossignol 1991; Kiehn et al. 1992). During ballistic contractions, surface EMG recordings have revealed that PD participants have an abnormal motor control strategy, such as EMG fractionation, when compared to control participants (Flowers 1975; Hallett and Khoshbin 1980). Interestingly, administration of L-DOPA and deep brain stimulation (DBS) significantly improves the pattern of muscle activation;

however, it is not completely restored to control values (Robichaud et al. 2002; Vaillancourt et al. 2004, 2006). This suggests that other neuromodulators may be involved. In these PD participants it may be interesting to determine whether administration of an SSRI or an SNRI can restore motor function to levels that are more closely matched with control participants. Preliminary clinical evaluation of PD participants taking citalopram has provided evidence that citalopram reduces bradykinesia; however, no quantitative measurements were made. Perhaps SSRIs and SNRIs could be used in conjunction with L-DOPA to restore motor function in individuals with Parkinson's disease.

5.6 Inhibition in the Spinal Cord: Future Directions

Complete and direct elimination of PICs by blocking constitutively-active receptors on the motoneurons is an extremely effective method to counteract the production of involuntary spasms. However, as mentioned earlier in the discussion, this strategy may be detrimental to overall recovery of function in individuals with incomplete SCIs who display residual motor function. As such, perhaps the restoration of lost inhibition provides a more suitable strategy to counteract the unchecked triggering and activation of PICs in these individuals.

5.6.1 Restoring inhibition via the KCC2 pump

Although *Chapter 3* provides substantial proof of the effectiveness of zolmitriptan in reducing sensory transmission to the motoneurons and reducing the triggering of involuntary muscle spasms its suitability as a potential anti-spastic remains questionable because of its unsuitability for long-term use, serious cardiovascular side effects and its ability to induce migraines. Another mechanism that could potentially be targeted to increase inhibition in the spinal cord after injury is the KCC2 pump. It has been shown that KCC2 expression can be increased via BDNF activation of TrkB receptors. It is interesting to note that BDNF is increased in response to training in animals after SCI (Cote et al. 2011). Rehabilitative training after injury could therefore be beneficial to indirectly

increase KCC2 expression and inhibition in the spinal cord. In fact, it has already been demonstrated that inhibition in the spinal cord is increased after treadmill training in individuals with incomplete SCI (Gorassini et al. 2009). Additionally, the use of either a 5HT_{2A} receptor agonist such as TCB-2 and/or activation of the Ca-independent secondary messenger PKC ϵ has been shown to upregulate expression of KCC2 and increase post-activation depression after SCI in animals (Bos et al. 2013). Although these results are promising, 5HT₂ agonists, such as DOI and LSD, are hallucinogenics and therefore are not suitable as a treatment for spasticity in humans. Additionally, the PKC agonist used in the animal study, FR266924, has not yet been developed for use in human participants therefore its effectiveness in restoring inhibition after SCI in humans cannot yet be evaluated.

5.7 Clinical Implications: Individualized Spasticity Treatment

5.7.1 Finding the Right Balance

Pharmacological manipulation of spinal cord excitability after SCI in this thesis has identified different targets for the treatment of spasticity. Although *Chapters 2 and 3* shed light on possible targets for future anti-spastic treatments, the question that still remains is whether or not it is desirable to eliminate excitability after injury? This question can most easily be answered by comparing the primary concerns of individuals with injuries of varying severity. In individuals with incomplete SCI, the drastic elimination of PICs and spasms by an inverse agonist such as cyproheptadine may be detrimental to any residual function and leave individuals feeling quite weak, however reduction of spasticity by cyproheptadine can also improve locomotor function (Norman et al. 1998; Wainberg et al. 1990). It may be beneficial if the dosage of cyproheptadine is titrated slowly over time to reach the optimal balance in these individuals. Another solution in individuals suffering incomplete SCIs would most likely be to lessen the triggering of involuntary spasms via inhibition of sensory afferent input to the motoneurons. Although zolmitriptan effectively reduced sensory input, it's

suitability as an anti-spastic treatment is questioned due to its severe cardiovascular side effects. However, perhaps combination treatment of clonidine, zolmitriptan and baclofen at lower doses than normally prescribed would limit the negative side effects of each drug while still additively reducing synaptic transmission to the motoneurons. On the other hand, individuals who have suffered a complete SCI and who no longer have residual motor function may consider spasticity reduction a higher priority with regards to their quality of life. In these participants cyproheptadine is likely the most suitable alternative to reduce involuntary muscle spasms since it acts directly at the site, the motoneurons. It is evident that as knowledge increases in the area of neuromodulation of excitability after injury potential new drug targets may be discovered that will greatly aid in the 'individualized' treatment of spasticity while limiting the negative side effects currently associated with traditional anti-spastic treatments.

5.7.2 Strategies to Limit Side Effects

It is well known that cyproheptadine and other commonly prescribed anti-spastics have undesirable side effects due to their lack of specificity and their effects on receptors outside of the spinal cord, such as in the brain and gastrointestinal system. Specific 5HT₂ and NA α 1 inverse agonists and neutral antagonists are available for animal use, and their effects have been examined in the rat SCI model (Murray et al. 2010; Rank et al. 2011). These drugs, such as SB206553, prazosin and WB4101, have increased receptor specificity when compared to cyproheptadine and therefore can effectively reduce PIC-mediated responses while limiting side effects. The safety and suitability of these drugs in humans has yet to be determined. However, it is interesting to note that prazosin is currently prescribed to treat high blood pressure and panic disorders in humans and therefore its safety for human use has been determined (Kerbage and Richa 2013). It would be interesting to examine the effects of this specific inverse agonist on spasticity while documenting potential side effects with its use.

Another strategy to limit potential side effects by localizing the effects of the drug to the spinal cord was recently presented by Kakinohana and colleagues. They utilized gene delivery and injected a GAD-65 lentivirus into specific spinal segments of the rat spinal cord. Alone, this treatment had no significant effect on spasticity. However, when used in combination with a low, ineffective dose of the GABA uptake inhibitor tiagabine, there was a drastic reduction spasticity that was segment-specific (Kakinohana et al. 2012). This method is extremely promising because it not only limits the effects of the drug to the spinal cord, but it also allows the use of extremely low doses of tiagabine, therefore resulting in less drug-mediated side effects. The use of gene therapy in humans is currently a fairly new area of research. Interestingly, gene delivery of GAD by an adeno-associated viral vector (AAV) has been utilized in individuals with Parkinson's disease and has been quite effective in reducing symptoms in individuals who are responsive to L-Dopa treatment over a 6 month period (Kaplitt et al. 2007). Additionally, in initial clinical trial studies there have been no adverse events related to this type of gene therapy in this population (Kaplitt et al. 2007). Therefore, perhaps the strategy developed by Kakinohana and colleagues may be promising in treating spasticity in individuals with SCI. Specifically, gene delivery via AAV vectors of either antisense mRNA or small interfering RNA (siRNA) specific to the 5HT_{2C} receptor isoform that is constitutively-active may produce silencing of this particular receptor isoform to a specific spinal segment. A similar strategy could most likely be utilized for the NA- α 1 receptors. This will ultimately result in decreased PIC activation and spasticity while localizing the effects to specific spinal segments and subsequently reducing the side effects which result from systemic drug administration. However, it remains important to note that, despite the lack of adverse events in initial trials treating Parkinson's disease, there are some cons related to the use of AAV vectors. Some issues include their small packaging capacity, the risk of insertional mutagenesis, immune responses against the vector and it still remains quite difficult to supply sufficiently high concentrations of the selected genes as is required for human clinical trials (Monahan and Samulski 2000).

5.8 Masseter Motoneuron Hyperexcitability in Bruxism

In *Chapter 4* we examined whether monoaminergic drive to motoneurons in individuals suffering from bruxism was increased when compared to non-bruxer control participants via indirect measurement of PICs in masseter motoneurons. We did not find increased PIC activation in participants with bruxism when compared to control participants, therefore it seems that there is not a persistent increase in monoaminergic drive to motoneurons in individuals suffering from bruxism during awake conditions.

5.9 Limitations of Bruxism Study

In this study we were unable to elucidate the mechanism behind increased motoneuron excitability in individuals suffering from bruxism. Specifically, because motor unit recordings were only performed in *awake* individuals performing voluntary contractions and *not* during periods of involuntary masseter muscle activity, which are typically associated with microarousals and increased monoaminergic drive, we are unable to rule out the possibility that there may still be heightened monoaminergic drive in bruxing individuals during periods of microarousals. This limitation illuminates the importance of recording data during pathological responses i.e. involuntary muscle activity/spasms. To examine this possibility further it would be necessary to record motor unit action potentials during various sleep states in bruxing individuals. In summary, the biggest limitation to this study was that it was not performed while participants were experiencing pathological, involuntary sustained masseter activity, therefore, it is most likely *essential* to examine the mechanisms behind bruxism during periods of bruxing or during microarousals to successfully elucidate the differences between non-bruxing and bruxing individuals.

5.10 Bruxism: Future Directions

5.10.1 Pain and Persistent Inward Currents?

Although there was no significant difference in ΔF values between non-bruxers and bruxers individuals, there was a trend for the most severe bruxers to display higher ΔF values. It is interesting to note that the only criterion differentiating this group of participants from others with bruxism was the presentation of chronic pain. It is of interest to note that various studies have shown that both Substance P and Glutamate, which are released at synapses responsible for pain signalling, can activate PICs in dorsal horn neurons (Russo et al. 1997). Animal studies specific to the trigeminal system have shown that there is co-localization of both substance P and serotonin receptors in the trigeminal motor nuclei (Nakamura et al. 2006), both known to be responsible for activation of persistent inward currents. Additionally, NK1 antagonists (antagonists to the substance P receptors) have been shown to inhibit the long latency jaw-opening reflex (JOR) in guinea pigs in vivo, indicating that these receptors do indeed play a role in the modulation of motoneuronal excitability in the trigeminal system (Alia et al. 1998). Furthermore, studies in the human masseter muscle have demonstrated that inhibitory reflexes are decreased in response to tonic, painful stimulation (Svensson et al. 1999), once again indicating that pain may indeed result in increased overall excitability of the trigeminal motoneurons. It is unknown however whether pain neuromodulators would directly increase PIC activation or result in decreased inhibition that allows the PICs to be triggered more easily therefore the effects of substances such as capsaicin, hypertonic saline injections and calcitonin-gene related peptide (CGRP) on PIC activation *and* sensory transmission to the masseter motoneurons should be examined in participants with severe bruxism to determine whether there is a causal link between pain and abnormal PIC activation or vice versa.

5.10.2 Other Currents that May be Involved

Besides the calcium and sodium PICs, intrinsic NMDA and glutamate receptor-mediated currents may also be responsible for the involuntary tonic and rhythmic bursting of masticatory muscles during sleep. Activation of NMDA receptors in trigeminal motoneurons produces both sustained plateau potentials and rhythmic discharge (Hsiao et al. 2002; Kim and Chandler 1995), the latter produced by the activation of a persistent inward current that is followed by the activation of a persistent calcium-activated SK- (Manuel et al. 2012) or Na-K ATPase- (Kim and Chandler 1995) mediated outward current. Trigeminal motoneurons also express metabotropic glutamate receptors (Turman and Chandler 1994, Turman et al. 1997) and receive glutamatergic input from rhythmically active premotoneurons (Turman and Chandler 1994; Katakura and Chandler 1990, Appenteng et al. 1995). Activation of both pre and postsynaptic glutamate receptors accentuates slow, synchronous inputs to the motoneuron by decreasing a potassium leak current to subsequently remove a magnesium block, allowing increased NMDA-mediated currents to rhythmically discharge the motoneuron (Del Negro and Chandler 1998). Modulation of inputs to the NMDA and glutamate receptors via GABAergic pathways also affects the autonomous activation of trigeminal motoneurons. For example drugs which act presynaptically to increase the efficacy of the GABA-signalling inhibitory pathways, such as tiagabine, clonazepam, gabapentin and clonidine, successfully reduces the occurrence of sleep bruxism (Lavigne et al. 2003; Lavigne et al. 2008; Kast 2005). Thus, there are several potential targets to reduce the occurrence of involuntary chewing and teeth clenching in bruxism, especially in severe cases where chronic pain and joint damage are present.

5.10.3 Reduced Inhibition to Masseter Motoneurons?

A second mechanism that has not yet been examined in human participants but could play a significant role in hyperexcitability in individuals with bruxism is reduced inhibition of sensory transmission to masseter

motoneurons. It would be beneficial to examine the duration of EPSPs and IPSPs to masseter motoneurons by recording PSFs in the masseter muscle in response to stimulation. It has already been shown that recurrent inhibition and reciprocal Ia inhibition to masseter muscles is absent. Additionally, animal studies have confirmed that masseter motoneurons receive a greater percentage of excitatory inputs to inhibitory inputs (50% to 48%) when compared to motoneurons in the lumbar spinal cord (33% to 60%) (Bae et al. 1999; Ornung et al. 1998). Therefore it seems that there may already be an inherent imbalance between excitatory and inhibitory inputs to masseter motoneurons, and perhaps this imbalance is more exaggerated in participants with bruxism. This may explain why periods of RMMA are more easily *triggered* in bruxer participants when compared to control participants when both groups are similarly experiencing microarousals during sleep.

5.11 Conclusion

This thesis has examined the mechanisms behind the development of involuntary sustained muscle activity in two distinctly different disorders. By utilizing pharmacological techniques to modulate motoneuron hyperexcitability in individuals with SCI we have been able to identify new drug targets to control spasticity. Additionally, new combinatorial treatments and drug delivery strategies have been discussed. It is evident that more work is needed to elucidate the mechanisms behind the development of bruxism. Once a sufficient understanding is reached, it may be possible to use similar pharmacological methods to identify treatments for this disorder. In conclusion, by increasing our understanding of the mechanisms that drive motoneuron hyperexcitability we hope to provide clinically relevant findings that can alleviate unwanted muscle activity while limiting potential drug-related side effects.

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APPENDIX 1:
ADDITIONAL PUBLICATIONS

- **Murray KC, Stephens MJ, Rank M, D'Amico J, Gorassini MA and Bennett DJ.** Polysynaptic excitatory postsynaptic potentials that trigger spasms after spinal cord injury in rats are inhibited by 5HT1B and 5HT1F receptors. *Journal of Neurophysiology* 106(2): 925-943, 2011.

Assisted in intracellular data collection and partial data analysis during Neuro 501 lab rotation with Dr. Bennett.

- **Rank MM, Murray KC, Stephens MJ, D'Amico J, Gorassini MA and Bennett DJ.** Adrenergic receptors modulate motoneuron excitability, sensory synaptic transmission and muscle spasms after chronic spinal cord injury. *Journal of Neurophysiology* 105(1): 410-422, 2011.

Assisted in intracellular data collection and partial data analysis during Neuro 501 lab rotation with Dr. Bennett.

- **Murray KC, Nakae A, Stephens MJ, Rank M, D'Amico J, Harvey PJ, Li X, Harris RL, Ballou EW, Anelli R, Heckman CJ, Mashimo T, Vavrek R, Sanelli L, Gorassini MA, Bennett DJ and Fouad K.** Recovery of motoneuron and locomotor function after spinal cord injury depends on constitutive activity in 5-HT_{2C} receptors. *Nature Medicine* 16(6): 694-700, 2010.

Collected all human experimental data and performed all data analysis examining effects of cyproheptadine in incomplete SCI participants.

- **Udina E, D'Amico J, Bergquist AJ and Gorassini MA.** Amphetamine increased persistent inward currents in human motoneurons estimated from paired motor-unit activity. *Journal of Neurophysiology* 103(3): 1295-1303, 2010.

Assisted in data collection, analysis of ΔF measurements and examination of the modulation depth of motor units.