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Estimates of Persistent Inward Current Decline in Human Soleus Motor Units during Fatigue

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Abstract

Fatigue is defined as any exercise induced reduction in strength or power, and can be attributed to central and peripheral components. Many central and peripheral mechanisms have been extensively studied, but few studies have looked at the changes in the intrinsic properties of motor neurons and their contribution to fatigue. Persistent inward current (PIC) is an important intrinsic property of motor neurons responsible for setting a large increase in the gain of motor output and may contribute to fatigue. Inhibitory inputs such as reciprocal inhibition (RI) have been shown to turn off PICs and reducing the gain of output. PIC measurements are typically done in animals but have recently been estimated in humans using the paired motor unit technique. Estimates of PIC were taken from paired motor unit recordings in the soleus. Estimates of PIC are calculated by using the difference (ΔF) between the instantaneous firing frequency of a control unit at the recruitment and derecruitment of a test unit during an isometric triangular ramp contraction. Inhibitory input via electrical stimulation of the common peroneal nerve was used to reduce PIC in the soleus. These isometric triangular ramp contractions used to calculated ΔF were performed with and without electrical stimulation after sets of 20 fatiguing contractions in order to assess ΔF estimates of PIC before and after fatigue. Maximum voluntary contractions (MVC) were performed after each set of fatiguing contractions to quantify the amount of fatigue. The experiment was terminated after a 30% reduction in MVC. It was hypothesized that there would be a decline in ΔF estimates of PIC during a fatiguing protocol and no change in PIC during a control day in ramps without electrical stimulation. In ramps with inhibitory input via electrical stimulation (RI), ΔF estimates of PIC would not decline as significantly as ramps without electrical stimulation over the course of a fatiguing protocol. On a control day, the ramps with electrical stimulation would have a lower ΔF than ramps without electrical stimulation, and also would not change over time. On the fatigue day, MVC dropped from 347.18N ± 96.54N to 220.57N ± 65.53N, $t(9) = 4.23$ ($p<0.01$) and did not change over a control day. ΔF decreased to 60.5% ($F=15.52(1,9)$, $p<0.01$) from initial values on a fatigue day in ramps without electrical stimulation and did not change significantly on a control day. The ramps with RI were significantly lower ($F=8.099$ (1,9), $p<0.05$) on a control day than the ramps without electrical stimulation and did not change over time. On a fatigue day, there is a trend ($p=0.068$) between the initial pre-fatigue values from the stimulation to no stimulation ramps. These results show a decrease in ΔF over a fatigue protocol and the application of RI adds validity to the paired motor unit technique. Further research warrants investigation of whether the changes in ΔF over fatigue are due to increases in inhibitory inputs or decreases in monoaminergic drive.

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I guess I have to thank my parents as well for raising me over these 24 long, arduous years. From arguing over spilled milk to life goals, we have talked and fought over it all. I cannot thank you guys enough for being the most caring and supportive parents in the world. I know I haven't been the easiest at times, and I hope that I have made you both proud as well.

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List of terms and abbreviations

PIC (Persistent inward current) – Long lasting inward current from monoaminergic-dependent, voltage-gated calcium and sodium channels located mainly on the dendrites of the motor neuron. PIC is responsible for amplifying and prolonging the effects of ionotropic inputs.

STA (Spike threshold accommodation) – The current required to bring a motor neuron to threshold increases as the rate of rise of the input current decreases.

SFA (Spike frequency adaption) - Decline in firing rate of a motor neuron in response to a constant current input over time.

RI (Reciprocal Inhibition) – Inhibition pathway through a 1a afferent neuron which causes inhibition of the antagonistic α-motor neuron. An inhibitory interneuron inhibits the antagonist muscle from working against the working agonist muscle.

PSTH (Post stimulus time histogram) – Histogram used to assess changes in motor neuron firing rates in relation to an external stimulus (e.g. reciprocal inhibition).

EMG (Electromyography) – Used to measure the electrical activity of a specific muscle.

ISI (Interspike interval) – The time between each successive action potential of one motor neuron.

ΔF (estimate of PIC) – Delta F is the estimate of PIC in humans, calculated from the difference in the instantaneous firing frequency in the control unit at the onset and offset of the test unit.

Fmax-Fmin - The difference between the maximal and minimal firing frequency of the control unit (discharge rate modulation).

CPN (Common peroneal nerve) – Nerve to the antagonist muscle of interest (soleus muscle) used to elicit reciprocal inhibition

MVC (Maximum voluntary contraction) – The greatest amount of force a muscle can generate.

Tlim – The time required to produce a given amount of fatigue.

Chapter 1: Literature Review

Literature Review

Motor neurons were once thought to be passive conduits for synaptic inputs, as these ionotropic inputs would be summated resulting in a given motor output. However, it has been recently discovered that certain intrinsic properties of motor neurons modulated by the neuromodulatory system, highly increase the gain of the motor neuron (Heckman, Mottram, Quinlan, Theiss, & Schuster, 2009). Motor neuron excitability is very complex and altered in different states, such as in sleep, and disease, and for different tasks. Understanding motor neuron excitability can give greater insight to the mechanisms of neuromuscular function.

There are 2 types of input to the motor neuron; ionotropic input and neuromodulatory input (Heckman et al., 2009). Ionotropic inputs release neurotransmitters that bind to ligand-gated ion channels. The opening of an ion channel allows ions to enter or exit the cell generating a synaptic current. These potentials are graded and are either excitatory (depolarizing) or inhibitory (hyperpolarizing). The summation of ionotropic inputs result in brief changes in the membrane potential of motor neurons that last as long as the synaptic input lasts. In contrast, neuromodulatory inputs have the potential to exert much longer-lasting effects on the motor neuron. For example, a persistent inward current (PIC) may increase the gain of motor excitability from 6 to 10 fold (Heckman, Johnson, Mottram, & Schuster, 2008) in response to serotoninergic or and noradrenergic neuromodulatory input. Neuromodulatory receptors are linked to G-proteins which activate a variety of intracellular signaling cascades. Some of these signaling cascades have a potent effect on motor neuron excitability that is slower to activate, but have a longer lasting effect.

There are several neurotransmitters that alter the gain of motor neurons, however the two monoamines; serotonin and norepinephrine are most influential on PIC (Heckman et al., 2008). Serotonin is a neuromodulator synthesized by raphe nuclei in the brainstem. Serotonergic neurons project supraspinally to various brain regions as well as to dorsal and ventral areas of the spinal cord (Perrier, Rasmussen, Christensen, & Petersen, 2013). These projections to the spinal cord have been shown to increase with increasing motor output (Jacobs, Martin-Cora, & Fornal, 2002) and are highly involved in the wide range of gain control of spinal motor neurons (Johnson & Heckman, 2014). Norepinephrine, released by the locus coeruleus in the brainstem, is another monoamine that also has a major role in locomotion. In vivo (Jordan, Liu, Hedlund, Akay, & Pearson, 2008) and in vitro (Merrywest, Fischer, & Sillar, 2002), norepinephrine has been shown to be extremely important for motor output and the modulation of reflexes. Increases in norepinephrine can also increase PIC and the excitability of a motor neuron (Lee & Heckman, 1999).

Not only does the type of neurotransmitter affect the excitability of a motor neuron, but so does the type of membrane receptor. Membrane receptors have several different subtypes that exhibit markedly different effects on a cell. The membrane receptors of serotonin are classified into 7 different subtypes (5HT1, 5HT2, 5HT3 etc.) and 14 total subclasses (5HT1a, 5HT1b, 5HT2a etc). Norepinphrine receptors are classified into alpha and beta. The different receptor subtype can have either excitatory or inhibitory effects on motor neuron excitability (Harvey, Li, Li, & Bennett, 2006). 5HT2 and norepinephrine alpha1 receptors have an excitatory effect and strongly facilitate PICs, while other receptor subtypes can have inhibitory actions such as 5HT1 receptors. The 5HT1 receptors are activated at high levels of serotonergic input

and is thought to serve as a braking mechanism for excessive excitability (Cotel, Exley, Cragg, & Perrier, 2013). Although these receptor subtypes have different actions on the motor neuron, the neurotransmitter that binds to them remains the same. Generally, receptors coupled to G-proteins have excitatory effects while receptors coupled to the Gs system have inhibitory effects (Garraway & Hochman, 2001). Other effects such as posttranscriptional modification can induce changes in receptor behavior, and there are several other effects that have yet to be understood (Nichols & Nichols, 2008).

PICs were first discovered in 1980 in reduced animal preparations (Schwindt & Crill, 1980). They are generated by voltage-gated Na^{+}, and Ca^{2+} channels and are activated by descending monoaminergic input (Alaburda, Perrier, & Hounsgaard, 2002). Channels responsible for mediating PICs reside mainly on the dendrites of spinal motor neurons but also on the soma of the motor neuron (Heckman et al., 2009). The channels responsible for generating PICs are a subtype of L-type Ca2+ channels (CaV1.2, 1.3), and fast-acting Na channels (NaV1.6) (Toledo-Rodriguez, El, Wallen, Svirskis, & Hounsgaard, 2005). The main function of PICs is to amplify and prolong the effects of synaptic inputs to the motor neuron. They are activated near spike threshold and stay active following removal of synaptic input. In Figure 1, steady excitatory input is generated by tendon vibration of muscle spindle 1a afferents in an animal model. When the cell is held or ‘clamped’ at a hyperpolarized potential in Figure 1A (green tracings) and is not brought close to its firing threshold where PIC is activated, the current generated from the tendon vibration returns to baseline after the synaptic input (tendon vibration) is removed. However, when the cell is brought closer to its firing threshold (red tracings), there is a significant increase in the current generated across the membrane due

to the activation of PIC. Not only does the PIC amplify the effects of the synaptic input but also prolongs the effects of the synaptic input after the synaptic input has been removed. The functional significance of this is shown in Figure 1C where voltage is shown on the x-axis instead of current in 1A. The green tracings represent the voltage across the membrane when the cell is held below its firing threshold. In the red tracings where the cell is brought closer to its firing threshold, PIC is activated and amplifies the effects of the synaptic input to result in subsequent motor unit firing. The PIC also prolongs the effects of the tendon vibration (synaptic input) resulting in self-sustained firing after the tendon vibration has been removed. Motor neurons capable of self-sustained firing are considered to be "bistable", one stable state at its resting potential, and the other during self-sustained firing (Hounsgaard, Hultborn, Jespersen, & Kiehn, 1988). Brief excitatory inputs activate PIC which induce self-sustained firing, and brief inhibitory inputs deactivate PIC and return the motor neuron to the quiescent state (Bennett, Hultborn, Fedirchuk, & Gorassini, 1998).

Self-sustained firing and 'bistability' are the main characteristic of PICs, quite active mainly in low-threshold 'type S' motor neurons during posture and gait (Heckman *et al.,* 2008). There are three types of motor units: 1) type S (slow); 2) type FR (fast, fatigue resistant); and 3) FF (fast fatigable). Slow, type S motor neurons have low force outputs, lower conduction velocities, and high resistance to fatigue. Faster, type FF motor neurons have high force outputs, higher conduction velocities, and a lower resistance to fatigue (Kernell, 1965). Type S to type FF motor neurons exhibit a range of about 10-fold in the current required to reach recruitment threshold (Figure 2). Type S motor neurons are more capable of self-sustained firing because only a small amount of synaptic input is needed for spike threshold, due to its

high input resistance (Lee & Heckman, 1998). PICs prolong the effects of synaptic input, especially in type S and FR motor neurons highly active during posture. Type S motor neurons are highly involved in postural tasks, where the prolonged effects of PICs are particularly important. The PICs are also involved in type FF motor neurons, however they do not exhibit the long term self-sustained firing that type S motor neurons do because of their low resistance to fatigue. Likely the PICs in type FF motor neurons are more responsible for greatly amplifying the response to synaptic inputs. Type FF motor units are responsible for generating large amounts of force. It has been estimated that in the absence of monoaminergic input, motor neurons would only be able to produce about 30-40% maximum motor output (Figure 4) (Heckman, 1994). Thus, PICs are especially important not only for posture and gait, but also generating a large and high range of forces.

A number of studies have quantified the magnitude of PIC in animal preparations through intracellular recordings, but this is not possible in humans. In humans, PIC can be estimated indirectly through a method called paired motor unit analysis (Gorassini, Yang, Siu, & Bennett, 2002). This method uses the difference between the firing rate of a lower threshold motor unit (control unit) at the time of the onset and offset of a higher threshold (test) motor unit. The control unit serves as indicator of net synaptic drive, and the difference in the firing frequencies of the control when the test unit turns on and off is mainly attributed to PIC This method is used during an isometric triangular ramp contraction and the control unit is compared to the test unit (Figure 3) (Heckman *et al.*, 2008).

There are a few assumptions and considerations to note when using the paired motor unit technique. The first assumption is that the control unit is a reliable indicator of changes in

the net excitatory input to the test motor unit. The later recruited, higher threshold test motor unit derecruits at a lower firing rate than which it recruits. The difference is thought to be mainly due to PIC as the net excitatory input drops below the level at which it needs to be recruited. To ensure that the control unit is a sensitive indicator of net excitatory input, the amount of discharge rate modulation of the control unit must not be within 0.5 pulses per second (pps) of △F (Stephenson *et al.*, 2011). The discharge rate modulation is the difference between the maximal and minimal firing frequency of the control unit (F_{max}-F_{min}). Anything below 0.5 pps may indicate that the test unit was recruited too close to the peak of the ramp thus any changes in firing rate would be too small, or the firing rate of the control unit was fully saturated (Stephenson *et al.*, 2011).

Another assumption is that the paired motor unit analysis is that both the control and test unit share similar synaptic input. To test common synaptic input, (Gorassini et al., 2002) have shown that the rate to rate correlation (r) between both units should be greater than 0.7 showing common synaptic input. Finally, for △F to be an accurate measure of PIC, the control motor unit PIC must be fully activated before the onset of the firing of the test unit. Full activation of PIC has been proposed to occur approximately 2s after recruitment motor unit (Udina *et al.,* 2010).

In addition to PIC, there are several intrinsic motor neuron properties that could contribute to △F. If △F is to be used as an estimate of PIC, then the contribution of other nonlinear firing properties must be minimized. Two other properties known to "contaminate" paired motor unit estimates of PIC include spike threshold accommodation (STA) and spike frequency adaptation (SFA). STA is the process by which the current required to bring a motor

neuron to threshold increases as the rate of rise of the input current decreases. There can be as much as a 2-fold difference in the amount of input current needed to depolarize a motor neuron with varying rates of rise of the input current (Revill & Fuglevand, 2011). SFA is a decline in firing rate of a motor neuron in response to a constant current input over time. SFA can inflate △F estimates in ramps of longer duration. Longer periods of time can lead to a decrease in firing rates, up to 60% of initial values (Button, Kalmar, Gardiner, Cahill, & Gardiner, 2007). A recent study by Revill & Fuglevand(2011) assessed the contributions of STA and SFA by varying the rates of rise and duration of simulated ramps. Ramps 10 seconds in duration with no plateau served to minimize STA and SFA, leading to greater accuracy of △F. To further validate the simulation study, another study was conducted in humans using the paired motor unit technique (Vandenberk & Kalmar, 2014). This study assessed the contribution of STA and SFA to △F. Vandenberk and Kalmar (2014) demonstrated that △F increases with decreasing rates of ramp rise and increasing ramp duration, likely due to SFA. The study further validated the 10s ramp profile and no plateau to minimize STA and SFA. This study also investigated changes in reciprocal inhibition (RI) to determine the extent to which △F could be attributed to PIC. Two opposing sets of muscles work in synchrony; when one muscle contracts, the other one relaxes due to RI. The 1a afferent of the working muscle enters the spinal cord, one branch synapsing onto the homonymous alpha motor neuron causing the muscle to contract, and one branch synapsing onto a 1a inhibitory interneuron of the antagonist muscle causing the muscle to relax. This RI pathway can be evoked through a sub-motor threshold electrical stimulus to the nerve to the antagonist muscle of interest. In this study RI was elicited through stimulation of the common peroneal nerve (CPN) to examine the influence on △F. There was a significant

correlation between RI and △F in shorter ramps with quicker rates of rise suggesting that PIC is the main contributor in △F, but also inversely correlated to RI. RI was found to be to be inversely correlated to △F, such that a greater increase in RI was associated with greater reductions in PIC as would be predicted by animal studies that have shown that PIC is "turned off" by hyperpolarizing inhibitory input (Hounsgaard *et al.*, 1998). RI has been shown in other studies to deactivate PIC (Kuo, Lee, Johnson, Heckman, & Heckman, 2003), and decrease PIC as much as 50% with as little as a 10 degree rotation in a joint through inhibitory pathways (Hyngstrom, Johnson, Miller, & Heckman, 2007). These inhibitory pathways serve as local gain control mechanisms in locomotion (Johnson & Heckman, 2014).

The paired motor unit technique can be used to investigate the changes in PIC in humans during altered states, such as during fatigue. Neuromuscular fatigue is defined as the inability to produce maximal levels of force, and can be a result of central or peripheral mechanisms (Gandevia, 2001). Central fatigue refers to the inability of the CNS to drive muscle. Central mechanisms of fatigue include, but are not limited to, loss of recruitment of high threshold motor units, increased inhibitory input to the motor cortex, motor neuron dropout, increased negative feedback from muscle afferent types III and IV sensory neurons, loss of positive feedback from muscle spindle type I sensory afferents. There are many sites of fatigue in the CNS all the way from the basal ganglia, and down to the motor neuron in the spinal cord. There are many inputs onto the alpha motor neuron (Figure 4), both excitatory and inhibitory, that affect the excitability of the motor neuron. Several studies have investigated electrical stimulation of type 1a sensory fibers (RI pathway) on PIC, but little research has investigated group III/IV afferents influence on PIC. Previous studies have shown the role of group III/IV

afferents inhibitory effects on spinal motor neurons (Amann et al., 2008), but not directly on PIC. A study by (Gandevia, Allen, Butler, & Taylor, 1996) has also shown decreases in spinal motor neuronal output and voluntary activation after 2 minutes of maximal voluntary elbow flexor contractions. Since group III/IV afferents have inhibitory effects on the motor neuron, potentially during fatigue these group III/IV afferents are turning off or reducing PIC. Fatigue has several sites of origin that have been examined, however one area that has not been examined but have been proposed to contribute to fatigue are changes in the intrinsic properties of the motor neuron (Gandevia 2001). Changes in monoaminergic drive, afferent inhibition, and reciprocal inhibition can all alter PIC during fatigue, however we are only investigating changes in PIC during fatigue. Plausibly, decreases in PIC can be a major contributing factor to fatigue.

Purpose and Hypotheses

The primary purpose of this study is to investigate the effects of fatigue on estimates of PIC in humans during isometric contractions. There are three main hypotheses:

1. Estimates of PIC will decrease during the course of a fatiguing protocol and will recover to baseline following a recovery period.
2. Estimates of PIC will not decline during a control day without fatiguing contractions.
3. When electrical stimulation is applied to the nerve of the antagonist (CPN) midway through a ramp contraction, initial estimates of PIC (before fatigue) will be lower, and will decline to a lesser extent during fatigue.

Chapter 2: Methods

Methods

Overview of Experimental Design

In the current study, PIC was estimated in human soleus motor units on two different days. On one day, PIC was estimated before and after an isometric plantarflexion fatigue protocol. On a second day, PIC was estimated at the same time points, but without fatiguing contractions. To provide evidence that fatigue-induced changes in paired motor unit △F values reflect changes in PIC, the nerve to the antagonist (common peroneal nerve) was electrically stimulated during alternate ramps each day to inhibiting the motor neuron, and diminish PIC, via reciprocal inhibition. To provide evidence that electrical stimulation of the common peroneal nerve elicited reciprocal inhibition of the test motor unit, post stimulus time histograms (PSTH), were produced for each test unit. A rightward shift in the PSTH would indicate inhibition.

Participant Recruitment

14 participants (4 females) ages 22.7 ± 2.7 years were recruited from Wilfrid Laurier University for this study, with 10 motor unit pairs used for analysis (n=10). Participants had to have no known neurological disorder, leg trauma (right leg), concussion, neurotrauma or any other problem indicated via self-report using an exclusion criteria checklist (Appendix B). The participants were also required to abstain for strenuous exercise the night before the experiment, and not to consume coffee the morning of the experiment. The participants were required to fill out an informed consent (Appendix C) outlining the details of this study. This study attained ethics approval from the University Research Ethics Board (REB#3849).

Before the experimental or control day, a 30 minute orientation day was completed to familiarize the participants with MVC's, electrical stimulation, and the isometric ramp contractions. The participants were informed about the details and procedures of the experiment during this orientation period.

Experimental Apparatus and Procedure

Participants were seated in a modified automobile chair with their right leg in a McComas boot dynamometer. Knee and ankle joint angles were each set to 90 degrees for this isometric protocol. An ankle brace was used to maintain the same foot position in the dynamometer to ensure that the participant's foot did not slide forward, changing the lever arm. A padded clamp was placed just above the knee to ensure an isometric contraction, preventing the heel from rising during plantarflexion contractions. A foot clamp was also placed over the cuneiforms to maintain foot position and also to maintain isometric contractions during dorsiflexion.

The hair from the skin of participants were shaved and cleaned with 99% isopropyl alcohol. surface electromyography (EMG) 0.5cm^2 Ag-AgCl electrodes (x60 amplification) with an interelectrode distance of 2.0 cm (EQ, Chalfont, PA) were placed on the lateral aspect of the soleus and the tibialis anterior and input to a custom-built variable gain amplifier (x20 setting used) (York University). To optimize the signal to noise ratio, a ground with electrode gel was placed on the medial tibia. Surface EMG data was sampled at 2000 Hz (Micro 1401-3, Cambridge Electronic Design) and filtered offline with a high pass filter at 15 Hz.

For intramuscular electromyography, three 50.8-µm Formvar-insulated stainless steel wires (California Fine Wire Company, Grover Beach, CA, USA) with three fine wires were inserted into the muscle of the participant's lateral aspect of the soleus using a 27-gauge sterilized hypodermic needle. Two of the three wires were input to an epoxy-embedded preamplifier assembly (x20 amplification, EQ Inc., Chalfont, PA) and connected to a second-stage custom-built variable gain amplifier. The intramuscular signal was sampled at 20 000 Hz (Micro 1401-3, Cambridge Electronic Design), and band pass filtered (3,000-30,000Hz) through a Neurolog system (Digitimer Inc.) to reduce noise and optimize the signal-to-noise ratio for spike discrimination. Motor unit mass action potentials in the intramuscular channel were sorted and coded offline as control or test motor units based on their distinct waveform shape and firing pattern (Figure 6) using Spike2 software (Cambridge Electronics Design).

Plantarflexion force from the McComas boot dynamometer was digitized at 1000 Hz (Micro 1401-3, Cambridge Electronic Design), then amplified 30x (1902 Amplifier, Cambridge Electronic Design) and low-pass filtered with a 50Hz cut-off offline (Spike2, Cambridge Electronics Design).

A 2.5cm^2 carbonized rubber electrode was used to stimulate the nerve to the antagonist (CPN) during reciprocal inhibition. A Digitimer constant current stimulator, model DS7AH (Digitimer Inc., Hertfordshire, England) was used to deliver electrical stimulation (1ms duration) for reciprocal inhibition (Figure 5).

Protocol

A schematic diagram of the protocol is shown in Figure 5. The experiment was run over 2 days (minimum separation of 2 days in between), approximately 2 hours each day following an initial orientation day to familiarize participants with the protocol and procedures. The 2 days involved one day with a fatiguing protocol and one day without a fatiguing protocol. The 'fatigue' day always occurred first. This was necessary to determine the number of sets each participant had to complete to adequately fatigue the plantarflexors (30% decrease in isometric plantarflexion maximum voluntary force). The same protocol and the same number of sets (without fatiguing contractions) was then repeated on the control day.

Participants began the experiment by steadily plantarflexing at varying force levels (10%,20%,30%) to ensure that good intramuscular motor unit recordings were observed. If the motor unit recordings were reliable and sortable, the experiment could commence. The first part of the protocol was to determine the appropriate intensity needed to elicit RI (Figure 6). The RI intensity needed to be above the threshold to activate sensory neurons, but not high enough to activate the motor axon. The stimulus intensity (mA) was set to 80% of soleus motor threshold. After the stimulus intensity was set, the participant was asked to hold a force level low enough to only activate 1 motor unit. On a screen in front of the participant was shown the firing rate of that motor unit in real time. Once the force level could be steadily maintained, 80 sub motor threshold stimuli were delivered to the CPN over 4 minutes. The participants were asked to maintain the current interspike interval as the firing rate was shown on a linear profile in front on them.

The participant would then begin by practicing plantarflexion triangular ramp contractions (Figure 5) needed to analyze estimates of PIC during the experiment. These triangular ramp contractions were 10s in duration (5s increasing plantarflexion force, 5s decreasing plantarflexion force), and the peak of the ramp set to 10% of the participant's plantarflexion maximum voluntary contraction (MVC). The participants also practiced a series of MVCs, until a reliable value of MVCs and accurate triangular ramp force tracings were observed. They would then proceed to perform several 'pre-fatigue' ramps, with and without electrical stimulation to elicit RI. After a minimum of 2 acceptable (minor deviations from a linear profile) ramps were completed in both the stimulation and non-stimulation ramps, the fatigue protocol began. 20 repeated fatiguing contractions (45% of MVC, 3s duration, 1s rest) were performed, and then followed by an MVC. The PIC ramps, fatiguing contractions, and MVC made up 1 set. The participants repeated these sets until a 30% reduction in MVC was observed, or 10 sets total was reached. The same protocol was then repeated on the control day, except the participants did not perform the fatiguing contractions.

Data Analyses

Evaluating strength of reciprocal inhibition via post stimulus time histograms

A post stimulus time histogram (PSTH) was used to quantify the amount of inhibition elicited by stimulating the nerve to the antagonist (Figure 6). 80 subthreshold stimuli were delivered to the CPN during a low level contraction. These stimuli were delivered every 3s and were triggered by discharge of the test motor unit. A PSTH was generated from these stimuli to assess the interspike intervals following antagonist nerve stimulation. 80 control triggers (markers with

the same delay, but no electrical stimulation) were delivered to generate PSTHs of the interspike intervals without stimulation. If stimulation of the nerve to the antagonist elicited inhibition of the soleus test motor unit, then the stimulation PSTH should exhibit a rightward shift relative to the control PSTH (denoting longer interspike intervals). Both PSTHs were set-up to count motor unit discharges in 5-ms bins for 400ms after each trigger. To quantify this rightward shift a difference PSTH was generated by subtracting the control PSTH counts from the stimulation PSTH counts for each bin. Each bin of the difference PSTH was then added to form a cumulative sum. The valley of the cumulative sum was used to quantify the amount of inhibition (Ellaway, 1978).

Estimating PIC from paired motor unit recordings

PIC was estimated using the paired motor unit technique (Gorassini, Yang, Siu, & Bennett, 2002) The firing rates of the control and test units were plotted over the course of each 10-s ramp contractions and fitted to a 4th order polynomials. The onset and offset of the test unit were used in the polynomic equation of the line fit to the firing rate of the control unit. The firing rate of the control unit at the offset of the test unit was subtracted from the firing rate of the control unit at the onset of the test unit to calculate $\triangle$F (Figure 3). The paired motor technique relies on a number of assumptions (Stephenson & Maluf, 2011). Therefore, only motor unit pairs that met the following criteria were included in analysis: 1) The test unit had to be recruited >1s after control unit recruitment to ensure that PIC was fully saturated in the control unit; 2) The rate-rate correlation had to be greater than 0.7 ($r > 0.7$) to ensure that there was common synaptic drive to the control and test units; and 3) When $\triangle$F estimates were

subtracted from the control unit firing frequency range (F_{max}-F_{min}), the difference had to be ≥ 0.5 to ensure that there was no control unit firing rate saturation.

Statistical Analyses

Differences between day (control, fatigue), and over time (pre-fatigue, 25% Tlim, 50% Tlim, 75% Tlim, Tlim, and recovery) were analyzed using a 2-way repeated measures ANOVA. Differences between ramps (stimulation, no-stimulation), and over time (pre-fatigue, 25% Tlim, 50% Tlim, 75% Tlim, Tlim, and recovery) were also analyzed using a 2-way repeated measures ANOVA. Differences between means were detected using Tukey's HSD test. Missing data (some participants had unusable ramps at a given time point during the fatigue protocol) were dealt with via mean substitution. Differences in the decline in MVC (prefatigue to the Tlim) on the fatigue day compared to the control day were assessed using a dependent t-test. A p-value of less than 0.05 was considered to be significant. All data are presented as mean±SD.

Chapter 3: Results

Results

MVC

On the fatigue day, MVC dropped from 347.18N ± 96.54N to 220.57N ± 65.53N $t(9) = 4.23$ ($p<0.01$) at the end of the fatigue protocol and recovered to prefatigue values within the 10-min recovery period (Figure 10). On the control day, there was no significant change in the MVC ($p=1.00$) over the course of the protocol.

Reciprocal Inhibition Analysis (PSTH)

On both days of the experiment, ramps were performed with and without electrical stimulation to the common peroneal nerve which was intended to elicit an inhibitory input to soleus motor neurons via the reciprocal inhibition pathway. Negative values were evident in the cumulative sum of each difference post-stimulus time histogram at time points coinciding with the first and second interspike intervals following stimulation on both the control (mean= -13.25 counts, SD=17.83), and fatigue day (mean =-28.5 counts, SD=14.62) (Table 1).

Paired Motor Unit Validation Criteria

Only motor unit pairs that exceeded previously published validation criteria (Stephenson & Maluf, 2011) for the paired motor unit technique were used (Table 2). The average Pearson's correlation (r^2) for the motor unit pairs was (0.91 ± 0.09) to ensure control unit and the test units to shared common synaptic drive. Test units were recruited 2.14 ± 0.64 s after the control unit to ensure that control unit PIC was fully saturated prior to test unit recruitment. Finally, the

average difference between the control unit firing range (rate modulation, $ff_{max} – ff_{min}$) and test unit estimates of ΔF were 3.18 ± 1.22.

Changes in △F estimates of PIC during fatigue without antagonist nerve stimulation

Figure 7 illustrates the fatigue-induced changes in △F on the fatigue day in ramps without RI. △F decreased to 60.5% ($F=15.52(1,9)$, $p<0.01$) of initial values by the end of the fatigue protocol, and returned to baseline following a 10 min recovery period. Post-hoc analysis (Tukey's HSD) revealed significant differences at 50% tlim ($p<0.05$), 75% tlim ($p<0.01$), and 100 tlim ($p<0.01$). Post-hoc analysis effect size was moderate ($d = 0.480$) and had low statistical power ($\alpha = 0.05$, power = 0.295) due to the small sample size (n=10).

Changes in △F estimates of PIC during fatigue with antagonist nerve stimulation

On the fatigue day, the initial pre-fatigue △F estimates of PIC were lower when the nerve to the antagonist was stimulated midway through the ramp compared to the ramps without antagonist stimulation (Figure 8). There is a trend ($p=0.068$) between the initial pre-fatigue values from the stim to no stim ramps. The ramps with electrical stimulation were lower than the ramps without electrical stimulation.

Changes in △F estimates of PIC during the control day

On the control day, △F values did not change from the start of the protocol until the end of the protocol. In addition, the ramps with antagonist nerve stimulation were significantly

lower ($F=8.099$ (1,9), $p<0.05$) than the ramps without stimulation and did not change over the course of the control protocol (Figure 9).

Chapter 4: Adderall Case Study

Background and Rationale

There is a 'wind-up' effect or delayed activation of PIC in response to repeated depolarizations. PIC activation increases with each subsequent depolarization, provided there is minimal time lapse between each contraction (Alaburda et al., 2002). A question remains if similar PIC measurements, more specifically ∆F measurements will be highly variable in humans with closely repeated triangular ramp contractions. Results from an earlier study by (Gorassini, Yang, Siu, & Bennett, 2002) showed reduced synaptic input in subsequent contractions to recruit a motor unit. However, there has been large calcium PIC variability in rat hypoglossal neurons in response to repeated brief stimuli (Moritz, Newkirk, Powers, & Binder, 2007). Amphetamine has been shown to double the calcium-mediated PIC in animals (Rank, Li, Bennett, & Gorassini, 2007) via increased presynaptic release of norepinephrine (Rank et al., 2007), and increase estimates of PIC by 62% in humans (Udina, D'Amico, Bergquist, & Gorassini, 2010).

Certain drugs have been shown to increase PIC due by increasing monoaminergic activity. In a study by (Udina et al., 2010) amphetamines allowed the test unit to fire at lower levels of synaptic input and shortened the amount of time of test unit activation, which led to greater ∆F values. A clinically used amphetamine-containing drug commonly taken is Adderall.

Adderall is a mixture of dextroamphetamine and levoamphetamine. It is mainly used to treat attention-deficit hyperactive disorder (ADHD) and narcolepsy (Heal, Smith, Gosden, & Nutt, 2013). ADHD is characterized by decreased cognitive abilities, such as reduced ability to focus (Froehlich 2000). Epidemiological data conservatively reveal that a staggering 5.3% of young adults are diagnosed with ADHD (Polanczyk & Rohde, 2007). Adderall affects the CNS by

increasing presynaptic release of norepinephrine in the prefrontal cortex and dopamine in the striatum. The drug is actively transported into the presynaptic terminal and releases norepinephrine and dopamine from newly synthesized and vesicular storage pools (Heal et al., 2009). Adderall also acts synergistically as a monoamine reuptake inhibitor (Heal et al., 2013). The structure of Adderall closely resembles monoamine neurotransmitters which explain the action of Adderall. The time to peak plasma levels for Adderall is 2-3 hours, but the effects can be observed 15-60 minutes after oral ingestion. The half-life of Adderall is 10 hours (Angrist, Corwin, Bartlik, & Cooper, 1987).

One very interesting phenomenon that occurs with any potentially beneficial treatment is the placebo effect. The expectation of beneficial treatment can elicit powerful, diverse physiological benefits regardless if the treatment itself has physiological benefits (Fuente-Fernandez, Schulzer, & Stoessl, 2002). Reward circuitry in the limbic system is activated with the sole expectation of a reward (Garris et al., 1999).The release of dopamine from the nucleus accumbens in the ventral striatum is associated with reward processing. Placebos have been used to treat numerous diseases such as Parkinson's (Mercado et al., 2006). Although dopamine is not a major neurotransmitter involved in generating PICs, it has been suggested that serotonin has a role in mediating placebo rewards as well (Fuente-Fernandez & Stoessl, 2004). Like most drugs, it is likely that Adderall elicits a placebo effect in addition to the intended clinical effects. This is particularly likely given that the drug acts on neurotransmitters that play an important role in both the reward and anticipation circuitry as well as the neural control of movement and central fatigue. We had originally intended to conduct dose-response placebo trials in this study, but time constraints will not permit this study design. In addition, it

would be difficult to design an ethical experiment that could replace the prescribed dose of Adderall with a placebo.

Purpose

The purpose of this study is to quantify the effects of Adderall and fatigue on motor unit firing rates, muscle activation, muscle contractile properties, and PIC. Since △F is an estimate of PIC, by adding a perturbation to the measure (adding a pharmacological drug), we can further validate the use of the paired motor unit technique.

Hypotheses

1) Estimates of PIC will increase following oral administration of Adderall due to an increase of presynaptic norepinephrine from the brainstem.
2) Adderall will decrease fatigability, force sensation, perceived exertion, and increase motor unit firing properties and force production.

Methods

Participant Recruitment

Participants were required to have a confirmed doctor's diagnosis of a neurological disorder (ADHD) requiring the use of Adderall. A minimum of ten participants, ages 18-25 will be used for the study on 2 different days (currently n=1). The participants have been recruited through posters around the Wilfrid Laurier University campus. Participants have been recruited through word of mouth. The project has been approved from the Research Ethics Board at Wilfrid Laurier University. Participants were required to sign informed consent documents before participating in the study. Participants with any neurological disorder, leg injury, use of

selective serotonin reuptake inhibitors, or not diagnosed with ADHD have been excluded from this study. Participants must also take the immediate release Adderall drug as their form of medication and not any other ADHD drug.

Set up and Procedures

The experiment was conducted over 2 days (1 control and 1 experimental), lasting approximately 3 hours each day following an initial orientation day to familiarize participants with the protocol and procedures. On the experimental day the participant completed the pre-test measures (described below), ingested their Adderall as prescribed, and one hour later complete the post-test measures. On the control day, the participant will complete pre-test measures, and one hour later complete the post-test measures. Once the control experiment is complete, the participant will be asked to take their Adderall as prescribed. Because Adderall is a stimulant and the drug is meant to be taken at the same time each morning, experiments will be scheduled around the participant’s usual drug administration time. As such, this experiment will not result in any change to the participant’s usual medication schedule (medication is not skipped, delayed, or adjusted in any way).

Experimental Apparatus and Procedure

Participants will be seated with their right leg in a McComas boot dynamometer for the triangular ramp contractions. Knee and ankle joint angles will be set to 90 degrees and 90 degrees respectively. The skin of participants will be cleaned with alcohol pads and ultrasound gel is applied to the surface of the skin. Surface electromyography (EMG) Ag-AgCl electrodes (x60 amplification) will be placed on the lateral aspect of the soleus and the tibialis anterior and connected to a custom-built variable gain amplifier (York University). For intramuscular electromyography, a 27-gauge needle with three fine wires will be inserted into the muscle of

the participant's lateral aspect of the soleus input to an epoxy-embedded preamplifier assembly (x20 amplification, EQ Inc., Chalfont, PA) and connected to a second-stage custom-built variable gain amplifier. The intramuscular signal is then band pass filtered (3,000-30,000Hz) through a Neurolog system (Digitimer Inc.) to reduce noise and optimize the signal-to-noise ratio for spike discrimination. Surface electromyography data will be digitized at 2000Hz and intramuscular at 20 000 Hz (Cambridge Electronic Design Micro 1401). Plantarflexion force from the McComas boot dynamometer is digitized at 1000 Hz (Cambridge Electronic Design Micro 1401), then amplified 30x and low-pass filtered with a 50Hz cut-off (Cambridge Electronic Design, 1902).

The data is analyzed through a program by Cambridge Electronics Design called Spike2. Spike2 allows for single and multi unit recordings online, and offline. It allows for motor unit action potential sorting based on their distinct waveform pattern (Figure 6). During each triangular ramp contraction each motor unit can be characterized by its unique action potential shape and spikes individually sorted and coded into control and test motor units.

Protocol

Participants will begin in the McComas boot dynamometer seated and will start by practicing a series of plantarflexion MVCs, and perform triangular ramp contractions until a reliable value of MVCs and good ramp force tracings are observed. Participants can see an increase in force with plantarflexion, and a decrease in force with dorsiflexion on a computer screen placed in front of them. Figure 15 shows a schematic diagram of the steps involved in the protocol. First, 3 sets of MVCs will be performed, measures of % voluntary activation, and twitch properties will be measured. Secondly, a series of 30 triangular ramp contractions with 1-2s rest in between will be performed to measure ΔF and see the 'wind-up' effect of PIC. The

ramps will be 10s in duration and set to 10% of the participants MVC. After a series of triangular ramps, an acute fatigue protocol will be performed, followed by a force sensation task. The acute fatigue protocol will be set to 70% of each participant's MVC and participants will be asked to hold for 10s for 5 repetitions. Participants will then be asked to hold a target force level (50% of MVC) with visual feedback, and asked to hold the same force level with the removal of the visual feedback. The target force level is seen on a computer screen in front of them and the screen turns blank after a few seconds of holding the target force level.

Adderall will be ingested immediately after the force sensation task and participants will be asked to wait an hour to allow for time to achieve elevated plasma levels of the drug. Blood pressure and heart rate will be recorded as a proxy measure for elevated drug plasma levels. The same steps will be repeated to observe the effects of the drug or the placebo on measures of the neuromuscular system such as % voluntary activation, motor unit firing rates and on subjective components of fatigue such as perceived exertion. Each participant will be tested on two separate days with either 100% of the drug, and the other day without the drug.

Analysis

Fatigue can occur centrally or peripherally. Central changes due to fatigue can be assessed using a twitch interpolation technique. A supramaximal stimulus is applied to a mixed nerve during an MVC, and immediately after the contraction. Voluntary activation can be measured by expressing the first twitch as a function of the second twitch after the MVC to estimate the percentage of the motor unit pool activated during that MVC.

Peripheral changes due to fatigue can be assessed by eliciting a mass action potential (M-wave) via supramaximal stimulation to the peroneal nerve. The maximum M-wave (M_{max}) increasing stimulus intensities until no increase in the M-wave are seen. M_{max} is the maximum

electrical activity that can be produced in a muscle, and is calculated from the peak-to-peak amplitude. Declines in M_{max} can be attributed to peripheral transmission failure.

To assess changes in spinal excitability induced by fatigue, the ratio of the slopes from the H reflex and M-wave recruitment curves are used. The protocol involves using a series of increasing intensity stimulations to 1a afferents to create an H-reflex recruitment curve. The peak of the H reflex recording is compared to the M-wave and the ratio represents the % of motor neurons activated by 1a afferent stimulation. The amplitudes of the H reflex and M waves are normalized to the maximum M wave amplitude and is plotted as a function of stimulus intensity. This creates H reflex and M wave recruitment curves that assess net spinal excitability.

A supramaximal stimulation to the peroneal nerve elicits a maximal twitch in the soleus. The half relaxation time (HRT) can be used to assess the rate of calcium reuptake. During fatigue there is a reduced rate of dissociation of cross-bridges after the removal of calcium back into the sarcoplasmic reticulum, or a reduced rate of calcium pumping by the sarcoplasmic reticulum (Dawson, Gadian, & Wilkie, 1980). It is calculated be taking the negative change in force over the change in time from the half of the peak tension. The time to peak tension following a supramaximal twitch indicates calcium availability in the sarcoplasmic reticulum (Figure 16).

The force sensation target will be set to 50% of participants MVC. Force sensation data will be fitted to an exponential decay function, $y = y_0 + ae^{-bx}$. The constant b is an estimate of sense of force.

Results

There were several variables investigated in this study. In all three cases, there was a decline in the MVC over the course of a fatigue protocol, from pre and post drug time periods (Table 1), and in both the Adderall and control group.

The $\triangle$F values pre and post fatigue with the ingestion of Adderall were much higher compared to a control day where no Adderall was taken (Table 3). On either the drug or control day, there change in $\triangle$F after fatigue declined in both the Adderall and control group. Comparing Ramp 2 to Ramp 4 to Ramp 7 pre-fatigue there was an increase in $\triangle$F in the Adderall and control group (Figure 13).

Chapter 5: Discussion

Discussion

In this study, ΔF estimates of PIC derived from paired motor unit recordings decreased over the course of a fatigue protocol compared to a control protocol that did not elicit fatigue. To determine whether changes in ΔF were due to changes in PIC, we used stimulation of the nerve to the antagonist during some ramps to elicit an inhibitory stimulus via the reciprocal inhibition pathway. As predicted, ΔF values were lower in ramps with electrical stimulation and declined much less over the course of the fatigue protocol. Therefore, fatigue-induced changes in the ΔF are likely to be due, at least in part, to changes in PIC. The fatigue-induced changes in PIC may be due to either a reduction in monoaminergic drive, or an increase in inhibitory input, but likely it is the latter of the two.

There several possible sites of failure during fatigue from central or peripheral origin, as high as the motor cortex and higher brain centers, and all the way down to the level of the muscle (Gandevia 2001). Corticospinal excitability is markedly reduced during fatigue (Pitcher *et al.* 2001), and can be due to spinal or supraspinal factors. During fatigue, an increase in the cortical motor evoked potential relative to the cervicomedullary motor evoked potential indicates an increase in motor cortex excitability, while a lengthening of the cortical silent period suggests intracortical inhibition (Fuhr, Agostino, & Hallett, 1991). Several experimental approaches have provided experimental evidence of a fatigue-associated decline in excitability at a spinal level. Evidence of reduced spinal excitability from these studies include a fatigue-induced reduction in H reflex (e.g. Kuchinad *et al.,* 2004), cervicomedullary motor evoked potential (McNeil *et al.,* 2009), and a reduction in group 1a (muscle spindle) activity (for review see Hagbarth and Macefield, 1995). In a 2001 review, Gandevia suggested that a reduction in

intrinsic motor neuron excitability may also contribute to a reduction in spinal motor neuron excitability following fatigue (Gandevia 2001). Persistent inward current (PIC) is an intrinsic motor neuron property that plays a key role in the gain of the motor neuron and may therefore contribute to the spinal motor neuron excitability changes that Gandevia proposed in 2001. This study is the first to assess fatigue-induced changes in estimates of PIC in human motor neurons.

At the level of the motor neuron there are many inputs that either excite or inhibit the cell. The inputs include, but are not limited to descending inputs from cortical and propriospinal inputs, ascending inputs from group I, II, III, and IV afferents, and local inputs in the spinal cord (Figure 4). PICs have been thoroughly examined in animal models (Hounsgaard *et al.* 1988), and was first shown in the decerabrate cat to be activated by descending monoaminergic input. These PICs increased the gain of the motor neuron and resulted in self-sustained firing. Descending monoaminergic input to the spinal cord is diffuse, meaning it affect many motor neuron pools simultaneously, including agonist-antagonist pairings. Reciprocal inhibition from 1a afferents and other inhibitory pathways act to reduce or completely turn off PICs. These local inhibitory circuits serve to act as a gain control mechanism for these diffuse and non-specific descending monoaminergic inputs to spinal motor neurons (Johnson & Heckman, 2014). In several animal studies, reciprocal inhibition via electrical stimulation to the nerve of the antagonist muscle (Kuo et al., 2003), and joint rotation which alters reciprocal inhibition (Hyngstrom et al., 2007), have been shown to reduce PIC. Accordingly, we report lower values for $\triangle$F estimates of PIC for the ramps with electrical stimulation compared to the ramps without electrical stimulation. Furthermore, there was a greater decline in $\triangle$F over the course

of the fatigue protocol in ramps without stimulation compared to ramps with stimulation. Thus, the inhibitory stimulus to the soleus motor neuron pool elicited by electrical stimulation was likely reducing the PIC, dialing down the gain of the motor neuron so further changes in fatigue would not decrease the PIC as sharply.

Group III/IV input from muscle also inhibits the motor neuron (Woods *et al.* 1987) and may contribute to fatigue-associated changes spinal excitability that have been demonstrated using the H reflex. However, modulation of PIC by group III, IV afferents has not been investigated. One of the main findings in this study was the decrease in ΔF estimates of PIC during fatigue. Plausibly, the decreases in ΔF during ramps without electrical stimulation are due to increased group III/IV inhibitory input to the motor neuron over the course of the fatigue protocol. Future studies should investigate group III/IV inhibitory activity and PIC during fatigue using ischemia (discussed in Future Studies). It is also possible that reciprocal inhibition changed over the fatigue protocol, as there could be increased coactivation or decreased reciprocal inhibition. However, this would be contrary to the findings of this study as decreased reciprocal inhibition would increase PIC (Vandenberk & Kalmar, 2014). The problem with analyzing reciprocal inhibition after fatigue is that the length of RI protocol makes it difficult to implement over a fatigue protocol.

Alternatively, the decreases in ΔF during ramps without electrical stimulation could have been due to decreases in monoaminergic input for supraspinal centers. However, likely this is not the case as reductions in neuromodulatory drive are likely to be a factor during whole body fatigue, and not from single joint fatigue during isometric contractions. One way to test if there is a reduction in neuromodulatory drive is to use a pharmacological agent that alters

monoaminergic input. Serotonin and NE are crucial in modulating gain control in the spinal cord, as the serotonergic system covaries with the intensity of the motor output system (Rasmussen *et al.* 1998), and the noradrenergic system covaries with arousal state (Aston *et al.*2002). In the case study we estimated PIC before and after fatigue in a participant who was prescribed a pharmacological agent (Adderall) known to increase presynaptic release of serotonin from the brainstem. △F values were significantly higher after ingestion of Adderall compared to a control where no Adderall was taken (Appendix A). Given these preliminary results, a full study using a stimulant such as Adderall may shed light on the role of neuromodulatory drive in spinal motor neuron excitability during acute bouts of neuromuscular fatigue.

There are several components of this study note considerable mention, although this is a case study from two participants. The first finding worth mentioning is there was a large increase in △F from the Adderall group on the experimental day after ingestion of Adderall as compared to the pre-fatigue measures. The △F values are also much higher than the control day (with no ingestion of Adderall), and also compared to the control group. This finding supports our original hypothesis, by using a pharmacological agent that alters monoaminergic input, specifically Adderall increase presynaptic release of norepinephrine, △F values should increase. The main purpose of this study was to test if monoaminergic drive was reduced during a fatigue protocol and likely this was not the case from our results. The △F still declined following a fatigue protocol after oral ingestion of Adderall suggesting that fatigue is not due to declines in monoaminergic drive. These findings support the notion that the fatigue may be caused by group III/IV inhibitory afferent input that is reducing PIC.

The second finding worth noting is the warm up effect of PIC. PIC has been shown in animal models to increase with closely repeated stimuli (Gorassini *et al.*, 2002b), and the same effect occurred in this study. Due to the nature of large variability in the accuracy of the isometric triangular ramp contractions, selective ramps had to be chosen that were consistent in all three groups. In a series of ten ramps, ramp 2, ramp 4, and ramp 7 were selected to compare in both participants as they were the most reliably accurate and suitable to analyze $\triangle$F. The pre-fatigue values comparing ramp 2 to ramp 4 to ramp 7 in either the Adderall group (experimental or control) and in the control group saw an increase in $\triangle$F values. Interestingly, there was no major increase in $\triangle$F post fatigue in the Adderall group, from ramp 2 to ramp 4 to ramp 7, but there was an increase in $\triangle$F post fatigue in the control group. Future participants will reveal if this finding proves to be a significant one.

Future studies can continue to investigate the contributors to neuromuscular fatigue, building off the current study. Group III/IV afferent inhibitory input is a likely a key player in reducing PIC, an intrinsic motor neuron property responsible for increasing the gain of a motor neuron. Also, the present study used a pharmacological agent to alter monoaminergic drive. Reward circuitry in the limbic system is activated with the sole expectation of a reward (Garris et al., 1999).The release of dopamine from the nucleus accumbens in the ventral striatum is associated with reward processing. The placebo effect may exist with the use of a pharmacological agent as it is a powerful phenomenon. Future studies should also look at the placebo effect of these drugs on neuromuscular function.

One limitation to this study was that there was no counterbalancing of the days in this study, as the fatigue day was always done before the control day. This was to determine the

number of sets until fatigue, so that could be replicated on the control day. However, this is unlikely to be a confounding variable in this study as there was a minimum of 2 days separation between the fatigue and control day. Also, the pre-fatigue ΔF and MVC measure were fairly consistent among participants. Another limitation is the number of participants in the study, however the same motor unit was followed throughout the whole protocol which increases the statistical power.

In summary, no study to date has investigated how fatigue is modulated by PIC. This study found that PIC is a significant contributor to fatigue, possibly due to group III/IV inhibition. Also, we found that electrically stimulating the nerve to the antagonist of the soleus (CPN) decreased Δf values, which suggest that the ΔF changes were due to changes in PIC. In the future, this contributes to the understanding of PIC in the neural control of movement. Hopefully in the future this study will serve a platform to investigate PICs in other populations such as aging and disease states.

Chapter 6: Future Directions

Future Directions

This present study found that changes in ΔF is a major contributor to fatigue, potentially due to group III/IV afferent input. Future studies should investigate the role of group III/IV afferent input and also changes in neuromodulatory inputs. There are many different excitatory and inhibitory inputs onto the motor neuron that may increase or decrease PIC. Many studies have looked at these inputs in isolation, but further studies require investigation of the interaction between these inputs and PICs. The present study found that the reductions in PIC may have been a contributor to fatigue, likely due to group III/IV inhibition. However, this study did not rule out other mechanisms that may have reduced PIC such as reduction is neuromodulatory input or an increase in afferent inhibition. Future studies can tackle both scenarios by 1) altering neuromodulatory input pharmacologically, and 2) altering afferent inhibition. Adderall is a known pharmacological agent that increases norepinephrine from the prefrontal cortex (Heal, Cheetham, & Smith, 2009). If decreases in PIC are due to reductions in monoaminergic drive, then a pharmacological agent such as Adderall that increases monoaminergic drive should be able to offset it. A case study (below) using Adderall has not seen this effect, as the reductions in the estimates of PIC will decline to a similar degree. A previous study by Garland SJ (2001) has shown there is some reflex inhibition during fatigue that decreases motor neuron activity, likely due to small diameter group III/IV afferents. This study used partial compression to block large diameter afferent input (group I,II) during a fatigue protocol. The EMG activity and MVC declined following a fatigue protocol even with the blockage of large diameter afferents, suggesting that group III/IV afferents was responsible for

mediating this reflex inhibition. A similar study should be done in the future investigating if this is the cause for the reductions in PIC.

While the current study provides insight in the role of PIC in the neural control of force production in young, healthy people during fatigue, future work could apply these findings to aging, pathology, and injury. PICs have been speculated to be involved in many diseases and injuries, such as amyotrophic lateral sclerosis (Kuo *et al.* 2004), hemiparetic cerebral stroke (Mottram *et al.* 2009), and in both acute and chronic spinal cord injury (Bennett *et al.* 1999). PICs have been shown to be minimized after acute spinal cord injury in an animal model (Hounsgaard *et al.* 1988), and show dramatic recovery in chronic spinal cord injuries in an animal model (Button *et al.* 2008) and in humans (Gorassini *et al.* 2004). The initial decrease in PIC is due to loss of neuromodulatory inputs from supraspinal centers, particularly serotonin and norepinephrine highly involved in activating PIC (Heckman *et al.* 2005). The rapid increase in PICs after several weeks of spinal cord injury is mainly due to increase in receptor sensitivity which can trigger muscle spasms (Bennett *et al.* 2004), although the mechanisms remain unknown (Heckman *et al.* 2010). The residual monoamines in the spinal cord are responsible for reactivating PIC, since descending monoaminergic input is no longer possible. Both the Ca^{2+} PIC and Na^{+} PIC adapt over time, but to what amplification remains to be seen in humans. These PIC channels are specific (Nav1.6, CaV1.2, CaV1.3) and potentially are expressed differently in different diseases (Toledo *et al.* 2005) making them suitable drug targets for rehab. Drugs that target 5HT2 and NE a1 receptors that activate PIC can be potentially be used in conjunction with 5HT1b and NE a2 receptors that inhibit PIC can be useful in controlling these spams. People with Parkinson's disease have degenerative monoaminergic inputs to the

spinal cord (Braak *et al.* 2003), and also lower strength in extensor muscles in the upper limb (Robichaud *et al.* 2004). Further understanding in the differences in PIC behavior between extensors and flexors may be useful for pharmacological and rehabilitation interventions for the upper limb. Previous studies (Wei *et al.*2014) have shown that modulating the efficacy of 5-HT through pharmacological agents on volitional and reflexive pathways is mainly of spinal mechanisms. Serotonin and other potentially other neurotransmitters are highly involved in gain control, and future studies should look at neuromodulatory gain control in the spinal cord as it is an important area for rehabilitation and motor control.

Finally, the method for estimating PIC in humans (paired motor unit technique) is not direct, and has certain limitations that will hopefully can be resolved in the future. The first limitation is that ΔF estimates of PIC are typically done seated, and isometrically. In an attempt to address this limitation, our lab recently investigated PIC during a standing forward sway postural task (unpublished work). This has been the first study known to estimate PIC in humans during a functional task, and is another step further in understanding PIC modulation in humans. Further studies should investigate PIC during a functional task, as it is unknown as what degree the change in PIC amplitude affects the overall gain of the entire motor pool. To what degree and what role does PIC play in functional tasks in humans remains to be seen. A second limitation of using the paired motor unit technique is that ΔF estimates of PIC may be due to other intrinsic motor neuron properties such as spike frequency adaptation, spike threshold accommodation, and after-hyperpolarization (AHP). Revill *et al.* (2011) used a simulation study to show that the contribution of spike frequency adaptation, spike threshold accommodation to ΔF estimates of PIC could be minimized by using short ramps with faster

rates of rise. Vandenberk *et al.* (2014) verified this in human soleus motor neurons and further strengthen the argument that PIC is a major contributor to ΔF by showing that altering joint angle to increase reciprocal inhibition diminished ΔF estimates of PIC. However, no study has definitively shown that ΔF estimates of PIC are influenced by AHP. A simulation study by (Powers & Heckman, 2015) have shown the changes in the AHP has little effect on ΔF, and ΔF is largely due to PIC. One way to further verify this is to estimate AHP duration using death-rate analysis in humans (MacDonell, Ivanova, & Garland, 2007) and correlating these estimates with ΔF estimates of PIC. Another method to further verify the paired motor unit technique is to use a pharmacological agent that reduces PIC. Baclofen, $GABA_b$ receptor agonist used as a muscle relaxer and antispastic agent used to treat muscle problems has been examined in animal studies and has been shown to reduce Ca^{2+} PIC (Li *et al.* 2004). Further studies can investigate if the same result occurs in humans, as △F should theoretically be zero, or close to zero after ingestion of Baclofen.

Tables and Figures

Tables

Table 1: PSTH results from reciprocal inhibition analysis. Reciprocal inhibition was analyzed from the difference in the control PSTH and stimulation PSTH, creating a cumulative sum (Figure 6). The 1st and 2nd valleys in the cumulative sum represent the reciprocal inhibition for the 1st and 2nd ISI. The fatigue and control day RI values are shown below.

FATIGUE

	Valley in Cumsum for 1st ISI	Valley in Cumsum for 2nd ISI
SSR1	-15	-41
SSR2	-60	-25
SSR3	-6	-12
SSR4	-19	-51
SSR5	-20	-23
SSR6	4	-19
mean	-19.3333	-28.5
SD	21.88759	14.61164

CONTROL

	Valley in Cumsum for 1st ISI	Valley in Cumsum for 2nd ISI
SSR1	-2	-14
SSR2	-34	-54
SSR3	4	-17
SSR4	-22	-48
SSR5	N/A	N/A
SSR6	-1	-17
mean	-13.25	-30
SD	17.83956	19.32615

Table 2: Criteria for the paired motor unit technique. For both the stimulation and no stimulation ramps, the criteria had to be met before final analysis. The table shows the time between control and test unit onset, Pearson's correlation between the control and test unit, and the difference between the control unit firing range (max-min firing rate) and ΔF.

Ramp Type	Time between control and test unit onset (s)	Pearson's correlation (r^2) (common synaptic drive)	Test unit ΔF - control unit firing range (max-min firing rate) (imp/s)
No Stimulation	2.34 ± 0.88 s	r^2=0.94 ± 0.06	3.02 ± 1.42
With Stimulation	2.01 ± 0.52 s	r^2=0.88 ± 0.12	3.61 ± 0.88

Table 3: MVC (Adderall Case Study). MVC values are shown below in Newtons for all three conditions, Adderall (experimental day), Adderall (control day), and the control (control day) from pre to post fatigue in both the pre drug and post drug time periods (no drug was taken during the Adderall control day or the control case).

	Pre Drug Period		Post Drug Period	
Group Type	Pre Fatigue	Post Fatigue	Pre Fatigue	Post Fatigue
Adderall (Experimental Day)	378.6483	346.0395	373.0103	320.3762
Adderall (Control Day)	359.5016	321.3374	366.473	349.5303
Control (Control Day)	341.776	305.6014	335.399	290.8318

Table 4: Contractile Properties (Adderall Case Study). The contractile properties are shown below before and after the pre and post drug 'periods'. These contractile properties are analyzed after the twitch.

	Pre Drug		Post Drug	
	ADD	CON	ADD	CON
Superimposed Twitch	0.005615	0.004162	0.010256	0.003791
Potentiated Twitch	0.130652	0.150651	0.2593	0.149222
Time to Peak Tension	0.140672	0.131572	0.10617	0.121576
Half-Relaxation Time	0.061286	0.060124	0.079189	0.065929
Peak Tension	0.130652	0.127432	0.37946	0.114412
M-wave amplitude	4.27158	4.12549	4.2602	4.10511
.+dF/dt	32.16029	28.1253	36.1291	26.1343
.-dF/dt	-11.646	-12.211	-13.8212	-11.921

Figures

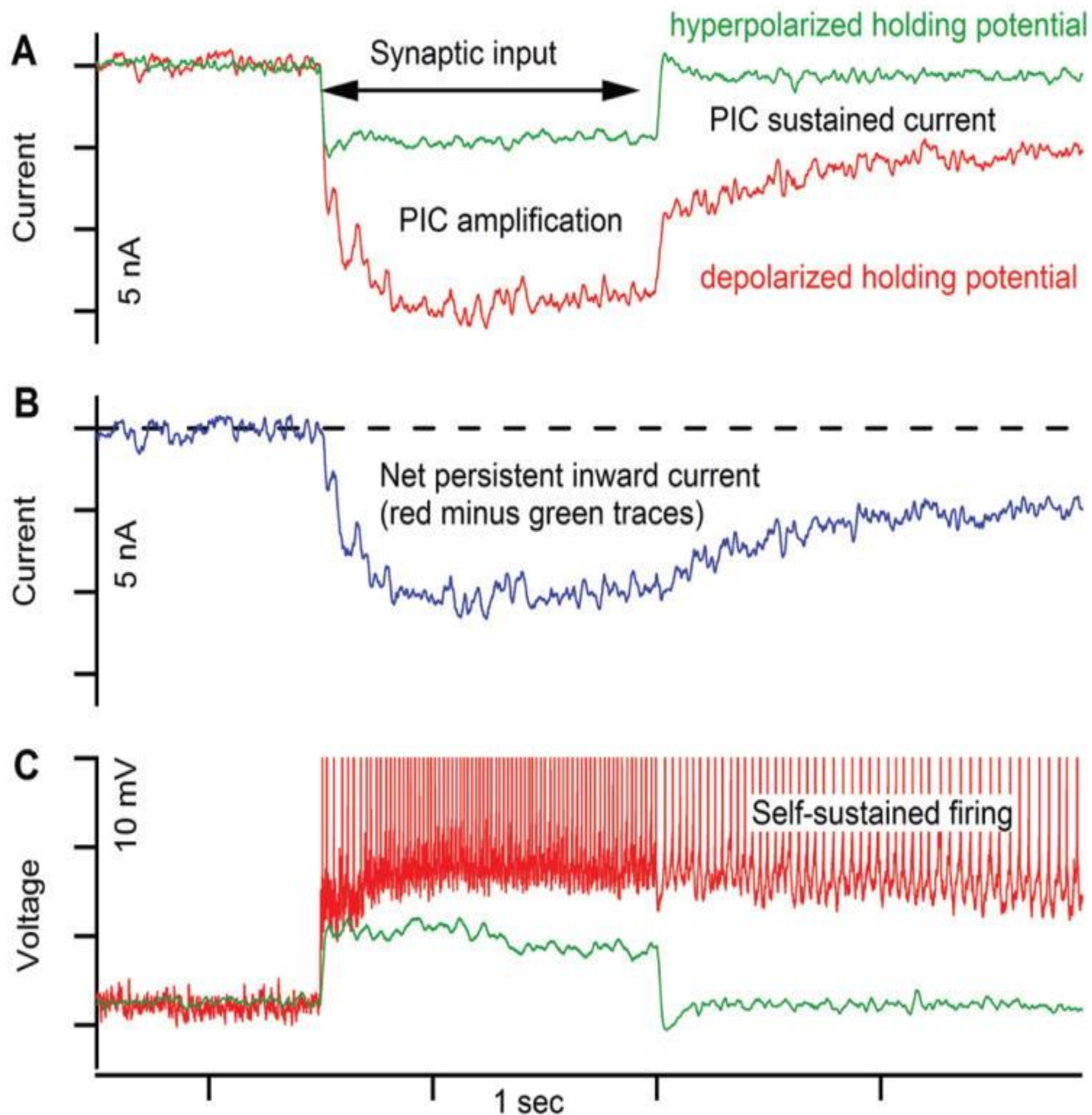


Figure 1 - PIC demonstrated in intracellular recording of cat hindlimb motor neuron during voltage-clamp

Synaptic input is generated from tendon vibration of 1a afferents in an animal model. The green tracings represent when the cell is held at a hyperpolarizing holding potential (not brought close enough to firing threshold, and the red tracings represent when the cell is unclamped, and brought closer to its firing threshold A) The x-axis represents time, while the y-axis represents current. The current generated across the membrane lasts. (Lee & Heckman, 1996).

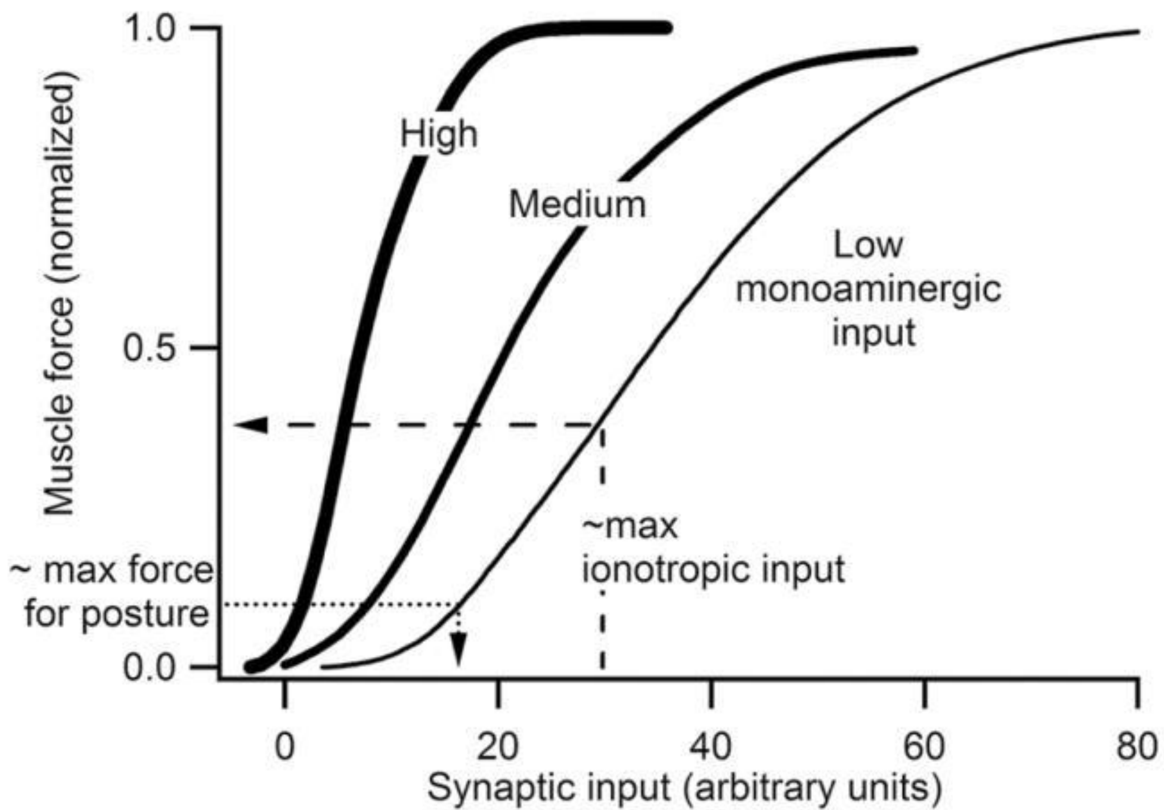


Figure 2: Ionotropic vs monoaminergic input for muscle force

Without monoaminergic input maximal muscle force output is substantially lower. Monoaminergic input allows for increased motor neuron excitability and much greater motor output. PIC is primarily responsible for the increase in motor output and is active even in low monoaminergic input. PIC is particularly important for locomotion such as walking and basic motor activity (Heckman, 1994).

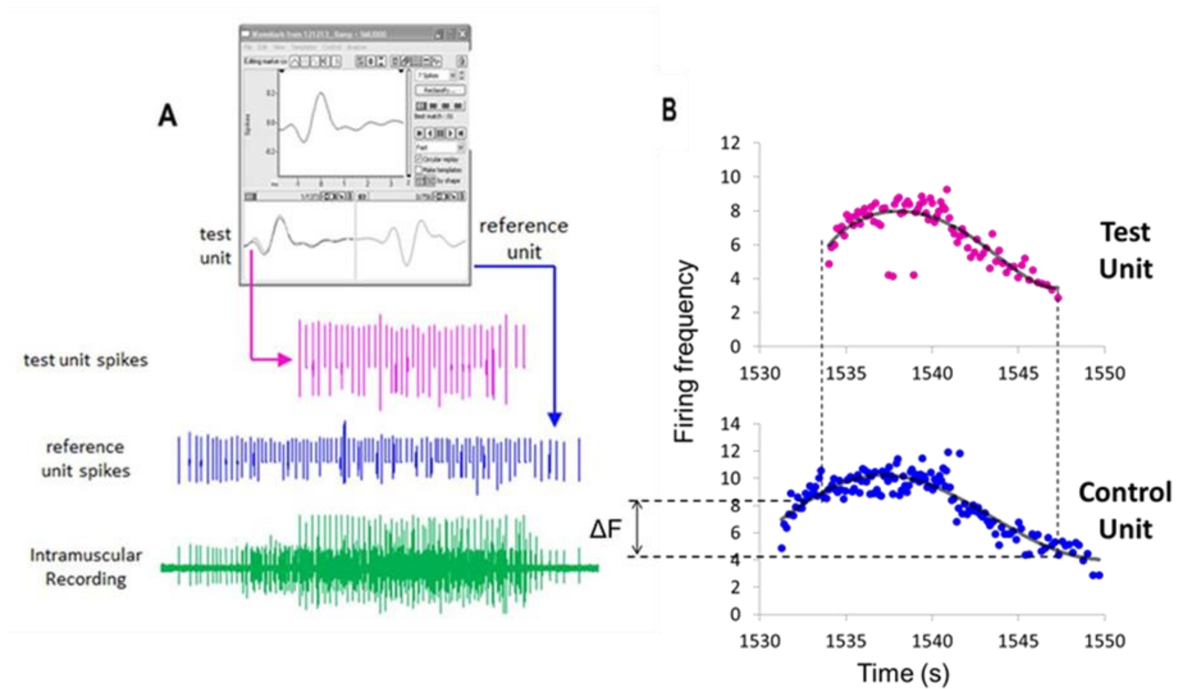


Figure 3: Paired motor unit technique

This sample recording for our laboratory illustrates the process of data acquisition and spike sorting used in the paired motor unit technique. In panel A, intramuscular recordings (green trace at bottom) are made from the soleus during a 10s isometric triangular plantarflexion contraction (5s to increase force and 5s to decrease force). Spike2 (version7, CED) is used to sort each action potential based on shape and amplitude (see insert figure at top of A). An earlier recruited motor unit (the reference unit in blue) and a later recruited motor unit (test unit in pink) are identified and used for the paired motor unit analysis is specific criteria are met (described in the review of literature and Methods). B) The instantaneous firing frequencies (Hz) are plotted and are fitted to a 4th order polynomial. The difference between the instantaneous firing frequency of the control unit at the onset and offset of the test unit is the estimate of ΔF.

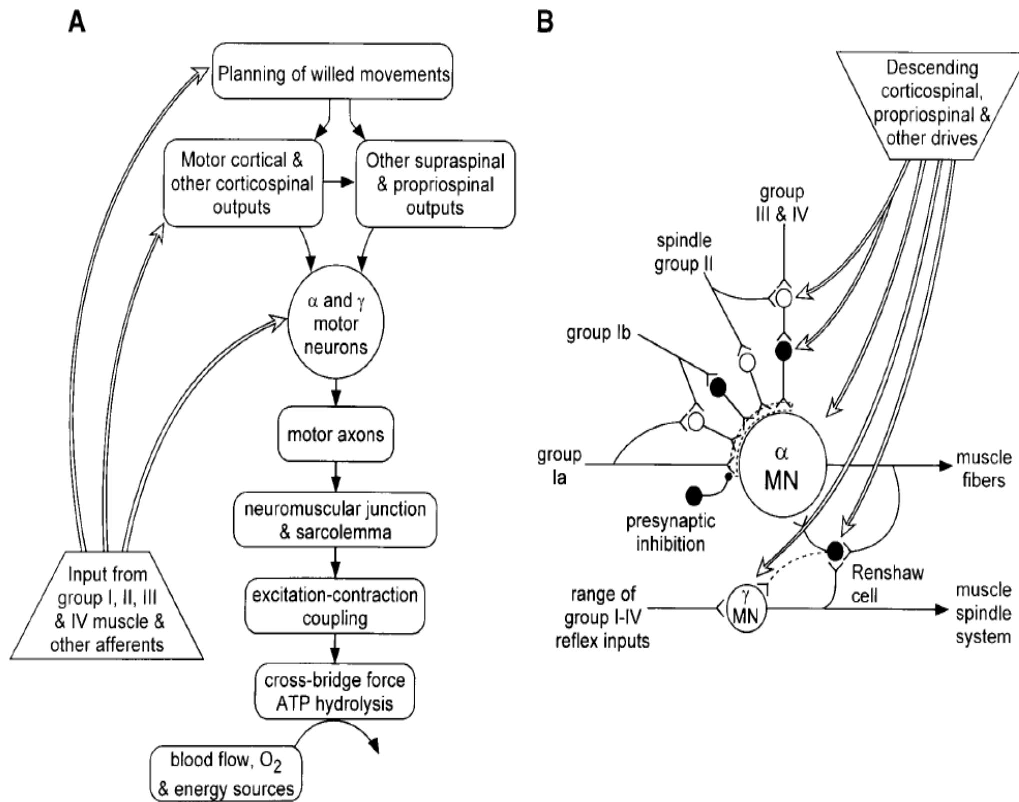


Figure 4: Levels of input from peripheral, spinal, and supraspinal sites of the neuromuscular system.

This diagram published in an extensive review of neuromuscular fatigue by Gandevia (2001) summarizes the pathways involved in muscle contraction including key afferent input to the motor neuron (panel B), as well as upstream drive to the motor neuron. In panel B, the solid circles are inhibitory while the clear circles are excitatory. This diagram illustrates the potential complexity of neuromuscular fatigue with multiple sites at which motor output can be regulated (Gandevia, 2001). As illustrated in both panel A and B, the alpha motor neuron is the final common pathway. Thus changes in the excitability of this neuron may contribute to changes in motor output during fatigue.

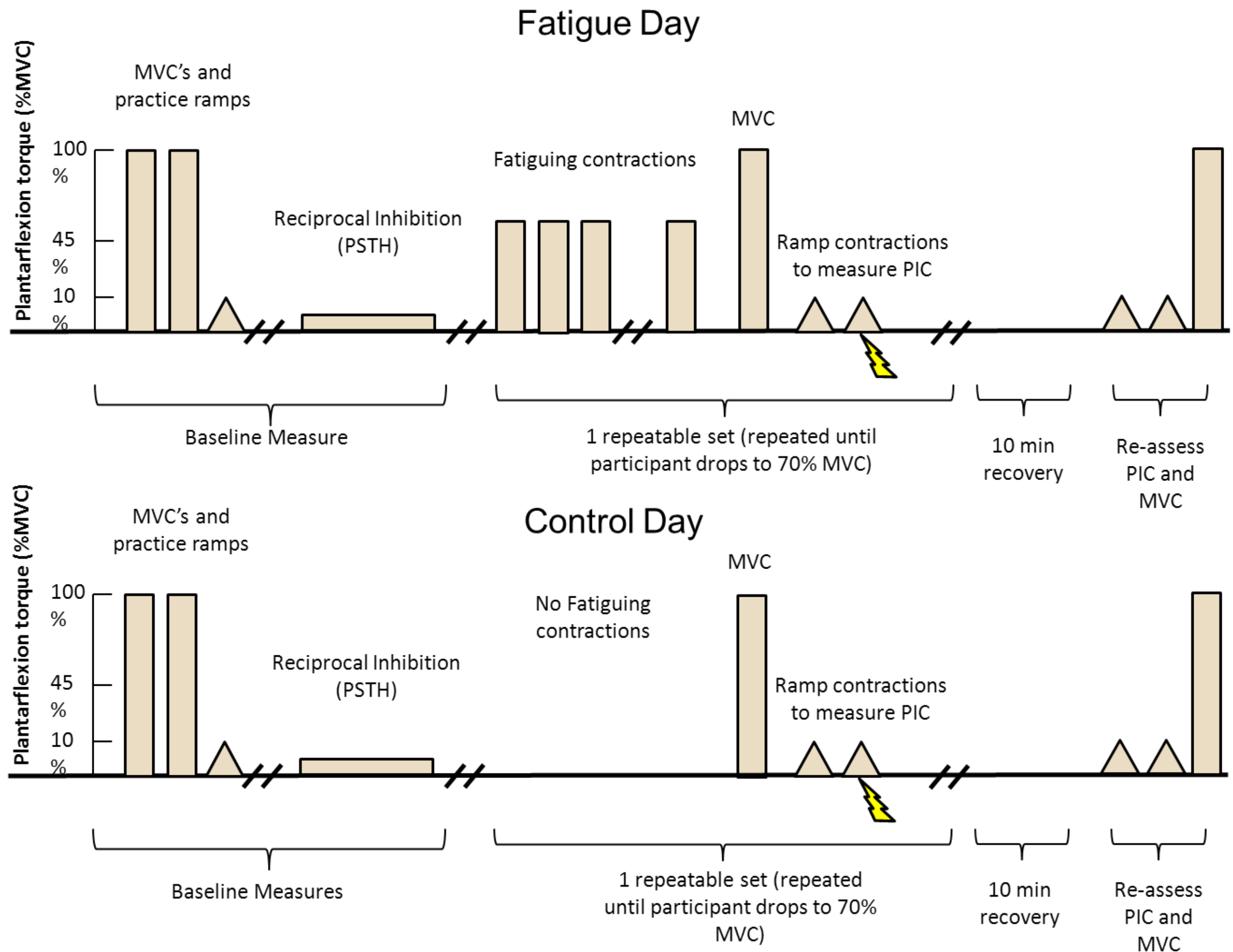
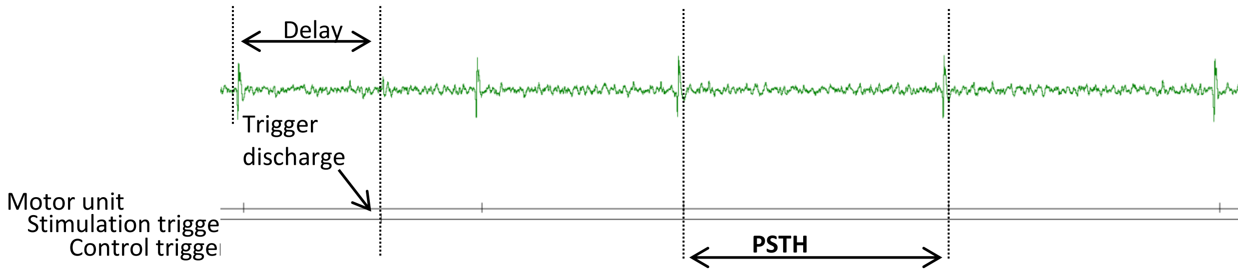


Figure 5: Experimental Protocol

A schematic diagram of the experimental protocol. A) The fatigue and control day will run 2 hours total time. The fatigue day will always begin before the control day to determine the number of sets until 'fatigue'. After a few plantarflexion MVCs and practice isometric ramp contractions, the protocol will begin with 20 fatiguing contractions. The intermittent fatiguing contractions will be set to 45% of maximum plantarflexion force, and will be 3s in duration with 1s of rest in between each contraction. After the fatiguing contractions, an MVC will be performed and then several PIC ramps will be performed with and without electrical stimulation to the CPN (RI). This protocol will be repeated until the participants declined to 70% of initial MVC force. The same protocol will be repeated on the control day, but without the fatiguing contractions. The same time period between each 'set' will remain the same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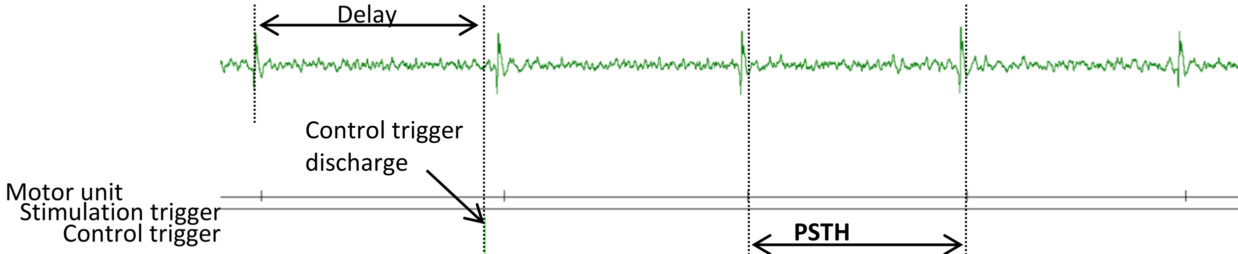
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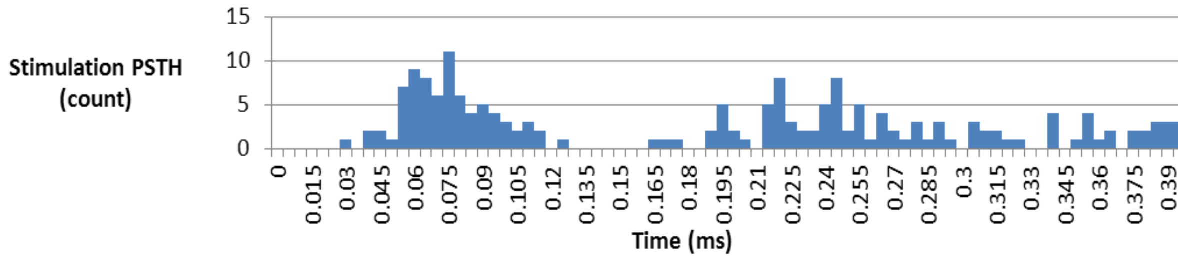
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Stimulation PSTH
(count)
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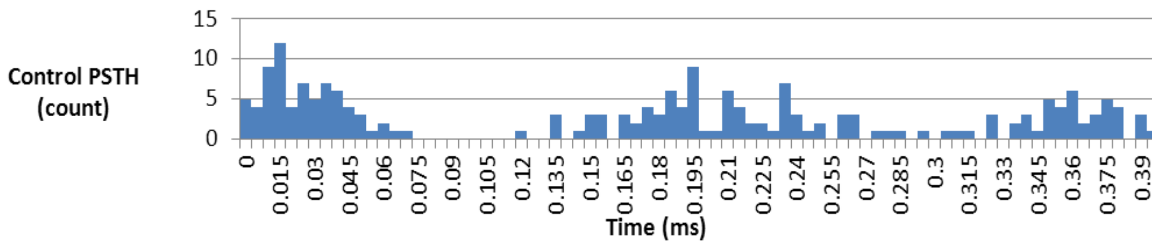
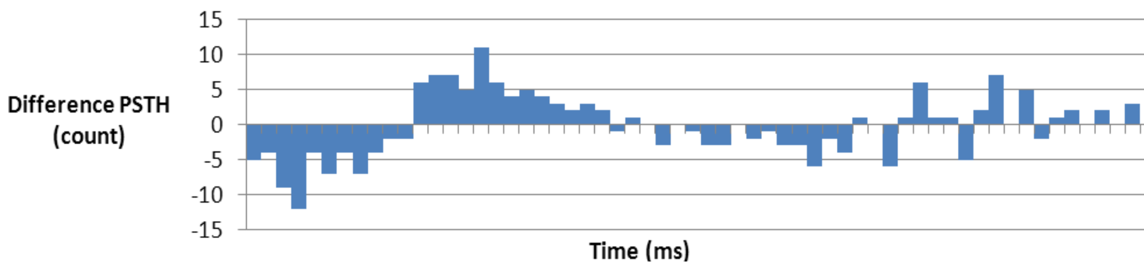

Control
Control PSTH
(count)
Time (ms)


Difference PSTH
Difference PSTH
(count)
Time (ms)

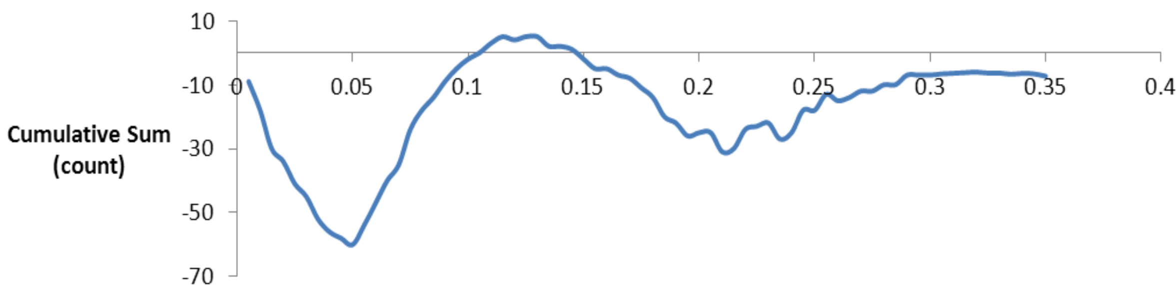

Cumulative Sum
(count)

Figure 6: Steps involved in quantifying reciprocal inhibition of each test unit.

A) Subthreshold electrical stimulation of the nerve to the antagonist (the CPN was used to elicit inhibition of the soleus test unit for each paired motor unit recording. The stimulation was triggered every 3 seconds by a soleus motor unit discharge. The timing was such that a stimulus was delivered to the CPN 130ms after a spike. The PSTH technique was used to determine the extent to which stimulation of the antagonist nerve delayed the next two spikes via reciprocal inhibition. B) A 'control' trigger was used to create a PSTH of interspike intervals (ISI) in the absence of electrical stimulation. C) The stimulation PSTH was derived from 80 subthreshold stimulations to the CPN. The number of spikes or 'counts' were placed in 5ms 'bins' for 400ms following the stimulus. D) The control PSTH was derived from 80 'control' triggers, or triggers placed with the same delay following a spike, but without electrical stimulation. E) The difference between the control PSTH and the stimulation PSTH was used to calculate a difference PSTH to quantify the amount of inhibition (a rightward shift from control to stimulation would denote inhibition or lengthening of the interspike intervals). F) The cumulative sum was derived from the difference PSTH, and a negative peak in the 2nd ISI indicates a stimulus-induced delay (inhibition).

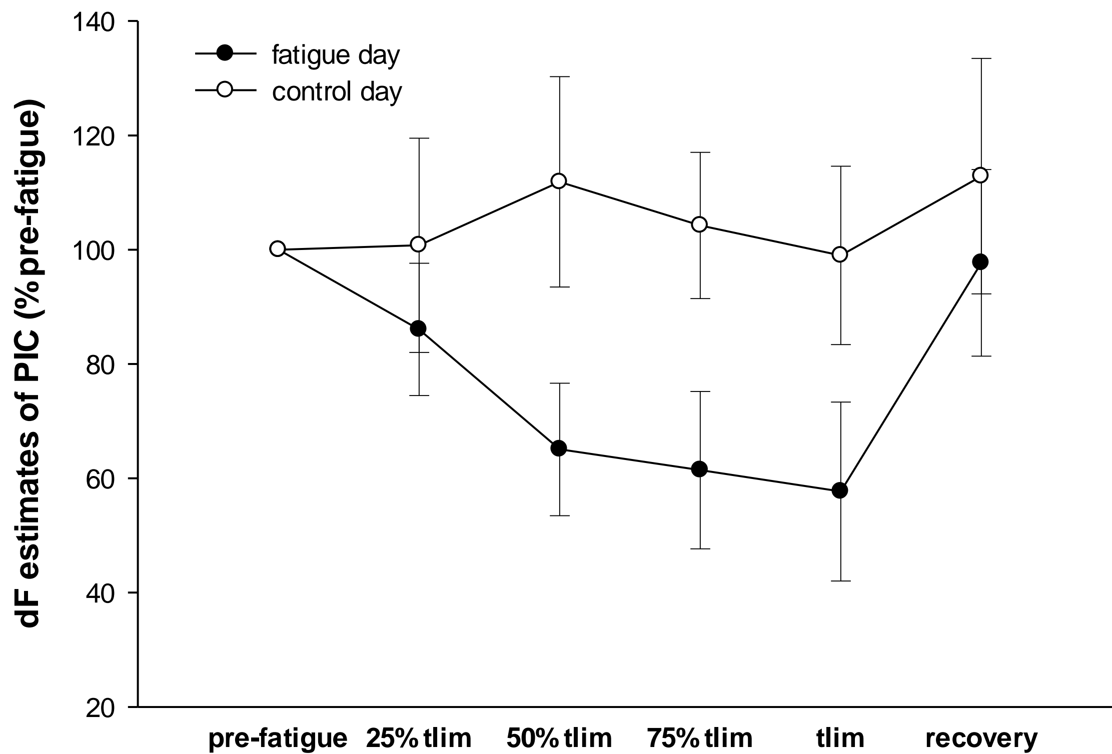


Figure 7: Change in ∆F over a fatigue protocol

Comparison of ∆F estimates of PIC (normalized to pre-fatigue values) made during ramps without electrical stimulation on both the control day and fatigue day. The x-axis represents the time normalized to the total time to fatigue (Tlim or a 30% decline from the pre-fatigue MVC). ∆F is significantly lower at50% tlim ($p<0.05$), 75% tlim ($p<0.05$), and tlim ($p<0.01$). After the recovery period, ∆F is not significantly different from the prefatigue value. There are no significant changes in ∆F on the control day.

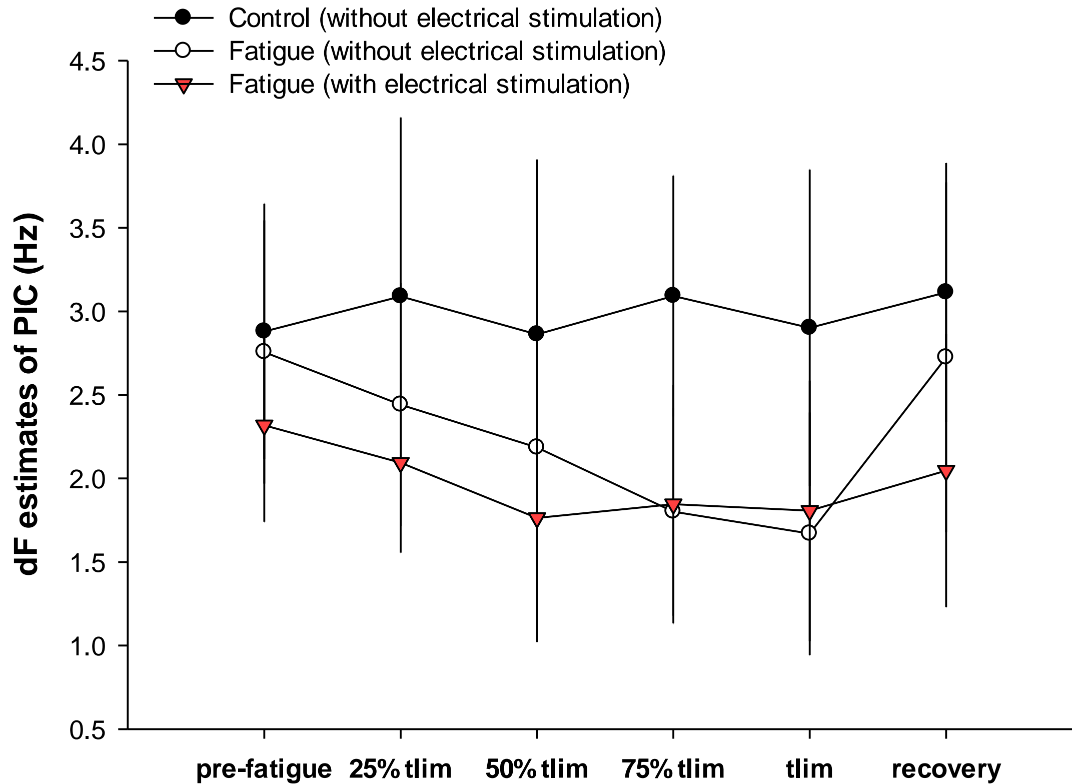


Figure 8: Change in ∆F estimates of PIC over a fatigue protocol (raw values)

Comparison of ∆F estimates of PIC made during ramps with and without electrical stimulation. Prefatigue estimates of PIC tended to be lower during ramps with stimulation compared to ramps without stimulation (p=0.068). Estimates of PIC made during ramps with stimulation did not change over the course of the fatigue protocol.

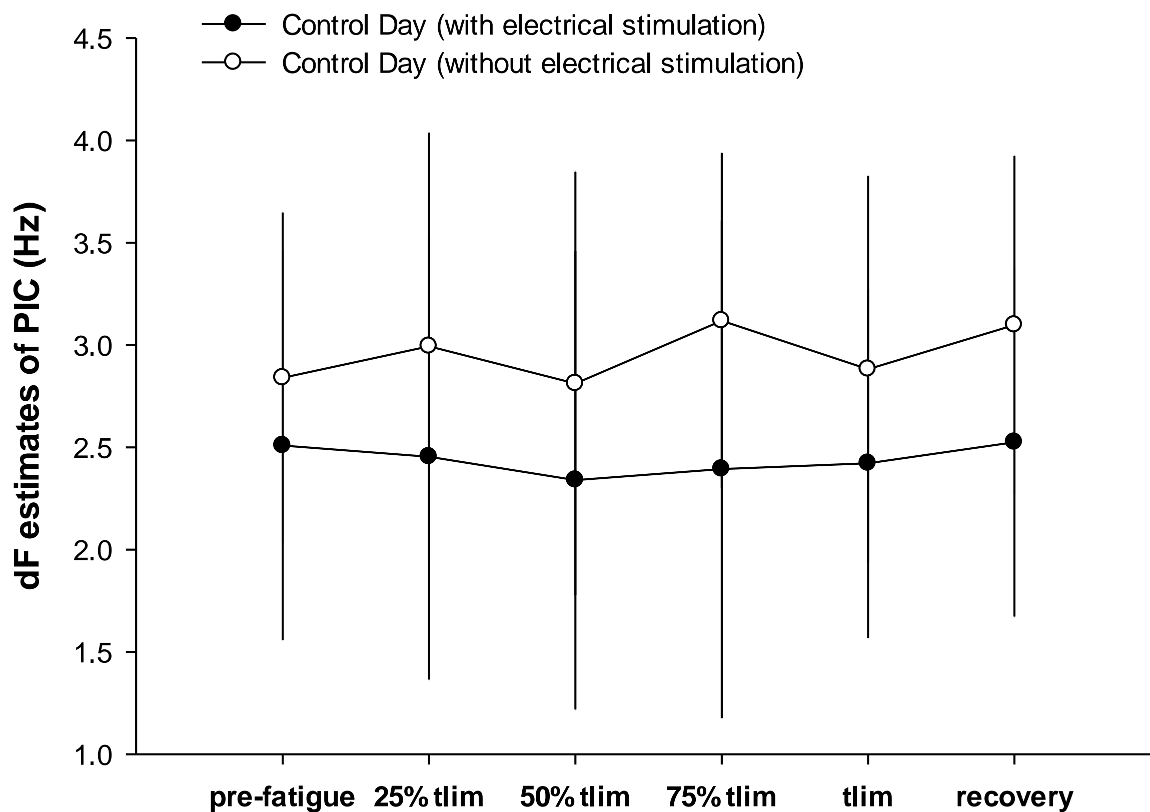


Figure 9: Change in ∆F during the control day (raw values)

Comparison of the raw data for PIC estimates on the control day with and without electrical stimulation. ∆F estimates of PIC during ramps with stimulation are significantly lower than the ramps without stimulation on the control day.

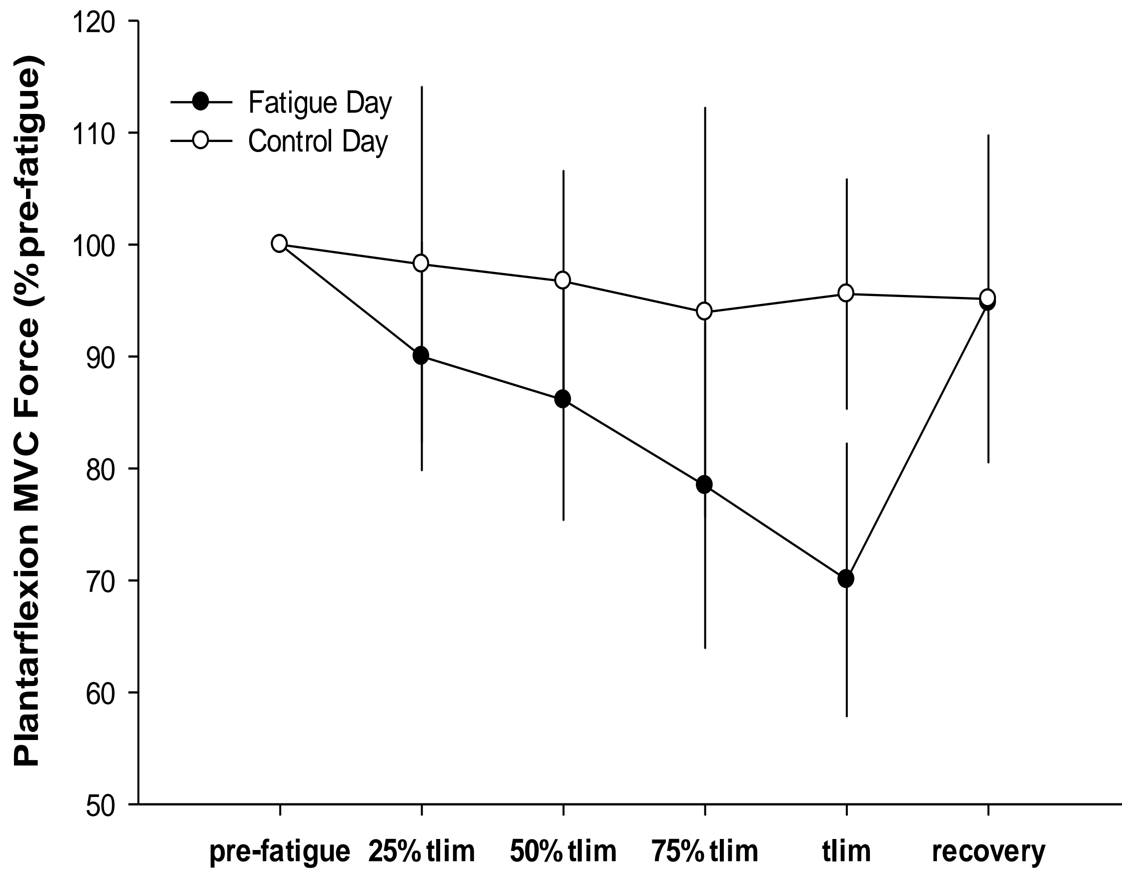


Figure 10: Changes in plantarflexion MVC over the fatigue protocol

MVC values on the fatigue day compared to control day. There is a significant drop in MVC on the fatigue day at 75% tlim ($p<0.05$) and tlim ($p<0.01$). After a recovery period, MVC force is not significantly different than the prefatigue value.

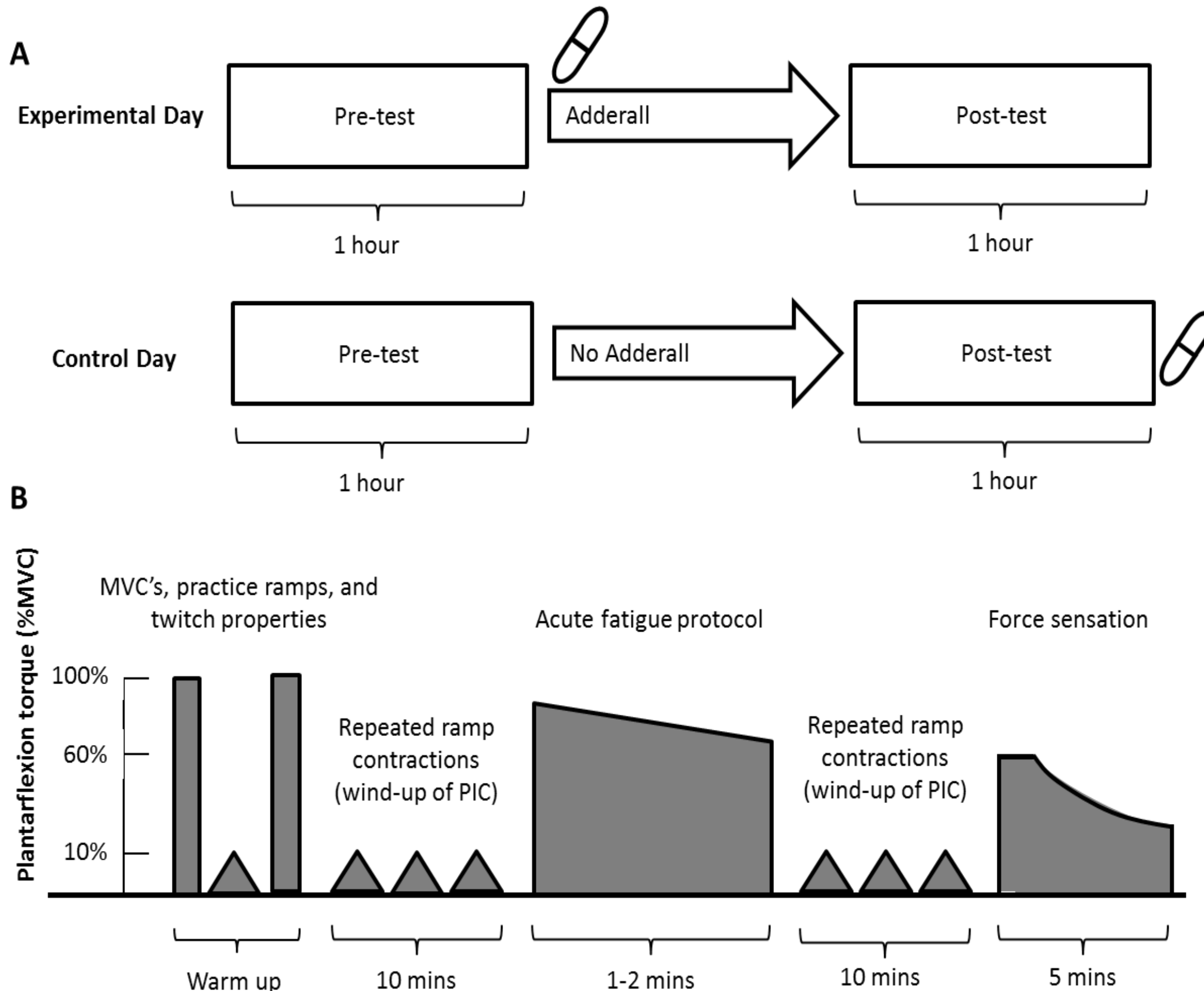


Figure 11: Experimental Protocol for Adderall Case Study

A schematic diagram of the experimental protocol. The experimental protocol (B) was completed before and 1 hour after administration of Adderall to allow the drug to reach peak plasma levels (experimental day). The same protocol was repeated by the Adderall case participant without Adderall ingestion during the one-hour rest period (control). The protocol was also completed by a control participant who is Adderall-naïve. B) On the control or experimental day, with the protocol begins with 3 sets of MVCs and measures of twitch properties, followed by a series of 30 ramp contractions. This is followed by an acute fatigue protocol and a force sensation task.

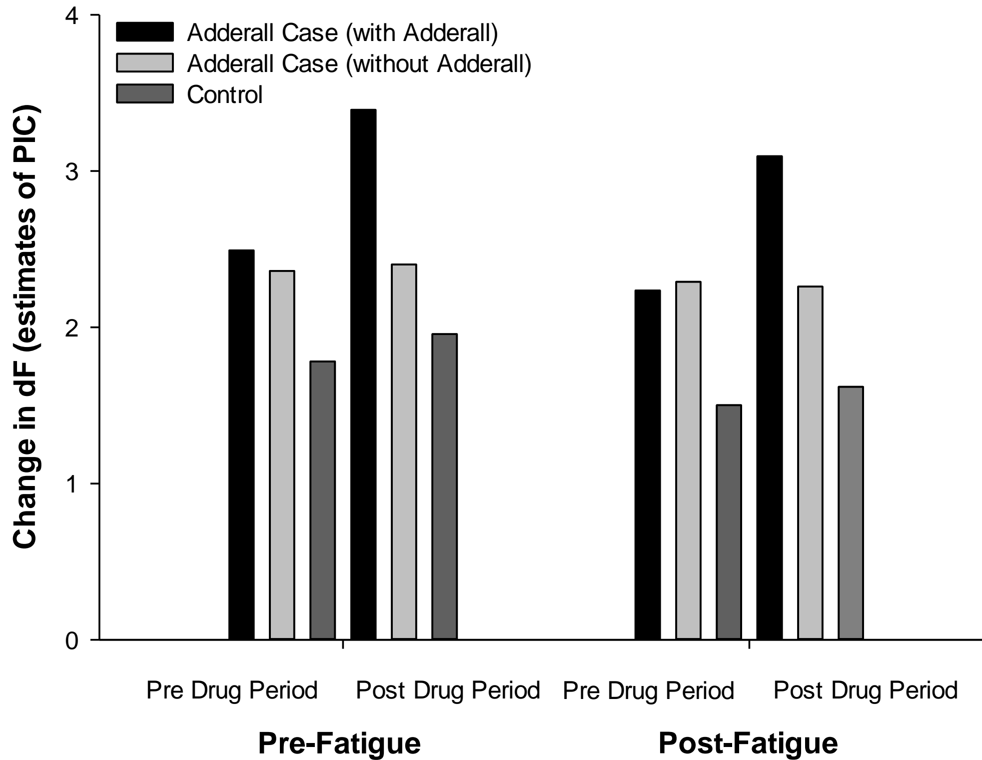


Figure 12: Changes in ΔF Estimates of PIC for Adderall Case Study

Comparison of the estimates of PIC in the Adderall case study. Prior to fatigue, there is a large increase in ΔF estimate of PIC after Adderall ingestion by the Adderall case participant compared to day when Adderall was not ingested by this participant and compared to the control participant. The data presented here was taken from ramp 2 in the experiment, prior to the potential warm-up of the PIC. In both the Adderall case (with Adderall), and the control participant, there is a large decrease in ΔF from pre to post fatigue in both the pre and post drug 'periods. However, there is no decrease in the Adderall case (without Adderall) after a fatigue protocol in either the pre or post drug 'periods'.

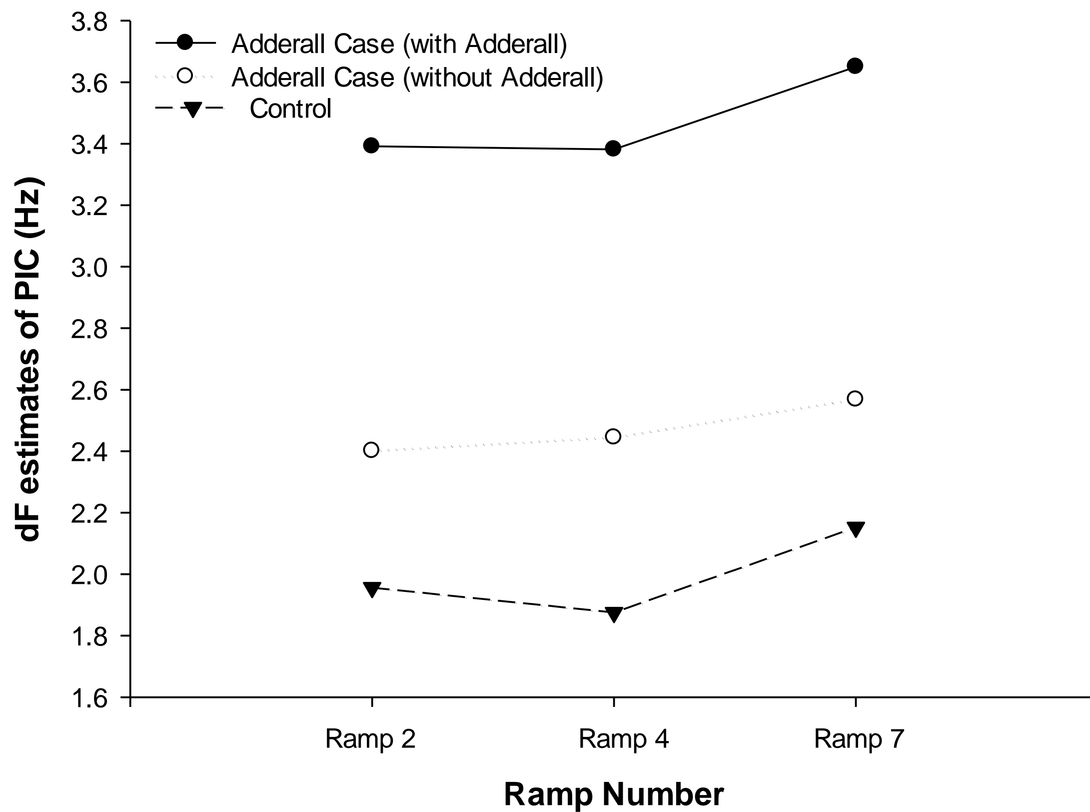


Figure 13: Warm-up of PIC during repetitive ramps

The warm-up effect of PIC during repetitive ramps is illustrated by the changes in ΔF from ramp 2, to ramp 4, to ramp 7. ΔF was always higher following Adderall ingestion, but in all three cases, increases in ΔF from ramp 2 to ramp 7 were evident.

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Appendices

Appendix A - Figure Permissions

Figure 1 and 2

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Author: C.J. Heckman, Michael Johnson, Carol Mottram, Jenna Schuster

Publication: The Neuroscientist

Publisher: SAGE Publications

Date: 06/01/2008

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Figure 3

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Title: Spinal and Supraspinal Factors in Human Muscle Fatigue
Author: S. C. Gandevia
Publication: Physiological Reviews
Publisher: The American Physiological Society
Date: Jan 10, 2001

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Appendix B – Exclusion Criteria

Exclusion and Contraindication Checklist

Participant: ____________ Age: __________ Date: _____________________

Please circle yes or no for the following activities, devices, or diagnosed medical conditions that are listed below. If uncomfortable or unsure about answering certain questions, direct your questions to the primary investigator.

Epilepsy	YES	NO
Seizures	YES	NO
Pacemaker	YES	NO
Heart arrhythmias	YES	NO
Cochlear implant(s)	YES	NO
Metal implants	YES	NO
Diabetes	YES	NO
Neurological disorder	YES	NO
Recent ankle or knee injury	YES	NO
Recent head injury	YES	NO
Ergogenic aids (sport supplements)	YES	NO
Use of centrally active drugs (serotonergic/dopaminergic drugs)	YES	NO
Smoking	YES	NO
Pregnancy	YES	NO
Caffeine consumption If "YES" how often (per day): _______________	YES	NO
Serious Allergy If "YES" please indicate: ____________	YES	NO

Investigator Initials: __________

Appendix C – Participant Instructions

Instructions for Experimental Subjects

Do not consume any of the following on the day of each experiment:

- Coffee
- Tea
- Chocolate
- Medications containing caffeine (e.g. diet pills, cold medications)
- Cola beverages (e.g. Coca-Cola, Pepsi etc.)
- Nutritional supplements (other than multivitamins), recreational drugs, and performance enhancing drugs

> **Note:** Be honest about your caffeine intake with the experimenter. Caffeine intake prior to the experiment may alter the results. However, if you are regular coffee drinker each day, you may maintain this activity on the test day.

Please abstain from these activities for 24 hours prior to the experiment:

- Strenuous physical activity of the right calf muscle (i.e. running, cycling, swimming, and weight lifting using the lower body such as squats, lunges, and toe raises)
- Excessive alcohol consumption

Contact the experimenter to reschedule your appointment if you:

- Fail to follow the above instructions
- Have been sick in the 48 hours prior to the experiment
- Have not had an adequate sleep the night before the experiment
- Simply need to reschedule due to a new and urgent commitment

Your experimental sessions have been scheduled for the following days:

Day 1: ____ from: ________________to_________________

Day 2: ____ from: ________________to_________________

Please arrive on time to Northdale campus off of Hickory Street. If you are running late please contact the experimenter. If you are running very late, the appointment may have to be rescheduled.

You can reach Kirby Mendes at mend2950@mylaurier.ca or at 519-884-0710 ext. 3334.

Appendix D – Informed Consent

WILFRID LAURIER UNIVERSITY
INFORMED CONSENT STATEMENT
The Relationship between Fatigue and Persistent Inward Current

You are invited to participate in a research study at Wilfrid Laurier University. The purpose of our study is to provide further insight into the relationship between fatigue and a motor neuron property known as persistent inward current.

Student Investigator:
Name: Kirby Mendes
Institution: Wilfrid Laurier University
Phone: (519) 884-0710 ext. 3334
Email: mend2950@mylaurier.ca

Supervisor:
Name: Dr. Jayne Kalmar
Institution: Wilfrid Laurier University
Phone: (519) 884-0710 ext. 2033
Email: jkalmar@wlu.ca

INFORMATION

Fifteen participants will take part in this research study. The aim of our study is to investigate the relationship between lower leg fatigue and a property of spinal motor neurons. Specifically, we will be studying the effects that muscle fatigue has on the properties of motor neurons. We are interested in how this fatigue acts to adjust the excitability of motor neurons during a fatigue task. The experiment will take place in room NC119 of the Northdale Campus at Wilfrid Laurier University, which is located on the corner of Hickory Street and Hazel Street. Upon arrival, you can dial the lab extension (3334) from the outdoor keypad at the main entrance and a member of the laboratory will meet you there at the entrance. Electrodes will be attached to the skin over a nerve in your leg and when stimulated it will cause muscles in your leg to contract. Intramuscular electrodes that are made out of very fine wires will be inserted into your leg to record the electrical activity within the muscle when you contract your leg voluntarily. These procedures are safe and have been used routinely in research settings for more than 40 years; however, some participants may find them unpleasant. If you find these procedures uncomfortable, you may withdraw from the study at any time. The first day that you visit our laboratory will be an orientation day. During this orientation session you will be introduced to the techniques employed in this study (nerve stimulation and intramuscular recordings). Following this orientation session, we will assess these preliminary recordings. If the recordings meet our criteria, we will contact you to schedule the experimental day. Each

experimental protocol will take approximately 1 hour; there will be two experimental protocols. You will be paid $20.00 for completing the experiment.

Initials________

RISKS

The electrical stimulation applied to the mixed nerve through a constant current stimulator will cause an involuntary muscle “twitch” in the target muscle. You may find this stimulation unpleasant; however, constant current stimulation is a noninvasive procedure that does not cause damage to the nerve or other tissues.

The initial insertion of the intramuscular electrodes may be associated with a stinging sensation due to the alcohol used to clean your skin. There is also a remote risk of infection with the insertion of intramuscular electrodes. To reduce this risk the needles and electrodes are sterilized using an autoclave and your skin is prepared with alcohol. The researcher will also be wearing latex gloves during the protocol. Needles and electrodes are never reused.
There occasionally may be localized bruising (<0.5cm diameter) around the site of electrode insertion similar to what you might observe following a blood test. This bruising subsides within 48 hours and is not typically associated with any discomfort.

BENEFITS

You will not benefit directly from participating in this study. However, this study will help us understand the neural control of muscles in healthy populations, which can later be applied to pathophysiological models such as aging and injury.

EXCLUSION CRITERIA

Ankle/knee injury, or have been diagnosed with a neurological disorder. Multiple sclerosis and sciatic nerve impingement would be two examples of neurological disorders that would confound our data (but not pose a risk to you as a participant.

CONFIDENTIALITY

All data collected in this study will be stored indefinitely in NC119 and will only be accessible by the investigators. All measures will be taken to ensure your privacy and all your data will be coded and identified by a participation code. Group results will be submitted for publishing in various research journals. Individual results will remain completely confidential and not published to ensure your privacy.

COMPENSATION

To compensate you for your participation you will receive $20 upon completion of this study.

CONTACT

If you have questions at any time about the study or the procedures you may contact the researcher or supervisor.
This project has been reviewed and approved by the University Research Ethics Board. If you feel you have not been treated according to the descriptions in this form, or your rights as a participant in research have been violated during the course of this project, you may contact Dr. Robert Basso, Chair, University Research Ethics Board, Wilfrid Laurier University, (519) 884-1970, extension 4994 or rbasso@wlu.ca.

Initials________

PARTICIPATION

Your participation in this study is voluntary; you may decline to participate without penalty. If you decide to participate, you may withdraw from the study at any time without penalty and without loss of benefits to which you are otherwise entitled.

FEEDBACK AND PUBLICATION

The results will be presented for completion of the undergraduate thesis project and at various neuroscience conferences. We will also be making submissions to appropriate scientific journal, such as the Journal of Neuroscience. IF YOU WISH, YOU MAY RECEIVE A BRIEF SUMMARY OF THE FINDINGS OF THIS STUDY AFTER IT IS COMPLETED. IF YOU WISH TO RECEIVE THIS SUMMARY, IT WILL BE SENT TO YOU IN JUNE 2014 (8).

If you would like to receive a summary of the results of this study, please indicate below:

☐ No Feedback ☐ Please email feedback to ______________________

CONSENT

I have read and understand the above information. I have received a copy of this form. I agree to participate in this study.

Participant's signature____________________________________Date _________________

Investigator's signature___________________________________Date _________________