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SPIKE FREQUENCY ADAPTATION IN MOTOR NEURONS AND JOINT
RESISTANCE IN RABBIT MODEL OF CEREBRAL PALSY

BY

PRESTON STEELE

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

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IN

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MASTER OF SCIENCE THESIS
OF
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2020

Abstract

Cerebral palsy (CP) is caused by a variety of factors attributed to early brain damage, resulting in permanently impaired motor control, marked by weakness and muscle stiffness. To find out if altered physiology of spinal motoneurons (MNs) could contribute to movement deficits, we performed whole cell patch clamp in neonatal rabbit spinal cord slices after developmental injury at 79% gestation. After preterm hypoxia-ischemia (HI), rabbits are born with motor deficits consistent with a spastic phenotype including hypertonia and hyperreflexia. There is a range in severity, thus kits are classified as severely affected, mildly affected, or unaffected based on modified Ashworth scores and other behavioral tests. At postnatal day (P)0-5, we recorded electrophysiological parameters of 40 MNs in transverse spinal cord slices using whole cell patch clamp. We found significant differences between groups (severe, mild, unaffected and sham control MNs). Severe HI MNs showed more sustained firing patterns, depolarized resting membrane potential, and fired action potentials at a higher frequency. In addition, HI cells also showed alteration in their ability to modulate their firing rates to sustained input. These properties could contribute to muscle stiffness and weakness, which are hallmarks of spastic CP. In summary, these changes we observed in spinal MN physiology likely contribute to the commonly observed phenotype in CP, and therapeutic strategies could target excitability of spinal MNs.

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First and foremost, I would like to acknowledge and thank my major professor, Dr Quinlan for taking me on as a graduate student in her lab and patiently explaining answers to all of my questions on either motor neurons, synaptic currents or anything else I had questions on, as well as, of course, supporting me in the work that I did in her lab. When I had applied to graduate school at URI, I was pretty burnt out of my last job and knew I needed to change something up, but wasn't sure at the time if I wanted to go to graduate school or find a new job in industry. Frankly, I'm not sure I would have followed through on graduate school if it weren't for this opportunity, so I'm very grateful to have been able to work with and learn from great people in Dr Quinlan's lab.

Next, I would like to thank my thesis committee members, Dr Angela Slitt and Dr Susan D'Andrea, for agreeing to be on my committee and taking the time to assess my work and give thoughtful feedback. Lastly, I'd like to thank Dr D' Andrea as well for giving me the opportunity to help with some of her labs research and learn gait analysis techniques and also for personal and professional guidance she gave me while working in her lab.

I enjoyed my time in graduate school at URI and feel as though I grew as a person and learned a lot not only about neuroscience and motor control but also about myself as a person. My committee members, other faculty members in the INP program and other INP students made my experience what it was and I'm glad I got to meet and learn from all of them.

Preface

This thesis is written in manuscript format and consists of two chapters and various appendices. Chapter 1 is a published work in *Frontiers in Cellular Neuroscience* and describes various altered electrical properties in HI MNs as compared to control MNS. Chapter 2 is a prospective manuscript and mainly highlights spike frequency adaptation and alterations in this property in HI MNs as well as the relevance to cerebral palsy. The first two chapters are followed by four appendices in the following order: introduction to the problem, wrap up discussion of the current work, tables from chapter 1 and MATLAB scripts used in analysis for chapter 2.

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Chapter 1: Altered Motoneuron Properties Contribute to Motor Deficits in a Rabbit Hypoxia Ischemia Model of Cerebral Palsy

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Abstract

Cerebral palsy (CP) is caused by a variety of factors attributed to early brain damage, resulting in permanently impaired motor control, marked by weakness and muscle stiffness. To find out if altered physiology of spinal motoneurons (MNs) could contribute to movement deficits, we performed whole cell patch clamp in neonatal rabbit spinal cord slices after developmental injury at 79% gestation. After preterm hypoxia-ischemia (HI), rabbits are born with motor deficits consistent with a spastic phenotype including hypertonia and hyperreflexia. There is a range in severity, thus kits are classified as severely affected, mildly affected, or unaffected based on modified Ashworth scores and other behavioral tests. At postnatal day (P)0-5, we recorded electrophysiological parameters of 40 MNs in transverse spinal cord slices using whole cell patch clamp. We found significant differences between groups (severe, mild, unaffected and sham control MNs). Severe HI MNs showed more sustained firing patterns, depolarized resting membrane potential, and fired action potentials at a higher frequency. These properties could contribute to muscle stiffness, a hallmark of spastic CP. Interestingly altered persistent inward currents (PICs) and morphology in severe HI MNs would dampen excitability (depolarized PIC onset and increased dendritic length). In summary, changes we observed in spinal MN physiology likely contribute to severity of the phenotype, and therapeutic strategies for CP could target excitability of spinal MNs.

Introduction

Cerebral palsy is not well understood, despite its prevalence and seriousness. There exist only a few evidence-based treatments for cerebral palsy: the effectiveness of many currently used therapeutic strategies is unclear (Novak et al., 2013; Wimalasundera and Stevenson, 2016). Recent clinical advances include use of magnesium sulfate and hypothermia after hypoxic-ischemic encephalopathy to acutely reduce neural damage (Magee et al., 2011; Rouse and Gibbins, 2013; Thoresen et al., 1996; Yager et al., 1993), but little basic research is devoted to addressing symptoms after they arise. Part of the problem in treating CP may be the diversity of causes including neonatal stroke, placental insufficiency, preterm birth, inflammation, traumatic injury, difficulties during birth and many other contributing factors (Graham et al., 2016; MacLennan and International Cerebral Palsy Task Force, 1999). Another problem could be that modeling the condition in animals is complicated, and while rodent models are useful for development of neuroprotective strategies, larger animal models are needed to study motor deficits (Cavarsan et al., 2019; Clowry et al., 2014).

Loss of corticospinal control of movement is considered causative of motor deficits in CP, but little investigation into the precise effect on spinal circuits has been conducted. A notable exception is the work of John H. Martin and colleagues, who have documented changes in corticospinal synaptic connectivity in specific spinal laminae and loss of cholinergic interneurons after either cortical silencing or lesioning the corticospinal tract (Friel et al., 2012; Friel and Martin, 2007, 2005; Jiang et al., 2016, 2018; Li and Martin, 2000; Martin et al., 1999). Another important study showed changes in parvalbumin-positive spinal interneurons after cortical silencing in development (Clowry, 2007; Clowry

et al., 2004). Both of these interneuron classes (parvalbumin positive and cholinergic) are synaptically connected to spinal MNs and could contribute to altered motor output after developmental injury. Based on these foundational studies, our hypothesis was that altering development with HI injury would also alter development of MNs, specifically the electrophysiological properties governing excitability in spinal MNs. We further hypothesized that changes in excitability would correspond / contribute to the severity of motor deficits. In short, that altered activity of spinal MNs could contribute to muscle stiffness and spasticity.

In order to assess changes in intrinsic properties of spinal MNs, we used the rabbit HI model of cerebral palsy (Derrick et al., 2004). It's been shown in previous studies that HI injury during late gestation in rabbits can result in a variety of neurologic and muscular damage, including muscle stiffness (Derrick et al., 2004), loss of neurons in cortical layers 3 and 5, white matter injury, thinning of the corticospinal tract (Buser et al., 2010), cell death in the spinal cord and decreased numbers of spinal MNs (Drobyshevsky and Quinlan, 2017), increased sarcomere length, decreased muscle mass and hyperreflexia (Synowiec et al., 2019). There is also an increase in spinal monoamines which could increase excitability of spinal neurons and thus promote spasticity (Bellot et al., 2014; Drobyshevsky et al., 2015). Thus, changes observed in spinal MNs in the rabbit model could be directly compared to motor deficits.

Changes in MN physiology are likely to contribute to motor impairment in cerebral palsy, yet this has not been directly assessed in any animal models. Thus, we assessed electrophysiological parameters in spinal MNs in neonatal rabbits after sham surgery or hypoxic-ischemic insult during development.

Methods

All rabbits were used according to National Institutes of Health guide for the care and use of Laboratory animals, and the University of Rhode Island's, Northwestern University's and Northshore University Health System's Animal Care and Use Committee guidelines. Pregnant New Zealand White rabbits (Charles River Laboratories, Inc, Wilmington MA), underwent HI procedures as described previously (Buser et al., 2010; Derrick et al., 2004). Briefly, the procedure was performed at ~80% gestation (day 25 of gestation, or E25), a time when HI has been found to result in the greatest degree of white matter injury and corticospinal tract thinning. Dams were anesthetized, and the left femoral artery was isolated. A Fogarty balloon catheter was inserted into the femoral and advanced to the level of the descending aorta, above the uterine arteries and inflated for 40 minutes. Sham animals underwent the same procedures but without inflation of the catheter. After the procedure, the dam recovered and later gave birth to kits with HI injuries. Categorization of the severity of the phenotype was performed by a blinded observer, using a modified Ashworth scale, observation / tests for activity, locomotion, posture, righting reflex, muscle tone (as described in Derrick et al., 2004). Kits could be given a maximum score of 6 (normal posture, righting and joint resistance). Since there was a large variation in the severity of motor deficits, HI kits were divided into 3 groups: HI unaffected (scores were the same range as control kits, 5-6), HI mild (scores 3-4), HI severe (scores 1-2). One rabbit kit which was affected by HI but displayed a phenotype of hypotonia instead of hypertonia was removed from the data set. All other kits included in this study displayed hypertonic phenotype if affected by HI.

Patch Clamp Whole cell patch clamp was performed similar to previously published work (Quinlan et al., 2011) from P0-5. Briefly, horizontal spinal cord slices 350µm thick were obtained using a Leica 1000 vibratome. Slices were incubated for one hour at 30°C and recordings were performed at room temperature. During recording, slices were perfused with oxygenated (95% O₂ and 5% CO₂) modified Ringer's solution containing (in mM): 111 NaCl, 3.09 KCl, 25.0 NaHCO₃, 1.10 KH₂PO₄, 1.26 MgSO₄, 2.52 CaCl₂, and 11.1 glucose at 2 ml/min. Whole cell patch electrodes (1-3 MΩ) contained (in mM) 138 K-gluconate, 10 HEPES, 5 ATP-Mg, 0.3 GTP-Li and Texas Red dextran (150 µM, 3000 MW). PICs were measured in voltage clamp mode with holding potential of -90 mV and depolarizing voltage ramps of both 36 mV/s and 11.25 mV/s bringing the cell to 0 mV in 2.5 s or 8 s, respectively and then back to the holding potential in the following 2.5 s or 8 s. Input resistance was measured from the slope of the leak current near the holding potential. Capacitance was measured with Multiclamp's whole cell capacitance compensation function. Resting membrane potential was measured in voltage clamp as the voltage at which there is 0 pA of injected current in the descending ramp. In current clamp, frequency – current measurements were obtained from current ramps.

Figure 1-1: Patch clamp of spinal MNs with dye filling via patch electrode as shown in panel (A). Scale bar = 100µm. **(B)** Placement of patch electrode (at arrow) within the slice is captured with a photo. **(C)** Map of recorded MNs within medial and lateral motor pools. Red circles = HI severe, blue triangles = HI mild, yellow stars = HI unaffected, gray rectangles = Sham Controls.

The first spike on the current ramp was used to measure properties of action potentials. Threshold voltage was defined as the voltage at which the action potential slope exceeds 10 V/s. Rate of rise and fall of the action potential were measured as peak and trough of the first derivative of the action potential. Duration of the action potential was measured at half-peak (defined as the midpoint between overshoot and threshold voltages). Depolarizing current steps of varying amplitude were used to find maximum firing rates (near depolarization block) and to measure after-spike after hyperpolarization (in single spikes elicited near threshold). Hyperpolarizing current steps (typically between -850 and -1250 pA) were used to measure hyperpolarization-activated sag currents (I_H). Neuron selection: Neurons were targeted in MN pools mainly from cervical and lumbar regions of the cord and were removed from the data set if their resting membrane potential was more depolarized than -45mV in current clamp.

Imaging After electrophysiological measurements were obtained, MNs were imaged to assess anatomical development, and photos were obtained of the electrode placement within the spinal cord slice, as shown in **Figure 1**. Images were acquired with a Nikon microscope fitted with a 40x water-dipping objective lens and two photon excitation fluorescence microscopy performed with a galvanometer-based Coherent Chameleon Ultra II laser. To optimize excitation of red/green fluorophores, the laser was tuned to 900 nm. 3D reconstructions of MNs were created using Neurolucida 360° software. It is likely that some processes extended past the surface of the slice and were excluded from reconstructions. However, since this was the case for all MNs in this study it is unlikely to have an impact on the findings.

Statistics All variables were checked for normality and homogeneity (using Shapiro Wilk and Levene's tests). The variables that were parametric (normal and homogenous) were run with one-way Analysis of Variance (ANOVA) and then assessed post-hoc with a Tukey test for between-group significance. The non-parametric variables were run with the Kruskal Wallis test followed by Dunn's test as a post-hoc analysis to assess significance between groups, adjusting the p-value for multiple comparisons. Analysis were done using R software for determining significance of parameters over groups, according to their injury classification (sham, HI unaffected, HI mildly affected, and HI severely affected). Injury classification, age of the kit (P0 – P5), and spinal cord region (cervical, thoracic, lumbar or sacral) were all tested. Significance was determined by p values ≤ 0.05 .

Results

After HI surgery was performed in pregnant dams at 79% gestation, kits were born naturally about a week later. At ages P0, neonates were rated as unaffected, mildly affected or severely affected. Since there was a large variation in the severity of motor deficits, HI kits were divided into 3 groups: HI unaffected, HI mild, HI severe. Experiments were all performed in the first 5 days of life. Over 40 spinal MNs were patched in transverse spinal cord slices, and over 40 parameters were measured from each. To determine significance of the variables, a one-way ANOVA was performed to find differences among 3 factors: 1) injury classification (sham control, HI unaffected, HI mildly affected, and HI severely affected), 2) age (postnatal day 0-5), and 3) spinal region (cervical through sacral). All data, including mean, standard deviation, group size and p value is included in table format (Tables 1 – 5 in appendix C and Supplementary Tables S1 and S2).

HI MNs show sustained firing and higher firing frequency

In rabbit kits severely injured by HI, MNs had significantly increased sustained firing. The frequency current (F-I) relationship was measured using current ramps, as shown in **Figure 2**. Depolarizing current ramps are used to evoke firing, and current at onset and offset of firing (I_{ON} and I_{OFF}) determine ΔI . In sham control MNs, ΔI was larger and always a positive value, indicating firing ceased at a higher current amplitude on the descending ramp than the current level that elicited firing on the ascending ramp (see figure 2A). Severe HI MNs had a smaller, and usually negative ΔI , revealing increasingly sustained firing (see figure 2B). In addition, resting membrane potential was significantly more depolarized in HI MNs than sham controls (see Figure 2F). Another significant difference in severe HI MNs is instantaneous firing rate, as shown in **Figure 3**. At the start of a depolarizing current step, the peak (instantaneous) firing rate is higher in severe HI MNs than sham controls (see figure 3B). Sustained firing is also apparent in Figure 3C, in the second current step which evokes a brief burst of action potentials followed by depolarization block in both MNs. The severe HI MN recovers and resumes firing while the sham control MN remains in depolarization block. Mean values for both instantaneous and steady state parameters are shown in bar graphs (Figure 3D, E). Significant changes in MN physiology were present in severely affected animals: posthoc analysis showed significant changes between sham and severe HI MNs in ΔI , RMP, and instantaneous firing rate. Only instantaneous firing rate also reached significance in mild HI MNs vs sham. No significant changes were present in HI unaffected MNs in any properties. While these parameters (ΔI , RMP, and instantaneous firing rate) suggest increased excitability in HI-injured MNs, there was no significant change in threshold, I_{ON} , or I_{OFF} in HI MNs. Thus,

MNs from severely affected kits should not be classified as hyperexcitable *per se*, since they begin firing with the same depolarizing input and at the same voltage threshold.

Changes in spike properties and subthreshold responses were not present

A complete analysis of I_H (sag and rebound currents), action potential parameters, and after-spike after-hyperpolarization (AHP) was performed and no significant differences in these parameters were found between groups. All data is included in supplementary data Tables S1 and S2.

Persistent inward currents suggest excitability is dampened after HI

PICs were significantly affected by hypoxia-ischemia, revealing that intrinsic excitability may be dampened. PICs were measured using both short (5 s) and long (16 s) protocols, which can preferentially activate and inactivate Na^+ and Ca^{2+} mediated PICs. The different protocols yielded different results. For example, as shown in **Figure 4** voltage dependence (PIC onset and PIC Max) was unchanged in the PICs evoked using a short 5 second voltage ramp HI severe MNs. Using longer voltage ramps (16 s), PIC onset was significantly depolarized in HI severe MNs compared to sham (see Tables 2 and 3 in appendix C). Since the change in PIC onset was more pronounced in longer ramps this could suggest an altered balance of Na^+ and Ca^{2+} channel activation or altered activation / inactivation of these channels (see discussion). Change in the magnitude of the PIC was not observed outright in either of the protocols: the magnitude of the currents was similar between groups (see figure 4 and Tables 2 and 3 in appendix C). Intrinsic properties including capacitance (which significantly increased in HI MNs compared to sham) and input resistance of all MNs are included in Table 2 in appendix C.

Morphology affected by HI injury

Morphology of MNs was assessed in all patched neurons, as shown in **Figure 5**. As suggested by the significantly larger whole cell capacitance, there were changes in MN morphology after HI injury. The soma size was unchanged: there were no significant differences between groups in soma largest cross-sectional area (Fig 5D) or other measurements of soma size (Table 4 in appendix C). There was, however, a significant increase in dendrite length in HI injured MNs compared to sham controls (Fig 5E), which could account for changes in electrical properties. Since we recorded from motor pools throughout the spinal cord (cervical through sacral), there was a large amount of variability within our data set. Future studies in our lab focus on analysis of specific motor pools. All data pertaining to dendritic morphology is included in Table 5 in appendix C.

Age and spinal region

No significant changes in MN properties were found due to spinal region. Postnatal age had a significant effect on the following properties: input resistance (decrease with age), action potential size (mV), rate of rise and rate of fall (all increase with age), 5s PIC amplitude (increase with age), and normalized PIC (PIC/Cap; current density increased with age for both 5 and 16 s ramps). These results are in line with previous studies on embryonic and postnatal maturation of MNs.

Discussion

Summary

Electrophysiological properties of spinal MNs are altered by developmental HI injury, and the magnitude of changes are correlated to severity of motor deficits. Specifically, these

changes include increased sustained firing and a higher firing rate. These changes could indicate increased excitability and would contribute to muscle stiffness that is common in

Figure 1-2: Severe HI MNs show more sustained firing than sham control MNs, and a more depolarized resting membrane potential. Control MNs (A) have larger values for ΔI compared to severe HI (B). Average ΔI and threshold are shown in panels (C) and (D) for all groups. The frequency current relationship (E) was not significantly different between groups. Resting membrane potential was significantly more depolarized in HI severe MNs than sham control MNs (F). Error bars = SEM. Scale bars in (A) = 20mV (vertical and 0.5s (horizontal) and applies to panels (A,B).

spastic cerebral palsy. However, concomitant changes in PIC onset and longer dendritic length could serve to dampen excitability and could contribute to weakness. Since traditional views of CP largely view motor dysfunction as a result of the damaged motor cortex improperly signaling to spinal neurons, our new evidence suggests this is only part of the problem. Spinal MNs are not developing the same after HI and show an overall change in intrinsic properties. Whether these changes are directly due to the HI insult or indirectly due to downstream effects must be determined by future work.

Contribution of spinal motoneurons to dysfunction in cerebral palsy

Here we show that MNs show altered excitability after HI injury, including elevated resting potential, and more sustained firing. Previous work showed that after HI injury in rabbits there were also fewer spinal MNs, and spinal interneurons in lamina VII were undergoing apoptosis (Drobyshevsky and Quinlan, 2017). After loss of corticospinal projections, it was recently found that the spinal cholinergic interneurons which give rise to C boutons on MNs are lost (Jiang et al., 2019, 2016, 2018).

Figure 1-3: Instantaneous firing frequency is increased in severe HI MNs. Depolarizing current steps (A) evoked action potentials (C) in a typical sham control (black trace) and severe HI (red trace) MNs. (B) Dotted lines show peak firing frequency of MNs in C. (D) Average instantaneous and (E) steady state firing frequency are shown for all groups. Error bars = SEM. Scale bar in C = 0.5 sec and applies to panels A, B, and C

Taken together this data suggests spinal circuits are 1) just as vulnerable to HI injury as the developing cortex and 2) potentially functioning with fewer neurons and altered circuitry. In addition to fewer neurons, there is also atrophy of the muscles which could contribute to motor deficits in cerebral palsy. In mice, rabbits, and humans, muscle atrophy appears along with losses in numbers of MNs (Brandenburg et al., 2018; Drobyshevsky and Quinlan, 2017; Han et al., 2013; Marciniak et al., 2015). Recent work has shown similar changes to muscle architecture in the rabbit HI model of CP to humans, including atrophy,

muscle shortening, and longer sarcomere length. Increased muscle stiffness in rabbits affected by HI was found even after administration of anesthetic – indicating some muscle stiffness is derived from mechanical changes in the muscles, though a large component of the muscle stiffness was diminished with anesthetic thus was driven neurally (Synowiec et al., 2019). During development, both feedback and feed-forward signaling can regulate growth and maturation, processes which may be disrupted in CP in both MNs and muscle fibers. It is likely the loss of spinal interneurons, MNs and muscle fibers reduces coordination and strength in those with cerebral palsy. Altered size and excitability of MNs is also a feature of other motor disorders including amyotrophic lateral sclerosis and spinal muscular atrophy (Dukkipati et al., 2018; Gogliotti et al., 2012; Quinlan et al., 2019, 2011; Shoenfeld et al., 2014). Since our data was collected from multiple motoneuron pools throughout the spinal cord, from control and HI injured rabbit kits, a relatively large variability in parameters was expected (Kanning et al., 2010), however consistent differences in electrical properties emerged in this data set. This suggests consistent changes are present across motor pools after injury and supports further exploration of interventions for CP and other motor disorders that target spinal MNs.

Figure 1-4: PICs are altered in HI injured motoneurons. (A) Typical current response to a 5 second voltage ramp in a sham (black trace) and HI severe (red) MN. (B) Leak-subtracted PICs from A are similar in amplitude. There is trend for depolarized PIC onset (C) in short ramps after HI injury, which reaches significance in long ramps (E). PIC Max did not reach significance (D) and (F). Error bars = SEM.

These treatments could include neuromodulators and therapies aimed at restoring balance between excitation and inhibition within spinal circuits for alleviation of spasticity.

Neuromodulation

The exact causes of the changes in MN physiology observed here are unclear, but they could result from the increase in spinal monoamines that occurs after developmental HI

injury in both rodents and rabbits (Bellot et al., 2014; Drobyshevsky et al., 2015). Serotonin is generally thought of as a neurotransmitter and neuromodulator, but developmental disruption in 5HT is associated with neurological disorders including autism, Rett syndrome, Down's syndrome and, more recently, cerebral palsy (Bar-Peled et al., 1991; Bellot et al., 2014; De Filippis et al., 2015; Drobyshevsky et al., 2015; Muller et al., 2016; Whittle et al., 2007; Wirth et al., 2017; Yang et al., 2014). Serotonin increases MN excitability in neonatal and juvenile mice, rats and guinea pigs (Hsiao et al., 1998, 1997; Wang and Dun, 1990; Ziskind-Conhaim et al., 1993), and likely has the same effect on rabbit MNs. Depolarization of the resting membrane potential, increased action potential firing through hyperpolarization of the voltage threshold and enhanced PIC, increased action potential height and reduction of high-voltage activated Ca^{2+} entry are all associated with 5HT receptor activation in neonatal and adult MNs (Bayliss et al., 1995; Elliot and Wallis, 1992; Gilmore and Fedirchuk, 2004; Hsiao et al., 1998, 1997; Inoue et al., 1999; Larkman and Kelly, 1992; Li et al., 2006; Lindsay and Feldman, 1993). Therefore increased 5HT could have a direct impact on excitability, though in HI rabbits the increase in 5HT was accompanied by decreased mRNA for 5HT₂ receptors and increased mRNA for the SERT serotonin transporter (Drobyshevsky et al., 2015). In light of that finding, it is not clear that neurons remain responsive to 5HT. In the experiments here, all MNs were recorded in spinal cord slices incubated and perfused in standard oxygenated aCSF without any serotonergic drugs present. Therefore, HI MNs *in vivo* could show different levels of excitability since they would be in the presence of elevated 5HT, while our MNs were all recorded in the same standard physiological solution. Thus, the contribution of 5HT to the altered excitability observed here is restricted to its chronic effects on neuron development,

namely morphological changes. Serotonin 5HT_{1A} and 5HT_{2A} receptor activation increases neurite outgrowth, dendritic branching, and spine formation

Figure 1-5: Morphology is affected by HI. Typical sham control (A) and HI severe (B) motoneurons filled with dye during patch clamp (electrodes visible on right). Average values of whole cell capacitance (C), soma largest cross-sectional area (D), total dendrite length (E), and number of stem dendrites (F) are included for all neurons. Scale bar in A = 100μm, applies to A and B. Error bars = SEM.

(Bou-Flores et al., 2000; Fricker et al., 2005; Mogha et al., 2012), findings that align well with the present finding of increased dendritic length and number of primary dendrites in the HI MNs. Future experiments will be needed to address the role of 5HT in enhancing

MN excitability and its effects on synaptically-evoked action potentials. Synaptic events in dendrites would more strongly evoke PICs, though both altered dendritic morphology and elevated 5HT could dampen them.

Possible mechanism of altered MN output

The mechanism for increased MN activity and thus muscle stiffness may be due to delayed Na^+ channel inactivation. In neonatal MNs, Na^+ channels generate the majority of the PIC and account for action potential initiation / repetitive firing. Specifically, Nav 1.1, 1.2 and 1.6 type Na^+ channels (Boiko et al., 2003, 2001; Rush et al., 2005) inactivate faster than Ca^{2+} channels that contribute to PICs (Li et al., 2006; Perrier and Hounsgaard, 2003). Short voltage ramps preferentially measure the Na^+ PIC for this reason: Na^+ channels inactivate quickly enough that even on the descending ramp of the short protocol, there is no longer a region of negative slope (see figure 4). Changes in Na^+ channel inactivation in adult MNs along with postnatal development of the longer-lasting Ca^{2+} PIC makes typical adult MNs display more negative ΔI values and longer lasting PICs (Harvey et al., 2006; Li et al., 2006; Quinlan et al., 2011). In the neonatal rabbit MNs, sham controls showed positive ΔI values that are quite typical for neonates, while HI MNs showed significantly more negative values. This could be due to slower Na^+ channel inactivation or increased contribution of Ca^{2+} channels to the PICs after injury. Interestingly the more depolarized resting potential found in HI severe MN would serve to increase Na^+ channel inactivation. To fully determine the altered mechanism of aberrant firing after HI injury future studies into the biophysical properties of Na^+ channels and maturation of Ca^{2+} channel expression must be pursued.

Severity in motor deficits and electrophysiology

Generally, unaffected and mildly affected MNs showed parameters that were intermediate between control MNs and severe MNs. There was only in one category in which mildly affected MNs become statistically significantly different from sham controls (instantaneous firing frequency). Thus electrophysiological changes were overwhelmingly in line with phenotype, suggesting aberrant MN properties contribute to the severity of the phenotype. It cannot be ruled out, however, that HI “unaffected” MNs may have a subtle phenotype that is not readily evident based on testing we performed here. Or perhaps abnormalities in these rabbits would develop later in life: in humans CP patients, diagnosis of CP is not made until 18-24 months of life and the peak of spasticity occurs around four years of age (Hadders-Algra, 2014; Hägglund and Wagner, 2008; Novak et al., 2017). In the rabbit model, deficits have not been characterized past P18, and a detailed analysis of the progression of motor deficits from P0 -18 is lacking. Future work is needed to assess maturation of the MN properties in different groups, the potential contribution of delayed Na⁺ channel inactivation in CP, progression of motor deficits with age, and the development of new therapeutic strategies that could target MNs.

Postnatal maturation and injury

The present study only extended from postnatal day 0-5, but even within this narrow window, significant changes that were apparent in MN electrical parameters. As MNs undergo postnatal maturation, they grow larger, with more complex dendritic arborizations, and gain the ability to fire action potentials at higher rates, as reviewed in

(Carrascal et al., 2005). We found that age had a significant effect on 5s PIC amplitude (increasing with age), normalized PIC amplitude (both 5 and 16 s PIC/Cap increased with age), input resistance (decreasing with age), and action potential size, rate of rise and rate of fall (all increasing with age). While neuron size increases during this period, the amplitude of the PIC typically increases more than proportionally (Quinlan et al., 2011). In other words, voltage gated ion channels are being inserted into the membrane at a faster rate than the cell is increasing in size, resulting in an increased normalized PIC amplitude with age. We suspect this parallels the ability of MNs to fire action potentials at higher rates during postnatal development, and the acquisition of coordinated motor control and weight bearing in developing animals.

Conclusion

Changes in MN physiology after developmental injury are consistent with motor deficits in rabbits. This suggests not only brain injuries but also changes in the spinal cord contribute to impaired function in cerebral palsy. Exploring both altered maturation of spinal neurons and loss of descending connectivity should be pursued to improve outcomes for individuals with cerebral palsy.

This manuscript has been released as a Pre-Print at BioRxiv (Steele et al., 2019).

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Chapter 2: Spike Frequency Adaptation, Joint Stiffness and CP

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Introduction

Cerebral Palsy

After an HI injury in rabbit and mouse models of CP, an increase in serotonin (5HT) has been shown in the spinal cord [Bellot et al. 2014, Drobyshevsky et al. 2015]. 5HT is known to increase Persistent Inward Currents (PICs) carried by both sodium (Na^+) and calcium (Ca^{2+}), which could have a direct influence on motor neuron (MN) excitability and influence spasticity [Li et al. 2007, Harvey et al. 2006]. PICs are voltage-sensitive inward currents carried by cations that depolarize the cell, bringing it closer to threshold and facilitating sustained firing. PICs are usually most pronounced in neurons which need to fire continuously, like motor neurons during sustained muscle contractions, and are very effectively shut off with synaptic inhibition. In CP, impaired development of descending tracts including the corticospinal tract (CST) likely means a reduction of synaptic inhibition. Since HI has been shown to increase 5HT in the spinal cord and 5HT has been shown to increase PICs, this may produce an unchecked excitatory effect on motor neurons allowing them to sustain firing pathologically. Further, 5HT has also been shown to influence a neuron's after hyperpolarization potential (AHP) which has implications for the cell's ability to adapt its firing frequency to sustained input. However, a decrease in 5HT₂ receptor mRNA has also been seen after HI injury [Drobyshevsky et al. 2015] and may reflect the body's attempt to compensate for the rise in 5HT levels and restore homeostasis in MNs. It was unclear if these injured MNs remained sensitive to 5HT.

Determinants of neuronal firing rates and Spike Frequency Adaptation (SFA)

Firing frequency of motor neurons is well characterized in normally developing MNs and is known to increase from birth to adulthood [Carrascal et al. 2005]. This developmental increase in firing frequency is attributed to multiple mechanisms including a decrease in the medium duration after hyperpolarization potential (mAHP) and an increase in sodium PICs. Under normal conditions, when a cell fires an action potential there is a rapid depolarization phase brought about by voltage gated Na^+ channels opening which in turn causes voltage gated calcium and potassium channels to open. This causes two things to occur: (1) as the potassium channels open, the membrane repolarizes and (2) as the calcium channels open and the intracellular calcium concentration increases, calcium-activated potassium channels open and the cell's membrane potential hyperpolarizes before returning to baseline. This hyperpolarization is the mAHP typically seen after an action potential and what is referred to above. A decreased duration of this hyperpolarization phase along with increased PICs would lead to the increase in firing frequency seen in development and, if further increased due to injury, could lead to hyperexcitable MNs and hypertonia. It is also one of the primary mechanisms that the phenomenon of SFA has been attributed to, although more recent work by Miles et al. calls this into question [Miles et al. 2005].

SFA in MNs plays an important role in the speed of force generation and its maintenance [Del Vecchio et al. 2019]. It also has implications for the energy cost related to AP generation and information coding [Yi et al. 2016]. While it's known that patients with CP often experience muscle weakness and impaired motor control, the literature regarding the role of SFA in symptoms of CP is non-existent. SFA describes a neurons

ability to dynamically alter its firing rate in response to a sustained *constant* input. This is not to be confused with rate modulation in neurons, which describes their ability to adjust firing rate in response to a *changing* input. Depending on the level of input, neurons exhibit different firing characteristics, and this even varies for similar neuron types. For example, when stimulated with a high level of current, a MN will begin firing at a very high frequency initially and then slow down its firing roughly following an exponential decay function [Binder et al. 2010]. This rapid initial firing followed by incrementally slower firing to a steady state is known as spike frequency adaptation. This adaptation is generally recognized to occur in 3 distinct phases: initial, early and late phases and each one is believed to be driven by distinct cellular mechanisms. The initial phase is characterized by a rapid, linear decrease in firing frequency and occurs within the first few inter-spike intervals [Binder et al. 2010]. The early and later phases however constitute a slower decrease in firing frequency which can be approximated by exponential functions with time constants on the order of 250ms and 10s of seconds, respectively [Binder et al. 2010]. The mechanisms driving SFA are not completely understood and have yet to be analyzed in the context of cerebral palsy. Therefore, the first aim of this paper is to assess the MNs ability to adapt their firing frequency in response to sustained *constant* input and correlate this with the level of injury observed. This will be done using a modified spike accommodation index as in Ha et al [Ha et al. 2017]. The second aim is to analyze the effects of 5HT on adaptation properties and understand differences seen between control and injured animals.

Methods

Hypoxia-ischemia surgery

All rabbits were used according to both the University of Rhode Island's and Northshore University Health System's Animal Care and Use Committee guidelines. Pregnant New Zealand White rabbits (Charles River Laboratories, Inc, Wilmington MA), underwent HI procedures as described in [Derrick et al. 2004]. Briefly, at 79% gestation (day 25 of gestation (E25) dams were anesthetized, and the left femoral artery was isolated. A Fogarty balloon catheter was inserted into the femoral and advanced to the level of the descending aorta, above the uterine arteries and inflated for 40 minutes. Sham animals underwent the same procedures but without inflation of the catheter. After the procedure, the dam recovered and later gave birth to kits with HI injuries. Categorization of the severity of the phenotype was performed by a blinded observer, using a modified Ashworth scale, observation / tests for activity, locomotion, posture, righting reflex, muscle tone (as described in [Derrick et al. 2004]).

Patch Clamp

Whole cell patch clamp is performed similar to previously published work [Quinlan et al. 2011]. Briefly, horizontal spinal cord slices 350 μm thick are obtained using a Leica 1000 vibratome. Slices are incubated for one hour at 30°C and perfused with oxygenated (95% O_2 and 5% CO_2) modified Ringer's solution containing (in mM): 111 NaCl, 3.09 KCl, 25.0 NaHCO_3 , 1.10 KH_2PO_4 , 1.26 MgSO_4 , 2.52 CaCl_2 , and 11.1 glucose at 2 ml/min. Whole cell patch electrodes (1-3 $\text{M}\Omega$) contain (in mM) 138 K-gluconate, 10 HEPES, 5 ATP-Mg, 0.3 GTP-Li and Texas Red dextran (150 μM , 3000 MW; used for a concurrent imaging

study). PICs are measured in voltage clamp mode with holding potential of -90 mV and depolarizing voltage ramps of both 36 mV/s and 11.25 mV/s bringing the cell to 0 mV in 2.5 s, and 8 s respectively, and then back to the holding potential in the following 2.5 or 8 seconds. In current clamp, frequency – current measurements are obtained from current ramps and steps, as well as maximum firing rates, spike accommodation and characteristics of action potentials and after-spike after hyperpolarization. Hyperpolarizing current steps are used to test I_h . Neuron selection: Neurons were targeted in the motoneuron pools mainly from cervical and lumbar regions of the cord, and were removed from the data set if their resting membrane potential was more depolarized than -45 mV at break in.

Drug application

Serotonergic drugs were applied to the bath and all electrophysiological parameters were recorded again. Either serotonin was applied at a concentration of 10 μ M or 0.3 μ M α -methyl 5 hydroxy tryptamine in combination with 10 μ M citalopram. Citalopram was included because of the previous finding that expression of SERT transporters could be upregulated [Drobyshevsky et al. 2015]. Since perfusion rate was 2.5 ml/minute and dead space plus the bath volume was 10 ml, all recordings were made 20 minutes after application, when the volume of the bath and tubing had been twice replaced.

SFA analysis

A custom MATLAB program was used to analyze data acquired from current clamp experiments and calculate firing rates and spike accommodation indices (SAI). The notion of a spike accommodation index originated from Shinomoto et al [Shinomoto et al. 2003] to describe spike train properties such as local variance. The formula was then simplified to be the inter-spike-interval (ISI) of the first two spikes in the train divided by the ISI of the last two spike in the train (Eq 1). A sample of data is also shown below to highlight the inter spike intervals used in the SAI calculation.

$$SAI = \frac{ISI_{first}}{ISI_{last}} \quad (1)$$

As such, a value of less than one and closer to zero indicates significant adaptation occurred and values closer to one or above one indicates less adaption occurred or even acceleration, respectively. The script created for this paper analyzes current step files and generates firing profiles like the ones published in Bikoff et al. (figure 4 in paper) [Bikhoff et al. 2016]. In order to generate these profiles, the channel containing action potentials was adaptively thresholded in order to identify which APs were at least 15mV in height. This threshold was based on the mean and standard deviation of the spike train data during a current step and was necessary since the baseline potential of the cell changes with different levels of injected current intensity. Once all peaks above 15mV were identified, ISI's were calculated and converted to frequencies. The first frequency point was taken as the max instantaneous firing frequency and the last 10 points in the profile were averaged

to calculate the steady-state firing frequency. The average firing rate was also calculated from the current steps that were not high enough to elicit spike frequency adaptation. In addition, several SAIs were calculated for analysis including the SAI over the total duration of the step as well as the SAI for the initial phase and the late phase of adaptation. To avoid confusion between initial phase adaptation and early phase adaptation, I will be referring to what is typically referred to in the literature as early phase adaptation as late phase adaptation in this paper. Since there were a range of current steps for each neuron that elicited SFA, a maximum, minimum and mean of the aforementioned variables was taken for each neuron. See the flow chart below to describe how the variables were calculated.

Figure 2-1: Flow chart outlining how analysis was carried out

These variables were then used in statistical analyses described in the *statistics* section.

Torque measurement

To measure joint stiffness in the rabbits, a torque device was constructed to measure the passive muscle resistance in control and HI kits. The device was modeled after the one used by Drobyshevsky et al. [Drobyshevsky et al. 2012] with the addition that the device runs automatically to produce timed sinusoidal movements instead of using the experimenter's hand which may introduce unwanted variability into the data. Force measurements were made with a 500g load cell and a servo motor was used to produce the sinusoidal movements between 0 and 80 degrees. From this force data, a torque-displacement graph with multiple torque measurements per angle is created similar to Drobyshevsky et al. [Drobyshevsky et al. 2012]. Each measurement was then averaged for a given angle and an average torque-displacement graph is created. The joint stiffness was calculated based on the slope of a linear regression of the average graph. In order to assure passive resistance was measured, kits were acclimated to the device for a few cycles of movement before recording measurements. Trials were discarded if the kits were visibly moving or trying to remove their limb from the device. With this setup, we will also be able to discern active and passive components to joint stiffness (as in Synoweic et al. 2019) by measuring torque before and after anesthesia administration. The device can be seen in the figure below.

Figure 2-2: Torque meter device used to measure muscles passive resistance to stretch

Statistics

All statistics were run using Rstudio. The data was checked for homogeneity and normality using Levene's test and The Shapiro-Wilkes test, respectively. For parametric variables, ANOVA was used to assess group differences and for non-parametric variables, the Kruskal-Wallis test was used. Data were grouped into sham control and HI animals for analysis. All HI animals were grouped together due to low number of neurons in each group. For the within group comparisons analyzing changes before and after 5HT administration, a paired t-test was used to assess statistical significance.

Results

After the surgery that was performed in the HI rabbit dams, kits were born naturally about a week later. All patch clamp experiments were performed in the first 5 days of life. Over 40 neurons are included in this analysis with just about 30 parameters calculated for each. A couple different statistical analyses were run with the variables to determine significance. First, between group differences were assessed in sham vs HI cells with parameters collected from cells in a bath of modified Ringers Solution. Second, the same between group differences were assessed with serotonin present in the bath. Finally, inter group differences were analyzed by finding significant differences before and after 5HT administration in each neuron.

Baseline data sham vs HI: no 5HT in bath

When comparing motor neurons from HI animals to neurons from control animals, we observed significant group differences in variables relating to the neurons steady state firing frequency. Both the minimum steady state frequencies from the HI group ($p=0.005$) and the mean steady-state firing frequencies ($p=0.01$) were significantly higher in the HI group than in the sham group (Figure 3 B,C). There were no between group differences in current levels injected or in spike accommodation variables indicating this result is not due to current levels used to inject cells and is instead present because of altered intrinsic mechanisms in the cells promoting hyperexcitability.

Figure 2-3: *Increased steady state firing frequency observed in HI cells as compared with controls with no 5HT in the bath.* (A) Raw data showing increased steady state firing frequency observed in HI MNs as compared to sham MNs in aCSF bath. (B) Statistically significant differences between groups minimum steady state firing frequency ($p=0.005$). (C) Statistically significant differences between groups mean steady state firing frequencies ($p=0.01$).

Before / after 5HT direct comparison (ie same cells before and after 5HT administration)

In contrast to the baseline analysis above, when comparing the direct effect of 5HT on specific sham and HI cells, we found a much larger effect of 5HT on HI cells than on sham. Interestingly, we did not observe any significant differences in firing frequency or spike accommodation in sham motor neurons in the presence of 5HT. There were quite a few interesting differences however in the cells from HI MNs. To start, the range of current steps that elicited adaptation in the cell was higher after 5HT administration ($p=0.0007$) and the minimum normalized current ($p=0.001$) and mean normalized currents that elicited SFA were lower with 5HT ($p=0.0004$). **These differences indicate that the HI cells adapted to a wider range of input current levels after 5HT administration.** In other words, low amplitude current steps that did not previously evoke SFA were capable of producing SFA in the presence of 5HT. This is in line with previous results showing serotonin's effect on motor neuron excitability by reducing their threshold for firing.

The maximum late phase spike accommodation indices were found to be significantly higher after 5HT administration and the mean spike accommodation indices were lower ($p=0.004$, $p=0.004$ respectively), indicating more overall adaptation of the firing rate in HI neurons but not in sham. In addition, the mean SAI during the initial phase of adaptation was significantly lower after 5HT ($p=0.007$), indicating more adaptation occurring in the initial phase of adaptation. A subset of variables pertaining to the cells firing frequency were also significantly different between before and after 5HT conditions. Specifically, the maximum ($p=0.01$), minimum ($p=0.02$) and mean instantaneous firing frequencies ($p=0.007$) and mean steady state firing frequency ($p=0.005$) was *decreased* after 5HT, indicating that neurons were firing more slowly in the presence of 5HT.

Although these results appear contradictory to the previous reports of 5HT *increasing* excitability, they are most likely a result of the fact that the cells fire at lower current levels in the presence of 5HT. The firing frequency is directly related to input level and if the current levels are lower with 5HT, the firing frequency variables would also be decreased.

Figure 2-4: Changes in various SAI's before and after administration of 5HT for HI cells only. (A) Significant difference in the maximum late phase accommodation index only in HI cells (0.004) showing higher tendency to accelerate firing after initial adaption with 5HT present. (B) Significant difference in the mean SAIs in the HI cells after 5HT administration showing more adaptation with 5HT ($p=0.04$). (C) Significantly lower mean SAI during the initial phase of adaptation with 5HT indicating more adaption taking place ($p=0.007$). (D) Significant difference found in the mean late phase SAI in addition to the max late phase SAI ($p=0.02$). Both indicate less adaptation occurring in the later phase, as shown by an increase in the SAI.

5HT data: 5HT added in bath

Because higher levels of 5HT are observed in the spinal cord after HI injury *in vivo*, when performing *in vitro* recording, it might be more accurate to compare properties of sham MNs to HI MNs in aCSF with added 5HT. When we did this, group differences were

observed in a completely different subset of variables than before. The only significant group differences were in the minimum average firing rates ($p=0.016$) and the maximum late phase accommodation indices (0.048), both of which were higher in the HI group than sham (Figure 5). This means that the lowest firing rates recorded when adaption did not occur were higher for HI cells than sham and that there was *less* adaptation occurring in the late phase adaptation in HI cells. Once again, no significant differences were found in variables reflecting the levels of injected current, indicating these results are not due to different current levels used between groups. The minimum average firing frequencies variable is measured in cases where the cell does not exhibit adaptation, so this means that even at low levels of input to the cell, it is still likely to fire at higher rates than controls. The fact that it is higher in the HI group shows trends toward hyperexcitability and could contribute to the impaired control of motor output experienced by patients. The late phase accommodation index is measured between the 5th spike and the last spike in the train when adaptation is present. The increase observed in this variable suggests that cellular mechanism involved in late phase adaptation is altered and promotes less adaptation and a higher likelihood of acceleration in HI cells.

Figure 2-5: Average firing frequency and late phase SAI results from cells recorded with 5HT in the bath. (A) Raw data trace of sham and HI MNs showing increased average firing rate in the HI MNs. (B) Statistically significant results between groups for minimum average firing frequency (0.016). (C) Raw data traces showing differences in late phase adaptation for sham (left) and HI (right) MNs. More variability observed in the HI MN and slight acceleration in the late adaptation phase. (D) Statistically significant results between groups in their maximum late phase accommodation index ($p=0.048$).

Torque

Torque was directly measured of the kits hip joint using a custom-built device. The data collected from this device was force data from a load cell that was converted to torque and stiffness was calculated using a custom MATLAB program. The method for calculating stiffness was the same as what was used in Drobyshevsky et al [Drobyshevsky et al. 2012], the slope of the torque displacement graph was used.

When analyzing torque data, no significant differences were found between HI and sham cells (figure 6). This is likely due to lack of severely affected kits in this data set and

it is expected that once more kits with hypertonia are included, group differences will reach significance and likely trend in the opposite direction. What these results indicate is that in injured kits that do not show obvious spasticity may actually have *hypotonic* muscles compared with controls. In children with perinatal brain injuries, hypotonia precedes hypertonia (more on this in the discussion), which could represent what we're observing in this study. This data will need further study and inclusion of more kits with more injury severity in the future.

Figure 2-6: Preliminary results showing stiffness comparison between sham and HI animals. (A) Average stiffness was lower in HI animals compared to controls, but the result is not significant. (B) An example torque-displacement graph measured by the torque meter. The stiffness is calculated as the slope of the regression line fit to the data.

We also evaluated stiffness in a subgroup of the kits analyzed above before and after administration of anesthesia to potentially assess active vs passive components of joint stiffness. There are only 3 animals so far for this analysis so no significance could be reached, but the trend does show a decrease in joint stiffness post-anesthesia, which is to be expected. What was unexpected however was that the HI kits tended to show lower

joint stiffness than controls, indicating some level of hypotonia may be present at least at late postnatal time points. This did not reach significance, however. In addition, the torque value reported here are an order of magnitude less than those previously reported with a similar device in Drobyshevsky et al. [Drobyshevsky et al. 2015]. This may be due to the different setup we used for measurement in which the experimenter was removed from the equation and a servo motor produced the limb rotation. In the future, this analysis will need to be repeated once more severe animals are included in the study.

Discussion

Effect of 5HT on sham and injured motor neurons

The data presented here on parameters measured from the cells simply in a bath solution are comparable to those recently published regarding the differences in firing frequency variables between sham and HI groups [Steele et al 2020]. We found increased steady state firing frequency and believe it to be a reflection of increased excitability in the HI cells especially since the current levels the cells were injected with did not come out significant between groups, consistent with results presented in Steele et al. [Steele et al. 2020/Chapter 1]. This instead suggests alterations of intrinsic mechanisms in motor neurons responsible for controlling firing frequency which could contribute to spasticity as the higher rate would cause a stronger contraction in vivo. Once in the presence of 5HT however, we found many more parameters affected in HI cells than in control cells and all of our results generally point toward a complex relationship between 5HT, HI injuries and SFA that this work alone is unable to tease apart. One complication in interpreting the results is that

there are differences between the environments in which HI motor neurons mature as compared to controls. For example, higher levels of 5HT have been reported in the spinal cord in kits subject to hypoxia ischemia in the womb. This is most likely because the hypoxic injury is incurred at a developmental timepoint when the serotonergic projections from the raphe nucleus are still developing connections in the spinal cord and respond to the injury by aberrantly sprouting [Drobyshevsky et al. 2015]. As such, there is an overabundance of serotonergic synapses on MNs that affect their development. It could be argued that adding 5HT to the bath represents an environment that is more comparable to one that HI motor neurons develop in and therefore may indicate that the HI cell results in the presence of 5HT best represent what is occurring *in vivo*. This is complicated by the fact that decreased 5HT₂ receptor mRNA and increased 5HT transporter (SERT) mRNA in the spinal cord have also been reported. However, this was measured in whole cord preparations, and altered mRNA levels do not necessarily translate into altered expression levels at the membrane surface so it is unclear if HI MNs remain sensitive to 5HT. More research on this topic is needed to clarify the role of 5HT receptors and transporters.

The present results comparing sham and HI neurons in a bath with 5HT show that even when the input is too small to elicit any adaptation, the lowest firing frequencies a cell can sustain are higher for HI cells than sham cells. This can be interpreted as cells showing hyperexcitability even at low levels of stimulation, comparable for example to *in vivo* cases where type 1 fibers are tonically recruited for balance or postural application. This has some clinical relevance in CP as a predominance of type 1 fibers has been reported previously [Marbini et al. 2002]. When the stimulus is large enough to evoke adaptation in the MN, there are no differences between groups in firing frequency or their overall

ability to adapt to the sustained input but the HI cells show larger maximum values for late phase adaptation indicating less adaptation occurring in the phase. Further, the mean values for the maximum accommodation indices are above a value of 1 which means that they even tend to accelerate in this late phase of adaptation more often than control cells. A value of 1 for the spike accommodation index (SAI) would indicate no adaptation occurred and values above 1 would indicate acceleration in firing. Values less than 1 indicate adaptation took place and the closer the values are to 0, the more adaptation occurred. One interpretation could be that this reflects a compensation mechanism if the cell over adapts in its initial phase, but since no variables representing initial adaptation came out significant that is unlikely to be the case. Instead, this more likely indicates that a mechanism or combination of mechanisms that contribute to late phase adaption may be altered in HI cells in the presence of 5HT. The mechanisms of late phase adaptation are thought to be related to the inactivation properties of fast, inactivating sodium channels responsible for the generation of an action potential and, as such, these channels should be investigated further in future research and may hold potential as a future therapeutic target.

When comparing within group results before and after 5HT administration, 5HT had very little effect on adaptation and firing properties of control MNs and a much larger effect on HI MNs. This alone is interesting and indicates that HI MNs are not only still sensitive to 5HT, but even more so than control cells. This seems counter intuitive in light of the previously reported results showing less 5HT_{2R} mRNA and more SERT mRNA in the spinal cord. However, as previously mentioned, altered mRNA levels do not necessarily translate into receptor changes on the cell's membrane. For example, there may be increased mRNA levels for the 5HT transporter, but if this mRNA isn't translated

and folded into a functional protein that is trafficked to the cell membrane, it will not have any effect on 5HT in the synapse. Alternatively, since these mRNA levels were measured from the whole spinal cord, it's possible that they don't reflect changes in the motor neuron pools specifically. There could also be different 5HT receptor subtypes expressed in HI MNs compared to controls which contribute to the increased affect we observe here. This will be covered in more depth later in the discussion. The first thing we observed was that HI cells exhibited spike frequency adaption at lower levels of current input after 5HT administration. This is in line with other reports of 5HT reducing the threshold for firing in MNs. In addition, the accommodation indices with 5HT present indicate *more* adaption taking place with 5HT present, both overall and in the initial phase. Judging by the mean variables in figure 4 (panels B, C, D), 5HT appears to have a corrective effect on HI cells and brings the accommodation indices closer to the levels of that seen in the controls without 5HT. This can be interpreted in a couple different ways. First it could be seen as more evidence that analyzing HI MNs in the context of 5HT is a more accurate way to interpret the data as it better represents the environment the cells are in *in vivo*. Alternatively, it could also suggest that there are still corrective mechanisms in place in injured cells that are controlled by serotonin and attempt to compensate for altered intrinsic cellular properties due to the injury. Either way, the results described here unequivocally show that injured MNs maintain their responsiveness to 5HT. However, the question remains, 'how and why does 5HT affect the adaptation ability of the injured cells only'? One mechanism could be that 5HT (specifically via 5HT₂Rs) increases PICs from calcium and sodium [Li et al. 2007, Harvey et al. 2006], which would increase excitability in motor neurons and likely cause them to adapt at lower levels of input. Although this explanation

intuitively makes sense and in fact has been previously hypothesized as a mechanism in late phase adaptation, there has been some evidence published indicating this is not what is occurring. Zeng et al. [Zeng et al. 2004] reported no effect of blocking sodium PICs on the late phase adaptation and concluded that there must be many different types of channels contributing to later phases of adaptation and that this may be the case since adaptation plays a very important role in force production in muscles. They reasoned that this property of adaptation would have been important enough that a motor neuron would have evolved to have multiple channels contributing to it so that it would still be able to adapt its firing rate in the presence of varying levels of neuromodulation. Importantly, the results of Zeng et al. do not rule out the possibility of slow inactivation of sodium channels being necessary for SFA as described by Miles et al. [Miles et al. 2005], since the sodium channels responsible for PICs are a different channel. They also don't rule out effects of calcium activated potassium conductance (pharmacologically distinct from mAHP) contributing to later phases of adaptation. This may be an interesting avenue to pursue in the future.

Another possibility is that there are different subtypes of 5HT receptors expressed in HI animals compared to controls. It's known that stimulation of 5HT₂ receptors have more excitatory effects on the cell whereas 5HT₁ receptors tend to be inhibitory [Ladewig et al. 2004]. Since it's been shown there is less 5HT_{2R} mRNA in the injured cord, the effect we're observing on adaptation may be due an increased expression of 5HT₁ receptors in injured MNs and this over expression could have a mild inhibitory effect on firing rate and increasing adaptation without effecting excitability. Alternatively, alterations in ion balance across the membrane in ions such as calcium or chloride would certainly influence firing rate and adaptation. For example, if the perinatal injury alters

expression of KCC2 (a potassium chloride co-transporter), then chloride balance across the membrane would be affected which could alter a neuron's firing alone. However, Synowiec et al. [Synowiec et al. 2019] did not find any differences in KCC2 between injured animals and controls so this explanation is unlikely.

Yet another possibility is if there are changes in calcium currents, as have been shown previously in response to 5HT [Li et al. 2007]. This could also affect both the initial and overall adaptation properties of the cell. 5HT has been shown to affect mAHP amplitude as well as the hyperpolarization-activated inward conductance, tending to decrease the former and increase the latter [Bayliss et al. 1995, Berger et al. 1992]. Both of these effects would allow the cell to fire at a sustained higher rate because less adaptation would occur. The decreased mAHP would cause less spike accommodation in the initial phase of adaptation and the increased hyperpolarization-activated inward currents would act to depolarize the cell, effectively blunting late phase adaptation. The increased hyperpolarization-activated inward current fits with our result of less late phase adaptation occurring because if there is an increase in a depolarizing current during the late phase, firing rate will adapt less or could even increase. However, the typical effect of 5HT decreasing the mAHP amplitude would have the opposite effect on the initial phase adaptation that we report here. This could only be possible if the effects of 5HT on mAHP and the hyperpolarization-activated currents are through different receptor types or if the initial phase of adaptation is not due to summation of the mAHP. Perhaps the lower mRNA levels of 5HT_{2R}'s observed in injured animals does translate to decreased expression in the membrane and eliminates the decrease in mAHP typically observed in response to 5HT and injured animals have a higher ratio of another receptor subtype. This of course is

speculation and more studies will need to be carried out assessing 5HT receptor subtypes expression in HI MNs. Finally, it should be noted that no significant correlations between SAI properties and severity of HI injury was found here, although trends of more adaptation in more severely injured animals was observed. The lack of significance could be due to small sample sizes but could also mean the neuron's ability to adapt to constant input is not a relevant contributor to spasticity. More research on this front will need to be carried out to verify the impact of SFA on spasticity and elucidate potential mechanisms behind the effects of 5HT observed here.

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Appendix A: Introduction and Review of the Problem

Cerebral Palsy (CP) is a developmental disorder which affects a person's ability to control their muscles and, more generally, their motor system. It occurs in roughly 1 in 400 live births and while a direct cause is not always known, certain events such as hypoxia ischemia or neonatal stroke have been shown to cause the disorder [Robertson et al. 1985]. CP is characterized by brain damage to specific areas related with movement and depending on the area affected is classified as spastic (cortical lesion), ataxic (cerebellar lesion) or athetoid (lesion in basal ganglia). The most common subtype of CP is spastic and is characterized by muscle weakness, hypertonia (rigidity) and hyperreflexia of muscles with peak spasticity occurring around 4-6 years of age. Other risk factors associated with the development of CP include premature birth and low birth weight, head trauma around time of birth and infection [Rosenbaum et al. 2007]. Current treatment options available are limited but include physical therapy, occupational therapy and certain medications including Baclofen and Botulinum toxin (Botox). The latter two options work by decreasing excitability in the spinal cord or paralyzing the muscle, respectively, which may offer relief from hypertonia but renders the person unable to control that muscle. Although these treatment options may be somewhat helpful, they are not ideal since they target symptoms after they've already developed instead of preventing or treating the underlying mechanism. This is because the mechanism driving spasticity (hypertonia and hyperreflexia) observed in people with CP is not well understood.

One reason for limited treatment options for CP is that historically there has not been an effective animal model that yields the motor impairments seen in humans. Some of the more commonly used models involve exposing developing fetuses or neonates to hypoxia-

ischemia (HI) and/or inflammation (often using lipopolysaccharide [LPS]). The rat/mouse models involving HI for example, can produce brain injury comparable to that seen in humans, but the animals do not present with severe motor deficits indicating that the brain injury alone may not be enough to produce the impairments in rodents (unlike humans). Likewise, the LPS models have also not yielded significant motor impairments but the use of both together has been shown to produce more reliable motor deficits in rats [Cavarsan et al. 2019]. The lack of motor impairments in rats and mice is likely due to when in the developmental process birth occurs compared to humans. The majority of CNS development in mice and rats occurs postnatally, so a perinatal CNS injury has less effect on their development compared to a postnatal injury. In fact, models using mice or rats inflict the injury at about one week of age to mimic the effects of that injury around the time of birth in humans. However, at one week of age, development of the neuromuscular connections in rodents are largely complete, thus there is a mismatch in development of the brain and spinal cord in humans and rodents. Rabbits on the other hand, are similar to humans in that they develop their CNS perinatally and, as such, a perinatal HI injury yields significant motor impairments along with other human characteristics of CP and may serve as a better model to study the motor deficits in CP [Graham et al. 2016, Synowiec et al. 2019]. Importantly, the rabbit model also shows similar brain injury as humans (with cell death in motor cortex) along with spasticity [Buser et al. 2010]. Derrick et al. [Derrick et al 2004] described a rabbit model of CP in which HI is induced in a pregnant dam at 80% gestation (E25) and the injured kits exhibit a phenotype very similar to that of humans – one characterized by hypertonia and hyperreflexia. The time course of the rabbit's nervous system development is more like that of a human in that spinal circuits involved in

movement, reflexes and sensory feedback are still forming perinatally and axons are still undergoing myelination. Myelination continues to occur into early adulthood in humans and up to the 2-month timepoint in rabbits (10-12 years in humans) and the peak spasticity in rabbits emerges right after birth around postnatal day 0 (P0) to P5, which corresponds to 4 years in humans [Franson et al. 1975, Hagglund et al. 2008]. The perinatal and postnatal developmental similarities between rabbits and humans is likely why the rabbit model expresses a similar phenotype of CP to that of humans and is why it is proving to be an essential tool in studying the etiology of the symptoms of the disease. Because of these similarities, the model is being used to study mechanisms driving spasticity in CP with the hope that one day, a therapeutic intervention can target these mechanisms and improve the quality of life of those suffering from the disorder.

Most CP research has focused on the brain lesions associated with the disorder, but the final pathway carrying the signal to the muscles is the spinal cord, specifically spinal motor neurons. The spinal cord has been largely overlooked but could be an important therapeutic target since there are also developmental perturbations in spinal circuits in CP. Currently it is thought that hypertonia in affected muscles is caused by disinhibition of MNs in the spinal cord, although there is some evidence that the hypertonia seen after birth is instead a direct result of muscle architecture [Synoweic et al. 2019]. However, this is somewhat of a ‘chicken or the egg’ problem as it presents the question ‘do the alterations of motor neuron firing appear first and then alter the development of muscle architecture or is it a separate mechanism that alters the muscle architecture first which has a retrograde effect on the MN firing pattern?’.

During development of the motor systems, projections from different brain regions

invade the spinal column in an orderly sequence. First is rubrospinal tracts around 9 weeks post conception in humans [Sundstrom et al. 1993, Williams et al. 2014] followed by the vestibulospinal tract and finally the corticospinal tract (CST around 24 weeks post-conception [Clowry et al. 2007, Eyre et al. 2000]). Despite these tracts being glutamatergic, they have a net inhibitory effect on the motor system since they synapse largely on inhibitory interneurons in the spinal cord [Jankowska et al. 1976]. The injuries occurring later in prenatal development will primarily affect the CST along with other circuits developing around this time which include those involved in reflex arcs and sensory feedback. Alterations of these circuits may be an explanation of why spasticity arises; spasticity arises from decreased inhibitory tone in the spinal cord which allows motor neurons to exhibit uninhibited sustained firing. Overactive reflex arcs are also thought to be involved in the generation of hyperactive MNs, again either due to increased synaptic excitation or alterations in their development.

After an HI injury in rabbit and mouse models of CP, an increase in serotonin (5HT) has been shown in the spinal cord [Bellot et al. 2014, Drobyshevsky et al. 2015]. This could influence spasticity since 5HT has been shown to increase Persistent Inward Currents (PICs) carried by both sodium (Na^+) and calcium (Ca^{2+}). PICs are voltage-sensitive inward currents carried by cations that depolarize the cell, bringing it closer to threshold which helps facilitate sustained firing and are seen in cells which need to fire continuously. For example, one could imagine an evolutionary scenario in which it'd be beneficial for motor neurons to fire repetitively for a muscle to contract and continue contracting whether it be trying to lift a heavy object or simply maintain an upright posture. Since HI has been shown to increase 5HT in the spinal cord and 5HT has been shown to increase PICs, this

increase in 5HT may have an unchecked excitatory effect on motor neurons allowing them to fire without inhibition due to the impaired development of later arriving tracts. A curve ball in the story, however, is that a decrease in 5HT₂ receptor mRNA has also been seen after HI injury [Drobyshevsky et al. 2015] and may reflect the body's attempt to compensate for the rise in 5HT levels and effectively cancel out the increase in excitability. In addition to affecting the function of voltage gated Na⁺ and Ca²⁺ channels, 5HT has also been shown to influence the after-spike after hyperpolarization (AHP) [Binder et al. 2010]. All these effects may have implications in the cell's firing frequency adaptation to sustained input discussed in chapter 2.

Appendix B: Final Discussion

In the context of CP

Until recently, most CP research has focused on the brain lesions associated with the disorder, but the final pathway carrying the signal to the muscles is the spinal cord. The spinal cord has been largely overlooked but, in light of recent results (including the ones presented here), could be an important therapeutic target since there are also developmental perturbations in these circuits in CP. It is currently thought that hypertonia in affected muscles is primarily caused by disinhibition of MNs in the spinal cord due to altered corticospinal development. During development, projections from different brain regions invade the spinal column in an orderly sequence. First is rubrospinal tracts around 9 weeks post conception in humans [Sundstrom et al. 1993, Williams et al. 2014] followed by the vestibulospinal tract and finally the corticospinal tract (CST around 24 weeks post-conception [Clowry et al. 2007, Eyre et al. 2000]). Despite these tracts being glutamatergic, they have a net inhibitory effect on the motor system since they synapse largely on inhibitory interneurons in the spinal cord [Janowska et al. 1976]. The injuries occurring later in prenatal development will primarily affect the CST along with other circuits developing around this time which include reflex arcs and sensory feedback loops, and likely the serotonergic projections from the Raphe nucleus. In addition to decreased inhibitory tone, Synoweic et al. [Synowiec et al. 2019] showed that altered muscle architecture is also a contributing factor to hypertonia by showing that injured kits still had more joint resistance under anesthesia than controls. This presents somewhat of a ‘chicken or the egg’ problem as it begs the question ‘do the alterations of motor neuron firing appear first and then alter the development of muscle architecture or is it a separate mechanism

that alters the muscle architecture first which has a retrograde effect on the MN firing pattern?'.

Muscle weakness

Interestingly, Rose and McGill [Rose and McGill 2005] found that muscle weakness in adults with CP is caused by the motor systems inability to recruit high threshold motor units and drive lower threshold units to fire at a higher frequency, perhaps due to an alteration of fiber type favoring type 1 fiber predominance [Marbini et al. 2002]. Type 1 fibers are part of lower threshold motor units which tend to fire at lower frequencies than higher threshold units. Rose and McGill did show less rate modulation in CP patients as shown by a significantly lower projected maximum firing rate, which is in line with data showing a switch from type 2 to type 1 fibers in response to tonic lower frequency stimulation in rabbits [Sreter et al. 1982] as well as in other conditions like stroke and Parkinson's Disease (PD) [Edstrom et al. 1973]. However, *Sreter et al* also showed that this switch can occur in response to sustained phasic high frequency stimulation, indicating the change in fiber type is in response to total activity instead of frequency- specific stimulation [Carrascal et al. 2005]. Taken together, these results may be indicative that sustained alterations in motor neuron firing frequencies *preceding* changes in muscle architecture could lead to weakness and spasticity. It has been traditionally believed that hypertonia results from overactive motor neurons due to disinhibition [Deon et al. 2010, Sanger 2003, Volpe 2001], however it could also be due to tonic recruitment of lower threshold type 1 fibers which over time leads to disuse atrophy of type 2 fibers, leading to simultaneously stiff but weak muscles. Importantly, these studies were not focused on children in early development so it remains possible that there could be a developmental

over-activity of MNs that lead to contractures and hypertonia leading to weakness and impaired muscle control later in life. Also, a relevant study in P1 rabbit kits by Synowiec et al. concluded that passive muscle properties are also a contributing factor to joint stiffness, although they did not directly measure motor neuron firing properties [Synowiec et al. 2019]. Currently, there is no characterization of the changes in MN firing frequencies during development in CP and, if the evolution of these patterns is tracked across development, we can gain valuable insight into mechanisms affecting MN development from an early age.

Our work highlighted in Chapter 1 adds to the body of literature supporting the rabbit model of CP as well as the idea that neurons in the spinal cord are also affected by prenatal HI injuries to the CNS. We found that there are indeed significant alterations in electrophysiological properties of MNs in the spinal cord following HI injury in parameters that contribute to hyperexcitability. For example, MNs in the injured animals displayed an increase in sustained firing as well as a depolarized resting membrane potential. Further, these changes correlated well with the severity of motor deficits which was characterized by the modified Ashworth Scale as described in Derrick et al. [Derrick et al. 2004]. Interestingly, normalized PIC values were found to be decreased in injured MNs which points toward decreased excitability. Upon further analysis, it was found that this was due to an increase of cell capacitance associated with the increase in dendritic length as well as an increased number of primary dendrites in the HI cells. This increase in dendritic length may reflect a compensatory mechanism of the spinal MNs in the absence of descending input and could also be affected by 5HT_{1A} and 5HT_{2A} receptors, which have been shown to increase dendritic outgrowth [Fricker et al. 2005]. In addition, the results showing

increased tendency toward sustained firing would produce spastic symptoms and the results of delayed PIC onset could have the opposite effect and dampen excitability, leading to weakness. Importantly, these results were recorded from neurons in an aCSF bath without any drugs added. Since increased 5HT has been shown in the spinal cord of HI rabbits, it may be more realistic to analyze these parameters recorded from motor neurons in a bath with 5HT which is what we did in chapter 2.

There is some potential overlap in implications from results in chapter 2 taken together with results from chapter 1 that may point toward alterations in sodium or calcium channels that affect their inactivation properties. In chapter 1, we showed that PIC onset in HI cells with the longer voltage ramp was significantly delayed whereas the onset from the shorter voltage ramps did not reach significance. This could suggest an altered balance of Na^+ and Ca^{2+} channels in HI cells or, alternatively, that the inactivation properties of these channels are affected. In chapter 2 we showed that spike accommodation index was altered in HI cells only in the presence of 5HT. The initial phase of adaptation has been attributed to the mAHP which is dependent on calcium influx into the cell, but the later phases of adaptation could also be affected by slowly activating or inactivating Ca^{2+} or Na^+ channels. In support of this, Miles et al. [Miles et al. 2005] found, both experimentally and through computer modelling, that slow inactivation of the fast-inactivating Na^+ channels is likely to be the key mechanisms involved in early phase adaptation. In addition, alteration in the alpha subunit of the Na^+ channel (which affects inactivation properties) could be responsible for different adaptation properties in various neuron types [Goldin 2001]. This, along with our data, highlights the importance of future studies focusing on

the biophysical properties of Na⁺ channels and the maturation of Ca²⁺ channels to assess their roles in the altered electrical properties we describe here.

Lastly, Jiang et al. [Jiang et al. 2016, Jiang et al. 2018] showed that spinal interneurons that give rise to C boutons on MNs are downregulated after a damage to the corticospinal tract. C boutons modulate the gain of the MN circuits through M2 muscarinic receptor activation, which increases excitability in MNs. Without this gain control mechanism on motor neurons, exhaustion of central drive would occur more rapidly and could produce muscle weakness and negatively impact skilled movements under cortical control. This loss of cholinergic drive on MNs may impact SFA as well. For example, muscarinic receptor activation has been shown to activate protein kinase C (PKC) which has downstream effects on inactivation properties of sodium channels and loss of this cholinergic drive could certainly impact a neuron's adaptation properties [Cantrell et al. 1996]. Another potential example is the calcium activated chloride channel TMEM16F in the MN membrane that localizes to C bouton synapses and affects the recruitment of delayed firing MNs. Interestingly however, other channels in this family have previously been associated with SFA in other neurons types, such as TMEM16B in thalamocortical neurons [Ha et al. 2016]. There is mixed evidence for 5HT's effect on chloride currents. For example, it has been previously reported that 5HT increases the calcium activated chloride conductance in *Xenopus* oocytes injected with rat brain mRNA [Gunderson et al. 1983], but also that chloride channels are not contributors to inward currents elicited by 5HT in rats [Takahashi et al. 1990]. It remains possible that alterations in the TMEM16B channel or the TMEM16F channel occur when C boutons are downregulated and are contributors to the changes in spike frequency adaptation we observe here, although this is

speculative. Future work will need to be done on this front in the rabbit model of CP to assess these channels' role in SFA alterations and spasticity.

Appendix C: Tables from Chapter 1

<i>Variable</i>	<i>Condition</i>	<i>Mean</i>	<i>SD</i>	<i>N</i>	<i>p</i>	<i>Table 1 Frequency – current characteristics</i>
I _{ON} (pA)	Sham	213	148.8	9		
	HI Unaffected	627	474.6	12	0.129	
	HI Mild	422	457.6	10	0.507	
	HI Severe	459	374.7	8	0.450	
I _{OFF} (pA)	Sham	416	357	9		
	HI Unaffected	768	538	12	0.545	
	HI Mild	509	319	10	1.000	
	HI Severe	443	452	8	1.000	
ΔI (pA)	Sham	219	224	9		
	HI Unaffected	141	159	12	0.496	
	HI Mild	88	279	10	0.106	
	HI Severe	-16	84	8	0.046*	
FI Slope (Hz/nA)	Sham	23	18	9		
	HI Unaffected	13	4	12	0.557	
	HI Mild	23	23	10	0.945	
	HI Severe	20	9	8	0.370	
Threshold (mV)	Sham	-35.8	7.2	9		
	HI Unaffected	-32.2	8.0	12	0.279	
	HI Mild	-36.3	7.1	10	0.879	
	HI Severe	-29.9	6.7	8	0.107	
Maximum instantaneous firing frequency (Hz)	Sham	98	28	9		
	HI Unaffected	106	32	12	0.556	
	HI Mild	129	33	10	0.025*	
	HI Severe	129	23	8	0.045*	
Maximum steady state firing frequency (Hz)	Sham	30	10	9		
	HI Unaffected	36	13	12	0.552	
	HI Mild	45	18	10	0.346	
	HI Severe	37	13	8	0.559	
Resting membrane potential (mV)	Sham	-60.8	6.4	9		
	HI Unaffected	-53.9	11.3	13	0.070	
	HI Mild	-58.5	4.3	10	0.556	
	HI Severe	-50.5	9.1	8	0.017*	

*Significant difference to sham animals

Table 2 Persistent inward current characteristics (5s ramp)

<i>Variable</i>	<i>Condition</i>	<i>Mean</i>	<i>SD</i>	<i>N</i>	<i>p</i>
Norm PIC amp (pA/pF)	Sham	-2.10	2.44	9	
	HI Unaffected	-1.45	0.81	13	0.852
	HI Mild	-1.86	1.02	9	0.988
	HI Severe	-1.22	0.41	8	0.923
PIC onset (mV)	Sham	-42.9	7.3	9	
	HI Unaffected	-37.4	6.7	13	0.200
	HI Mild	-38.7	8.1	9	0.337
	HI Severe	-38.1	10.6	8	0.278
PIC max (mV)	Sham	-30.0	7.3	9	
	HI Unaffected	-22.9	7.6	13	0.081
	HI Mild	-22.6	7.4	9	0.066
	HI Severe	-23.1	9.2	8	0.093
PIC negative slope range (mV)	Sham	12.9	3.1	9	
	HI Unaffected	14.4	2.7	13	0.458
	HI Mild	16.0	2.8	9	0.250
	HI Severe	15.0	3.1	8	0.462
PIC amplitude (pA)	Sham	-280	185.2	9	
	HI Unaffected	-366	194.6	13	0.344
	HI Mild	-417	272.9	9	0.162
	HI Severe	-344	114.5	8	0.525
Capacitance (pF)	Sham	192	98	9	
	HI Unaffected	259	45	13	0.167
	HI Mild	214	77	10	0.471
	HI Severe	282	0	8	0.028*
Input resistance (MΩ)	Sham	60	35.0	9	
	HI Unaffected	40	15.9	13	1.00
	HI Mild	56	33.8	10	1.00
	HI Severe	54	30.2	8	0.965

*Significant difference to sham animals

Table 3 Persistent inward current characteristics (16s ramp)

<i>Variable</i>	<i>Condition</i>	<i>Mean</i>	<i>SD</i>	<i>N</i>	<i>p</i>
Norm PIC amp (pA/pF)	Sham	-2.32	2.77	6	
	HI Unaffected	-1.58	.90	13	0.953
	HI Mild	-1.73	.94	9	0.706
	HI Severe	-1.03	.50	8	0.735
PIC onset (mV)	Sham	-47.2	6.0	6	
	HI Unaffected	-39.7	6.3	13	0.086
	HI Mild	-40.9	9.7	9	0.169
	HI Severe	-35.0	11.5	8	0.013*
PIC max (mV)	Sham	-27.8	9.4	6	
	HI Unaffected	-21.4	7.4	13	0.175
	HI Mild	-24.2	9.9	9	0.479
	HI Severe	-18.3	11.1	8	0.071
PIC negative slope range (mV)	Sham	19.5	6.0	6	
	HI Unaffected	18.3	3.2	13	0.927
	HI Mild	16.6	2.4	9	1.000
	HI Severe	16.7	4.1	8	0.872
PIC amplitude (pA)	Sham	-315	189.3	6	
	HI Unaffected	-389	226.7	13	0.215
	HI Mild	-397	253.9	9	0.310
	HI Severe	-323	112.6	8	0.802

*Significant difference to sham animals

Table 4 Soma morphology characteristics

<i>Variable</i>	<i>Condition</i>	<i>Mean</i>	<i>SD</i>	<i>N</i>	<i>p</i>
Largest x Sectional Area (μm^2)	Sham	577	316	9	
	HI Unaffected	756	287	12	1.000
	HI Mild	676	307	10	0.277
	HI Severe	694	338	8	1.000
Surface Area (μm^2)	Sham	2599	1153	9	
	HI Unaffected	3609	2083	12	0.591
	HI Mild	2489	1129	10	0.447
	HI Severe	2582	851	8	0.155
Volume (μm^3)	Sham	12306	8219	9	
	HI Unaffected	23735	28313	12	0.716
	HI Mild	12445	7963	10	0.255
	HI Severe	12264	6308	8	0.826

*Significant difference to sham animals

Table 5 Dendrite characteristics

Variable	Condition	Mean	SD	N	p
Number of Dendrites	Sham	7.7	2.8	9	
	HI Unaffected	9.5	1.4	12	0.089
	HI Mild	10.1	2.9	10	0.360
	HI Severe	10.1	2.5	8	0.128
Nodes	Sham	14.8	14.1	9	
	HI Unaffected	14.7	11.5	12	0.909
	HI Mild	13.5	5.7	10	0.800
	HI Severe	17.0	8.9	8	1.000
Length (μm)	Sham	1103	649	9	
	HI Unaffected	1422	664	12	0.346
	HI Mild	1443	537	10	0.591
	HI Severe	1744	521	8	0.050*
Mean Length (μm)	Sham	140	67	9	
	HI Unaffected	149	67	12	0.947
	HI Mild	144	32	10	0.992
	HI Severe	178	57	8	0.169
Surface Area (μm ²)	Sham	6264	3842	9	
	HI Unaffected	9667	7000	12	0.649
	HI Mild	7537	3293	10	0.526
	HI Severe	9868	5384	8	0.698
Mean Surface Area (μm ²)	Sham	792	414	9	
	HI Unaffected	1024	772	12	0.919
	HI Mild	740	159	10	0.537
	HI Severe	1012	603	8	0.612
Volume (μm ³)	Sham	3614	2642	9	
	HI Unaffected	6926	7512	12	0.807
	HI Mild	4073	2406	10	1.000
	HI Severe	5844	5239	8	0.664
Mean Volume (μm ³)	Sham	451	295	9	
	HI Unaffected	743	836	12	0.771
	HI Mild	390	129	10	0.847
	HI Severe	605	587	8	0.917

*Significant difference to sham animals

Appendix D: MATLAB Analysis Scripts

Script 1: Load Spike2 files and calculate firing frequencies for each current step

```
%This script was used in Preston Steele's thesis and was
co-written by
%Preston and Lynn McCane. Lynn wrote the part pertaining
to loading Spike2 files from the CED and Preston wrote the
analysis part

%This script is step 1 in the firing frequency and spike
frequency adaptation analysis
%
%to use this script, all the .smr files from Spike2 must be
in the same
%directory that the script is in and the script will run
the following
%steps automatically.
%1: load in all .smr files in directory
%2: find current steps of fixed length (in this case 1s),
and find AP
% peaks during each current step
%3: Once peaks are identified, firing rates are calculated
based on the
% inter-spike interval and saved to structure named in
the following
% format:
%
% 'MN#''5HT or no 5HT''date Recorded' -> example
MN15HT_161208 is MN1
% recorded on 122/08/16 with 5HT in the bath solution
%
% step amplitude, spike amplitude, firing frequencies,
AP peaks and
% locations of AP peaks
%
% finally, the structure is saved to the directory the
script is located
% in and this structure is loaded into the step 2 script
titled
% 'MATLABscript4thesisPt2'
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%% Script starts below
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

%clear all variables currently in MATLAB and close any
figure windows
```



```

clc
clear all
close all

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%% Open Spike2
files%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%% Get the SMR file names
wd = 'C:\Users\Preston\Documents\MATLAB\ePhys
data\MATLAB_CED\CP project\HI severe\161208\MN1_5HT';
%folder where script looks for .smr files
files =
dir(fullfile('C:\Users\Preston\Documents\MATLAB\ePhys
data\MATLAB_CED\CP project\HI
severe\161208\MN1_5HT\*.smr')); %find all .smr files
[num_f1, ~] = size(files) %outputs how many files were
found in directory

%%% Load to
CED%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%required code from documentaion to access library
cedpath = 'c:\CEDMATLAB\CEDS64ML'; %must be path that the
CED library was downloaded to
addpath( cedpath );
CEDS64LoadLib( cedpath );

%LOAD CHANNELS FOR SPIKE DATA BELOW
num_f = num_f1;
SpikeCh= 1; % define which channel in data is spike
channel, typically 1, but to be sure plot all channels and
verify
for i = 1: num_f
    fhand{i} = CEDS64Open(files(i).name) ;
%load file
    maxTimeTicks{i} = CEDS64ChanMaxTime(fhand{(i)}, 1 )+1;
%extract timepoints
    [ fRead{i}, fVals{i}, fTime{i} ] = CEDS64ReadWaveF(
fhand{(i)},...
        SpikeCh, 100000, 0, maxTimeTicks{i} ); %read the
wavform data for SPIKE data
end

%below loop concatenates all spike channels head to tail
m= 1; %dummy variable to store spikes data in chSpikes
variables
for i= 1:num_f

```

```

        nextFile4mat = cell2mat(fVals(i));
        l= length(nextFile4mat);
        chSpikes(m:m+(l-1),1) = [nextFile4mat-nextFile4mat(1)];
%remove any offset between previous channel end and current
channel start
        m= m+l;
end
%below 2 lines can plot channel loaded above to verify it's
spike channel
% figure(1)
% plot(chSpikes)

%LOAD CHANNELS FOR CURRENT STEP DATA
StepCh= 2;
for i = 1: num_f
    %      i
    fhand2{i} = CEDS64Open(files(i).name) ;
%load file
    maxTimeTicks2{i} = CEDS64ChanMaxTime(fhand2{(i)}, 1
)+1; %extract timepoints
    [ fRead2{i}, fVals2{i}, fTime2{i} ] = CEDS64ReadWaveF(
fhand2{(i)}, ...
        StepCh, 100000, 0, maxTimeTicks2{i} ); %read the
wavform data for STEP data
end

n= 1; %dummy var
for i= 1:num_f

    nextFile4mat2 = cell2mat(fVals2(i));
    l2= length(nextFile4mat2);
    chSteps(n:n+(l2-1),1) = [nextFile4mat2-
nextFile4mat2(1)];
    n= n+l2;
end
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%% END file loading, next is analysis below
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
% ANALYSIS SCRIPT to identify current steps and find
sample ranges of each step

%sample frequency
fs = 10000;

%Initial AP thresh height to try to identify all AP pks

```

```

APthresh = 20;%mV, may need to adjust depending on if all
AP's are identified

%real AP thresh from min in between AP
APthreshReal = 15; %min AP height threshold

%ENTER PULSE LENGTH (seconds)
PulseLength = 1; %adjust if current step duration is
different than 1 second

%%% ANALYSIS PART 1: Find points of current injection,
stores all data
%%% indices where the sample value is above baseline+20

%define baseline segment
baseline = mean(chSteps(1:1000)); %baseline from first 1000
samples, ~100ms
%initialize variables for storing inidices
m = 1;
for i = 2:length(chSteps)
    idiff = abs(chSteps(i) - chSteps(i-1));
    if idiff<5&&abs(chSteps(i))>baseline+20 %identifies
steps by any points that are at least points above baseline
        istep(m) = i; %%%index of samples DURING current
step
        m = m+1;
    else
        non_ind = i;
    end
end

% separates pulse indices from above into indiv steps
ipulstart(1) = istep(1); %initialize ipulstart variable
k = 2;
k1 = 1;
for i = 2:length(istep)

    %find pulse start points
    istepdiffstart = istep(i) - istep(i-1);
    if istepdiffstart>5000 %if difference in indices is
>5000, determined to be start of different step, change if
inter-step interval is <1s
        ipulstart(k) = istep(i);%index where pulse starts
        k = k+1;
    else

```

```

        dummy = istep(i);
    end

end

%define end of current pulse
ipulseend = (PulseLength*fs)+ipulstart;
% indices for step start and step end are calculated and
can be used to
% create sample ranges for each step. The AP pk finding
script will 'look'
% only between these sample ranges for AP peaks

%Define sample ranges for each step
for i = 1:length(ipulseend)
    rangeint(i,:) = ipulstart(i):ipulseend(i)+100;
    %     range = ipulstart(i):ipulseend(i)+100;

end

%Save sample ranges for each step to the data structure
for i = 1:length(ipulseend)
    range = rangeint(i,:);
    MN15HT_161208.amplitudes(i) =
mean(chSteps(rangeint(i,:)));
    MN15HT_161208.spikes(:,i) = chSpikes(range);
end

%% ANALYSIS to find AP peaks during each step and calculate
firing
%%initialize variables below since these values will be
calculated each
%%time through the loop
MN15HT_161208.pks(:, :) = zeros(120,1);
MN15HT_161208.frequencies(:, :) = zeros(120,1);
MN15HT_161208.spikeAmps(:, :) = zeros(120,1);

%main analysis loop
for i = 1:length(ipulseend)

    %Sample range for current current step
    range = rangeint(i,:);

    %data for the current loop is the spike channel data
only for the
    %samples of the current current step
    spikeDataForLoop =MN15HT_161208.spikes(:,i);

```

```

%find peaks
[pks1, locs1] = findpeaks(spikeDataForLoop);

%%% find all local mins for AHP max calculation further
down
oneifmin = islocalmin(spikeDataForLoop);
idxmin = find(oneifmin==1);
locmin = range(idxmin);

%      m = 1; %might not need??

%   calculate average baseline of AP data
avgbaseline = mean(chSpikes(locmin));
avgbase2plot(i,:) = avgbaseline*ones(1,length(range));
%plotting baseline line variable for manual check that it
is correctly identified
thresh2plot =
(avgbaseline+APthresh)*ones(1,length(range)); %plotting
threshold for manual check

%%% This for loop checks amplitude of all identified
peaks and only
%%% saves ones that are above previously defined AP
threshold
n = 1;
d = 1;
for k = 1:length(pks1)
    height = pks1(k) - avgbaseline;

    if height>APthresh
        pks(n) = pks1(k);
        locs(n) = locs1(k);
        n = n+1;
    else
        fakelocs(d) = 1;
        d = d+1;
    end
end

```

```

end

dminus1 = d-1;
if dminus1==length(locs1) % if no peaks are above
threshold, still save all below vairables, but just saved
as zeros
    %define all structure variable again assigning all
zeros
    MN15HT_161208.frequencies(:,i) = [0, zeros(1,120-
1)];
    MN15HT_161208.spikeAmps(:,i) = [0, zeros(1,120-1)];
    MN15HT_161208.pks(:,i) = [0, zeros(1,120-1)];
    MN15HT_161208.locs(:,i) = [0, zeros(1,120-1)];
else % if there are peaks above threshold, confirm only
1 pk identified per AP, first AP height check
    g = 2;
    y = 1;
    rllocs(1) = locs(1);
    rlpks(1) = pks(1);
    for k = 2:length(pks)
        dist = locs(k) - locs(k-1);
        if dist>30
            rlpks(g) = pks(k);
            rllocs(g) = locs(k);
            g = g+1;
        else
            doublepk(y)= pks(k);
            y = y+1;
        end
    end

    end
    yminus1 = y-1;
    if yminus1==length(pks)||length(rlpks)==1 % if only
1 peak identified, can't calculate frequency so ignore it
and
        %redefine all structure variables again
assigning all zeros
        MN15HT_161208.frequencies(:,i) = [0,
zeros(1,120-1)];
        MN15HT_161208.spikeAmps(:,i) = [0, zeros(1,120-
1)];
        MN15HT_161208.pks(:,i) = [0, zeros(1,120-1)];
        MN15HT_161208.locs(:,i) = [0, zeros(1,120-1)];
    else % now it's confirmed that for steps below,
there are at least 2 APs and no double peaks

```

```

        %% FIND MINS BETWEEN APs for AP height
calculation
    for p = 1:length(rllocs)-1

        [minimum idxMin] =
min(spikeDataForLoop((rllocs(p)):rllocs(p+1)));

        minAP(p) = minimum;
        locMinAP(p) = idxMin+rllocs(p);

    end

    %% second AP height check to make sure AP
height (max - min) is
    %%above threshold - APthreshReal defined above
    u = 1;
    o = 1;
    f = 1;
    for k = 1:length(rlpks)

        %bc of length disparity (one less min
identified than pks), use first minAP for first 2 rlpks
        if k==1

            height2(o) = rlpks(k) - minAP(1);
            if height2(o)>APthreshReal
                rlpks2(u) = rlpks(k);
                rllocs2(u) = rllocs(k);
                kind(u) = k;
                u = u+1;
            else
                fakepks(f) = rlpks(k);
                f = f+1;
            end
            o=o+1;
        elseif k==2
            height2(o) = rlpks(k) - minAP(1);
            if height2(o)>APthreshReal %if height
above thresh, keep, if not save to fakePks variables and
don't use

                rlpks2(u) = rlpks(k);
                rllocs2(u) = rllocs(k);
                kind(u) = k;
                u = u+1;
            else
                fakepks(f) = rlpks(k);

```

```

        f = f+1;
    end
    o=o+1;
else
    height2(o) = rlpks(k) - minAP(k-1);
    if height2(o)>APthreshReal
        %           realAP(o) = 1;
        rlpks2(u) = rlpks(k);
        rllocs2(u) = rllocs(k);
        kind(u) = k;
        u = u+1;
        %           o = o+1;
    else
        %           realAP(o) = 0;
        fakepks(f) = rlpks(k);
        f = f+1;
        %           o = o+1;
    end
    o=o+1;
end

%           pause

end %%%end second AP height check

%dummy var for final loop below
fminus1 = f-1;

%%assign rlpks2 to MN15HT_161208 Structure
if fminus1==length(rlpks) %if no peaks
identified above 'real' threshold, then save zero array to
these vars
        MN15HT_161208.pks(:,i) = [0, zeros(1,120-
1)];
        MN15HT_161208.locs(:,i) = [0, zeros(1,120-
1)];

    else %else save the actual values for real phks
and rl locations of the peaks
        MN15HT_161208.pks(:,i) = [rlpks2,
zeros(1,120-length(rlpks2))];
        MN15HT_161208.locs(:,i) = [rllocs2,
zeros(1,120-length(rllocs2))];
    end
end

```



```

        %%% plots for visualization/troubleshooting %%%
        %%% verify that the script identified all peaks
that you would
        %%% deem real AP's.... if not, adjust threshold
variable or
        %%% minimum distance between peaks (dist
variable on line 229)
        figure(1)
        subplot(2,1,1)
        plot(chSteps)
        hold on
        plot(istep, chSteps(istep), 'go')
        subplot(2,1,2)
        plot(chSpikes)
        hold on
        plot(range, thresh2plot, 'r')
        plot(range(rllocs), rlpks, 'ro')
        if fminus1==length(rlpks) %if current step does
not produce APs above threshold, do not plot, if there are
APs above thresh, plot

        else
            plot(range(rllocs2), rlpks2, 'go')
        end
        plot(range, avgbase2plot(i,:), 'k')
        plot(range(locMinAP), minAP, 'ko')


        %%% calculate firing frequencies

        if fminus1==length(rlpks)

        else
            if length(rlpks2)>1
                freqVect(1) = 0;
                for j = 2:length(rlpks2)
                    freqVect(j) = 1/((1/fs)*(rllocs2(j)
- rllocs2(j-1)));

                end
            else
                disp('only one spike')
                freqVect = 0;

```

```

        end
    end
    %     length(freqs)
    %     pause

    %%%ASSIGN MORE VARIABLES TO STRUCTURE
    if fminus1==length(rlpks)
        MN15HT_161208.frequencies(:,i) = [0,
zeros(1,120-1)];
        MN15HT_161208.spikeAmps(:,i) = [0,
zeros(1,120-1)];
    else
        MN15HT_161208.frequencies(:,i) = [freqVect,
zeros(1,120-length(freqVect))];
        MN15HT_161208.spikeAmps(:,i) =
[height2(kind), zeros(1,120-length(height2(kind)))];
    end

    end
end

    %%% clear all variables for next loop through to avoid
problems with
    %%% saving over local variables
    clear locmin
    clear pks1
    clear locs1
    clear rlpks
    clear rllocs
    clear rlpks2
    clear rllocs2
    clear minAP
    clear locMinAP
    clear height2
    clear pks
    clear locs
    clear freqVect
    clear kind
    clear fakepks
    clear minimum
    clear idxmin

end

    %%% finally clear all variabls except structure and save it

```

```
clearvars -except MN15HT_161208
save MN15HT_161208
```

Script 2: Plot firing frequency profiles and calculate firing rates and SFA

```
% Plot firing freq profiles and calculate FF and SFA
variables for analysis
%This script will load in the structure data from the Pt 1
script and use
%the frequency points to plot the firing frequency profile
for each current
%step
% From frequency profile, the following variables are
calculated
%currentsMean - average current amplitude injected for
given neuron
%NumCurrentsSFA - # of current steps, the neuron exhibited
SFA for
%NumStepsTotal - total # steps the current was injected
with
%SAIinitMean - average initial phase accommodation index
%SAIlateMean - average late phase accommodation index
%InstFFMean - average Inst firing rate
%SSffMean - Average steady state firing rate
%SAIMean - average overall accommodation index
%InstFFRange - max and min inst firing rates for the neuron
%SSffRange - max and min steady state firing rates for the
neuron
%avgFFRange - max and min average firing rate for the
neuron.. *only
%
% calculated for current injection that did NOT
elicit SFA
%SAIRange - max and min accommodation indices for the
neuron, only
%
% calculated for current amplitudes that elicited
adaptation
%SAIbyCurrent - SAI for minimum current amplitude injected
and SAI for
%
% maximum current amplitude injected
%currents - max and min current amplitudes the cell was
injected with
%SAIallRange - max and min SAI, similar to SAIRange
variable except this
%
% variable is calculated for ALL current
amplitudes, mainly
%
% used to identify if cell accelerates firing
%SAIinitRange - max and min initial phase SAI for the
neuron
```

```

%SAIlateRange - max and min late phase SAI for the neuron

% Script below
%clear workspace before running script and close all
figures
clear all
clc
close all

%load data set
load MN15HT_161208
%structure that includes amplitudes, spikes, pks,
frequencies, spikeAmps,
locs

%define some variables
amps = MN15HT_161208.amplitudes;
spikes = MN15HT_161208.spikes;
pks = MN15HT_161208.pks;
frequencies = MN15HT_161208.frequencies;
spikeAmps = MN15HT_161208.spikeAmps;
locs = MN15HT_161208.locs;

%Save current amplitudes with step number to find specific
steps that had a
%specific accommodation index...only used for making
figures
for i = 1:length(amps)

    Amps_stepNums(i,:) = [amps(i) i];

end

%sort Amplitudes Vector, and along with it frequencies from
smallest to
%largest
[ampsOrdered,idxOrdered] = sort(amps);

for i= 1:length(idxOrdered)
    freqsOrdered(:,i) = frequencies(:,idxOrdered(i));
end

% the loop below runs through all current steps that neuron
was injected
% with
k = 1;

```

```

f = 1;
for i= 1:size(pks,2) %is to say 1:100

    %first, find all frequency points that are 0
    idx = find(freqsOrdered(:,i)==0);

    %remove current pulses with only 1 frequency (should
    correspond to 2
    %spikes)
    if
length(idx)==length(freqsOrdered(:,i))||length(idx)==(length
h(freqsOrdered(:,i))-1)
        %do nothing
        l=1;
        zeroFreqs = freqsOrdered(:,i);
    else%if there are at least 2 frequency points
        amps4Real(f) = ampsOrdered(i);%keep track of curent
        amplitude that elicited >2 freq points
        f = f+1;
        %initialize m for following loop
        m =1;
        for j = 1:length(freqsOrdered(:,i)) %remove all
zero points from
            %frequency array...remember in
            %part 1, we saved all frequency
            %points to an array that was
            %1x120 points, so if there were
            %30 freq points, the first 30
            %frequencies would be real
            %frequencies and the last 90
            %points would just be zeros
            if freqsOrdered(j,i)==0
                idxzero = j;
            else
                o = 1;
                freq2plot(m,k) = freqsOrdered(j,i);
            %frequency to plot for frequency profile
                m = m+1;
            end

        end

        k = k+1;
    end
end
end

```

```

p=1;
b=1;
i4Avg = 1;
accomIdx = 1;
AvgFF(1) = 0;
i4SAI = 1;

%now run through loop for all REAL frequency profiles
(profiles w zero's
%removed and at least 2 freq points
for i = 1:size(freq2plot,2)
    % i
    freq2plotCurrent = freq2plot(:,i);%frequencies to plot
    for the current pass through the loop

        g = 1;
        for k = 1:length(freq2plotCurrent)
            freq2plotCurrent(k);
            if freq2plotCurrent(k)==0 %remove 0 entries in
frequency variables
                %nothing - ignore these entries
                l=1;

            else
                freq2plot4Real(g) = freq2plotCurrent(k);
                g = g+1;

            end
        end
    end

    %diff between first and last freq point to determine if
adaptation
    %occured
    firstLastDiff = freq2plot4Real(length(freq2plot4Real))
- freq2plot4Real(1);
    diff(p) = freq2plot4Real(length(freq2plot4Real)) -
freq2plot4Real(1);

    %If first/last diff is negative (indicating adaptation
occured),
    %calculate max inst firing rate
    if firstLastDiff<0
        freq4maxFF(p) = max(freq2plot4Real);
        idxFreqMax =
find(freq2plot4Real==max(freq2plot4Real));

```

```

else
    display('Cell accelerates')%if first/last diff>0,
cell accelerates
    %         freq4maxFF(p) = freq2plot4Real(1);
    idxFreqMax =
find(freq2plot4Real==max(freq2plot4Real));
end

    if firstLastDiff>-4 %if the difference between first
and last freq point >-4,
        %adaptation did not occur, so calculate avg
        %firing rate and SAIall variable
        AvgFF(i4Avg) = mean(freq2plot4Real);
        SAIall(i4SAI) =
(1/freq2plot4Real(length(freq2plot4Real)))/(1/freq2plot4Rea
l(1));
        i4Avg = i4Avg+1;
        i4SAI = i4SAI+1;
        p=p+1;
    elseif firstLastDiff<0%should be for all traces that
exhibit SFA

        %spike accomodation index calculation
        if idxFreqMax==1||2
            SpikeAccomIdx(accomIdx) =
(1/freq2plot4Real(idxFreqMax))/(1/freq2plot4Real(length(fre
q2plot4Real)));
            SAIall(i4SAI) =
(1/freq2plot4Real(idxFreqMax))/(1/freq2plot4Real(length(fre
q2plot4Real)));
            i4SAI = i4SAI+1;
            accomIdx = accomIdx+1;
            if length(freq2plot4Real)>13 % if/else loops to
calculate initial and late phase
                                                    % accommodation
indices, if full
                                                    % profile,
initial is firstISI/4th
                                                    % ISI and late
is 5th ISI/last ISI
                                                    %^this changes
if freq profile has
                                                    %less points
though

```

```

        SAIinit(p) =
(1/freq2plot4Real(idxFreqMax))/(1/freq2plot4Real(4));
        SAIlate(p) =
(1/freq2plot4Real(5))/(1/freq2plot4Real(length(freq2plot4Re
al)));
        SSff(p) =
mean(freq2plot4Real(length(freq2plot4Real)-
10:length(freq2plot4Real)));
        p = p+1;
        elseif length(freq2plot4Real)>8
            SAIinit(p) =
(1/freq2plot4Real(idxFreqMax))/(1/freq2plot4Real(4));
            SAIlate(p) =
(1/freq2plot4Real(5))/(1/freq2plot4Real(length(freq2plot4Re
al)));
            SSff(p) =
mean(freq2plot4Real(length(freq2plot4Real)-
4:length(freq2plot4Real)));
            p = p+1;
            elseif length(freq2plot4Real)>3 %if there are
only between 3 and 8 frequency points, don't calculate late
phase SAI
                SAIinit(p) =
(1/freq2plot4Real(1))/(1/freq2plot4Real(4));
                p = p+1;
            else
                SAIinit(p) =
(1/freq2plot4Real(1))/(1/freq2plot4Real(2));
                p = p+1;
            end
            else %if the index of the max fre point is NOT the
first or second point, rarely occurs
                SpikeAccomIdx(accomIdx) =
(1/freq2plot4Real(1))/(1/freq2plot4Real(length(freq2plot4Re
al)));
                SAIall(i4SAI) =
(1/freq2plot4Real(1))/(1/freq2plot4Real(length(freq2plot4Re
al)));
                i4SAI = i4SAI+1;
                accomIdx = accomIdx+1;
                if length(freq2plot4Real)>13
                    SAIinit(p) =
(1/freq2plot4Real(idxFreqMax))/(1/freq2plot4Real(4));
                    SAIlate(p) =
(1/freq2plot4Real(5))/(1/freq2plot4Real(length(freq2plot4Re
al)));

```



```

        SSff(p) =
mean(freq2plot4Real(length(freq2plot4Real)-
10:length(freq2plot4Real)));
        p = p+1;
        elseif length(freq2plot4Real)>8
            SAIinit(p) =
(1/freq2plot4Real(idxFreqMax))/(1/freq2plot4Real(4));
            SAIlate(p) =
(1/freq2plot4Real(5))/(1/freq2plot4Real(length(freq2plot4Re
al)));
            SSff(p) =
mean(freq2plot4Real(length(freq2plot4Real)-
6:length(freq2plot4Real)));
            p = p+1;
            elseif length(freq2plot4Real)>3
                SAIinit(p) =
(1/freq2plot4Real(1))/(1/freq2plot4Real(4));
                p = p+1;
            else
                SAIinit(p) =
(1/freq2plot4Real(1))/(1/freq2plot4Real(2));
                p = p+1;
            end
        end
        else %for all traces exhibiting acceleration where last
freq point is at least 5Hz higher than first, rarely
happens

            SAIall(i4SAI) =
(1/freq2plot4Real(1))/(1/freq2plot4Real(length(freq2plot4Re
al)));
            i4SAI = i4SAI+1;

        end

%general plot fo basic script
figure(1)
plot(freq2plot4Real,'-o','color',rand(1,3))
legendEntries{i} = [num2str(amps4RealCurrent)];
legend([legendEntries, num2str(amps4RealCurrent)]);
hold on

%clear vars
clear freq2plot4Real
clear freq2plotCurrent

```

```

end

%find indices where firstLastDiff<-4 - traces that
underwent SFA in order to
%find currents that elicited SFA, also indices where
idxSlope = find(diff<-4);
idxSlopeNoAdapt = find(diff>-4);

%current amplitudes that elicited SFA and didn't
Amps4AvgWslopeUnder10 = amps4Real(idxSlopeNoAdapt);
currentAmps4sfa = amps4Real(idxSlope);
currentAmps4avg = amps4Real(idxSlopeNoAdapt);

%inst FF max and min
InstFFRange = [min(freq4maxFF(idxSlope)) max(freq4maxFF)];

%idx for SSff calculation
idxSSffNonZero = find(SSff>0);
SSFF = SSff(idxSSffNonZero);

%indices for currents used for SAI initial and late
calcualtion
idxSAIinitNonzero = find(SAIinit>0);
idxSAIlateNonzero = find(SAIlate>0);

%assign variables to structure to data structure
MN15HT_161208.FFslopes = InstFFSLOPErange;
MN15HT_161208.FFRange = InstFFRange;
MN15HT_161208.SSff = SSff;
MN15HT_161208.CurrentAmps4slopesUnder10 =
Amps4AvgWslopeUnder10;
MN15HT_161208.FFavg = AvgFF;
MN15HT_161208.spikeAccomodationIndex = SpikeAccomIdx;
MN15HT_161208.currents4sfa = currentAmps4sfa;
MN15HT_161208.SAIlate = SAIlate;
MN15HT_161208.currents4avg = currentAmps4avg;

%for loop to also save the step Number that a prticular SAI
%was calculated for...mainly for making figures to make it
easier to find
%specific steps that had Avg firing rates
for i = 1:length(SAIlate)
    stepNum(i) = idxOrdered(idxAmps4Real(i));
end

```

```

for i = 1:length(AvgFF)
    stepNum4AvgFF(i) = idxOrdered(idxAmps4Real(i));
end
MN15HT_161208.StepNums = stepNum;
MN15HT_161208.AmpsWstepNums = Amps_stepNums;

%more variables to save to structure
avgFFwCurrents =
[transpose(AvgFF),transpose(amps4Real(idxSlopeNoAdapt))];
MN15HT_161208.avgFFwCurrents =
[transpose(AvgFF),transpose(amps4Real(idxSlopeNoAdapt))];
SAIinit(idxSAIinitNonzero);
SAIlate(idxSAIlateNonzero);
SAIinitRange = [(min(SAIinit(idxSAIinitNonzero)))
(max(SAIinit))];
SAIlateRange = [(min(SAIlate(idxSAIlateNonzero)))
(max(SAIlate))];
SSffRange = [min(SSFF) max(SSFF)];
avgFFRange = [min(AvgFF) max(AvgFF)];
SAIRange = [(min(SpikeAccomIdx)) (max(SpikeAccomIdx))];
SAIbyCurrent = [(SpikeAccomIdx(1))
(SpikeAccomIdx(length(SpikeAccomIdx)))]; %by current
currents = [currentAmps4sfa(1)
currentAmps4sfa(length(currentAmps4sfa))];
SAIallRange = [(min(SAIall)) (max(SAIall))];

%means for variables
SAIinitMean = mean(SAIinit(idxSAIinitNonzero));
SAIlateMean = mean(SAIlate(idxSAIlateNonzero));
InstFFMean = mean(freq4maxFF(idxSlope));
SSffMean = mean(SSFF);
SAIMean = mean(SpikeAccomIdx);
currentsMean = mean(currentAmps4sfa);
NumCurrentsSFA = length(currentAmps4sfa);
NumStepsTotal = length(amps4Real);

%data for excel export, can display to MATLAB command
window to verify
my_table = table(currentsMean, NumCurrentsSFA,
NumStepsTotal, SAIinitMean, ...
SAIlateMean, InstFFMean, SSffMean, SAIMean, InstFFRange,
SSffRange, ...
avgFFRange, SAIRange, SAIbyCurrent, currents
,SAIallRange, ...
SAIinitRange, SAIlateRange)

```

```

%export to excel sheet
filename = 'MN15HT_161208.xlsx'; %will be saved to excel
sheet with this filename
writetable(my_table,filename,'Sheet',1,'Range','B1')

%clear all variables except neuron structure dataset
clearvars -except MN15HT_161208
save MN15HT_161208sfaMeans

```

Script 3: Script used for calculating muscle stiffness from torque meter data

```

%% Script info
%written by Preston Steele as part of masters thesis
%
% This script allows analysis of torque data from the
torque meter
% run through script first with leg=L and then again with
leg=R. You will
% need to change which leg it runs through manually. It
will run through
% all files for one leg, then you change the leg and it
will run through
% all files for the next leg
%
% input the kitNums and numFiles is the number of files
PER LEG
%
% the script will plot the data and allow you to click on
the graph
% to create upper and lower thresholds. It will then bring
up another plot
% and if the thresholded data looks good, left click on the
graph and stiffness will be analyzed
% if the data still needs additional thresholding, right
click on the graph and
% you will be able to threshold the data again to remove
outliers/noise

% stiffness is analyzed by doing linear regression of
torque data and finding the slope

```

```

% of the regression line - absolute value of slope is used
as stiffness
% measure

%% Main script
%%% clear all variables in workspace and close figures
clc
close all
clear all

%define armlength and conversion factor for grms to newtons
gConvertN = .0098/10;% 1 g~= 0.0098 N, div by 10 to get
units same as Drobyshevsky paper
ArmLength =7; %cm

%define kit numbers
kitNums = [1 2 3];
numFiles =length(kitNums);

%filename info for excel sheet the script will write to
filename2write = 'TorqueData.xlsx';

%Define leg script will analyze data for - CHANGE
leg = 'R';%CHANGE depending on leg

%for right leg, data will be saved in sheet 1 of excel
sheet and sheet 2
%for left leg
if leg=='R'
    cellNum = 6; % cell number to start writing to - 6 for
RIGHT, 7 for LEFT
    sheet=1;
else
    cellNum =7;
    sheet=2;
end

%initialize avgLoopSham85 variable before loop...This
variable iis mainly
%for making an average figure for presentation and i
typically would change
% the 'Sham85 part to reflect whether the data is from
ssham or HI kit and
% the birth date
avgLoopSham85 = zeros(length(kitNums),80);

```

```

% main loop begin
for i = 1:numFiles %run through each file, 1 by 1

    %filename to load
    filename = ['kit' num2str(kitNums(i)) 'rear' leg
'.xlsx']

    %read file and extract angle and force data
    data = readtable(filename);
    angle1 = table2array(data(:,2)); %angle1 in degrees
from 100-180
    Forceg = table2array(data(:,3)) -
mean(table2array(data(:,3)));%force in grams

    %convert angle1 to angle range from -40 to 40 degrees
    for j=1:length(angle1)

        angleReal(j) = -140+angle1(j);

    end

    %this is the main thresholding loop, h is arbitrarily
set to 100
    %so the maximum amount of times you can re-adjust the
threshold is 100
    %100 should be sufficiently high that you will never
need to readjust
    %the threshold that many times, but it allows you to
readjust as many
    %times as you want depending on how the data looks
    for h=1:100

        %find avg and sd of force data to set threshold at
mean+/- 2*sd
        %This is just to aid you in seeing the data falling
out of that
        %range, but you can still include points out of
this range if it
        %looks accurate
        avg = mean(Forceg);
        sd = std(Forceg);
        threshUpper = avg+2*sd;
        threshUpper2plot =
threshUpper*ones(1,length(angleReal));
        threshLower = avg-2*sd;
        threshLower2plot =
threshLower*ones(1,length(angleReal));

```

```

%figure that displays the force/angle plo
figure(1)
plot(angleReal,Forceg)
hold on
plot(angleReal, threshUpper2plot,'r')
plot(angleReal, threshLower2plot,'r')
[x,y] = ginput(2);%require user input to graph by
clicking graph first
                                %on the upper threshold, then
lower threshold

    upperThresh = y(1);%the y value of the first point
you click on the graph on
    lowerThresh = y(2);%the y value of the first point
you click on the graph on
    uT2plot =
ones(1,length(angle1))*upperThresh;%convert threshold value
to liens to plot on graph
    lT2plot = ones(1,length(angle1))*lowerThresh;
    k=1;

    if h<3
        replaceNum = 5;
        %remove poioints above threshold by seting them
equal to the
        %value of the point 5 points before or after,
depending on
        %replaceNum variable
        for g = 1:length(angle1)

            dataPt = Forceg(g);
            if dataPt > upperThresh%check force points
above upper threshold
                if k<replaceNum+1%if current force
index is less than replaceNum, then replace it with the
forec 5 samples ahead
                    forceReal(k) =
Forceg(g+replaceNum);
                    k = k+1;
                else%if the current force index is
great than replaceNum, replace it with force 5 samples
previous
                    forceReal(k) = Forceg(g-
replaceNum);
                    k = k+1;

```

```

        end
        elseif dataPt < lowerThresh %check points
below lower threshold
            if k<replaceNum
                forceReal(k) =
Forceceg(g+replaceNum);
                k = k+1;
            else
                forceReal(k) = Forceceg(g-
replaceNum);
                k = k+1;
            end
        else %if the force is within the bounds,
keep as actual force recording
            forceReal(k) = Forceceg(g);
            k = k+1;
        end

    end
    else %if there are certain points that remain above
thresholds after 3 adjustments, just set thhem equal to the
mean force
        for g = 1:length(angle1)

            dataPt = Forceceg(g);
            if dataPt > upperThresh
                forceReal(k) = mean(Forceceg);
            elseif dataPt < lowerThresh
                forceReal(k) = mean(Forceceg);
            else
                forceReal(k) = Forceceg(g);
                k = k+1;
            end

        end
    end

    %finl check on torque graph
    %figure below plots thresholded angles and if data
looks good,
    %click left mouse button on the graph, if need to
threshold again,
    %right click and it will go back in script to
threshold again
    figure(2)
    subplot(2,1,1)
    plot(angleReal,Forceceg)

```



```

hold on
plot(angleReal,uT2plot,'r')
plot(angleReal,lT2plot,'r')
hold off
subplot(2,1,2)
plot(angleReal,forceReal)
[x1,y1,button] = ginput(1);
if button==3 %corresponds to left click
    Forcecg = forceReal;
    close all
elseif any besides left click, script will go back
to thresholding adjustment again (previous loop)
    break1=1;
    break;
end
end

%next loop segments data into each -40 to 40 degree
cycle, since there
%are multiple cycles for each kit, this is done so we
can calculate an
%average cycle in the neext part
n = 1;
for p = 1:floor(length(angle1)/80)

    newAngle(:,p) = angleReal(n:n+(80-1));
    newData(:,p) = forceReal(n:n+(80-1));
    n = n+80;
end

if floor(length(angle1)/80)==1 %if only 1 cycle in
data, shouldn't happen often
    avgLoop = transpose(newData);%avg loop just equals
the data, since only 1 loop
    avgAngle = transpose(newAngle);

    %again, the beloww variable is mostly for
preesentaiton figures,
    %change name depending on sham/HI and birthdate
    avgLoopSham85(i,:) = avgLoop;

    %regression calculation, multiple ways to do this,
but this
    %function makes it easy

```

```

        [r,m1,b] = regression(angleReal(1:80),
(transpose(newData(1:80)).*gConvertN).*(ArmLength/100));%convert
force to newtons and arm length to meters
        regFit = m1.*angleReal(1:80)+b;

        %plot averaged torque data (Nm) and regression line
        figure(3)
        plot(avgAngle,
(avgLoop.*gConvertN).*(ArmLength/100))
        hold on
        plot(angleReal(1:80),regFit,'r')
        hold off

        pause

    else
        avgLoop = mean(transpose(newData));
        avgAngle = mean(transpose(newAngle));
        avgLoopSham85(i,:) = avgLoop;
        sizeAvgHI = size(avgLoopSham85)
    %        pause

        %regression
        [r,m1,b] = regression(angleReal(1:80),
(avgLoop(1:80).*gConvertN).*(ArmLength/100));
        regFit = m1.*angleReal(1:80)+b;

        figure(3)
        plot(avgAngle, avgLoop.*ArmLength)
        plot(angleReal(1:80),regFit,'r')
        hold off
        pause
    end

    %calculate stiffness
    stiffness = abs(m1); %stiffness taken as absolute value
of regression slope
                                %units would be Nm/degree)

    %write all stiffness values to excel sheet
    cell2write = ['I' num2str(cellNum)];
    xlswrite(filename2write,stiffness,sheet,cell2write);
    cellNum = cellNum+2;

```

```

        %final if statement that clears all variables for next
time through the
        %loop and, if it's in the final loop, it just closes
figures
        if i == length(kitNums)
            close all
        else

            close all
            clear angle1
            clear angleReal
            clear Forceg
            clear forceReal
            clear newAngle
            clear newData
            clear avgLoop
        end
    end

    %average torque variable created for presentation figures
    newAvg = mean(avgLoopSham85);
    %can plot the above variable to see what it looks lilke or
    troubleshoot
    figure
    plot(avgAngle, newAvg)

    %clear all variables except the two stated ->only important
    for
    %presentation figures
    clearvars -except newAvg avgAngle

```

Appendix E: R Scripts

Script 1: t-testing for before/after 5HT comparisons

script used for paired (or unpaired) t -testing between before and after 5HT conditions

#####set working directory and load data from directory

setwd("C:/Users/Preston/Documents/R/CPsfaData")

my.data <- read.csv(file.choose()) #this file load will be the one that graphs are made from,
typically i've used a csv file that included both control

#and HI data so it shows control before, control after, HI b4, HI after

attach(my.data)

dim(my.data)

Load sham and HI data to be split into 4 groups total -> sham b4,
sham after, HI b4, HI after

#the below data sets should ONLY contain shamB4/shamAfter OR HIb4/HI after data, not
all combined -> if all combined, this won't work

DatSham <- read.csv(file.choose())

DatHlall<- read.csv(file.choose())

```
#calcualte length of data sets to split them in half
```

```
lengthDatSham<-nrow(DatSham)
```

```
lengthDatHIall<-nrow(DatHIall)
```

```
splitVar1<-lengthDatSham/2
```

```
splitVar2<-lengthDatHIall/2
```

```
v1<-c(splitVar1+1,lengthDatSham+1)
```

```
v2<-c(splitVar2+1,lengthDatHIall+1)
```

```
#separate into b4 and after 5HT vars
```

```
dfTestSham<-split(DatSham, cumsum(1:lengthDatSham %in% v1))
```

```
dfTestHIall<-split(DatHIall, cumsum(1:lengthDatHIall %in% v2))
```

```
#sham and HIall before/after 5HT
```

```
shamB4<-data.frame(dfTestSham$`0`)
```

```
HIallB4<-data.frame(dfTestHIall$`0`)
```

```
shamAfter<-data.frame(dfTestSham$`1`)
```

```
HIallAfter<-data.frame(dfTestHIall$`1`)
```

```
#dim check
```

```
dim(shamB4)
```

```
dim(HIallB4)
```

```
dim(shamAfter)
```

```
dim(HIallAfter)
```

```
#####
```

```
#split data in 4 groups, sham and HIall b4/after 5HT, make sure the typeCode data column  
has 4 levels in this case
```

```
Type2<-ordered(my.data$typeCode, levels = c("CTR before","CTR after", "HI before",  
"HI after"))
```

```
rdata<-factor(my.data$typeCode,labels=c("CTR before","CTR after", "HI before", "HI  
after"))
```

```
CellType<-ordered(rdata, levels = c("CTR before","CTR after", "HI before", "HI after"))
```

```
CellType
```

```
typeCode
```

```
#####
```

```
##### BELOW WORKS FOR PLOT!!!!!!!
```

```
### instal plot packages and libraries
```

```
install.packages("dplyr")
```

```
library(dplyr)
```

```
install.packages("ggpubr")
```

```
library("ggpubr")
```

```
install.packages("Rmisc")
```

```
library(Rmisc)
```

```
install.packages("extrafont")
```

```
library(extrafont)
```

```
font_import()
```

```
yloadfonts(device="win")    #Register fonts for Windows bitmap output
```

```
fonts()
```

```
##### Actual plot script #####
```

```
#i usually just do a find and replace for whatever variable is in the measureVar field below  
in order to plot different variables
```

```
myDataSummary      <-      summarySE(my.data,      measurevar="SAIMean",  
groupvars=c("CellType"), na.rm =TRUE, conf.interval = 0.95)
```

```
myDataSummary
```

```
attach(myDataSummary)
```

```
#####33
```

```
dodge <- position_dodge(width=0.9)
```

```
limits <- aes(ymax=SAIMean+se, ymin=SAIMean-se) #Set up the error bars
```



```

ggplot(myDataSummary, aes(y=SAIMean, x=CellType, fill=CellType), colour =
"CellType") +

geom_bar(position=dodge, stat="identity") +

scale_fill_manual(values = c("#C0C0C0", "#FF8000", "#0080FF", "#FF0000"))+

geom_errorbar(limits, position=dodge, width=.5)+

geom_point(data=my.data,aes(rdata,SAIMean), position=position_jitter(width=.15))+

ggtitle("Mean SAI before and after 5HT in CTR and HI group")+

xlab("Cell Type and Condition") +

ylab("Mean SAI") +

theme(plot.title = element_text(hjust = 0.5))+

theme(text=element_text(family="Times New Roman", face="bold", size=20)) #Times
New Roman, 12pt, Bold

#####End plot #####

##### running the t tests and normality tests

#####

#initialize stats lists

resultShapiro <- list()

```

```
resultShapiroB4 <- list()
```

```
resultShapiroAfter <- list()
```

```
tTestResults<-list()
```

```
WilcTest<-list()
```

```
#install and call necessary libiraries
```

```
library(car)
```

```
install.packages("FSA")
```

```
library(FSA)
```

```
install.packages("RVAideMemoire")
```

```
library(RVAideMemoire)
```

```
#define the following two variables depending on the type of t test you want to run
```

```
myDatb4<-shamB4
```

```
myDatAfter<-HIallAfter
```

```
#define which variables you'd like to test, based on column numbers in dataset
```

```
testVar<-names(myDatb4) #contains ALL column names
```

```
testVar
```

```
names2<-testVar[51:88]#Just the column you want to test
```

```
names2
```

```
### BEGIN FOR LOOP to run paired t test on all vars
```

```
for (i in names2){
```

```
  #find difference in observations to run normammlity test for pair t-test
```

```
  #d<- myDatAfter[[i]] - myDatb4[[i]]
```

```
  #normality test for paired t-test
```

```
  #resultShapiro[[i]] <- shapiro.test(d)
```

```
  #normality test for unpaired t-test?
```

```
  resultShapiroB4[[i]] <- shapiro.test(myDatb4[[i]])
```

```
  resultShapiroAfter[[i]] <- shapiro.test(myDatAfter[[i]])
```

```
###Paired t-test comparison, t test for normally distributed and wilcoxon signed rank test  
for non-normal: t test should be fine even for non normal data
```

```
# only if the sample size is large
```

```
#tTestResults[[i]] <- t.test(myDatb4[[i]], myDatAfter[[i]], paired = TRUE, alternative =  
"two.sided")
```

```
#WilcTest[[i]]<-wilcox.test(myDatb4[[i]], myDatAfter[[i]], paired = TRUE, alternative =  
"two.sided")
```

```
###Unpaired t-test and wilcoxon test
```

```
tTestResults[[i]] <- t.test(myDatb4[[i]], myDatAfter[[i]], paired = FALSE, alternative =  
"two.sided")
```

```
WilcTest[[i]]<-wilcox.test(myDatb4[[i]], myDatAfter[[i]], paired = FALSE, alternative =  
"two.sided")
```

```
}
```

```
#results
```

```
resultShapiro #if sig, NOT NORMAL
```

```
resultShapiroB4 #if sig, NOT NORMAL
```

```
resultShapiroAfter #if sig, NOT NORMAL
```

```
tTestResults
```

```
WilcTest
```

Script 2: Parametric and non-parametric group comparisons

#this script loads in group data and separates groups based on column of choice. It then runs normality tests and group comparisons to assess significance

```
setwd("C:/Users/Preston/Documents/R/CPsfaData") #set working directory
```

```
my.data <- read.csv(file.choose())
```

```
names(my.data) #display column names
```

```
dim(my.data) #dimensions check of data
```

#define indices of data of interest - i usually just included the separating variable (column 4 in this case) and then all variables i wanted to test

```
idx<-c(4,36:90)
```

```
my.data<-my.data[,idx]#redefine my.data
```

```
names(my.data)#recheck names of new my.data
```

```
dim(my.data) #dim check of new my.data
```

```
attach(my.data) #attach my.data so from here on you can refer to variables just by using their names and not my.data$$varname
```

```
## group data into groups of your choosing groups
```

```
# sham vs HIall based on typeCode column
```

```
Type4<-ordered(my.data$typeCode, levels = c("Sham MNs", "HI MNs"))
```

```
rdata<-factor(typeCode, labels=c("Sham MNs", "HI MNs"))
```

```
CellType<-ordered(rdata, levels = c("Sham MNs", "HI MNs"))
```

```
CellType
```

```
#group data into 4 groups
```

```
Type4<-ordered(my.data$typeCode, levels = c("sham","HI unaffected", "HI mild", "HI  
severe"))
```

```
rdata<-factor(typeCode, labels=c("sham","HI unaffected", "HI mild", "HI severe"))
```

```
injury<-ordered(rdata, levels = c("sham","HI unaffected", "HI mild", "HI severe"))
```

```
# can group based on age if you want
```

```
Type4<-ordered(my.data$AGE, levels = c("P1","P2", "P3","P4","P5"))
```

```
rdata<-factor(AGE, labels=c("P1","P2", "P3","P4","P5"))
```

```
injury<-ordered(rdata, levels = c("P1","P2", "P3","P4","P5"))
```

```
#####
```

```
##### PLOTTING PART BELOW
```

```
## import libraries for plotting
```

```
install.packages("dplyr")
```

```
library(dplyr)
```

```
#install and load library for plots
```

```
install.packages("ggpubr")
```

```
library("ggpubr")
```

```
### bar graph stuff below
```

```
install.packages("Rmisc")
```

```
library(Rmisc)
```

```
install.packages("extrafont")
```

```
library(extrafont)
```

```
font_import()
```

```
loadfonts(device="win")    #Register fonts for Windows bitmap output
```

```
fonts()
```

```
##### start plotting ##### change var below in measureVar field to plot  
different variable
```



```
myDataSummary <- summarySE(my.data, measurevar="AHPstartingPot",
groupvars=c("CellType"), na.rm =TRUE, conf.interval = 0.95)
```

```
myDataSummary
```

```
attach(myDataSummary)
```

```
#####33
```

```
dodge <- position_dodge(width=0.9)
```

```
limits <- aes(ymax=AHPstartingPot+se, ymin=AHPstartingPot-se) #Set up the error bars
based on standard error
```

```
ggplot(myDataSummary, aes(y=AHPstartingPot, x=CellType, fill=CellType), colour =
"Cell Type") +
```

```
geom_bar(position=dodge, stat="identity") +
```

```
scale_fill_manual(values = c("#C0C0C0", "#FF8000", "#0080FF", "#FF0000"))+
```

```
geom_errorbar(limits, position=dodge, width=.5)+
```

```
geom_point(data=my.data,aes(rdata,AHPstartingPot), position=position_jitter(width
=.15))+
```

```
ggtitle("AHPstartingPot")+
```

```

xlab("Cell type") +

ylab("AHPstartingPot") +

theme(plot.title = element_text(hjust = 0.5))+

theme(text=element_text(family="Times New Roman", face="bold", size=20)) #Times
New Roman, 12pt, Bold

#####

##### normality, ANOVA, kruskall wallis annd post-hoc tests below

#create test vaar with all names in dataset

testVar<-names(my.data)

testVar

IdxVars<-c(2:length(my.data))#index of variables you want to test

names2<-testVar[IdxVars] #defining the variables you want to test

names2

```

```
#initialize non parametric stats results
```

```
resultsKW<-list()
```

```
DT <- list()
```

```
#normality test initialize
```

```
resultsLevene <- list()
```

```
resultShapiro <- list()
```

```
#anova initialize
```

```
resultsPvalANOVA <- list()
```

```
resultsTukey<-list()
```

```
#import libraries to run these tests
```

```
library(car)
```

```
install.packages("FSA")
```

```
library(FSA)
```

```
install.packages("RVAideMemoire")
```

```
library(RVAideMemoire)
```

```
### BEGIN FOR LOOP
```

```
for (i in names2){
```

```
  #normality tests
```

```
  resultsLevene[[i]] <- leveneTest(my.data[[i]]~ rdata, data = my.data)
```

```
  resultShapiro[[i]] <- byf.shapiro(my.data[[i]]~ rdata, data=my.data)
```

```
  #non parametric tests
```

```
  resultsKW[[i]] <- kruskal.test( my.data[[i]]~rdata, data = my.data)
```

```
  # DT[[i]] = dunnTest(my.data[[i]]~ rdata, #dunn test post hoc test, comment out if only 2  
  groups being compared
```

```
  #           data=my.data,
```

```
  #           method="bh")
```

```
#parametric tests
```

```
##ANOVA testing below and post hoc
```

```
anova<-aov(my.data[[i]]~rdata, data=my.data)
```

```
resultsPvalANOVA[[i]]<- summary(anova)[[1]][["Pr(>F)"]][[1]]
```

```
#resultsTukey[[i]]<-TukeyHSD(anova) #tukey post hoc test, comment out if only 2  
ggroups being compared
```

```
}
```

```
#normality results
```

```
resultsLevene##significant = UNEQUAL VARIANCES
```

```
resultShapiro#significant = NOT NORMAL
```

```
#parametric results
```

```
resultsPvalANOVA
```

```
resultsTukey[["SSffRange_min"]]  
#<-can check specific variable like this
```

```
#non parm results
```

```
resultsKW
```

```
DT
```

```
##### lin regression testing below
```

```
datIdx<-c(3, 8, 13:14,20:21 )
```

```
dat<-my.data
```

```
dim(dat)
```

```
linearMod <- lm(typeCode ~  
SAIinitRange_1+SAIinitRange_2+SAIlateRange_1+SAIlateRange_2+SAIRange_1+SAI  
Range_2, data=dat)  
  
summary(linearMod)
```

Appendix F: Arduino Script for Torque Measuring Device

Script 1: Calibrate the load cell

//Load cell calibration sketch to be used to make sure load cell is outputting desired force level. Taken directly from the author below and used as they describe

/*

Example using the SparkFun HX711 breakout board with a scale

By: Nathan Seidle

SparkFun Electronics

Date: November 19th, 2014

License: This code is public domain but you buy me a beer if you use this and we meet someday (Beerware license).

This is the calibration sketch. Use it to determine the calibration_factor that the main example uses. It also

outputs the zero_factor useful for projects that have a permanent mass on the scale in between power cycles.

Setup your scale and start the sketch WITHOUT a weight on the scale

Once readings are displayed place the weight on the scale

Press +/- or a/z to adjust the calibration_factor until the output readings match the known weight

Use this calibration_factor on the example sketch

This example assumes pounds (lbs). If you prefer kilograms, change the Serial.print("lbs"); line to kg. The

calibration factor will be significantly different but it will be linearly related to lbs (1 lbs = 0.453592 kg).

Your calibration factor may be very positive or very negative. It all depends on the setup of your scale system

and the direction the sensors deflect from zero state

This example code uses bogde's excellent library: <https://github.com/bogde/HX711>

bogde's library is released under a GNU GENERAL PUBLIC LICENSE

Arduino pin 2 -> HX711 CLK

3 -> DOUT

5V -> VCC

GND -> GND

Most any pin on the Arduino Uno will be compatible with DOUT/CLK.

The HX711 board can be powered from 2.7V to 5V so the Arduino 5V power should be fine.

```
*/
```

```
#include "HX711.h"
```

```
#define DOUT 3
```

```
#define CLK 2
```

```
HX711 scale;
```

```
float calibration_factor = 3019;// worked for my 440lb max scale setup, 3014 works for  
500g max load cell and with 100g weight
```

```
void setup() {
```

```

Serial.begin(9600);

Serial.println("HX711 calibration sketch");

Serial.println("Remove all weight from scale");

Serial.println("After readings begin, place known weight on scale");

Serial.println("Press + or a to increase calibration factor");

Serial.println("Press - or z to decrease calibration factor");


scale.begin(DOUT, CLK);

scale.set_scale();

scale.tare(); //Reset the scale to 0


long zero_factor = scale.read_average(); //Get a baseline reading

Serial.print("Zero factor: "); //This can be used to remove the need to tare the scale. Useful
in permanent scale projects.

Serial.println(zero_factor);

}

void loop() {

```

```

scale.set_scale(calibration_factor); //Adjust to this calibration factor


Serial.print("Reading: ");

Serial.print(scale.get_units(), 1);

Serial.print(" lb"); //Change this to kg and re-adjust the calibration factor if you follow SI
units like a sane person

Serial.print(" calibration_factor: ");

Serial.print(calibration_factor);

Serial.println();


if(Serial.available())

{

    char temp = Serial.read();

    if(temp == '+' || temp == 'a')

        calibration_factor += 1;

    else if(temp == '-' || temp == 'z')

        calibration_factor -= 1;

}

}

```

Script 2: Operate Servo and record force from load cell into PLXDAQ excel sheet

// This script controls a servo motor for the torque meter and measures force outputs from the load cell

// It is meant to be used in conjunction with PLXDAQ program which allows the serial data from the load cell amplifier to be read directly into an excel spreadsheet.

// This script needs to be uploaded to the Arduino board, and then PLXDAQ must be opened and the device can be controlled from there

//

//

//

// loading necessary header files and important data and clock definitions -> do not edit

#include <AFMotor.h>

#include <Servo.h> //Servo library

#include <Wire.h>

#include "HX711.h" //Header file for the load cell amplifier/serial data converter

```
#define DOUT 3
```

```
#define CLK 2
```

```
////////////////////////////////
```

```
HX711 cell;
```

```
float calibration_factor = 3019; //should work to output 100g, if not seeming right, can  
recalibrate using the calibration script
```

```
Servo servo_test; //initialize a servo object for the connected servo
```

```
//define some variables that will be used throughout the script
```

```
float LoadVal = 0;
```

```
int angleStep = 2; //1 for 0.5 Hz, 2 for 1Hz, 4 for 2Hz<- CHANGE depending on freq  
desired
```

```
int angle = 100;
```

```
int HighLow;
```

```
float HighLowconvert;
```

```
float HLVolt;
```

```

//setup loop to be run once on start up

void setup()

{

    Serial.begin(38400); //make sure this matches the data rate set in PLXDAQ!

    Serial.println("ServosketchLoadCell sketch");

    cell.begin(DOUT, CLK);

    cell.set_scale();

    cell.tare(); //Reset the scale to 0

    cell.set_scale(calibration_factor);

    pinMode(11, INPUT); //define pin 11 as input to arduino

    // //initialize stuff for PLX DAQ ///

    Serial.println("CLEARDATA"); \

    Serial.println("LABEL, comp time, angle, Force (g)"); //Set up first row of spreadsheet for
label names

    Serial.println("RESETTIMER");

```

```

servo_test.attach(10);    // attach the signal pin of servo to pin 10 of arduino motor shield

// servo_test.writeMicroseconds(1500);    // 1.5 ms stay still signal

servo_test.write(100);//This corresponds to our initial angle of -40 degrees, needs to be
this since the platform impedes the servo from rotating to it's angle 0

//initialize variables

int angle = 100;

//long val = 0;

delay(5000);

}

//long val1 = 0;

//float count = 0;

//this loop is run through indefinitely

```

```

void loop()

{

  HighLow = digitalRead(11); //Read the output of the push button


  if (HighLow==HIGH) { //if HIGH, move torque arm and record torque, if LOW, do nothing


    for (angle = 100; angle < 180; angle += angleStep)  // command to move from 100
degrees to 180 degrees

    {

      servo_test.write(angle);          //command to rotate the servo to the specified angle


      Serial.print("DATA,TIME,");


      Serial.print(angle);


      Serial.print(",");


      Serial.println(cell.get_units(), 1);


      //delay to match the data output rate of the HX711


      delay(12.5); //must be 12.5 to be fit the 80Hz output rate


    }

```



```

    for (angle = 180; angle >= 101; angle -= angleStep) // command to move from 180
degrees to 100 degrees

    {

        servo_test.write(angle);          //command to rotate the servo to the specified angle

        //  Serial.print("angle:");

        Serial.print("DATA,TIME,");

        Serial.print(angle);

        Serial.print(",");

        Serial.println(cell.get_units(), 1);

        delay(12.5); //delay must be 12.5 to be consistent with 80Hz output rate of HX711

    }

}

else { //if button is not being pressed, idle at 100 degrees

```

```
servo_test.write(100);  
  
}  
  
}
```