THE EFFECTS OF AN ACUTE BOUT OF NEUROMUSCULAR ELECTRICAL STIMULATION ON ANABOLIC SIGNALING OF THE mTORC1 PATHWAY IN INDIVIDUALS WITH CHRONIC STROKE

by

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DEDICATION

We dedicate this thesis to Dr. Joni Mettler's grandfather, Leo Mettler, and Sydney's father-in-law, Bernie Richard.

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First and foremost I would like to thank Dr. Joni Mettler. I am thankful that she has been there every step of this thesis journey, for the invaluable experiences, knowledge and skills she has taught me. I am especially thankful she allowed me to piggyback on to her research. I would also like to thank my committee members, Dr. Williams and Dr. McCurdy. Dr. Williams, specifically for his help during muscle biopsies, his support and his feedback. I would like to thank Dr. McCurdy for his willingness to join my committee on short notice as well as his support and feedback.

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Finally, I thank my mom, Paula, and boyfriend, Brandon, for their unconditional support and love through this journey. Thank you God for I would not have been able to do any of this without your love, will and wisdom.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

ABSTRACT

Stroke causes limited ability to produce voluntary muscle contraction and movement on one side of the body leading to further muscle wasting and weakness. Neuromuscular electrical stimulation (NMES) is often used to facilitate involuntary muscle contraction; however, the effect of NMES on muscle growth in hemiparetic muscle is not clear. **PURPOSE:** To determine the skeletal muscle anabolic response of an acute bout of NMES in individuals with chronic stroke and healthy older adults. **METHODS:** This study employed a two-group, pretest/posttest design. Ten individuals (59 ± 2.81 years old) were divided into two groups, a chronic stroke group (STROKE: n = 4) or a healthy older adult control group (CON: n = 6). A muscle biopsy was obtained from the *vastus lateralis* of the hemiparetic leg for STROKE and the right leg for CON before and 30 min after the NMES intervention. The NMES protocol consisted of a 60 Hz stimulation train of 10 seconds on and 15 seconds off which was repeated for 60 minutes. Phosphorylation of mTOR and p70S6K were analyzed using the SDS-PAGE Western blot technique. Phosphorylation is expressed as the ratio of phosphorylated to total protein content. Data were analyzed using two-way repeated measures analysis of variance. Data are reported as mean ± SE with statistical significance set at $p < 0.05$. **RESULTS:** An acute bout of NMES increased the phosphorylation of mTOR after stimulation (CON: 0.67 ± 0.10 vs. 1.01 ± 0.06; STROKE: 0.64 ± 0.15 vs. 0.99 ± 0.14; $p < 0.01$) and p70S6K (CON: 0.85 ± 0.13 vs. 2.18 ± 0.32; STROKE: 1.03 ± 0.29 vs. 2.56 ±

0.96; $p < 0.01$) from resting levels to 30 min following the NMES treatment, respectively. Phosphorylated protein content was not different between STROKE and CON ($p > 0.05$) following NMES. **CONCLUSIONS:** These findings suggest that NMES, in addition to facilitating muscle contraction, may initiate cellular processes that facilitate skeletal muscle growth and strengthening in healthy older and post-stroke populations.

Key Words: neuromuscular, anabolic, stimulation, stroke

CHAPTER 1

Introduction

According to the American Heart Association, stroke is a leading cause of disability and is ranked fourth in causes of death in the United States (Go et al. 2013). It is estimated that approximately 795,000 Americans suffer from a new or recurring stroke each year (Go et al. 2013). Individuals suffering from stroke commonly experience hemiparesis, partial paralysis on one side of the body, leading to decreased participation in activities of daily living, decreased mobility and physical function as well as cognitive deficits (Kelly-Hayes et al. 2003; Mayo et al. 2002; Jin et al. 2006). Muscle function is often significantly impaired, leading to a reduction in muscle mass, strength, and power (Snyder-Mackler et al. 1995; Durigan et al. 2014). Rehabilitation for individuals who have suffered a stroke commonly includes physical, occupational, speech and cognitive therapies. Strength training, a voluntary exercise, is commonly included in physical rehabilitation programs following stroke to increase muscle strength and function (Ouillette et al. 2004; Pak and Patten 2008; Merring and Gobert 2011; Yang et al. 2006); however, following a stroke the individual can be left with little or no ability to perform voluntary muscle contractions. An alternative to strength training is neuromuscular electrical stimulation (NMES). Neuromuscular electrical stimulation is a therapeutic modality frequently used in physical rehabilitation to artificially induce muscle contraction. However, inconsistencies in the effectiveness of this treatment for muscle growth and strengthening are apparent throughout the literature.

In the late 1700s, Luigi Galvani performed the first observed motion caused by electrical stimulation by attaching electrical wire to the muscle of a severed frog leg

(Cambridge 1977). Then, again in 1831, Michael Faraday demonstrated that electrical current could trigger nerves in the muscle to produce a movement (Cambridge 1977). Voluntary muscle contraction is created by a signal in the motor cortex that is sent to the spinal cord where the signal synapses with the alpha motor neurons to produce a muscle contraction. However, NMES bypasses the motor cortex and the spinal cord, as muscle activation is initiated directly at the level of the muscle by depolarization of the sarcolemma via the electrical current delivered from the stimulating unit. In this way, the electrical stimulation creates an involuntary muscle contraction. Today, NMES is commonly used for muscle building and strengthening in a variety of settings including, but not limited to, physical therapy, occupational therapy, athletic training and for exercise training purposes (Minetto et al. 2013). Neuromuscular electrical stimulation is used as a treatment for a variety of neuromuscular diseases and disabilities, such as stroke (Glinsky et al. 2007; Newsam and Baker 2004), spinal cord injuries (Belanger et al. 2000; Crameri et al. 2000), cerebral palsy (Merrill 2009; Stackhouse et al. 2007) and orthopedic injuries (Durigan et al. 2014; Kim et al. 2010), to list a few. NMES has also been used to aid in pain management, increase circulation, and reduce edema, among other ailments (Maffiuletti 2010).

Several functional outcome studies suggest that NMES application may maintain muscle mass and/or restore function of the muscle during extended periods of immobilization (Gibson et al. 1988; Qin et al. 1997), prolonged hospital stays (Snyder-Mackler et al. 1995), and other circumstances that inhibit movement (Minetto et al. 2013). Research thus far, however, has been inconsistent in terms of muscular strength and muscle mass improvements in response to NMES treatment (Qin et al. 1997; Durigan

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Several studies, that measured animal and human skeletal muscle strength and/or mass, show NMES increases or maintains strength and or mass (Qin et al. 1997; Babault et al. 2010; Gibson et al. 1988; Broucherie et al. 2005; Kim et al. 2010; Bax et al. 2005; Gondin et al. 2005), whereas some do not (Durigan et al. 2014b; Kim et al. 2010; Bax et al. 2005; Gondin et al. 2005). Neuromuscular electrical stimulation initially prevented muscle mass loss in rats with an anterior cruciate ligament rupture, but later saw a decrease in muscle mass (Durigan et al. 2014b). Following three weeks of lower limb immobilization in rabbits, NMES prevented muscle atrophy (Qin et al. 1997). Human skeletal muscle strength and power were found to increase in young adult athletes following NMES application (Baboult et al. 2007), however, strength did not change in young and middle-aged adults following NMES treatment (Bax et al. 2005). As seen, data are inconclusive as to whether NMES increases or maintains skeletal muscle strength and or mass.

In regard to individuals with stroke, inconsistent data have also been found for functional grasp, a measure of muscle strength, in case studies with some indicating an increase in strength while some decreased or had no change following ≥ 7 months of percutaneous intramuscular electrical stimulation training in the hand (Chae and Hart 2003). Hand grip strength, following a four week NMES intervention, increased in subjects who received high frequency stimulation with no change in those who received low frequency NMES in older adults with chronic stroke (Doucet and Griffin 2013).

Additionally, following a three-week intervention that included 60 minutes of NMES five days per week, older adults who suffered from a stroke significantly increased maximal voluntary contraction (MVC), an indicator of strength, of the dorsiflexor in comparison to the placebo stimulation group (Yan et al. 2009). Functional mobility via the Timed Up and Go (TUG) test was also measured and no significant differences within or between groups were found (Yan et al. 2009). Walking speed has also been evaluated using a 20-meter walk test in stroke survivors and a significant increase in walking speed was found following a 12-week electrical stimulation intervention (Sabut et al. 2010). The findings of these studies (Yan et al. 2009; Sabut et al. 2010; Doucet and Griffin 2013; Price and Pandyan 2001; Chae and Hart 2003) show inconsistent data in walking function, skeletal muscle strength and muscle mass following treatment of electrical stimulation in those who have had a stroke and individuals with other neuromuscular dysfunction. Stroke patients are variable day to day in their functional ability making it difficult to determine if the inconsistent data observed in functional outcome studies are due to the day-to-day variability of these individuals or the study interventions.

Due to inconsistencies in the literature and variability in day-to-day physical function in clinical patients, there is a need to study muscle building processes at the metabolic level in human skeletal muscle tissue in response to NMES. Most studies that have examined anabolic response use a voluntary resistance training intervention (Dreyer et al. 2006; Drummond et al. 2009; Walker et al. 2011; Dreyer et al. 2010) with only two studies examining this response with NMES as the treatment (Wall et al. 2010; Dirks et al. 2015). Strengthening exercises are a conventional mode of rehabilitation for stroke

patients and several studies have examined anabolic signaling of the mammalian target of rapamycin complex 1 (mTORC1) pathway following voluntary resistance training exercise (Dreyer et al. 2006; Drummond et al. 2009; Walker et al. 2011; Dreyer et al. 2010). This complex is a primary pathway that affects skeletal muscle growth. Some of the specific proteins in mTORC1 pathway that stimulate or inhibit muscle growth in response to muscular exercise include: protein kinase B (Akt), mammalian target of rapamycin (mTOR), ribosomal protein S6 kinase beta-1 (S6K1), and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). Immediately following ten to 11 sets of ten repetitions of knee extension resistance exercise at 70% of one repetition maximum, it was found that skeletal muscle anabolism in healthy young adults decreased or demonstrated no change for Akt, mTOR, and 4E-BP1, however, significant increases in Akt, mTOR, and S6K1 were seen one and/or two hours post exercise, respectively (Dreyer et al. 2006; Dreyer et al. 2010; Drummond et al. 2009). Also, increased muscle protein synthesis, following an acute bout of resistance training, was found to be associated with increased mTORC1 signaling (Walker et al. 2011). Although several studies demonstrate an increase in anabolic signaling activity following voluntary resistance training in healthy adults (Dreyer et al. 2006; Dreyer et al. 2010; Drummond et al. 2009; Walker et al. 2011), often, stroke patients cannot perform voluntary muscle contractions, thus creating the need for NMES for increasing muscle mass, strength and function following a stroke. To our knowledge, no research on strength training and anabolic signaling in human hemiparetic skeletal muscle exists.

Similar to Doucet and Griffin (2013), an animal model found Akt and mTOR, anabolic signaling proteins, to significantly increase after high frequency stimulation,

whereas no significant change was noted for low frequency stimulation (Atherton et al. 2005). Only two studies, however, have examined metabolic mechanisms of the mTORC1 pathway of muscle growth in response to NMES in human skeletal muscle (Wall et al. 2012; Dirks et al. 2015). Wall and colleagues (2012) were the first to observe anabolic-signaling following NMES in skeletal muscle of six older type 2 diabetic men. Stimulating electrodes were adhered to the *quadriceps* musculature of both legs, while stimulation was only delivered to one randomly selected leg. Subjects underwent a one time bout of electrical stimulation that was set to stimulate for three seconds with a three second rest interval, for 60 minutes at a frequency of 60Hz. Stimulation intensity was set at a maximal level the subject was comfortable with to produce a visible muscle contraction and was increased for the first 30 minutes to maintain a visible muscle contraction. Muscle biopsies were obtained from the *vastus lateralis* of both the stimulated and control legs at 5 minutes and 2 and 4 hours post-NMES. No significant difference between control and stimulated legs for these anabolic signaling proteins, S6K1, mTOR, and 4E-BP1 was found at any time point (Wall et al. 2012). However, trends were observed for increased phosphorylated protein content of S6K1 (5 times higher) and mTOR (75% higher) and a 40% decrease in 4E-BP1 in the stimulated leg compared to the control leg. The second study observed the anabolic response to NMES in six comatose patients (Dirks et al. 2015). Patients were sedated, as part of a medically necessary treatment, for a minimum of three days in order to be eligible to participate in the study. NMES was administered two times a day, for 3.5 to 7.5 days depending on length of time the patient was sedated, for 30 minutes per treatment at a frequency of 100Hz and stimulation intensity was increased every 3 minutes to maintain visible

muscle contraction in the *quadriceps* musculature (Dirks et al. 2015). Significant changes were observed in the stimulated leg for phosphorylated mTOR (19 ± 5 % increase) and in both legs for total P70S6K (decrease), whereas no significant change was found in either leg for phosphorylated P70S6K and Akt as well as total mTOR and Akt following NMES. Again, this study observed the effects of NMES on anabolic signaling in middle-aged and older comatose adults. This study, however, has several limitations including a small sample size and the unknown effects of sedation and medications each patient received. Additionally, muscle fiber cross sectional area in young adults was preserved during five days of leg immobilization following NMES treatment (Dirks et al. 2014).

In an animal model, it has been shown that in paralyzed, immobilized and denervated skeletal muscles the anabolic responses decreased or were maintained following stimulation, supporting that this type of muscle is different than healthy skeletal muscle (Dreyer et al. 2008). While only two studies have examined the effects of NMES on anabolic signaling (Wall et al. 2012; Dirks et al. 2015) in humans, no studies have examined this response in older healthy or hemiparetic skeletal muscle. Furthermore, to our knowledge, no study has investigated how basal muscle anabolism is affected in human hemiparetic muscle of individuals affected by stroke.

Statement of Purpose

The primary purpose of this study was to 1) investigate basal anabolic signaling activity of the mTORC1 signaling pathway in skeletal muscle of chronic stroke individuals (hemiparetic) compared to healthy older adults and 2) examine the anabolic signaling response following a single bout of NMES in the *vastus lateralis* muscle in chronic stroke compared to healthy muscle of older adults. The relationship between

basal anabolic signaling in the *vastus lateralis* muscle and maximal knee extension strength in stroke individuals and healthy older adults was also assessed.

Hypotheses

This study addressed the following hypotheses:

1. Basal anabolic signaling of the mTORC1 pathway will be blunted in older hemiparetic muscle compared to older healthy muscle.
2. A single bout of NMES will increase anabolic activation of the mTORC1 pathway similarly in older hemiparetic muscle and older healthy muscle.
3. There will be a positive relationship between basal level anabolic signaling of the mTORC1 pathway and maximal knee extension strength in older hemiparetic and healthy muscle.

Operational Definitions

1. Chronic Stroke: Individuals who suffered a stroke at least six months prior to joining the study.
2. NMES Protocol: The protocol consisted of a single bout of 60 minutes with a duty cycle of 10 seconds on and 15 seconds off at a frequency of 60 Hz with a pulse duration of 200 μs at an intensity (mA) that produced a torque at 15% of subject's MVC.

Limitations and Delimitations

Limitations

1. This study was limited by the severity of the stroke and specific areas of the brain that were affected by the stroke.

Delimitations

1. This study was delimited to the 40 to 85 year old age range of the subjects.
2. This study was delimited to skeletal muscle of individuals with hemiparesis and healthy older adults.
3. This study was delimited to a 60 minute NMES protocol at a frequency of 60 Hz.

Significance

As previously stated, stroke is a leading cause of disability and ranked fourth in causes of death in the United States (Go et al. 2013). Stroke causes limited ability to produce voluntary muscle contraction and movement on one side of the body. A lack of voluntary movement causes muscles to atrophy further causing physical deficits, ultimately resulting in physical decline and an increased need for caregivers.

Resistance training is known to activate signaling pathways. The problem is that stroke patients may have impaired ability to perform voluntary strength training. Thus, NMES is often used to facilitate the contraction of muscles involuntarily. Mixed results have been reported pertaining to the effects of NMES on muscle growth, strength and function in stroke patients and individuals with other neuromuscular conditions. The proposed study may provide new information regarding the effectiveness of a single bout of NMES on hemiparetic muscle, specifically, how it will affect anabolic signaling activities, which are necessary for muscle hypertrophy and muscle function. Additionally, it may supply an understanding of basal anabolic signaling in hemiparetic and healthy adult muscle. The information gained will be used to enhance the efficacy of physical rehabilitation and determine if NMES is an effective treatment modality for recovery of

muscle mass, strength and function in those who have suffered from a stroke and those with neuromuscular dysfunction.

CHAPTER 2

Manuscript

The Effects of an Acute Bout of Neuromuscular Electrical Stimulation on Anabolic Signaling of the mTORC1 Pathway in Individuals with Chronic Stroke

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Kevin McCurdy. Ph.D.

ABSTRACT

Stroke causes limited ability to produce voluntary muscle contraction and movement on one side of the body leading to further muscle wasting and weakness. Neuromuscular electrical stimulation (NMES) is often used to facilitate involuntary muscle contraction; however, the effect of NMES on muscle growth in hemiparetic muscle is not clear. **PURPOSE:** To determine the skeletal muscle anabolic response of an acute bout of NMES in individuals with chronic stroke and healthy older adults. **METHODS:** This study employed a two-group, pretest/posttest design. Ten individuals (59 ± 2.81 years old) were divided into two groups, a chronic stroke group (STROKE: n = 4) or a healthy older adult control group (CON: n = 6). A muscle biopsy was obtained from the *vastus lateralis* of the hemiparetic leg for STROKE and the right leg for CON before and 30 min after the NMES intervention. The NMES protocol consisted of a 60 Hz stimulation train of 10 seconds on and 15 seconds off which was repeated for 60 minutes. Phosphorylation of mTOR and p70S6K were analyzed using the SDS-PAGE Western blot technique. Phosphorylation is expressed as the ratio of phosphorylated to total protein content. Data were analyzed using two-way repeated measures analysis of variance. Data are reported as mean ± SE with statistical significance set at $p < 0.05$. **RESULTS:** An acute bout of NMES increased the phosphorylation of mTOR after stimulation (CON: 0.67 ± 0.10 vs. 1.01 ± 0.06; STROKE: 0.64 ± 0.15 vs. 0.99 ± 0.14; $p < 0.01$) and p70S6K (CON: 0.85 ± 0.13 vs. 2.18 ± 0.32; STROKE: 1.03 ± 0.29 vs. 2.56 ± 0.96; $p < 0.01$) from resting levels to 30 min following the NMES treatment, respectively. Phosphorylated protein content was not different between STROKE and CON ($p > 0.05$) following NMES. **CONCLUSIONS:** These findings suggest that

NMES, in addition to facilitating muscle contraction, may initiate cellular processes that facilitate skeletal muscle growth and strengthening in healthy older and post-stroke populations.

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According to the American Heart Association, stroke is a leading cause of disability and is ranked fourth in causes of death in the United States (Go et al. 2013). It is estimated that approximately 795,000 Americans suffer from a new or recurring stroke each year (Go et al. 2013). Individuals suffering from stroke commonly experience hemiparesis, partial paralysis on one side of the body, leading to decreased participation in activities of daily living, decreased mobility and physical function as well as cognitive deficits (Kelly-Hayes et al. 2003; Mayo et al. 2002; Jin et al. 2006). Muscle function is often significantly impaired, leading to a reduction in muscle mass, strength, and power (Snyder-Mackler et al. 1995; Durigan et al. 2014). Rehabilitation for individuals who have suffered a stroke commonly includes physical, occupational, speech and cognitive therapies. Strength training, a voluntary exercise, is commonly included in physical rehabilitation programs following stroke to increase muscle strength and function (Ouillette et al. 2004; Pak and Patten 2008; Merring and Gobert 2011; Yang et al. 2006); however, following a stroke the individual can be left with little or no ability to perform voluntary muscle contractions. An alternative to strength training is neuromuscular electrical stimulation (NMES). Neuromuscular electrical stimulation is a therapeutic modality frequently used in physical rehabilitation to artificially induce muscle contraction. However, inconsistencies in the effectiveness of this treatment for muscle growth and strengthening are apparent throughout the literature.

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Due to inconsistencies in the literature and variability in day-to-day physical function in clinical patients, there is a need to study muscle building processes at the metabolic level in human skeletal muscle tissue in response to NMES. Most studies that have examined anabolic response use a voluntary resistance training intervention (Dreyer et al. 2006; Drummond et al. 2009; Walker et al. 2011; Dreyer et al. 2010) with only two studies examining this response with NMES as the treatment (Wall et al. 2010; Dirks et al. 2015). Strengthening exercises are a conventional mode of rehabilitation for stroke

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muscle contraction in the *quadriceps* musculature (Dirks et al. 2015). Significant changes were observed in the stimulated leg for phosphorylated mTOR (19 ± 5 % increase) and in both legs for total P70S6K (decrease), whereas no significant change was found in either leg for phosphorylated P70S6K and Akt as well as total mTOR and Akt following NMES. Again, this study observed the effects of NMES on anabolic signaling in middle-aged and older comatose adults. This study, however, has several limitations including a small sample size and the unknown effects of sedation and medications each patient received. Additionally, muscle fiber cross sectional area in young adults was preserved during five days of leg immobilization following NMES treatment (Dirks et al. 2014).

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Methods

Participants

This study employed a two-group, pretest/posttest design. Eleven individuals (59.8 ± 2.67 years old) were divided into two groups, a chronic stroke group (STROKE: n = 5) or a control group (healthy males and females; CON: n = 6). The average time since the onset of the stroke was 4.7 ± 0.57 years prior to study enrollment. Participants were recruited from San Marcos and surrounding areas via flyers, newspaper ads and meetings at stroke support groups. All participants met the following criteria: Inclusion criteria. Participants were 1) 40 to 85 years of age; 2) for the stroke group, had a stroke onset ≥ 6 months prior to the study start date; 3) able to communicate orally and provide informed consent; 4) able to follow three-step instructions; and 5) able to comprehend the responsibilities and procedures related to the study. Exclusion criteria. Participants were not enrolled if 1) contraindicating conditions for muscle biopsy were present, i.e., currently taking warfarin (Coumadin) or other anticoagulants; 2) contraindicating conditions for electrical stimulation were present, i.e., swollen, infected or painful areas on lower limbs, implanted pacemaker, or surgical hardware implants in the lower limbs; 3) currently taking medications that might affect metabolic data (i.e. insulin); or 4) if participating in therapy or a regular, rigorous strength training program involving the lower extremity within 2 months of the study. A health history phone screening was used to determine eligibility of participants based on the inclusion and exclusion criteria. Participants also obtained medical clearance (Appendix A) to participate in the study from their personal physician. All subjects provided informed consent and all procedures were approved by the Texas State University Institutional Review Board.

Data Collection

If participants qualified by the health history phone screen, they scheduled on a day and time that worked best for them to visit the Neuromuscular Physiology Laboratory at Texas State University on three different study days.

Study Day 1: Body Mass Index and Functional Assessment

On Study Day 1 participants signed the consent form, provided emergency contact information and completed payment forms. Day 1 also included body mass index assessment, functional testing, and muscle performance familiarization. Participants' height and weight were obtained with shoes off (Health-O-Meter Professional 500KL, Alsip, IL).

Following height and weight measures, participants were given a five-minute rest interval before proceeding to the functional tests. Participants performed three functional measures that were used as a comparison of functional mobility between the two study groups (CON and STROKE). The first functional measure was the Five Repetition Sit-to-Stand test. For this test, the participant transitioned from a seated position in a chair to fully standing (i.e., knees in full extension) and back to seated five times as fast, safely and accurately as they could. Participants performed three timed trials with a 30-second rest interval between each trial (Gee 2005; Bohannon 2006). Specific instructions for the Five Repetition Sit-to-Stand Test for healthy and stroke subjects are included in Appendix B.1. To reduce effects of fatigue, a ten-minute rest interval preceded the second and third functional measures, the Grip Strength test and the Timed Up and Go Test (TUG). Four minutes into the ten-minute rest interval the participants performed

three trials with each hand for upper body strength measure with a 30second rest interval between trials. Specific instructions for the Grip Strength test are included in Appendix B.2. The TUG test is used with older individuals as a measure of overall mobility. Participants were timed on how long it took to rise from a chair, walk three meters, turn around, walk back to the chair and sit back down on the chair (Shumaway-Cook 2000). Participants performed three trials with a 30 second rest interval between trials. Specific instructions for the TUG Test for CON and STROKE subjects are included in Appendix B.3.

Lastly, participants went through an orientation session on the isokinetic dynamometer (Biodex Systems 4 Pro, Shirley, NY) for muscle performance testing and practiced performing submaximal and maximal effort muscle contractions on both legs using the quadriceps for knee extension and hamstring muscles for knee flexion. The participant was properly seated and stabilized in the Biodex chair. Chair settings were recorded for use on Days 2 and 3. The familiarization protocol is in Appendix C.

Study Day 2: Muscle Function Testing

Study Day 2 occurred at least 48 hours after Day 1 to allow for muscle recovery following Day 1 testing. Quadriceps and hamstring muscle strength were measured using an isometric contraction with the knee angle set at 60 degrees on an isokinetic dynamometer (Biodex Systems 4 Pro, Shirley, NY). The Biodex System 4 has been found to be a reliable measure of strength (Gediminas et al. 2013; Alvares et al. 2015; Gunnarsson et al. 2011). All testing was performed on both legs and was conducted in random order. Leg order was determined using the Research Randomizer software

(Urbaniak & Plous 2013). Torque data was recorded by the Biodex software (Version 4.5, Shirley, New York) to a personal computer with LabChart software (Version 8, Bella Vista, Australia) through PowerLab 16/35 (ADInstruments, Colorado Springs, CO) via a specific cord for more accurate data analysis. Participants had five straps in total; two shoulder straps crossing the torso, one strap across the waist, one strap across the thigh of the leg being tested and one strap tightly securing the lower leg above the ankle (Figure 1). Before testing, participants completed a short orientation session before performing maximal voluntary contractions (MVC). Prior to performing MVCs subjects were given a ten-minute rest period to allow the muscles to return to a rested/baseline state. MVC, a measure of maximal strength, was performed isometrically for knee extension, first, and then knee flexion. Participants were instructed to kick out, for knee extension, as fast and forcefully as possible and hold that until told to relax (~4s). For knee flexion, participants were instructed to pull back as fast and forcefully as possible and hold that until they were instructed to relax (~4s). Participants performed three MVC's, with approximately four seconds of rest between contractions. Maximal torque was achieved if the participant's torque did not continue to increase over the three contractions. If maximal torque was not achieved, a fourth MVC was performed. Participants were given these specific instructions, "3-2-1 Go…go, go, go." Verbal encouragement was provided for the duration of each muscle contraction.

Study Day 3: Metabolic Study

Study Day 3 occurred at least one week after Day 2 to ensure there was no muscle fatigue, soreness, or residual metabolic effects from Day 2 testing. On Day 3, participants

arrived to the lab in the morning under fasted conditions (see Figure 2 for Study Day 3 timeline schematic).

Participants did not consume any food or beverages, other than water, after midnight the night prior to the study. When the participant arrived, they rested comfortably in a bed in the Neuromuscular Physiology lab for two hours to control for metabolic activity level. Muscle biopsy #1 was obtained two hours after the participant arrived to the lab (Figure 2). For the CON group, the muscle biopsy was obtained from the *vastus lateralis* muscle of the right leg. For the STROKE group, the muscle biopsy was obtained from the *vastus lateralis* muscle of the hemiparetic leg. Muscle biopsies were obtained by a trained specialist under aseptic conditions following standard techniques (Bergstrom 1975). The biopsy site was cleaned with sterile gauze, saturated in Betadine, starting at the incision site and working outward in a circular motion. In brief, each muscle biopsy (approximately 50 mg) was obtained under local anesthesia (1% lidocaine) from an incision in the *vastus lateralis* approximately 10-15 centimeters above the mid-patella, using a 5-mm Bergstrom biopsy needle. The incision was closed with steri-strips, bacitracin was applied and then covered with sterile gauze until the second biopsy was obtained. The second biopsy was obtained from the same incision and closed with medical grade glue or a single suture. Immediately after the muscle biopsy was obtained, the muscle sample was removed of adipose tissue, connective tissue and blood clots and then immediately preserved in liquid nitrogen. Frozen muscle tissue was placed in a labeled cryotube and stored at -80°C for later analysis.

Neuromuscular Electrical Stimulation Intervention

Following the muscle biopsy #1, to minimize effects of exercise, the participant was transported via a wheelchair to the Biomechanics Laboratory for NMES application. The participant was seated on the Biodex chair with knee at a 60 degree angle and strapped in with two shoulder straps, one waist strap, and a padded ankle strap (Figure 1). These straps were used to stabilize the participants' body. The NMES protocol was applied to the same leg from which the muscle biopsy was obtained. The leg was cleaned with alcohol swabs where the stimulating electrodes were placed. The participant was asked to shave the front of the thigh in the region of electrode placement prior to the visit.

Four, 3 X 5 inch, stimulating electrodes (ValuTrode Neurostimulation Electrodes, Fallbrook, CA) were placed at the proximal and distal ends of the *vastus lateralis* and *vastus medialis* muscles to artificially activate the quadriceps musculature (Figure 3). Stimulating electrodes were placed on the hemiparetic limb of persons with STROKE and to the right limb of the healthy CON group. The NMES intervention consisted of a single bout of electrical stimulation to the quadriceps musculature for a duration of 60 minutes. Neuromuscular electrical stimulation protocol used a direct current square waveform and was delivered with a duty cycle of 10 seconds on and 15 seconds off at a frequency of 60 Hz with a pulse width of 200 μs (Digitimer DS7A, Garden City, England). Neuromuscular electrical stimulation was delivered at an intensity that produced torque equal to 15% MVC monitored via the Biodex torque output. The MVC torque was determined from Day 2 MVC values. If this intensity was intolerable to the participant, the intensity that produced the highest amount of force and was tolerable was used. Torque production from the muscle declines during NMES programs due to natural

fatigue of the muscles; therefore, torque production was monitored every five minutes and the intensity of the stimulation was increased as needed every five minutes within subject tolerance to maintain an initial torque at 15% of the participant's MVC (See Appendix D). Torque output was recorded on an isokinetic dynamometer and the signal was transferred in real-time to the PowerLab analogue to digital (A/D) converter hardware and LabChart software for digital data output. The NMES stimulation protocol was also programmed and sent to the Digitimer stimulator system via the PowerLab A/D converter. Stimulation intensity was adjusted on the Digitimer system.

Data Analysis

Physical Function Measure Analysis

For both functional tests (Five Repetition Sit-to-Stand & TUG), the fastest time, out of three trials, was used to compare baseline physical function between the CON and STROKE groups. For the Grip Strength test, the largest measurement, out of the three trials, for each hand, was used to assess upper body strength. Grip strength data are expressed as a ratio of paretic to nonparetic limb for the STROKE group and for the CON group, limb side was matched to the paretic limb of the stroke group. Ratios for grip strength were used to account for any bilateral limb discrepancies in strength.

Body Mass Index Analysis

To obtain body mass index (BMI), height was converted from centimeters to meters (m) and body mass was converted from pounds to kilograms (kg). The equation used to determine BMI was mass divided by height squared (kg/m^2). Body mass index

values were classified according to American College of Sports Medicine standards (Pescatello et al. 2013).

Muscle Strength Analysis

MVC torque output (strength) was analyzed using LabChart software. MVC torque was determined by measuring all three contractions, or all four if there were four. The MVC with the highest torque was used for analysis. Maximum torque values were measured by evaluating the middle two seconds of each contraction. MVC data were normalized to body weight (kg) for analysis. This variable was used to compare baseline strength between study groups (CON and STROKE).

Western Blot Analysis

Muscle tissue from both biopsies was used to measure cell signaling for total mTOR, phosphorylated mTOR, total S6K1, and phosphorylated S6K1 (Cell Signaling Technology, Danvers, MA), as previously described (Dreyer et al. 2006). Briefly, samples of frozen muscle tissue were homogenized and the supernatant was removed. The supernatant was diluted in a 1:1 2x sample buffer and was boiled for 3 minutes. A Bradford assay analysis was used to quantify the protein concentration of each sample in duplicate (Bio-Rad SmartSpec plus spectrophotometer, Hercules, CA). Fifty micrograms (μg) of total protein for each sample was loaded in duplicate into each well of the gel, and samples were separated via SDS-PAGE gel electrophoresis (Criterion Blotter; Bio-Rad, Hercules, CA) at 150 V for 60 minutes. A standard and a molecular weight marker were added to each gel. Following electrophoresis, proteins were transferred to a membrane. The membrane was blocked for 60 minutes in either a 5% non-fat dairy milk solution or

bovine serum albumin solution, depending on primary protein being identified, to block other proteins. The membrane was incubated overnight on a rocker at 4°C with primary anti-body. Secondary anti-body was then applied and placed on a rocker for 60 minutes at room temperature for each protein. Blots were then incubated for 5 minutes with enhanced chemiluminescence reagent (ECL plus Western Blotting Detection System; Amersham Biosciences) and were imaged using an optical density imager (Fotodyne Inc., Hartland, WI) to determine optical density of each protein band. Band density was analyzed with Quantity One 1-D analysis Software (Version 4.5.2; Bio-Rad). Activity of each protein is expressed as a ratio of phosphorylated to total protein content. Biopsy #1 serves as the basal (pre-NMES) measure and biopsy #2 serves as the post-NMES measure.

Statistical Analysis

Statistical analysis was performed using SPSS statistical software (Version 22, IBM) and Microsoft Excel (Version 14.5.5). This study employed a two-way repeated measures analysis of variance (ANOVA) with group (CON and STROKE) and time (pre-NMES and post-NMES) as the independent factors to compare anabolic signaling activity in response to the NMES intervention. A Pearson Product Moment Correlation was used to examine the relationship between knee extensor strength (MVC) and basal (pre-NMES) anabolic signaling activity for mTOR and S6K1 for both CON and STROKE groups. Grip strength and MVC data are expressed as a ratio of paretic to nonparetic limb for the STROKE group and for the CON group, limb side was matched to the paretic limb of the stroke group. Descriptive data, physical function measures and

body mass index were compared by independent *t*-test with group as the independent factor. Data are reported as mean ± SE and statistical significance was set at $P \leq 0.05$.

Results

Subject Characteristics and Body Mass Index

Subject characteristics are displayed in Table 1. BMI was not significantly different between groups ($P = 0.11$). However, according to the ACSM BMI standards (Pescatello et al. 2013), average BMI for the STROKE group was classified as overweight and at increased risk for disease (28.72 ± 2.78 $kg \cdot m^{-2}$) while the CON group was classified as normal with no increased risk for disease (23.75 ± 1.05 $kg \cdot m^{-2}$).

Physical Function and Muscle Strength

For the TUG test, the STROKE group was significantly slower compared to the CON group (CON: 6.57 ± 0.19 s; STROKE: 9.16 ± 1.14 s; $P = 0.03$) (Table 2). Five Repetition Sit-to-Stand time was also significantly slower in the STROKE compared to the CON group (CON: 5.98 ± 0.55 s; STROKE: 8.52 ± 1.09 s; $P = 0.05$) (Table 2). Discrepancy in grip strength was not significantly different between groups ($P = 0.07$) when expressed as a ratio between limbs (Table 2).

The strength discrepancy between limbs for knee extension strength, as measured by MVC, was significantly greater in the STROKE group compared to the CON group ($P = 0.05$) when as expressed as a ratio to measure strength discrepancy between limbs (Figure 4). One subject from the STROKE group had severe osteoarthritis in the non-paretic knee; therefore, data from this subject were excluded from this analysis.

Anabolic Signaling

Phosphorylated protein content of key anabolic signaling proteins obtained from the *vastus lateralis* muscle of the CON and STROKE groups is displayed in Figure 5. Signaling protein data are expressed as a ratio of phosphorylated protein to total protein. One STROKE subject was not included in this analysis because muscle biopsy was not obtained (n = 4). An acute bout of NMES increased phosphorylation of mTOR after stimulation (CON: 0.67 ± 0.10 vs. 1.01 ± 0.06; STROKE: 0.64 ± 0.15 vs. 0.99 ± 0.14; P = 0.006) and S6K1 (CON: 0.85 ± 0.13 vs. 2.18 ± 0.32; STROKE: 1.03 ± 0.29 vs. 2.56 ± 0.96; P = 0.007) from basal levels to 30 minutes following the NMES treatment, respectively (Figure 5). An acute bout of NMES resulted in a 32.64% change for phosphorylated mTOR in the STROKE group and a 34.82% change in the CON group. Additionally, there was a 36.46% change for phosphorylated S6K1 in the STROKE group and a 60.79% change in the CON group. No significant differences for phosphorylated protein content between STROKE and CON groups were observed for mTOR (P = 0.85) or S6K1 (P = 0.59) and the interaction (group x time) for mTOR and S6K1 phosphorylated protein content was not significant (P > 0.05) (Figure 5).

MVC and Signaling Protein Relationship

A strong positive correlation was found between MVC knee extensor strength and pre-NMES phosphorylated protein content of S6K1 for the STROKE group (r = 0.999; P = 0.001) and a moderate negative correlation was found for the CON group (r = -0.494; P = 0.32) (Figure 76a). Negative weak and positive moderate correlations, respectively, were found between MVC knee extensor strength and pre-NMES phosphorylation

protein content of mTOR for CON (r = -0.288; P = 0.58) and STROKE groups (r = 0.439; P = 0.56) (Figure 6b).

Discussion

This is the first study to demonstrate that a single, 60-minute bout of NMES increases anabolic signaling activity in hemiparetic and healthy older adult skeletal muscle. The anabolic response was similar in hemiparetic and healthy older skeletal muscle despite impaired physical function in the STROKE group compared to the CON group for the knee extensor strength, TUG test and Five Repetition Sit-to-Stand test. To our knowledge, only two other studies have examined anabolic signaling in response to NMES in humans, but the stimulation protocols and participant populations were different in these studies (Wall et al. 2012; Dirks et al. 2015).

Anabolic Signaling

The present study demonstrates that following a single bout of NMES, skeletal muscle anabolic signaling activity increased. The increase in muscle anabolism observed in the present study is consistent with previous research which reported that 60 minutes of a single bout of NMES, set at a frequency of 60Hz and a stimulation train of three seconds on and three seconds off increased phosphorylated protein content of mTOR and S6K1 in diabetic men, however, the increase was not significant (Wall et al. 2012). The lack of a significant increase post-stimulation may be due to not having a controlled stimulation intensity during the NMES protocol. Participants received stimulation that was set at a level the subject was comfortable with and which produced a visible muscle

contraction. The intensity was increased for the first 30 minutes, of the 60 minute protocol, in order to maintain a visible muscle contraction (Wall et al. 2012).

In contrast, in the present study, the stimulation intensity was adjusted to achieve 15% MVC for all subjects, within subject tolerance, and torque output was monitored and increased every five minutes when torque produced by the quadriceps was less than 15% MVC. The stimulation intensity necessary to achieve a specific level of muscle activation may vary between subjects for a number of reasons, including, but not limited to rate of muscle fatigue, force potentiation, and subcutaneous fat (Mettler and Griffin 2010; Sayenko et al. 2014). In the present study, the muscle biopsy was obtained 30 minutes post-NMES, whereas Wall and colleagues (2012) obtained the muscle biopsies at 5 minutes and at 2 and 4 hours post-NMES. Anabolic protein phosphorylation status changes over the course of time following voluntary resistance training (Burd et al. 2010) and following NMES treatment (Wall et al. 2012); therefore, of the duration of time elapsed between completion of the NMES protocol and the muscle biopsy is important to note. The increase in muscle anabolism observed in the present study is also supported by previous research, in which it was found that a single bout of NMES increased skeletal muscle protein synthesis in diabetic men (Wall et al. 2012).

In comatose patients, a significant increase in phosphorylated mTOR was observed (19 ± 5 % increase), whereas no significant change was found for phosphorylated P70S6K status following a three to seven day NMES intervention that consisted of stimulation two times per day for 30 minutes at a frequency of 100Hz (Dirks et al. 2015). The study with comatose patients is different than the present study in that the patients were in a comatose state in which catabolic and inflammatory processes are

present. In addition, it was not specified at what time point the post-NMES biopsy was taken, other than it was taken on the last day of sedation (Dirks et al. 2015). Additionally, it is important to note that the comatose patients were nourished with a nutritional supplement, but it is not clear if the subjects were in a fasted or post-prandial state in relation to time of muscle biopsy. Skeletal muscle anabolism is greater in a post-prandial state compared to a fasted state in healthy adults (Symons et al. 2007). In the present study, subjects were fasted.

The data from the current study support the hypothesis that a single bout of NMES increases anabolic activation key signaling proteins of the mTORC1 pathway similarly in older hemiparetic and older healthy muscle. Based on the findings of the present study, NMES is a modality that could be used to prevent muscle atrophy and to improve muscle strength and function for individuals suffering from chronic stroke and long-term illnesses that cause prolonged limb disuse.

Physical Function

Several functional outcome studies suggest that NMES application may maintain muscle mass and/or restore function of skeletal muscle during extended periods of immobilization (Gibson et al. 1988; Qin et al. 1997), prolonged hospital stays (Snyder-Mackler et al. 1995), and other circumstances that inhibit movement, such as stroke (Minetto et al. 2013). In the present study, the STROKE group was significantly slower compared to the CON group in the Five Repetition Sit-to-Stand and TUG test times. These physical function data demonstrate that individuals in the STROKE group were physically impaired in terms of muscle function compared to individuals in the healthy

older control group. Regardless of differences between STROKE and CON group for the Five Repetition Sit-to-Stand and TUG test, neither group was classified at an increased risk for falls (Bohannon 2006). As for grip strength, there was no significant difference between groups. This may be due to the heterogeneous nature of the severity of physical impairments following a stroke. These results differ from previous research between stroke and control participants (Boissy et al. 1999). There was a significant difference between groups, with the control group being stronger than the stroke group; and there was no significant difference in hand grip strength between hands for the control group. However, there was a significant difference between hands for the stroke group (Boissy et al. 1999). The paretic side was weaker for maximal knee extensor strength compared to the non-paretic side in the STROKE group, which was expected. This finding is in line with another study in which knee extensor strength was also different between affected and non-affected legs in individuals who suffered a stroke (Watanabe et al. 2015). A strong positive correlation was observed between MVC knee extensor strength and basal phosphorylated protein content for S6K1 for the STROKE group. This illustrates, for the STROKE group, that greater MVC appears to be related to greater phosphorylated S6K1 protein content in the muscle at basal levels of anabolism. These data support the hypothesis that there would be a positive relationship between basal level anabolic signaling of the mTORC1 pathway and maximal knee extensor strength in older hemiparetic muscle. To our knowledge, no previous research has addressed the relationship between strength and anabolic activation. Based on these limited data, it is possible that individuals who have greater muscular strength have higher levels of anabolism during rest and fasting periods which could contribute muscle growth and to

the greater strength levels these individuals are able to produce. Further investigation of this relationship is warranted with more subjects who are healthy and with neurological impairment.

Limitations

We acknowledge that there were several limitations to this study. Although an inherent limitation to stroke research in humans, this study was limited by differences in the specific areas of the brain that were affected by the stroke and by the degree of physical dysfunction of the subjects with chronic stroke. Additionally, this study was limited by the small sample size. It was difficult to recruit older subjects and individuals who have had a stroke, who were healthy enough, to meet the study inclusion and exclusion criteria. Although a larger sample size would have resulted in greater statistical power and may have resulted in significant differences for some additional tests, we did find a significant increase in our primary dependent variable, phosphorylated protein content of key anabolic signaling proteins, following a single bout of NMES. Additionally, several tests of physical function were significantly different between the stroke and control groups.

Conclusions

The findings of the present study suggest that a 60-minute bout of NMES, in addition to facilitating muscle contraction, is an effective treatment for initiating skeletal muscle growth and strengthening processes in individuals with neurologically impaired muscle and older healthy skeletal muscle. Both the STROKE and CON groups demonstrated a significant increase in both phosphorylated mTOR and S6K1 protein

content following a single bout of NMES. In conclusion, NMES appears to be an effective physical rehabilitation treatment to promote skeletal muscle growth and strengthening in paretic and healthy older skeletal muscle.

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CHAPTER 3

Summary and Recommendations for Future Research

Summary

The primary purpose of this study was to 1) investigate basal anabolic signaling activity of the mTORC1 signaling pathway in skeletal muscle of chronic stroke individuals (hemiparetic) compared to healthy older adults and 2) examine the anabolic signaling response following a single bout of NMES in the *vastus lateralis* muscle in hemiparetic muscle of individuals with chronic stroke compared to healthy muscle of older adults. The relationship between basal anabolic signaling in the *vastus lateralis* muscle and maximal knee extension strength in stroke individuals and healthy older adults was also assessed.

While only two studies have examined the effects of NMES on anabolic signaling (Wall et al. 2012; Dirks et al. 2015) in humans, no studies have examined this response in older healthy or hemiparetic skeletal muscle. Furthermore, to our knowledge, no study has investigated how basal muscle anabolism is affected in human hemiparetic muscle of individuals affected by stroke.

This study employed a two-group, pretest/posttest design. Eleven individuals were divided into two groups, a chronic stroke group or a healthy older adult control group. Several functional outcome measures were evaluated, which included a TUG test, Five Repetition Sit-to-Stand test and Grip Strength test to evaluate physical impairments in the stroke group compared to the control group. Strength of lower limbs was assessed via MVC of knee extensors to compare between STROKE and CON groups. A muscle biopsy was obtained from the *vastus lateralis* of the hemiparetic leg for STROKE and the

right leg for CON before and 30 min after the NMES intervention. The NMES protocol consisted of a 60 Hz stimulation train of 10 seconds on and 15 seconds off which was repeated for 60 minutes. Phosphorylation of mTOR and p70S6K were analyzed using the SDS-PAGE Western blot technique. Phosphorylated protein content of key anabolic signaling proteins in the mTORC1 pathway were analyzed using two-way repeated measures analysis of variance. Physical function data were analyzed by *t*-test. Data are reported as mean ± SE with statistical significance set at $P \leq 0.05$.

The findings of the present study suggest that a 60-minute bout of NMES, in addition to facilitating muscle contraction, is an effective treatment for initiating skeletal muscle growth and strengthening processes in individuals with neurologically impaired muscle and older healthy skeletal muscle. Both the stroke and older healthy adult groups demonstrated a significant increase in both phosphorylated mTOR and S6K1 protein content, key anabolic signaling proteins, following a single bout of NMES. In conclusion, NMES appears to be an effective rehabilitation treatment to aid in muscle mass and strength preservation as well as initiate skeletal muscle growth during periods of immobilization or limited limb use. Additionally, the stroke group was significantly slower compared to the control group for the TUG and Five Repetition Sit-to-Stand tests.

We acknowledge that there were several limitations to this study. Although an inherent limitation to stroke research in humans, this study was limited by differences in the specific areas of the brain that were affected by the stroke and by the degree of physical dysfunction of the subjects with chronic stroke. Additionally, this study was limited by the small sample size, although significance was achieved for several variables.

Recommendations for Future Research

Future directions for this research include implementing a long-term NMES intervention with chronic stroke survivors. Administer a NMES intervention for 30 minutes rather than 60 minutes is suggested as it would be more practical in rehabilitation settings and determine if this shortened treatment would result in an anabolic response. It would also be interesting to evaluate the anabolic signaling response in the non-paretic leg in individuals with stroke after a single bout of NMES to determine if the response would be similar or different compared to the paretic leg. Other anabolic signaling proteins should also be evaluated to gain a better understanding of the metabolic processes occurring in older hemiparetic and healthy skeletal muscle.

Tables

Table 1. Subject characteristics. Data are presented as mean ± SE. Statistical significance is set at $P \leq 0.05$.

	n	Age (yrs)	Height (m)	Weight (kg)	BMI
Stroke	5	61.8 ± 5.44	1.66 ± 0.03	78.56 ± 6.95	28.72 ± 2.78
Control	6	58.17 ± 2.3	1.69 ± 0.05	68.21 ± 4.08	23.75 ± 1.05
***p*-value**		0.52	0.53	0.21	0.10

Table 2. Physical function. Data are presented as mean ± SE. Statistical significance is set at $P \leq 0.05$. * indicates STROKE significantly slower than CON. Grip strength data are expressed as a ratio of paretic to nonparetic limb for the STROKE group and for the CON group, limb side was matched to the paretic limb of the stroke group. Ratios for grip strength were used to account for any bilateral limb discrepancies. 5-STS (Five Repetition Sit-to-Stand).

	n	TUG (sec)	5-STS (sec)	Grip Strength Ratio
Stroke	5	9.16 ± 1.14	8.52 ± 1.09	0.93 ± 0.07
Control	6	6.57 ± 0.19	5.98 ± 0.55	1.25 ± 0.17
***p*-value**		0.03 *	0.05 *	0.07

Figures

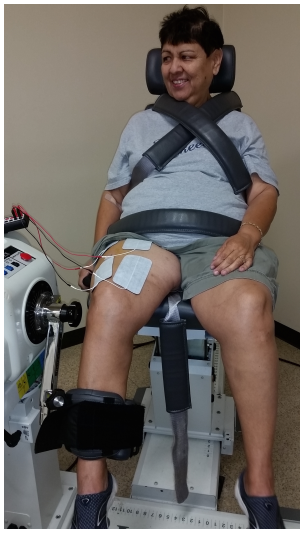

Figure 1. **Experimental Setup**: Biodex isokinetic dynamometer with five securing straps and stimulating electrode placement for NMES application. (Permission obtained for use of photograph)

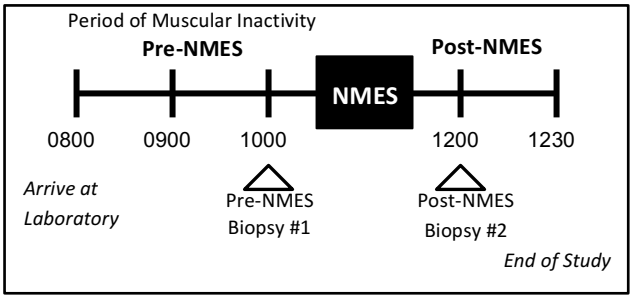

Figure 2. Day 3 Metabolic Study Timeline Schematic.

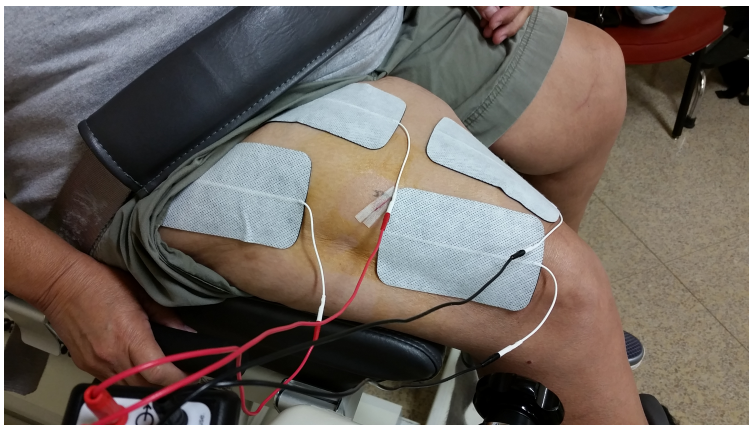

Figure 3. Stimulating Electrode Placement.

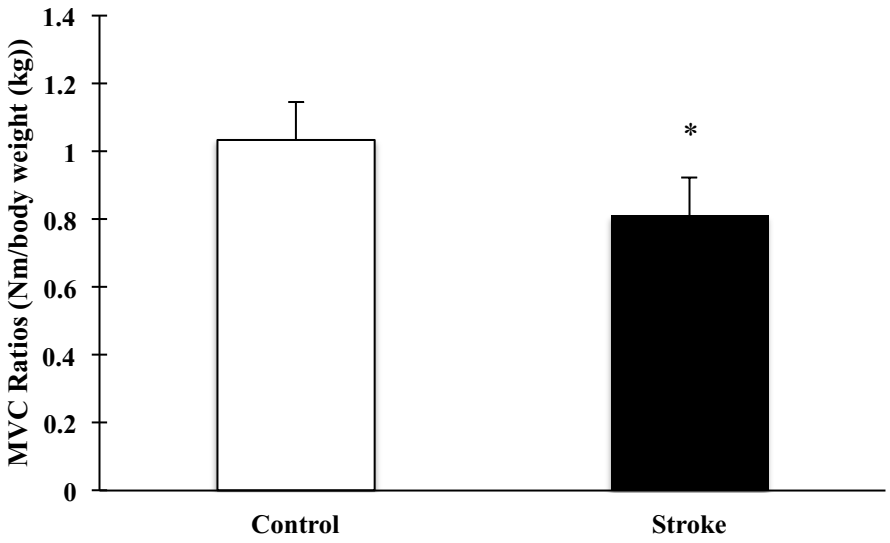

Figure 4. Knee Extensor Strength. MVC values are normalized to subject's body weight and expressed as a ratio of paretic to nonparetic limb for the STROKE group and for the CON group, limb side was matched to the paretic limb of the stroke group. Ratios for MVC knee extensor strength were used to account for any bilateral limb discrepancies. STROKE: n = 4; CON: n = 6. Data are presented as mean ± SE. Significance is set at $P \leq 0.05$.

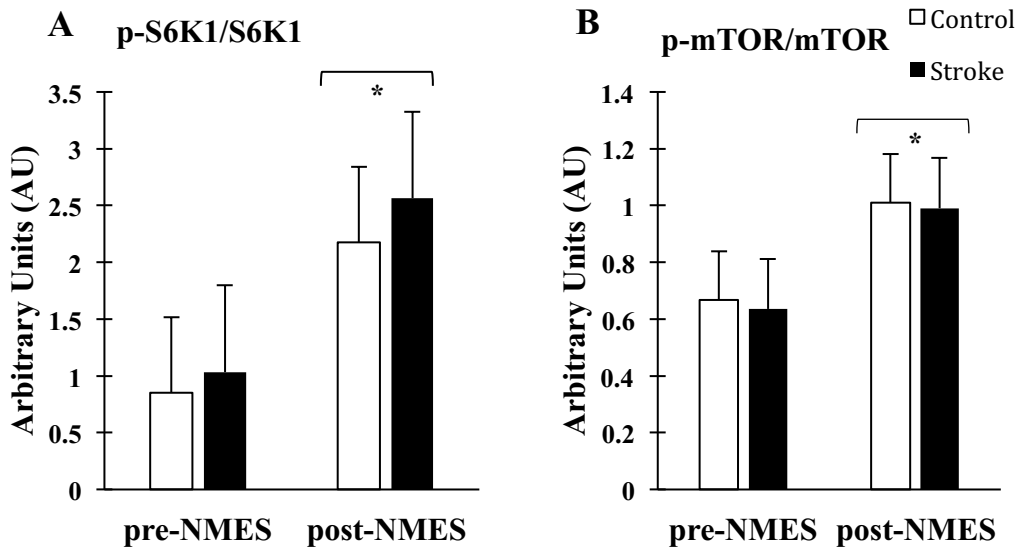

Figure 5. Phosphorylated protein content for S6K1 and mTOR. * indicates significant main effect for time (pre- vs. post-NMES). STROKE: n = 4; CON: n = 6. Data are presented as mean ± SE. Significance is set at $P \leq 0.05$.

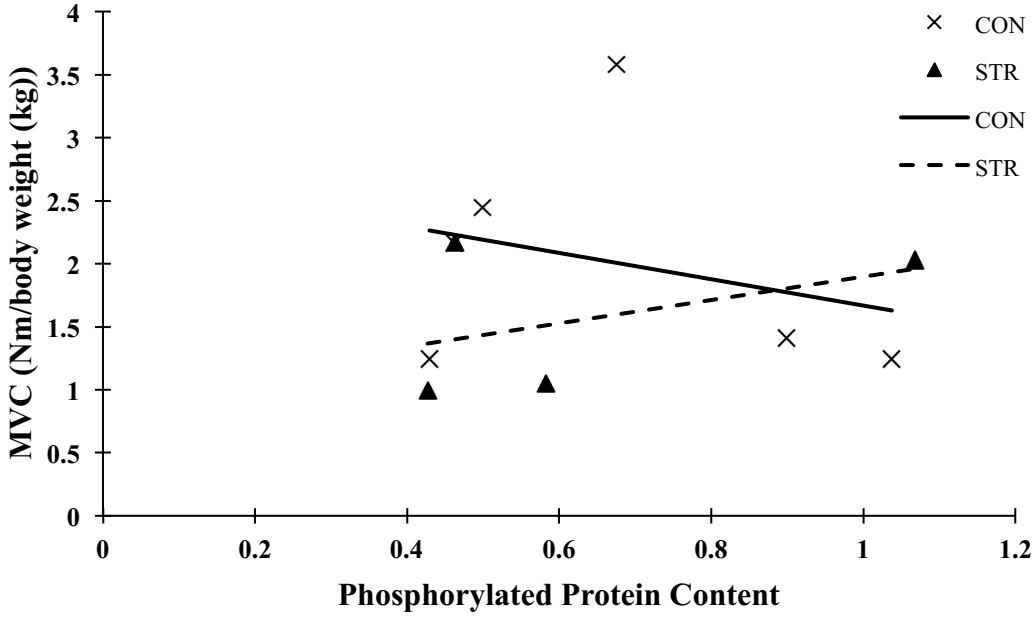

Figure 6a. **Relationship between knee extensor strength and phosphorylated mTOR protein content.** CON (y = -1.044x + 2.7122; $R^2 = 0.0827$; $r = -0.288$) and STROKE (y = 0.9254x + 0.9717; $R^2 = 0.1924$; $r = 0.439$). Data are fit to a linear trendline. STROKE: n = 4; CON: n = 6.

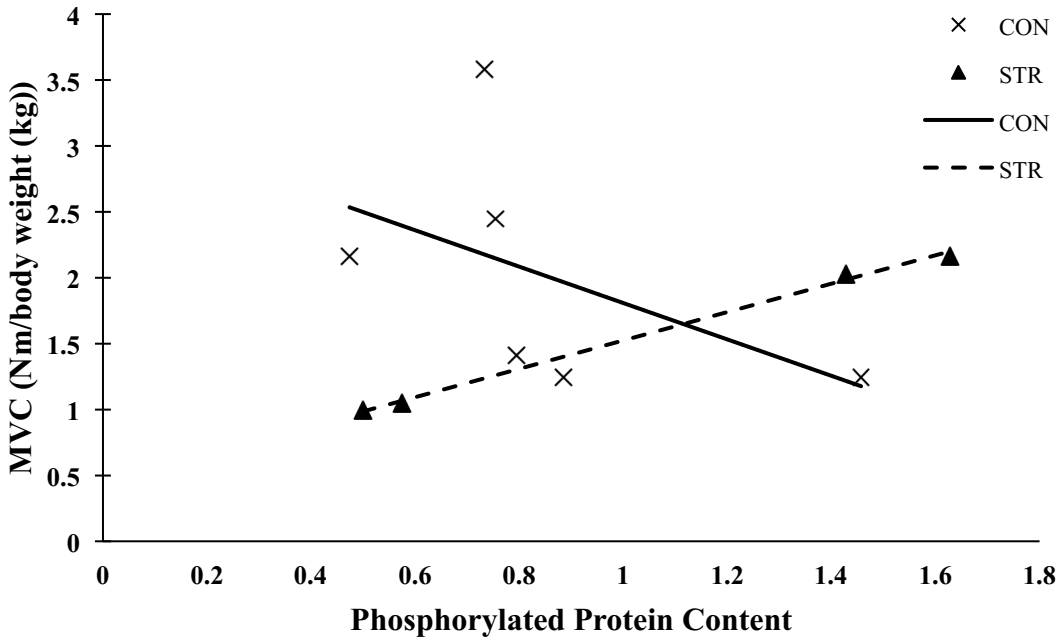

Figure 6b. Relationship between knee extensor strength and phosphorylated S6K1 protein content. CON ($y = -1.3808x + 3.1889$; $R^2 = 0.2439$; $r = -0.494$) and STROKE ($y = 1.077x + 0.4472$; $R^2 = 0.9971$; $r = 0.999$). Data are fit to a linear trendline. STROKE: n = 4; CON: n = 6.

APPENDIX SECTION

Appendix A.1—Telephone Screen Information

TELEPHONE SCREEN INFORMATION

Participant Full Name: **Date:**

DOB: **Age:** **Address:**

Primary Phone:

Secondary phone (in case we can't reach you):

Email:

Primary or Neuro Physician Name and Phone:

Name of person giving information:

Relationship to participant:

Height: _____ Weight: _____ BMI: _____

Y	**N**	**Have you had a stroke?** **How long ago did your stroke occur? Side affected?**
		What type of stroke did you have? (ischemic or hemorrhagic)
Y	**N**	**Did you participate in any therapies? For how long?**
Y	**N**	**Has your Dr. ever told you not to exercise? Why?**
Y	**N**	**Do you still see a Dr. for your stroke symptoms?** **What would you say is your most significant limitation since having your stroke?**
Y	**N**	**Is the affected leg swollen, infected, or painful?**
		How would you describe your ability to stand, walk and transfer?
		How would you describe your leg strength?
		How would you describe your overall endurance? Do you tire easily?

ASK EVERYONE

Y	**N**	**Do you exercise or participate in any therapies (PT, OT)?**

What type of exercise (walk, resistance training)? How often?

Y N Do you have any knee or hip issues (pain, swelling)?
Y N Have you lost or gained any weight in the last 3 months? If yes, how much?
Y N Do you smoke?
Y N Have you ever smoked? If so, how long and how many packs/day?
Y N Do you have a pacemaker?
Y N Do you use a walking aid (wheelchair, cane, walker)?
Y N Have you had any surgeries? What for? When?

Y N Do you have any surgical hardware implants? If yes, where? What for?

Do you have any other medical conditions?

	Yes	No
Diabetes		
Clotting disorders		
Autoimmune conditions		
Seizures		
Kidney or liver disease		
High blood pressure		
Heart Disease		
Thyroid condition		
Cancer in the last 5 years		

What prescription medications do you take?		
Medication:	**Dosage:**	**For:**

Y N Are you currently taking warfarin (Coumadin) or other anticoagulants including aspirin?
Are you taking any supplements, vitamins, herbals?

Y N Do you have any allergies?
Y N Do you eat shellfish?
Y N Are you allergic to iodine?

Have you previously (currently) participated in a research study? When?

If you don't qualify for this study, would you be interested in participating in other studies as they become available? Yes No

Appendix A.2—Medical Clearance Form

MEDICAL CLEARANCE FORM

Your client, _____ is interested in participating in a research study being conducted by Texas State University, Department of Health and Human Performance, in San Marcos, TX. The study is entitled "Effects of Neuromuscular Electrical Stimulation on Cellular Markers of Skeletal Muscle Growth and Degradation in Stroke Patients". This study will investigate the effects of a single bout of neuromuscular electrical stimulation (NMES), a technique commonly used for physical rehabilitation, on cellular markers of muscle growth in hemiparetic muscle of persons with stroke and in healthy age-matched control subjects. Additionally, we will compare muscle function, physical function, muscle morphology and cellular markers of muscle breakdown between chronic stroke patients to age-matched control subjects. This project is being conducted by Joni Mettler, PhD, ATC, CSCS, from the Health and Human Performance Department at Texas State University. In order for the above named individual to become a participant in this project, we are seeking your medical clearance.

Your patient will be assigned to the stroke group or control group. Study related procedures are the same for both groups. Subjects will perform muscle function tests of the lower extremity on a Biodex machine. Electromyography (EMG) will be recorded during these tests. During the study, about 6 teaspoons of blood will be drawn from the antecubital vein of the subject's arm via venipuncture. A single bout of NMES, consistent with that used in physical therapy for muscle strengthening, will be administered to the *quadriceps* muscle of the affected leg in the stroke group and to the right leg in the control group. A muscle biopsy will also be obtained from the lateral portion of the vastus lateralis muscle before and after the NMES intervention. (See consent form for study details).

Individuals with hemorrhagic stroke are excluded from the study. Individuals with swollen, infected, or inflamed areas (e.g. Phlebitis, thrombophlebitis, varicose veins and cancerous lesions) or pain syndromes affecting the legs and feet will not be included in the study. Persons who have implanted electronics (e.g., pacemakers, defibrillators, transcerebral or carotid sinus electrode placement) or surgical hardware in the legs or feet and those with epilepsy or who are pregnant are also not eligible to participate. Caution should be taken for individuals with heart problems or those who have a tendency to hemorrhage following trauma or fracture. Due to the muscle biopsy procedure and increased risk of bleeding, we ask that participants currently on aspirin or Plavix to discontinue use for 7 days prior to the biopsy study. Subjects taking Coumadin are excluded from the study. In order to obtain data that has not been influenced by medications, we ask that subjects hold all medications on the day of the muscle biopsy study until the final biopsy has been obtained (until approximately 12:00pm). Subjects must be of sound mind, capable of giving consent to be involved in the study, and in addition, must understand the project requirements and agree to perform project activities as directed. (See consent form for more details and risks associated with muscle biopsy).

If a participant meets inclusion criteria, they will be informed of the specifics of the study. A consent form will be given to them to read; the participant can take whatever amount of time is necessary to read the document. At the first session, any questions about the study will be answered and the individual will be asked to sign the consent form indicating their willingness to participate. They are under no obligation to provide their consent. If they do consent, they will be provided an orientation to the study and demonstration of the muscle testing device as well as the NMES.

The NMES also carries minimal risk; the lowest possible intensity will be used and then gradually increased to allow participants to habituate to the sensation of the stimulation. The electrical stimulation treatment, consistent with programs currently used in physical rehabilitation of stroke patients, will consist of two surface electrodes being placed on the surface of the skin over the quadriceps muscle, producing a tetanized, intermittent contraction of knee extension. Electrical stimulation of the lower extremity produces tingling-type sensations when administered. This may be uncomfortable for some individuals, however the overall risks are minimal and stimulation is tolerated well by most subjects. The intensity of the stimulation is completely at the tolerance of the subject who can request to increase or decrease the intensity at any time. The stimulation can also be interrupted or discontinued immediately at any time.

Should the participant decide that he/she does not like or is not able to tolerate the activities of the study, they are free to withdraw at any time without consequence. If you have any questions, concerns, or would like to discuss the procedures of the project in more detail, please contact Dr. Joni Mettler, Principal Investigator, at 512.245.9691.

MEDICAL CLEARANCE

I _____, MD, have reviewed this medical clearance form
(print physician name)
and I give medical clearance for _____, a client
(print patient name)

under my care, to participate in the "Effects of Neuromuscular Electrical Stimulation on Cellular Markers of Skeletal Muscle Growth and Degradation in Stroke Patients" research study being conducted by Joni Mettler, PhD, ATC, CSCS, of Texas State University. I am aware of the above listed procedures associated with participation in this research study and attest that the client named above currently has no known medical condition(s) that would contraindicate their participation in the muscle performance testing, muscle biopsy procedure or the application of electrical stimulation to the affected lower extremity.

_____ _____
Treating Physician Signature Date

Appendix B.1—Timed Sit-to-Stand Test

Timed Sit to Stand Test
(reproduced from Gee 2005 and Bohannon 2006)

I. EQUIPMENT
   a. Chair
      i. Standard chair with arms
      ii. Height: 43-45 cm
      iii. Chair should not be against the wall or on a mat
      iv. Use same chair for entire study
   b. Timer

II. TEST
   a. Starting Position
      i. Begin with subject sitting with arms folded across chest
      ii. Back against chair
      iii. *Stroke subjects can have the impaired arm at the side*
   b. Instruct the subject to stand FULLY between reps and NOT to touch the back of the chair during each rep (it's ok if the subject does touch the back of the chair but is not recommended)
   c. One Practice Trial
      i. This includes 2 reps
      ii. If you are concerned about fatigue with a practice trial, it is ok to demonstrate for the subject.
   d. Subject Instructions: "I want you to stand up and sit down 5 times as quickly as you can when I say 'Go'"

III. TIMING
   a. Begins at "Go"
   b. Stops when the subjects buttocks touch the chair on the 5th rep
   c. 30 second rest period between each of the 3 trials

IV. TIPS
   a. Inability to complete 5 reps without assistance or use of an upper extremity support indicates failure of test (any modification should be documented)
   b. Try not to talk to the subject during the test (this can affect performance)

Appendix B.2—Grip Strength Test

Grip Strength Test with Hand Dynamometer:

I. ADJUST DYNAMOMETER
   a. The second joint of the hand should fit snugly under the handle, which should be gripped between the fingers and the palm at the base of the thumb. 2nd rung.

II. TEST
   a. Test both hands
   b. Position
      i. Sit in an armless chair (demonstrate proper position)
      ii. Ankles, knees, elbows should be at 90 degrees
      iii. Sit as erect as possible toward edge of chair with feet firmly on floor
      iv. Elbow stays against the thorax (Patients with stroke can rest the device on their thigh if needed, but this needs to be consistent and tested in the same way for repeat testing).
      v. The dial can be visible for motivational purposes
      vi. Alternate between left and right giving a 30 second break after each set
   c. Direction to be read to subject: "Squeeze your hand as hard as you can for about 2 seconds. We will repeat this 3-4 times." "Ready, 3, 2, 1 SQUEEZE…RELEASE"

III. SCORE
   a. How to score
      i. Average of the tests of each hand
         1. Pick the best 3 of 4 (or 2 of 3) for each hand
      ii. Units are read in kilograms (kg)

Appendix B.3—Timed Up and Go (TUG) Test

Timed Up and Go (TUG) Test
(reproduced from Shumway-Cook et al. 2000)

Name:_____ MR: _____ Date:_____

1. Equipment: arm chair, tape measure, tape, stop watch.

2. Begin the test with the subject sitting correctly (hips all of the way to the back of the seat) in a chair with arm rests. The chair should be stable and positioned such that it will not move when the subject moves from sit to stand. The subject is allowed to use the arm rests during the sit stand and stand – sit movements.

3. Place a piece of tape or other marker on the floor 3 meters away from the chair so that it is easily seen by the subject.

4. Instructions: "On the word GO you will stand up, walk to the line on the floor, turn around and walk back to the chair and sit down. Walk at your regular pace.

5. Start timing on the word "GO" and stop timing when the subject is seated again correctly in the chair with their back resting on the back of the chair.

6. The subject wears their regular footwear, may use any gait aid that they normally use during ambulation, but may not be assisted by another person. There is no time limit. They may stop and rest (but not sit down) if they need to.

7. Normal healthy elderly usually complete the task in ten seconds or less. Very frail or weak elderly with poor mobility may take 2 minutes or more.

8. The subject should be given a practice trial that is not timed before testing.

9. Results correlate with gait speed, balance, functional level, the ability to go out, and can follow change over time.

Appendix C—Biodex Orientation Protocol

Biodex Orientation Protocol

1. Set Chair
2. Leg 1
    a. Quadriceps
    b. Hamstrings
3. Check Chair Settings
4. Leg 2
    a. Quadriceps
    b. Hamstrings

Quadriceps

Explain how to produce force—PUSH

Target Practice
- 20 N-m 2x hold for 5 s
- 40 N-m 2x hold for 5 s
- 50 N-m 2x hold for 4 s

3 MVC Practice

Target Practice
- 60 N-m 2x hold for 3 s
- 40 N-m 2x hold for 3 s
- 25 N-m 2x hold for 5 s

Hamstrings

Explain how to produce force—PULL

Target Practice
- 10 N-m 2x hold for 5 s
- 20 N-m 2x hold for 5 s
- 25 N-m 2x hold for 4 s

3 MVC Practice

Target Practice
- 25 N-m 2x hold for 3 s
- 20 N-m 2x hold for 3 s
- 15 N-m 2x hold for 5 s

Appendix D—NMES Data Sheet

Subject ID:_____ **MVC:_____** **15% MVC Target Torque:_____**

NMES Data Sheet

	0 (min)	5	10	15	20	25	30
mA							
Beg. Torque							
End Torque							

	35	40	45	50	55	60
mA						
Beg. Torque						
End Torque						

Appendix E—Metabolic Study Flow Sheet

Date:	
Subject	**STR**

Biopsy	Time
1	
2	

Real Time	Goal Time	Study Time	Sample Label	Procedures			Notes/Problems
	8:00 AM			Subject arrived to lab			
	10:00 AM		**M1**	**Biopsy 1**			
	10:30 AM	**0.00**		**60-min NMES Intervention**	start time _____		
	11:30 AM			**60-min NMES intervention**	end time _____		
	12:00 PM	NMES+ 30 min	**M2**	**Biopsy 2**			

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