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Modulation of Spasticity by Trans-Spinal Direct Current Stimulation in Animals with Spinal Cord Injury

Wagdy Mekhail

*The Graduate Center, City University of New York*

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MODULATION OF SPASTICITY BY TRANS-SPINAL DIRECT CURRENT STIMULATION IN ANIMALS WITH SPINAL CORD INJURY

by

WAGDY W. MEKHAEL, DPT

A dissertation submitted to the Graduate Faculty in biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2017
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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Executive Officer

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Bassem El-Khodor, Ph.D.

THE CITY UNIVERSITY OF NEW YORK
ABSTRACT

Modulation of Spasticity by Trans-Spinal Direct Current Stimulation in Animals with Spinal Cord Injury

by

Wagdy W. Mekhail, DPT

Advisor: Zaghloul Ahmed, PT, Ph.D.

Central nervous system injuries usually produce motor impairments that are exacerbated by pathologically altered muscle tone. Abnormal muscle tone interferes with voluntary movement and is associated with loss of dexterity. Prior work in our laboratory demonstrated that 30-second trans-spinal direct current (DC) stimulation can temporarily modify muscle tone in anesthetized spastic mice after spinal cord injury (SCI). These experiments described DC-induced muscle tone responses to be polarity-dependent. That is, anodal stimulation (current passed from the lumbar spine to sciatic nerve) decreased muscle tone, and cathodal stimulation (current passed from the sciatic nerve to the lumbar spine) increased it. The present study investigates the therapeutic potential of noninvasive trans-spinal DC stimulation (tsDCS) to modulate spasticity in awake mice with SCI. The study aims to investigate the following: 1- The effects of short-term tsDCS and repeated-sessions of tsDCS on muscle tone and on skilled and unskilled locomotion. 2- The long-term modulatory effects of tsDCS on the stretch reflex of the triceps surae muscle (TS), using the rate-dependent depression (RDD) of Hoffmann’s reflex. 3- The changes in muscle tone as a result of combining tsDCS with several
pharmacological agents, including gabapentin, morphine and MK-801. To assess spasticity in the awake SCI mouse model, an innovative computer-controlled stretch apparatus with a mouse holder was designed and constructed in our laboratory. The design permitted spasticity assessment by stretching the TS at different speeds, while the animal received noninvasive tsDCS treatment. The treatment protocol consisted of seven consecutive daily tsDCS sessions (20 min/day), followed by spasticity evaluations for four weeks after stimulation ended. The mice were also evaluated for two different types of locomotion: 1- unskilled locomotion using the DigiGait treadmill system, and 2- skilled locomotion using a computer-controlled ladder wheel, constructed in our laboratory. Results show that anodal tsDCS significantly reduced several indices of spasticity during treatment, and for at least four weeks after simulation ended. Additionally, anodal-tsDCS treated animals exhibited significantly improved unskilled and skilled locomotion, and significant increase in RDD. Anodal treatment also improved the spasticity-reducing effects of gabapentin and MK-801, and decreased morphine-induced rigidity. In conclusion, these results show anodal tsDCS to be therapeutically effective in ameliorating SCI-induced spasticity in mice when used alone, or in combination with gabapentin or MK-801.
Acknowledgment

During my Ph.D. endeavor, I have met so many people who helped me, and without their invaluable assistance, I would have been lost. To those people “thank you” can never be enough. But for one person, words cannot begin to express my gratitude. My mentor, Dr. Zaghloul Ahmed, knew exactly what to be at the right time: He was an instructor when I needed to learn; he was a listener when I needed an ear for my ideas, regardless of how outrageous they were; he was a friend when times were rough. Before I met him, I liked neuroscience, and through his mentorship, neuroscience became my passion. He taught me independence in the laboratory, and gave me his feedback professionally and respectfully.

I am forever thankful to my family: my wife Theresa, and my two children, Samantha and Christian. During the years I was working on this project, they had been supportive, gracious and tolerant.

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I would like to thank my research assistants: Mazen Hassan, Alexis Gorin, Malik Ahmed, Michael Maisano, Mark Ayad, and Marina Andrawis for their valuable contribution to the study.

I wish to thank my fellow neuroscience graduate students for providing rich and stimulating discussions.

Finally, I wish to thank my patients for their support, and thank all patients with spinal cord injury and spasticity as their inspiring resilience continues to fuel the efforts to find a cure.
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<th>Description</th>
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<tbody>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>DCS</td>
<td>Direct current stimulation</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglia</td>
</tr>
<tr>
<td>E1</td>
<td>Evaluation 1</td>
</tr>
<tr>
<td>EMG</td>
<td>Electromyography</td>
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<tr>
<td>EPSP</td>
<td>Excitatory postsynaptic potential</td>
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<td>GABAp</td>
<td>Gabapentin</td>
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<td>HR</td>
<td>Heart rate</td>
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<tr>
<td>H-Reflex</td>
<td>Hoffmann Reflex</td>
</tr>
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<td>Hz</td>
<td>Hertz</td>
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<tr>
<td>i.p</td>
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<td>ID</td>
<td>inside diameter</td>
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</tr>
<tr>
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<td>kilogram</td>
</tr>
<tr>
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<td>Lumbar vertebra 13</td>
</tr>
<tr>
<td>L6</td>
<td>Lumber vertebra 6</td>
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<tr>
<td>LTD</td>
<td>Long-term depression</td>
</tr>
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<td>left hindlimb</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>mA</td>
<td>Milliamp</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MK801</td>
<td>Dizocilpine</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-Methyl-D-aspartate receptor</td>
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<tr>
<td>OD</td>
<td>Outside diameter</td>
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<tr>
<td>RDD</td>
<td>Rate-dependent depression</td>
</tr>
<tr>
<td>RE</td>
<td>Recording electrode</td>
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<tr>
<td>RHL</td>
<td>Right hindlimb</td>
</tr>
<tr>
<td>RR</td>
<td>Respiratory rate</td>
</tr>
<tr>
<td>SE</td>
<td>Simulating electrode</td>
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<td>tsDCS</td>
<td>Trans-spinal direct current stimulation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>T10</td>
<td>Thoracic Vertebra 10</td>
</tr>
<tr>
<td>T13</td>
<td>Thoracic Vertebra 13</td>
</tr>
<tr>
<td>TS</td>
<td>Triceps Surae muscle</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometer</td>
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Chapter 1 – Introduction

Overview of Muscle Tone Abnormalities and Management

Hypertonia is a common muscle tone abnormality associated with spinal cord injury (SCI). Clinically, muscle tone is determined by the amount of resistance conveyed by the muscle during a passive stretch. A normal muscle typically possesses a healthy level of resting muscle tone, which is usually active in the background of normal voluntary contractions. A normal level of muscle tone is essential for coordination, preparedness to initiate movement, and maintenance of the proper position of body joints at rest. While low muscle tone, hypotonicity, is a condition marked by reduced resistance to a passive muscle stretch, hypertonicity is an increase in muscle tone associated with an enhanced stretch reflex (Katz and Rymer, 1989). And spasticity is a velocity dependent sensorymotor system disorder, recognized when increases in the stretch speed lead to subsequent increases in muscle resistance, and vice versa (Lance, 1980). The exaggerated tendon jerks associated with spasticity are attributed to hyperexcitability of spinal interneurons (Elbasiouny et al., 2010), and alpha motor neurons (Mayer, 1997). Hypertonicity can vary greatly in severity, from mild muscle stiffness to a painfully and debilitating sustained muscle contraction (Adams and Hicks, 2005). When spasticity interferes with functional movement after SCI, medications are usually prescribed. However, pharmacologic treatments of spasticity come with a wide variety of side effects. For example, Botox, a well-known and widely-used spasticity drug, is actually botulinum, an extremely potent biological toxin, which acts on the motor neurons via inhibiting acetylcholine release. Botox administration is an invasive procedure that require muscular injections, provides only temporary spasticity control, and has been reported to produce adverse effects (Cote et al., 2005). Moreover, physical and surgical interventions do not provide a
cure for spasticity (Gracies et al., 1997). Therefore, for the past few decades, a large body of research has focused on testing safer methods to control spasticity.

**Direct Current Stimulation Affects Muscle Tone**

One promising approach has been the use of direct current (DC) to improve the functional motor activities of patients with spasticity. Recent work supports the modulatory effects of DC stimulation on CNS activity (Aguilar et al., 2011; Ahmed, 2016; Ranieri et al., 2012; Song and Martin, 2017). The support is compelling in humans (Cogiamanian et al., 2008; Nitsche and Paulus, 2001), and animals (Ahmed, 2014b; Bolzoni and Jankowska, 2015). For example, when applied transcranially, anodal DC current significantly improved motor control functions in stroke patients (Boggio et al., 2007), and reduced spasticity for at least two days after stimulation stopped in children and adolescents with cerebral palsy (Aree-uea et al., 2014). Additionally, animal studies demonstrated the ability of DC stimulation to induce plastic changes in CNS circuitry (Ahmed, 2013b; Ahmed, 2014b; Song and Martin, 2017).

It has also been suggested that anodal trans-spinal DC current stimulation (tsDCS) reduces spasticity by modulating the activity of the stretch reflex circuit (Winkler et al., 2010). The electrophysiological mechanisms through which tsDCS influences the stretch reflex synapse have been explained in the mid-20th century (Eccles et al., 1962). Working on cats, Eccles and his group determined that when tsDCS passed across the spinal cord, it generated changes in the membrane potential of the sensory afferent neurons, which in turn caused excitability changes in their associated motor neurons. Namely, anodal tsDCS depolarized the presynaptic terminals of the sensory afferents, decreasing their corresponding excitatory post-synaptic potential (EPSP). And cathodal tsDCS hyperpolarized the presynaptic terminals of the sensory afferents, increasing their EPSPs. The same findings were seen in other types of synapses: for example, in the neuromuscular
junction of the frog, cathodal-induced hyperpolarization increased neurotransmitter release (Del Castillo and Katz, 1954). And in the skeletal muscles of rat, cathodal DC induced hyperpolarization of the presynaptic terminals, causing increased acetylcholine output. Anodal DC, however, caused the opposite effect (Hubbard and Willis, 1962a). Recent animal studies have confirmed these modulatory effects of DC stimulation. For example, intraspinal cathodal DC stimuli, applied locally at the dorsal horn or within the hindlimb motor nuclei, reduced afferent and motor neuron activity (Bolzoni and Jankowska, 2015). In other experiments, anodal tsDCS reduced the amplitude of synaptically mediated responses, and cathodal current caused them to increase (Ahmed, 2014b). Additionally, motor neurons showed decreased responses to anodal and increased responses to cathodal tsDCS (Ahmed, 2016; Song and Martin, 2017). In humans, anodal tsDCS was found to decrease the activity of ascending nociceptive tracts within the spinal cord, reducing pain conduction signals (Truini et al., 2011). It also decreased the amplitude of the cervico-medullary somatosensory potentials of the posterior tibial nerve; a reduction that continued for 20 minutes after stimulation stopped (Cogiamanian et al., 2008). These encouraging findings prompted this current study, which aims to test the potential noninvasive therapeutic potential of tsDCS in treating muscle tone abnormalities in the SCI-mouse model.

**Direct Current Stimulation Circuit**

Two known neuronal types contribute to abnormal muscle tone and spasticity: the alpha motor neurons (Hiersemenzel et al., 2000; Li et al., 2004) and the spinal interneurons (Kitzman, 2006; Mailis and Ashby, 1990). Ahmed, (2014a) explained a procedure designed to target the hyperactivity of both types of neurons, utilizing two different loops in one DC circuit setup (figure 1-1a). First, by targeting the DC flow between the spinal cord (a1 branch) and the sciatic nerve (c2 branch), the excitability of both spinal motor neurons, and afferent neurons was modulated.
Second, by targeting the DC flow between the spinal cord (a1) and the abdominal skin (c1), the excitability of the interneurons in the spinal cord was also influenced. The author also explained that the a2-c2 loop was designed to provide a charge balance, and deliver relatively higher DC values to the spinal cord, and only sub-threshold DC values to the sciatic nerve. This loop was crucial to avoid the deleterious effects of monopolar stimulation. However, this circuit was designed for anesthetized mice to receive DC immediately post-SCI, through electrodes placed invasively (surgically) under the skin. This current study proposes a new noninvasive method of DC delivery in awake mice, utilizing over-skin electrodes. Prior studies that tested the effects of tsDCS on neuronal circuits in mice utilized anesthetized animals. And while these studies provided great deal of information about the effects of DC, trials on anesthetized animals carry less clinical potential. Therefore, in this current study, we utilize awake mice to eliminate the side effects of anesthesia and bring tsDCS closer to clinical application.
Figure 1-1

a

b
Figure 1-1  DC stimulation circuits

a, trans-spinal DC stimulation circuit was previously developed in our laboratory to modulate muscle tone in anesthetized mice using trans-spinal DC stimulation (Ahmed, 2014a; Ahmed, 2016). The circuit permits DC current to pass un-attenuated to the spinal cord, and attenuated to the sciatic nerve. b, a modified rendition of the original circuit in (a), used with over-skin noninvasive tsDCS in awake animals, as a permanent component of the mouse holder (figure 2-2). This was accomplished by keeping the anode (a) conductor intact, and dividing the cathode into two branches: one branch (c1), connected directly to the abdominal electrode, second branch (c2), connected to the sciatic nerve electrode. DC in the c2 branch was attenuated by passing through a 300 KΩ resistor to produce 0.3 mA at the sciatic nerve.
Hypothesis and Aims

This study hypothesizes that noninvasive tsDCS (delivered through over-skin electrodes) can be used to reduce abnormal muscle tone and stretch responses (spasticity) tested in the triceps surae muscle (TS), and will improve recovery of locomotion and skilled movement in mice after SCI. The aims of this study are as follows: 1- To test the immediate and short-term effects (60 minutes) of anodal and cathodal tsDCS on muscle tone, in awake mice with spasticity, following SCI. 2- To compare the effects of invasive (implanted electrodes) and noninvasive (over-skin) tsDCS on muscle tone in mice with spasticity, following SCI. 3- To test the long-term effects of repeated, longer-duration anodal and cathodal tsDCS on muscle tone in awake mice with spasticity, following SCI. For the latter aim, the mice will receive daily, 20-minute, sessions of tsDCS for 7 days, and will be followed for 30 days. 4- To test the modulatory effects of longer-duration, anodal and cathodal tsDCS on the stretch reflex circuit (using the rate-dependent depression (RDD) of the Hoffman reflex) in lightly anesthetized mice with spasticity, following SCI. 5- To test long-term effects of repeated-sessions anodal and cathodal tsDCS on spasticity and recovery of skilled locomotion in animals with SCI. 6- To test the immediate and short-term effects of tsDCS on muscle tone after the injection of the following drugs: Neurontin (gabapentin), morphine, and Dizocilpine (MK-801).

Considering the strong evidence obtained from the preliminary experiments on anesthetized mice in our laboratory (Ahmed, 2014a; Ahmed, 2014b), it can be expected that the tsDCS modulatory effects on spinal cord activity can have a long-term effect, and maybe used to control muscle tone abnormalities in the SCI mouse model. Controlling muscle tone abnormalities is expected to improve motor function of mice presenting with neurological impairments after SCI.
These experiments will also help investigate the possible interactions and combined effects of tsDCS with some drugs that are known to have an impact on muscle tone.
Chapter 2 – Methods and Material

Animals

Adult female CD-1 mice (n = 90, weight 27-37 g) were used for all experiments (table 2-1). In this study, “sham” refers to mice that had received SCI, went through the protocol steps of the experiment, but did not receive tsDCS treatment. “Control” or “healthy” refers to mice that did not receive SCI, went through the protocol steps of the experiment, and did not receive tsDCS.

In the pharmacologic studies, “experimental” refers to animals that received the drug being tested with tsDCS, while “control” refers to mice that received the drug, but no tsDCS.

“Anodal group” identifies mice that received anodal tsDCS. In this group, the anodal electrode was positioned over the SCI site, and the current passed from the spinal cord to the sciatic nerve. “Cathodal group” identifies mice that received cathodal tsDCS. In this group, the cathodal electrode was positioned over the SCI site, and current passed from the sciatic nerve to the spinal cord. Both the anodal and cathodal groups are called the treatment groups. All study protocols were approved by the College of Staten Island’s Institutional Animal Care and Use Committee.

For each stretch parameter tested using the apparatus, two sets of data were collected: a set collected while the animal was awake, and another set collected while the animal was under full isoflurane anesthesia. The data collected while the animal was anesthetized represented the passive resistive forces of muscle and connective tissue. To isolate the neuronal component of the stretch resistance from that caused by passive tissue, the value of the tested parameter collected during anesthesia was deducted from that collected while the animal was awake. This method ensured that only the neuronal contribution of the stretch response will be analyzed.
Charles River Laboratories (USA) supplied the Breeder CD-1 mice. The College of Staten Island vivarium conducted the pairing and breeding process. All animals were placed in polycarbonate ‘Shoebox’ cages, and housed in a standard facility, where they were kept at 12 h light/dark cycle, and room temperature set to 70 °F and humidity averaged 30-70%. Each cage measured 7.25” W × 11.5” D × 5”. The animals were housed 3-5 per cage before the beginning of the study, then individually immediately after they received the spinal cord injuries. No environmental enrichments, such running wheels or toys were supplied in the cages. Cage maintenance included cleaning twice a week, and daily checks for food, water and wellness. Cage bedding was supplied by Andersons Bed-o’Cobs (USA). Mice had ad libitum access to filtered water and food during the entire length of the experiment, Purina Mouse Chow #5015 (LabDiet, USA).
<table>
<thead>
<tr>
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<tr>
<td>Long-term effect of tsDCS</td>
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<tr>
<td>Immediate effect of tsDCS</td>
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<tr>
<td>Effect of tsDCS using implanted electrodes</td>
<td>4</td>
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<tr>
<td>Morphine and effect of tsDCS</td>
<td>6</td>
</tr>
<tr>
<td>MK-801 and effect of tsDCS</td>
<td>8</td>
</tr>
<tr>
<td>Gabapentin and effect of tsDCS</td>
<td>7</td>
</tr>
</tbody>
</table>

**Table 2-1**  The number of animals assigned to each experiment

A total of 90, female, CD-1 mice were used in all experiments.
Spinal Cord Contusion Injury

A ketamine/xylazine cocktail (90/10 mg/kg, i.p.) was used to deeply anaesthetize the mice. A laminectomy at thoracic (T) 10 vertebra was conducted to expose T13 spinal segment. The IH Spinal Cord Impactor (Precision Systems & Instrumentation, IH-0400; VA, USA) was used to standardize the contusion SCI force for all mice. The impactor was fully computerized once the desired contusion force level was selected using the system’s software. Impact force level was selected at 60 kdyn. This impact force level was determined based on data collected from a preliminary SCI contusion study using the impactor. The aim of the preliminary study was to determine the most ideal SCI contusion force needed to produce spastic mice. This was accomplished by creating contusion injuries in mice utilizing progressive impact forces. Mice impacted at 60 kdyn produced ideal spastic behavior, approximately 2-3 weeks post-injury (the number of animals used in the preliminary study is not included in table 2-1). All contusion impacts were conducted using the standard mouse tip (diameter = 1.3mm) at T13 spinal cord level, which was exposed by the laminectomy. Overlying muscles and skin were sutured after the contusion procedure. Next, the surgical site was covered with a layer of gentamicin sulfate ointment to prevent infection. The mouse then received a number and was placed on a warm pad (30 °C) for recovery.
**Stretch Apparatus**

A computer controlled stretch device was assembled from a stepper motor (ARCUS Technology INC, DMX-UMD-23, CA USA), a foot presser mounted on a force-displacement transducer (GRASS Technologies/NATUS, FT03, CA USA) connected to a bridge amplifier (Kent Scientific Corporation, Torrington, CT) (figure 2-1a, left), and a mouse holder (figure 2-2). The foot presser (figure 2-1a, right) was manufactured in our laboratory using 4.5 cm, number 8 threaded rod, screwed at one end into a rigid 0.5x1.5 poly (vinyl chloride) (PVC) pad. The PVC pad was then heat-molded into a U-shaped plate to accommodate the flat surface of a mouse’s hind foot. The other end of the rod was screwed into the receiving inlet of the force-displacement transducer. The transducer was permanently fixed to a linear rail-guided carriage (ZNT Automatic Technology Co, SGB15NUU; Zhejiang, China). The rotational movement of the stepper motor is converted into linear displacement at the carriage using a set of jointed flat-aluminum rods.

Two computer software programs were utilized to control the stepper motor and perform the online (during) stretch and electromyogram (EMG) recordings: The first, a stepper motor control software (ARCUS Technology INC, DMX-UMD GUI, CA USA) was re-written to permit the execution of three consecutive timed stretches, separated by two 10-second recovery intervals. The stepper motor was programed to provide three different stretch speeds: 18,180, and 1800˚/s, which correspond to 3, 30, and 300 RPM, respectively. The second, LabChart-7 bundled with PowerLab data acquisition system software (ADInstruments, Colorado Springs, CO) was used for gathering and analyzing of the data.

The following stretch parameters were collected: Active resistance peak amplitude, active slope, EMG area, and EMG amplitude. Active peak resistance is calculated as the height of the resistance trace relative to baseline (before the stretch). Active slope is calculated as the first
derivative of the rising phase of the muscle resistance in response to stretching. EMG amplitude is calculated as the height of the EMG trace relative to baseline. EMG area is calculated as the area covered by EMG activity traces, as calculated by the LabChart software.
Figure 2-1

(a) [Diagram of a setup with labels: Carriage, Controller and driver, Stepper motor, Force transducer, Power supply, Movement arm, Mouse holder, Ankle stabilizer, EMG electrodes, Knee stabilizer.]

(b) Graphs showing awake and isoflurane conditions:
- Awake:
  - Speed 1: 30 g
  - Speed 2
  - Speed 3
- Isoflurane:
  - Force
  - EMG
  - Averaged EMG

(c) Graphs showing peak and rising slope:
- Awake
- Isoflurane
Figure 2-1  Set up and procedure for testing spasticity in mice

a, the stretch apparatus, constructed from stepper motor, movement arm, force-displacement transducer, and mouse holder. The system was controlled by computer software. Right image shows the positioned hindlimb mounted on the mouse holder, being tested for spasticity. b, Top: examples of muscle resistance traces recorded at three speeds: Speed 1 = 18 °/s; Speed 2 = 180 °/s; Speed 3 = 1800 °/s; the bottom two traces show raw (blue) and root squared (green) EMG. Left recordings are taken during awake condition, and right recordings are taken while the animal was fully anesthetized with isoflurane. * denotes EMG traces presence during deep anesthesia. c, Left: graph depicts the measured rising slope and peak force. Right: overlaid traces recorded during speed 1 showing the effect of isoflurane.
Mouse Holder

The mouse restraining system, fabricated in our laboratory (figure 2-2), is made of three components: 1- A clear Plexiglas acrylic tube that served as the mouse holding chamber. 2- An internal adjustable support system made of a clear acrylic concave stabilization plate. The location of the plate could be adjusted along the length of the chamber linearly via a handle that protruded through a cutout made into the entire length of the tube. The concave surface of the plate was designed to contour to the back of the animal, securing it dorsally, and could be adjusted externally to different mouse sizes. 3- Four over-skin stimulating electrodes. Each electrode was made of wick-covered 1x1.5 cm stainless-steel plate. One of the electrodes was permanently fixed to the floor of the holding chamber, serving as the abdominal conductor. The second electrode was permanently fixed to the middle of the stabilization plate, serving as the dorsal conductor. This electrode could be aligned with the desired stimulation site on the spinal cord by moving the handle. The remaining two electrodes could be adjusted linearly by sliding into cutouts on the long sides of the stabilization plate, serving as the left and right sciatic nerve conductors. The design of the sciatic nerve electrodes enabled the positioning of the electrode directly over the sciatic nerve location on the skin. All electrodes had external connectors through the acrylic surface of the mouse holder to the current source. Clear acrylic was chosen to permit continuous visualization of the animal, and ease of cleaning. The abdominal surface of the holding chamber contained two openings, one for each of the animal’s hindlimbs. Each opening equipped with knee stabilizer pad to ensure full knee extension during testing. A third smaller opening on the abdominal surface of the holding chamber served as an injection access point for intraperitoneal (i.p.) drug administration. Before testing, a cap with an opening in the center was placed on the anterior end of the holding chamber for breathing and isoflurane administration.
The detachable holding chamber was mounted on a support system made of an acrylic base and stand. The stand ended in an acrylic tubular clamp on which the holding chamber was inserted. The tubular clamp permitted rotational adjustment of the animals paw under the foot presser, and the selection of either hindlimb to be tested. To limit the hindlimb movement to the ankle joint during stretching; another acrylic stand was created to secure the distal leg. The stand ended in an adjustable stainless steel ankle stabilizer clamp that could be moved in the x, y and z axes. This was necessary for proper alignment of the foot under the presser, and adjusting the hip angle of the animal before stretching.
Figure 2-2

Abdominal electrode connector
Abdominal electrode
Stabilization plate
Knee stabilizer
Spinal electrode connector
Right sciatic nerve connector
Right sciatic nerve electrode
Figure 2-2  Assembled mouse holder

Designed to permit concurrent stretch and EMG testing during tsDCS, the mouse holder consists of a chamber, internal adjustable support system, and stimulating electrodes. Mounted on an acrylic stand, the holder contains four internal over-skin electrodes with external connectors to a power source: one abdominal (not shown), one spinal, and two sciatic nerve electrodes. Left sciatic nerve electrode is not shown.
Direct Current Stimulation Circuit and Electrodes

Circuit and power source

The tsDCS protocols of this study required the modification of a trans-spinal circuit that was originally designed in our laboratory to be used with anesthetized animals (Ahmed, 2014a). The original circuit was modified to pass DC to the spinal cord, and the sciatic nerve of the most affected limb, noninvasively, using over-skin electrodes. To prevent damage to the sciatic nerve, the current passing into the sciatic nerve electrode was attenuated. As shown in figure 1-1b, this was accomplished by keeping the anode (a) conductor intact, and dividing the cathode into two branches: the first branch (c1), connected directly to the abdominal electrode, carried un-attenuated current. The second branch (c2) was passed through a 300 KΩ resistor to attenuate current to the sciatic nerve. The same circuit was used for the two different protocols of tsDCS utilized in our experiments: Over-skin, and Implanted-electrodes. As a current supplier, a GRASS stimulator with a dedicated DC unit was used (GRASS Technologies/NATUS, S88, CA USA) (Ahmed, 2016). By switching the polarity of the current source, the circuit design permitted instant reversal of the current direction from anodal to cathodal, and vice versa. The circuit design was effective in attenuating sciatic nerve current: For each 1.5 mA passing through the spinal-abdominal electrodes, there was only 300 μA passing through the sciatic nerve circuit. Monitoring of current parameters and the verification of DC attenuation was performed in the beginning, during, and at the end of each experiment using a bench-top digital multi-meter (Agilent/Keysight Technologies, 34401A, CA, USA).
Electrodes

Over-skin mouse-holder electrodes

Four mouse-holder electrodes designed for over-skin tsDCS were fabricated to permit concurrent over-skin tsDCS while testing the animal by the stretch apparatus (figure 2-1). The first electrode was permanently fixed to the middle of the stabilization plate, serving as the dorsal conductor, and connected to the anode (a). The second electrode was permanently fixed to the floor of the holding chamber, serving as the abdominal conductor, and connected to the (c1) branch. The remaining two were the sciatic nerve stimulating electrodes. Depending on which sciatic nerve was being stimulated, the corresponding electrode would be connected to the (c2) branch. To protect the sciatic nerve, current passing into the c2 branch was attenuated using a split current circuit (figure 1-1b). Each electrode was made of wick-covered 1x1.5 cm stainless-steel plate.

Implantable electrodes system

To provide under-skin trans-spinal DCS in awake animals, an implantable electrode system was fabricated according to (Ahmed, 2016). The system is made of spinal, abdominal and cuff electrodes.

Spinal and abdominal electrodes

Each electrode was fabricated using a stainless-steel plate (1.5 mm wide, 3 mm long, 50 μm thick), placed in between soft cotton-wick fabric (0.5 mm thick) and silicone rubber (178 μm thick) (Ahmed, 2016). Silicone adhesive was used to fuse all three layers. The electrode was, then, left to dry in room temperature overnight. Both spinal and abdominal electrodes were identical and differ only in implant location. The ready to use electrode (10 mm wide and 15 mm long) was
soaked in sterile 0.9% saline solution for 3 hours before implanted. The spinal electrode was placed over the spinal column between T13-L6 vertebrae. The abdominal electrode was placed between the skin and the abdominal wall.

**Cuff electrode**

The cuff electrode was fabricated according to Ahmed, 2016. A class of silicone tubing suitable for surgical implanting was used (2-mm outer diameter (OD), 3 mm long, 1.5-mm inner diameter (ID); AlliedSil) (figure 2-3). The conductors were made of stainless-steel wire (280-µm overall diameter, 10 strands, 45-Ω/foot impedance, nylon insulation material; Cooner Wire) (Ahmed, 2016). This wire was chosen due to the high degree of flexibility needed for implantation. In order to pass the wires into the silicone tubing, the hub of a 27-gauge needle was snipped and its shaft was passed through the silicone tubing. Then the stainless-steel wires were passed through shaft’s lumen (Ahmed, 2016; Akay, 2014). A soldering iron was used to melt and remove the insulation from the portion of the wire that would act as the conductor within the silicone tube. The ends of the wires were then tied to prevent slipping from the silicone tubing. The distance between the two sciatic nerve conductors within the cuff was fixed at 2 mm for all electrodes. A (6 cm), number 6–0, silk suture was tethered between the two wires through the silicone tube to secure it during implanting. To prevent electrical current conduction in the tissue that surrounds the cuff, the silicone tubing, wire knots, and suture, were covered with silicone adhesive. To prevent tangling of the remaining wires during animal movement, they were clamped using a thinner silicone tubing (51-µm inner diameter, 5-mm long) (Ahmed, 2016). silicone adhesive was then used to fill the thin tube. Next, a cut was made along one side of the 4mm length of the silicone tubing to allow the cuffing of the sciatic nerve by the electrode. Using a fine-tipped solder, the
loose end of each wire was then connected to a mini connector. The connectors from the nerve cuff, the spinal and abdominal electrodes were bonded together using super glue.

Implanting the cuff electrode system

The electrode system was then sterilized with 100% alcohol in preparation for implantation. To induce deep anesthesia, the animals were injected with a ketamine-xylazine cocktail (90% ketamine/10% xylazine, mg/kg, i.p). Deep anesthesia was verified when the animal stopped exhibiting withdrawal reflex response to hind foot stimulations, using forceps (Ahmed, 2016). They were then shaved on the thoracic back, and the most visually spastic hindlimb. Next, the area was disinfected with betadine before surgery started. Two incisions were made to the skin of the thoracic back, and the skin of the most spastic hindlimb, overlaying the sciatic nerve. To implant the electrode system, the spinal electrode was sutured with the silicone rubber side up, and the fabric side down, in complete contact with the spinal cord, covering vertebral levels between T13 to L6. The abdominal electrode was laid with the silicone side against the lateral abdominal wall and the fabric side pointing towards the deep side of the skin.

To attach the cuff electrode around the sciatic nerve, the sciatic nerve had to be exposed. This was done by dissecting the fascia between two muscles, the gluteus superficialis and biceps femoris. Once the muscles were separated, the sciatic nerve was freed from the surrounding connective tissue, and meticulously placed within the cuff (Ahmed, 2016). The cuff was, then, anchored to the gluteus superficialis muscle with one suture. To enhance the conductivity of the electrodes, sterile saline was used to moisten the tsDCS electrodes and completely fill the silicone tubing of the cuff. The surgical site was, then, covered with a layer of gentamicin sulfate ointment to prevent infection. The mouse then received a number and was placed on a heated tray (30 °C) for recovery.
Figure 2-3  Implantable electrode system

a, the cuff electrode. b, left, the assembled implantable electrode system. Right, the animal moved freely after the electrode system was implanted with no abnormalities (with permission from Dr. Zaghloul Ahmed)
The Effects of Direct Current Stimulation on Spasticity in Combination with Pharmacological Agents

Drug administration and protocols

Each animal was weighed then mounted to the stretch apparatus, and a baseline recording was taken before drug administration. Drug dose was calculated based on body weight. All control animals of the drug studies received sham tsDCS by going through the exact process as the experimental animals without turning the current on.

Isoflurane

In order to separate the passive and neuronal components of stretch resistance, a set of data were collected while the animals were fully anesthetized. Isoflurane was chosen as the drug of choice for this step due to its fast elimination half-time. Isoflurane was administered to all animals at the end of each study via inhalation: A Q-tip was soaked in isoflurane until saturated, then brought in contact with the animal’s nose through an opening in the cap of the mouse holding chamber.

Morphine

Morphine was dissolved in a sterile 0.9% saline solution, and administered in 30 mg/kg, i.p. Two groups of uninjured mice were used for this study: 1) an experimental group (n=5), received anodal tsDCS, 2) a non-stimulated, control group (n=2). All animals were tested using the stretch apparatus for stretch resistance and corresponding EMG activity. The study protocol proceeded as follows: a baseline recording was taken, followed by morphine injection. Then another recording was taken 35 minutes after injection to test morphine effect before tsDCS.
Subsequently, to test responses to morphine rigidity during tsDCS, more recordings were taken immediately, 10 minutes, and 20 minutes after anodal tsDCS treatment started.

**Gabapentin**

Gabapentin (GABA\textsubscript{p}) was dissolved in a sterile 0.9\% saline solution, and administered in 50 mg/kg, i.p. Two groups of mice with SCI were used for this study: 1) an experimental group (n=6) received anodal tsDCS, 2) a non-stimulated, control group (n=2). All animals were tested using the stretch apparatus for stretch resistance and corresponding EMG activity. Initially, a baseline recording was taken before GABA\textsubscript{p} injection. Then another recording was taken 30 minutes after injection to test GABA\textsubscript{p} effect before tsDCS. Subsequently, to test responses to spasticity during tsDCS, more recordings were taken as follows: during anodal tsDCS, when the anodal current was turned off, during cathodal tsDCS, and when the cathodal current was turned off. Similarly, the same animal group was used to test the effect of a longer duration (20 minute) of anodal tsDCS treatment after GABA\textsubscript{p} injection.

**MK-801**

MK-801 was dissolved in a sterile 0.9\% saline solution, and administered in 0.6 mg/kg, i.p. Two groups of mice with SCI were used for this study: 1) an experimental group (n=6), received anodal tsDCS, 2) a non-stimulated, control group (n=2). All animals were tested using the stretch apparatus for stretch resistance and corresponding EMG activity. Initially, a baseline recording was taken before MK-801 injection. Then another recording was taken 55 minutes after injection to test MK-801 effect before tsDCS. Subsequently, to test responses to spasticity during tsDCS, more recordings were taken as follows: during anodal tsDCS, when the anodal current was turned off, during cathodal tsDCS, and when the cathodal current was turned off.
**During and Short-Term Effects of Direct Current Stimulation**

To examine changes in spasticity in response to the immediate (during) and short-duration tsDCS, SCI-female CD-1 mice were randomly selected to one of three groups: anodal (n=5), cathodal (n=5), or sham (n=6). The animals of the anodal and cathodal groups received one tsDCS treatment according to their group assignment for 20 minutes, at 1.5 mA. Animals of the sham group went through the same procedure as the other two groups, but received no tsDCS. Prior to testing and tsDCS, the skin areas covering the SCI injury site and the sciatic nerve of the most affected hindlimb were shaved. The shaved skin of the animal and the mouse holder electrodes were wetted with 3% NaCl solution to promote DC conduction. Then the animal was placed in the mouse holder, mounted on the stretch apparatus, and received a baseline recording before tsDCS started. Once a baseline recording was taken, the tsDCS, whether cathodal or anodal, was turned on and went uninterrupted for the entire treatment period of 20 minutes. More recordings were taken while the animal was receiving tsDCS (during), immediately after the current was terminated, and every 10 minutes for 60 minutes.

**Procedure of Repeated-Session (Long-Term) Direct Current Stimulation**

This study was conducted to investigate the long-term effects of repeated-sessions anodal and cathodal tsDCS on spasticity in animals with SCI. Female CD-1 mice with SCI were randomly selected to one of three treatment groups: anodal (n=16), cathodal (n=15), or sham (n=10). A fourth group (n=8), did not receive SCI, served as the non-injured control (healthy group). All animals received initial baseline testing, which included the following: peak resistances at 3 different stretch speeds, EMG activity, skilled locomotion testing, and locomotion recovery testing. A total of nine mouse holders were fabricated to provide tsDCS to multiple animals, simultaneously. All animals were shaved in the same manner discussed above in the short-term
study. One shave on the first day of the long-term study was sufficient for the entire week of tsDCS sessions. The shaved skin of the animal and the mouse holder electrodes were wetted with 3% NaCl before each session. Animals of the anodal and cathodal groups received daily 20-minute tsDCS (1.5 mA) treatments for 7 days, according to their group assignment. Animals of the sham and control groups went through the same procedure as the treatment groups, but received no tsDCS intervention. Using the same baseline testing parameters, the animals were reevaluated immediately after the conclusion of the 7-day tsDCS treatment period, then at two weeks, and four weeks post-treatment. Additionally, following the last evaluation, the animals were tested for rate-dependent depression of the Hoffman reflex.

**Locomotion Recovery Testing**

The DigiGait system (Mouse Specifics Inc., Framingham, MA) was used to quantify locomotor recovery following SCI and tsDCS treatment (figure 3-4a). The system films the ventral aspect of the animals through a transparent treadmill belt. The system includes software that employs algorithms to quantify the characteristics of animal locomotion. All groups were tested four times by the DigiGait system: before, immediately after, two, and three weeks after tsDCS interventions. The control group was tested twice, two weeks apart. During testing, a minimum of 20 steps were collected at two speeds: 10 cm/s, and 20 cm/s. Three parameters measured during the stance phase of the mouse locomotion were chosen. These parameters are sensitive to changes in locomotion as a response to changes in muscle tone after tsDCS. These parameters are as follows: the peak paw area, rising slope and falling slope. Peak paw area is calculated as the maximum contact area of the foot of the mouse with the DigiGait treadmill belt. This rising slope is an indicator of the speed of locomotion during the first half of the stance phase of the mouse locomotion cycle. The stance phase is the segment of the locomotion cycle when the foot of the
mouse is in contact with the treadmill belt. The falling slope is an indicator of the speed of locomotion during the second half of the stance phase of the mouse locomotion cycle.

**Skilled-Locomotion Recovery Testing**

The ladder-wheel (figure 3-5a) was assembled in our laboratory from a stepper motor, a driver, and a controller (ARCUS Technology INC, DMX-UMD-23, CA, USA). The speed and direction of the wheel is controlled by custom-written software. The wheel is made from Plexiglas to allow side visualization. Spaces between rungs were variable to prevent memorization. A camera was placed under the animal to record foot faults. Animals were tested before stimulation started and once a week for three weeks after stimulation ended.

**Video analysis of the ladder-wheel task**

For each recorded session, a continuous 90 second video underwent a frame-by-frame analysis using a foot fault scoring system previously described by Metz and Whishaw (2009) (see, table 2-2). The score for each of the animal’s hindlimbs was calculated by multiplying the number of foot fault by its corresponding score for each criterion according to table 2-2 to get the criterion’s score. Then the scores for all criteria are added to find the total score for each of the hindlimbs.
<table>
<thead>
<tr>
<th>Foot Fault</th>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total miss</td>
<td>0</td>
<td>Assigned when a limb completely misses a rung and a fall occurs. A fall is defined as a limb deeply falling in-between rungs with body posture and balance disturbed.</td>
</tr>
<tr>
<td>Deep slip</td>
<td>1</td>
<td>Assigned when a limb is initially placed on a rung, then it slips off upon weight bearing and this causes a fall.</td>
</tr>
<tr>
<td>Slight slip</td>
<td>2</td>
<td>The limb is placed on a rung and then slips off upon weight bearing. However, the slip does not result in a fall and the animal is able to maintain balance and a coordinated gait.</td>
</tr>
<tr>
<td>Replacement</td>
<td>3</td>
<td>The limb is placed on another rung in the same step (score 3) and then slips and falls in-between rungs (score 1). For this a score of 1 is recorded. When a fall occurs, only the limb initiating the error is rated placed on a rung, but before it is weight bearing it is quickly lifted and placed on another rung.</td>
</tr>
<tr>
<td>Correction</td>
<td>4</td>
<td>When a limb aims for one rung yet is placed on another rung without touching the original rung. Alternatively, if a limb is placed on a rung and is quickly repositioned while remaining on the same rung.</td>
</tr>
<tr>
<td>Partial</td>
<td>5</td>
<td>The limb is placed on a rung with either the wrist or digits of the forelimb, or with the heel or toes of the hindlimb.</td>
</tr>
<tr>
<td>placement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correct</td>
<td>6</td>
<td>The mid-portion of the palm of a limb is placed on a rung and can bear the animal’s full weight.</td>
</tr>
<tr>
<td>placement</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2-2  Ladder wheel error scores**

Scoring system used to evaluate skilled walking using the ladder wheel apparatus (Metz and Whishaw, 2009).
Rate-Dependent Depression of the Hoffman Reflex (RDD)

Following the last evaluation, animals were anesthetized using mixture of ketamine and xylazine (90 mg/kg). The animals were shaved at the hindlimb and pelvic area. Two sets of recording electrodes were used to record EMG, and heart rate and breathing activity. Stimulation of the tibial nerve was achieved using concentric needle stimulating electrode (figure 3-6a, right). Following positioning the animal on the recording station, the heart (HR) and respiratory (RR) rates were monitored online. In deeply anesthetized animals, typically HR and RR were faster and RR was shallower. At that level of anesthesia, the reflexes were difficult to evoke and if evoked, the RDD was very high even in animals expressing severe spasticity. Therefore, in exploratory experiments, the procedure was standardized by monitoring the relationship between both HR and RR and the levels of awareness of the animal and RDD. For example, as animals awaken the HR first becomes regular at 2 Hz then becomes more irregular between 2 and 4 Hz, and RR becomes deeper and slower (figure 3-6b, c, and d). At the beginning of the irregular HR and deeper RR phase, the RDD protocol was started. It consists of five trains of 5 pulses. Frequencies of the trains were at 0.1, 0.5, 1, 2, and 5 Hz. The waiting intervals between the trains were 1-minute long. Testing was done on both hindlimbs. Stimulation intensity was gradually increased until the maximum H-wave relative to M-wave is seen, followed by the RDD protocol. Data was collected using the PowerLab system (ADInstruments, Colorado Springs, CO), recorded at sample rate of 10 KHz, and filtered at low pass of 2 kHz and high pass of 100 Hz. The data were averaged using the root square mean function with a 1-ms window using a built-in function in the LabChart software (ADInstruments, Colorado Springs, CO). Peaks of the averaged H-wave were measured and percent of change was calculated relative to the baseline values. RDD was calculated as the percentage reduction = difference between the H-wave amplitude, induced by the fifth pulse and
the first pulse in a train divided by the amplitude of the H-wave induced by the first pulse, multiplied by 100, then multiplied by -1 to inverse the values.
**Statistical Analysis**

Repeated measures ANOVA (RM ANOVA) was used to analyze the data for the following experiments: 1- the implanted system, 2- the during and short-term effects of tsDCS on muscle peak resistance, 3- the long-term effects of tsDCS, 4- the paw area responses to tsDCS, 5- the effect of tsDCS on the rising and falling slope in locomotion, 6- effects of tsDCS on RDD, 7- the effect of tsDCS on peak resistance with GABA\textsubscript{p} 7- The effect of tsDCS in MK-801-injected mice. All assumptions for the use of repeated-measures ANOVA were fulfilled. Holm–Sidak post-hoc correction was used to test differences between speeds in the stretch responses, the skilled motion, the GABA\textsubscript{p}, the morphine and MK-801 experiments. Post-hoc analysis was used to analyze the difference between the Anode and cathode in tsDCS treated animals. Mixed factorial ANOVA was used to assess the difference in EMG amplitude. The two-tailed paired t-test was used to analyze the difference between the two evaluations of the control groups in the skilled locomotion study. Bonferroni test was used to analyze the control group evaluations. Mixed two way repeated measures ANOVA was used to analyze the main effects between groups for the skilled locomotion. Friedman RM ANOVA was used to analyze the tsDCS on morphine rigidity, and Chi square to assess the difference between speeds. SPSS software was used to perform all statistical tests (IBM Statistics, SPSS version 23). And Sigma plot software to perform the statistics.
**Data Collection and Blindness**

A total of six research assistants gathered and filed data and calculated the scores for each animal: four research assistants transferred the stretch apparatus data from the LabChart software to Excel sheets, and calculated the scores for each animal using embedded Excel sheets formulas. One research assistant analyzed the DigiGait (unskilled locomotion) and ladder wheel (skilled locomotion) videos. And another assistant ran the data into embedded excel sheet formula to calculate animal scores. All assistants were blinded to the study hypothesis. Root mean square (RMS) equations were embedded in LabChart with window of 0.5 ms to generate the RMS for EMG and stretch data.
Chapter 3 – Results

Immediate Effects of Direct Current Stimulation on Spasticity using Implanted Electrodes

To investigate the immediate changes in spasticity as a result of tsDCS in mice with SCI, electrode systems were implanted in SCI animals (figure 2-3). After a recovery period of 48 hours, the animals were placed in the mouse holder, and mounted on the stretch apparatus for testing. Recordings of the animal’s peak resistance and EMG were taken before and after tsDCS (1.5 mA). Stretch testing shows that spinal anodal tsDCS treatment for 20 minutes caused significant reduction of muscle peak resistance in all speeds (figure 3-1). The reduced peak resistance was evident during stimulation, and continued for 60 minutes after the current stopped. The third speed showed the most reduction of peak resistance. Contrarily, cathodal tsDCS caused significant increase in peak muscle resistance during stimulation compared to baseline, but not afterward. Sham treatment, on the other hand, showed no changes in muscle peak resistance in any of the speeds. These results indicate that anodal and cathodal tsDCS have opposite effects on spasticity: cathodal current increases stretch responses only while the current is applied, with no significant carryover effect after the current is terminated. However, anodal current decreases stretch responses significantly; a response that was maintained for at least a full hour after the termination of current.
Figure 3-1

[Graph showing the effect of tsDCS on peak resistance across different speeds over time. The graph includes line graphs for Speed 1, Speed 2, and Speed 3, with error bars indicating variability. The x-axis represents time after tsDCS in minutes (0 to 60), and the y-axis represents peak resistance as a percentage of baseline.]
Applying anodal tsDCS (n = 4) results in significant reduction in muscle peak resistance (RM ANOVA, for speed 1: F = 5.99, p < 0.001; for speed 2: F = 8.61, p < 0.001; for speed 3: F = 17.07, p < 0.001). *p < 0.001 (Holm-Sidak method).
Immediate Effects of Direct Current Stimulation on Spasticity using Over-Skin Electrodes

Unlike the mice with implanted electrode systems, these animals received tsDCS via over-skin electrodes within the mouse holder to investigate immediate changes in spasticity as a result of tsDCS. In corroboration with the findings from the implanted electrode study (figure 3-1), over-skin tsDCS for 20 minutes caused significant reduction of muscle peak resistance in all speeds (figure 3-2a). The reduced peak resistance was evident during stimulation, and continued for the testing period of 60 minutes after the current stopped. The third speed showed the most reduction of peak resistance. Once more, cathodal tsDCS caused significant increase in peak muscle resistance during stimulation compared to baseline, but not afterward (figure 3-2b). Sham treatment, on the other hand, showed no changes in muscle peak resistance in any of the speeds (figure 3-2c). Comparable to the findings of the implanted electrodes study, these results confirm that anodal and cathodal tsDCS have opposite effects on spasticity responses. While increases in stretch responses following cathodal stimulation occur significantly during stimulation, they subside after termination of current. And anodal current, again, results in a significant reduction of stretch responses that persisted after termination of current. These findings indicate that the results obtained from tsDCS using implanted electrodes are reproducible using surface electrodes.
Figure 3-2

(a) Peak resistance (% baseline) over time for different speeds.

(b) Peak resistance (% baseline) for three different speeds (Speed 1, Speed 2, Speed 3).

(c) Comparison of peak resistance (% baseline) before (BL) and during tsDCS, and after tsDCS for different times (min).
Figure 3-2  During and short-term effects of tsDCS on muscle peak resistance in animals with spasticity after SCI

a, anodal tsDCS (n = 6) causes reduction of muscle peak resistance in all speeds, during and for 60 min after application (RM ANOVA, for speed 1, F = 3.57, p = 0.003; for speed 2, F = 3.96, p = 0.002; for speed 3, F = 13.44, p < 0.001). *p < 0.01 compared to baseline, Holm-Sidak method. b, cathodal tsDCS (n = 5) causes significant increase in peak muscle resistance during stimulation (During) compared to baseline (BL), but not afterward (RM ANOVA, for speed 1, F = 3.98, p = 0.002; for speed 2, F = 3.74, p = 0.002; for speed 3, F = 3.35, p = 0.005). *p < 0.05 compared to baseline, Holm-Sidak method. c, Sham treatment (n = 5) shows no changes in muscle peak resistance in all speeds (RM ANOVA, for speed 1, F = 1.0, p = 0.455; for speed 2, F = 0.83, p = 0.582; for speed 3, F = 1.55, p = 0.178).
Repeated Sessions of Direct Current Stimulation Produce Long-Term Effects on Spasticity

This study was conducted to investigate the long-term effects of repeated-sessions tsDCS on spasticity in animals with SCI. The animals of this study were divided into 4 groups:

1- Anodal tsDCS (n = 16); received 20-minute daily anodal tsDCS for 7 days.

2- Cathodal tsDCS (n = 15); received 20-minute daily cathodal tsDCS for 7 days.

3- Sham (n = 10), SCI mice, received 20-minute daily sham tsDCS for 7 days.

4- Control (n = 8), uninjured, received 20-minute daily sham tsDCS for 7 days.

The data of four parameters were collected: Active resistance peak, active slope, EMG area, and EMG amplitude. All four parameters were calculated by subtracting the raw amplitude value obtained while the animal was anesthetized with isoflurane from the raw amplitude value while the animal was awake. The differences in amplitude of active muscle resistance was assessed using mixed factorial ANOVA using time (4 levels: pre, E1, E2, and E3), and speed (3 levels: speeds 1, 2, and 3) as within subjects variables, and condition (three levels: anode, cathode, and sham) as between subjects variable. There is no significant interaction between the factor of condition and the factor of speed. The effect of different level of speed does not depend on what level of condition is present (F (6,114) = 0.4, p = 0.81). However, there is a significant interaction between condition and time of evaluation (F (6,114) = 2.36, p = 0.03). After adjustment for multiple comparison (Bonferroni), there is significant marginal mean difference between anode and cathode treated animals (p < 0.001), and between anode and sham treated animals (p < 0.001), but no significant difference between cathode and sham treated animals (p = 0.13) in E2, E3, and E4, but shows no significance difference between anode, cathode, and sham treated animals at E1 (p > 0.05).
After 7 days of 20-minute daily treatment with anodal tsDCS, SCI mice showed significant long-term depression of peak muscle resistance, and significant reduction in rising slope (figure 3-3, a and b). These significant depressions were evident in all three stretch speeds. On the other hand, cathodal or sham treatments resulted in no significant difference. Additionally, anodal tsDCS resulted in a significant reduction in EMG amplitude and EMG area (figure 3-3, c and d), which was evident in all three stretch speeds. However, the cathodal or sham treatments results in no significant difference. These results indicate that repeated stimulations using anodal current have long-term reduction in spasticity in mice with SCI.
Figure 3-3

(a) Active resistance peak (Awake-sleep) [g]
(b) Active elongation (Awake-sleep) [g/cm]
(c) EMG area (Awake-sleep) [µV]
(d) EMG amplitude (Awake-sleep) [µV]

Speed 1  | Speed 2  | Speed 3
---|---|---
Pre  | E1  | E2  | E3
Anode | Cathode | Sham | Control

* indicates significant difference
Figure 3-3  Treatment with anodal tsDCS results in long-term depression of spasticity in mice with SCI

a, animals treated with anodal tsDCS had significant reductions in the amplitude of active muscle resistance. The mean scores among the different levels of time within speed1 is statistically significant compared to pre-treatment score (E1, p < 0.001; E2, p = 0.003; E3, p = 0.002, Holm-Sidak method). The mean scores among the different levels of time within speed 2 is statistically significant compared to pre-treatment score (E1, p < 0.001; E2, p = 0.038; E3, p = 0.027, Holm-Sidak method). The mean scores among the different levels of time within speed 3 is statistically significant compared to pre-treatment score (E1, p < 0.001; E2, p = 0.004; E3, p = 0.046, Holm-Sidak method). b, Anodal tsDCS results in significant reduction in rising slope. The difference in active slope was also assessed using mixed factorial ANOVA. The analysis shows significant interaction between time and treatments (F (2, 114) = 3.59, p = 0.03). There was no interaction between speed and treatments. Post-hoc analysis shows significant main difference between Anode and cathode treated animals (p = 0.015), and between anode and sham (p = 0.04) during E2, E3, and E4. There was no difference between sham and cathode treated animals (p = 0.829). c, Anodal tsDCS results in significant reduction in EMG area. d, Anodal tsDCS results in a significant reduction in EMG amplitude. Both EMG area and amplitude analysis show similar results: the difference in EMG amplitude was assessed using mixed factorial ANOVA. The analysis shows significant interaction between time and treatments (F4, 218) = 3.63, p = 0.007). There was no interaction between speed and treatments (F (4,218) = 0.61, p = 0.66) nor between speed, time, and treatments (F (8,218) = 0.19, p = 0.9). Post-hoc analysis shows significant difference between anode and cathode treated animals (p <0.001), and between anode and sham treated animals (p = 0.001). There was no difference between cathode and sham treated animals (p = 0.96).
**Locomotion Recovery**

The DigiGait system was used to quantify locomotor recovery following SCI and tsDCS treatment (figure 3-4). In general, there was significant correlation between peak area, rising, and falling slopes in all groups (Pearson product moment correlation, p < 0.05). This indicates that the three parameters are mechanistically and functionally linked. Anodal tsDCS treated animals show significant increase in paw area at all evaluations during slow speed (E1-E3) for the left hindlimb (LHL) and E1 and E2 only for the right hindlimb (RHL). Paw area is significantly increased in E1 during fast speed for the LHL, and for the RHL. Rising slope is significantly increased during slow speed in E1 and E2 for RHL, but not for LHL. Rising slope is significantly increased in E1 during fast speed for RHL only. Falling slope is significantly increased in E1 and E2 during slow speed for LHL, and for RHL. Falling slope is significantly increased in E1 during fast speed for RHL only (figure 3-4d).

Control animals show no difference in the paw area, rising slope, or falling slope between the two evaluations of the two limbs (figure 3-4b). However, sham treated animals show significant decrease in evaluations (E1-E3) in paw area of the left hindlimb (LHL) during slow speed, but not for right hindlimb (RHL). Paw area is significantly decreased in all evaluations (E1-E3) during fast speed for LHL, but not significant change for RHL. Rising slope is significantly decreased in E1 during slow speed for the LHL, but not for the RHL (figure 3-4c). Rising slope is not changed significantly during fast speed for both hindlimbs. Falling slope is not significantly decreased for both hindlimbs during the slow speed. Falling slope is significantly decreased in all evaluation during fast speed for the LHL, but not for RHL.

The cathodal tsDCS treated animals show significant increase in paw area in E1 during slow speed compared to baseline but not in E2 or E3 (figure 3-4e). No significant change is seen in
paw area in all evaluations during fast speed. Rising slope is not changed during slow or fast speed. Falling slope is significantly increased in E1 and E2 for the LHL for slow speed, and for E1 only for the RHL for slow speed. There is no significant change in falling slope during fast speed.
Figure 3-4

a) DigiGait
Belt
Camera
Paw images
Control
Sham
Anode
Cathode

b) Speed = 10 cm/s
Speed = 20 cm/s

C) Speed = 10 cm/s
Speed = 20 cm/s

Sham
Anode
Cathode
**Figure 3-4  Repeated treatment with anodal tsDCS results in changes in spastic animals’ locomotion patterns**

a, schematic of the DigiGait system showing the general setup. Animal locomotion was tested at two speeds: 10 cm/s, and 20 cm/s. A typical recording is shown on the top right. Three parameters were taken for analysis: rising slope, peak paw area, and falling slope. The bottom figure shows recordings of stance phases from a representative animal from each group. b, control healthy animals were evaluated twice separated by 2 weeks. Gray bar for left hindlimb (LHL); blue bar for right hindlimb (RHL); E1: evaluation 1; E2: evaluation 2. Control animals show no difference in the paw area, rising slope, or falling slope between the two evaluations of the two limbs (p >0.05; two-tailed paired t-test). c, sham treated animals show significant decrease in evaluations (E1-E3) in paw area of the LHL during slow speed (RM ANOVA, $F = 4.75, p = 0.009$), but not for RHL. Paw area is significantly decreased in all evaluations (E1-E3) during fast speed for LHL (RM ANOVA, $F = 5.54, p = 0.005$), but not significant change for RHL. Rising slope is significantly decreased in E1 during slow speed for the LHL (RM ANOVA, $F = 3.20, p = 0.039$), but not for the RHL. Rising slope is not changed significantly during fast speed for both hindlimbs. Falling slope is not significantly decreased for both hindlimbs during the slow speed. Falling slope is significantly decreased in all evaluation during fast speed for the LHL (RM ANOVA, $F = 4.47, p = 0.012$), but not for RHL. *p < 0.05, Holm-Sidak method. d, anodal tsDCS treated animals show significant increase in paw area at all evaluations during slow speed (E1-E3) for LHL and E1 and E2 only for RHL (RM ANOVA, for LHL: $F = 12.70, p < 0.001$; for RHL: $F = 17.32, p < 0.001$). Paw area is significantly increased in E1 during fast speed for the LHL (RM ANOVA, $F = 7.04, p < 0.001$), and for the RHL (RM ANOVA, $F = 13.32, p < 0.001$). Rising slope is significantly increased during slow speed in E1 and E2 for RHL (RM ANOVA, $F = 6.06, p = 0.002$), but not for
LHL. Rising slope is significantly increased in E1 during fast speed for RHL only (RM ANOVA, \( F = 4.19, p = 0.011 \)). Falling slope is significantly increased in E1 and E2 during slow speed for LHL (RM ANOVA, \( F = 10.03, p < 0.001 \)), and for RHL (RM ANOVA, \( F = 13.48, p < 0.001 \)). Falling slope is significantly increased in E1 during fast speed for RHL only (RM ANOVA, \( F = 9.06, p < 0.001 \)). *p < 0.05, Holm-Sidak method. e, cathodal tsDCS treated animals show significant increase in paw area in E1 during slow speed compared to baseline (RM ANOVA, for LHL: \( F = 7.92, p < 0.001 \); for RHL: \( F = 8.46, p < 0.001 \)) but not in E2 or E3. No significant change is seen in paw area in all evaluations during fast speed. Rising slope is not changed during slow or fast speed. Falling slope is significantly increased in E1 and E2 for the LHL (RM ANOVA, for slow speed: \( F = 6.72, p < 0.001 \)), and for E1 only for the RHL (RM ANOVA, for slow speed: \( F = 3.47, p = 0.025 \)). *p < 0.05, Holm-Sidak method. There is no significant change in falling slope during fast speed.
Anodal Treatment Enhances Recovery of Motor Control of Skilled Locomotion

The effect of the repeated tsDCS on skilled locomotion was investigated using a computer controlled ladder wheel (Figure 3-5A). There was a significant difference across the four time points in the anode treated group ($F = 6.49, p = 0.002$). The RHL anode treated group showed statistically significant higher mean score in E2 compared to E1 ($p = 0.001$, Bonferroni). The LHL of the anode treated group showed statistically significant higher score in E2 and E4 compared to E1 ($p < 0.01$, Bonferroni) (Figure 3-5B). No other group showed significant difference across the four time points ($p > 0.05$, Bonferroni).

Next, the percent change (from pre-evaluation) between groups (Figure 3-5C) was compared. Mixed two way repeated measures ANOVA showed significant main effects among the groups ($F = 5.97, p = 0.016$). Anode group showed significantly higher percent change in scores compared to cathode group ($p = 0.027$, Bonferroni), and healthy group ($p = 0.048$, Bonferroni). Anode group showed higher percent change than sham group, however, not statistically significant ($p > 0.05$). There were no significant differences among other the groups ($p > 0.05$).

Overall, these findings reveal enhanced recovery of motor control of skilled locomotion in anode-treated animals. Two major findings can be deduced from the above results: 1) since the healthy group did not show changes in the skilled locomotion scores, this indicates no motor learning is possible using our motorized ladder-wheel system. And that the motorized ladder-wheel test is probing motor control and not motor learning. 2) By reducing spasticity, anodal treatment enhances motor control.
Figure 3-5  Enhancement of skilled locomotion following repeated anodal tsDCS treatment

a, left: an image of the ladder-wheel; right: consecutive frames showing a total miss occurred by the hindlimb. b, summary plots showing mean score of skilled locomotion. Sham, SCI group that was sham treated; healthy group, no injury/no treatment; anode, SCI group that was treated repeatedly for 7 days; cathode, SCI group that was treated for 7 days. c, summary plots showing percent change (of pre-treatment evaluation or first evaluation in healthy controls). Data shows as means ± S.E. * p < 0.05 compared to pre.
Rate-Dependent Depression of the Hoffman Reflex (RDD)

Mixed two way repeated measures ANOVA was conducted to assess the changes in RDD. The treatment condition (independent factor, 4 levels: control, sham, anode, and cathode) shows significant difference in the main effect among the different levels of treatment ($F = 3.0$, $p = 0.038$). There is also significant main effect of frequency (within subjects repeated factor, 5 levels: 0.1, .5, 1, 2, and 5 Hz) ($F = 13.8$, $p < 0.001$). There is not a statistically significant interaction between frequency and treatment condition ($F = 1.35$, $p = 0.192$). The effect of different levels of treatment does not depend on what level of frequency is present.

Multiple comparison test (Tukey) was applied to isolate which group(s) differ from the others. As shown in figure 3-6, within control, the mean scores of RDD at frequencies 1, 2, and 5 Hz are significantly higher compared to RDD at 0.1 Hz (5 vs. 1 Hz, $p <0.001$; 4 vs. 1Hz, $p = 0.006$; 3 vs.1, $p = 0.024$). Within sham, the mean scores of RDD at all frequencies are not significant compared to RDD at 0.1 Hz ($p > 0.05$). Within anode, the mean scores of RDD at frequencies 2, and 5 Hz are significantly higher compared to RDD at 0.1 Hz (5 vs. 1 Hz, $p <0.001$; 4 vs. 1Hz, $p <0.001$). Within cathode, the mean scores of RDD at 1 Hz is significantly higher compared to RDD at 0.1 Hz ($p = 0.017$).

Between groups comparison (Tukey) shows significant differences of RDD scores at 5Hz. RDD score at 5 Hz is significantly higher in the anode group compared to both sham and cathode groups ($p < 0.05$). The control group shows significantly higher RDD at 5 Hz compared to cathode group ($p = 0.016$). Overall, these findings provide physiological mechanism that underlying the reduction of spasticity in response to repeated anode stimulation.
a, left photograph: visible attributes of spasticity in animals with SCI; right photograph showing the recording setup. SE, stimulating electrode; RE recording electrode. The two recording traces on the right in (a) are the raw trace (top), and the root-mean square calculated trace (bottom). b, the respiratory rate (RR) during deep anesthesia (blue), and 45 min after anesthetic injection (orange). c, Top: heart rate (HR) during deep anesthesia (blue), and after 45 min of injection (orange); bottom: the full recording, with breaks, showing the HR at which RDD testing took place. d, showing changes when RDD was tested during deep anesthesia versus during light anesthesia. e, representative recordings from different groups. f, left: summary blots showing the average RDD in different groups. RDD is significantly higher in control group (n = 12) for rate 1-5 Hz compared to rate 0.1 Hz (RM ANOVA, F = 8.815, p < 0.001). RDD in sham group is significantly higher at rates 2 and 5 compared to 0.1 Hz (RM ANOVA, F = 3.725, DF = 16, p = 0.009). RDD in anodal tsDCS group is significantly higher at rates 1-5 Hz compared to 0.1 Hz (RM ANOVA, F = 8.598, FD = 16, p < 0.001). RDD in cathodal tsDCS group is significantly higher at rates 1 and 5 Hz only (RM ANOVA on Ranks, Chi-square= 16.933, n = 12, p = 0.002. *significant from RDD at 0.1Hz in the same group (Holm-Sidak method; **significant from respective RDD in sham group (t-test). Right: cumulative probability distribution of the groups.
**Effect of Direct Current Stimulation after Gabapentin Injection**

Gabapentin (GABAp) is a drug with many pharmacologically therapeutic effects: it has been found to treat epilepsy and reduce spasticity after SCI through multiple cellular mechanisms. Although not a GABA receptor agonist, it has been suggested that GABAp works by increasing GABA synthesis and inhibiting glutamatergic transmission (Kitzman et al., 2007).

This study aimed to investigate tsDCS effect on GABAp’s ability to reduce stretch peak resistance in mice with SCI, during and after 20-minutes of anodal tsDCS. Two groups of mice with SCI were used for this study: 1) an experimental group (n=6), received anodal tsDCS, 2) a non-stimulated, control group (n=2). Data show that there was significant decrease after 30 min of GABAp injection and further significant decrease during anodal tsDCS (figure 3-7b). In the same group of animals, 20 min of anodal tsDCS results in further significant depression of peak muscle resistance for speed 1, but not for the other two speeds (figure 3-7c). There was a steady reduction of peak muscle resistance during sham treatment in the control group (figure 3-7d). The data indicate that anodal tsDCS adds further depression to peak resistance in addition to the depression induced by GABAp injection.
Figure 3-7

(a) Graph showing force over time with different speeds indicated by blue, black, and red lines. The x-axis represents different speeds, and the y-axis represents force.

(b) Bar graph showing peak resistance across different conditions and speeds. The x-axis represents conditions (Baseline, GABA, Anode, Cathode), and the y-axis represents peak resistance (g). The graph includes error bars.

(c) Bar graph showing peak resistance across conditions and speeds. The x-axis represents conditions (Baseline, Anode 20 min, Baseline, Anode 20 min), and the y-axis represents peak resistance (g). The graph includes error bars.

(d) Line graph showing peak resistance over time with different speeds. The x-axis represents time (min), and the y-axis represents peak resistance (g). The graph includes markers for Speed 1, Speed 2, and Speed 3.
Figure 3-7  Anodal tsDCS adds further depression to peak muscle resistance following the depression induced by injecting GABAp

a, examples of traces of muscle force recording and their respective EMG. b, Summary plot shows significant decrease of peak resistance after 30 min of gabapentin injection (n = 6; RM ANOVA, for speed 1, F = 14.66, p < 0.001; for speed 2, F = 11.14, p < 0.001; for speed 3, F = 16.43, p < 0.001), and further significant decrease during anodal tsDCS (RM ANOVA, for speed 1, F = 8.96, p < 0.001; for speed 2, F = 11.68, p < 0.001; for speed 3, F = 18.20, p < 0.001). *p < 0.001 Holm-Sidak method. c, In the same group of animals, 20 min of anodal tsDCS results in significant depression of peak muscle resistance for speed 1 but not the other two speeds (RM ANOVA for speed 1, F = 8.63, p = 0.007). *p < 0.01 Holm-Sidak method. d, control group (n = 2) shows steady reduction of peak muscle resistance during sham treatment.
Effects of Direct Current Stimulation after Morphine Injection

Morphine injection has long been associated with catatonic rigidity (Widdowson et al., 1986). This study aimed to investigate whether tsDCS has an effect on morphine-induced increase in the stretch responses in mice with SCI, during, 10, and 20 minutes after anodal tsDCS. Two groups of mice with SCI were used for this study: 1) an experimental group (n=5), received anodal tsDCS, 2) a non-stimulated, control group (n=2). The data show that morphine caused significant increase in muscle resistance, 35 min after injection (figure 3-8a). And although immediate anodal tsDCS resulted in reduction of morphine-induced muscle resistance in all three speeds, the reduction was not significant (figure 3-8b). Additionally, there was no significance between baseline and after 10 or 20 min of anodal tsDCS. Morphine-induced muscle resistance continued to increase steadily during a sham treatment (no tsDCS) (figure 3-8c).
Figure 3-8

(a) [Image of a biological study setup]

(b) [Bar graph showing peak resistance (% baseline) with different conditions and comparisons marked with asterisks (*) indicating significance.]

(c) [Graphs showing force and RMS-EMG measurements with 23 g and 100 µV scales, indicating conditions like baseline, morphine, and anode, and speed conditions 1, 2, and 3.]

Speed 1  Speed 2  Speed 3
Figure 3-8  Anodal tsDCS reduces morphine-induced muscle resistance

a, photographs showing fanning of the toes, and extension pattern of the paws. Right: strong resistance was manually felt. Also the tail was rigid. Bottom, example of force and RMS-EMG recorded before morphine (blue), after 35 min of morphine injection, and during the application of anodal tsDCS (red). b, morphine caused significant increase in muscle resistance 35 min after injection (n = 5) and this increase was reduced but is still significant by immediate application of anodal tsDCS (Friedman RM ANOVA, for speed 1, chi square = 14.8, p = 0.005; for speed 2, chi = 13.92, p = 0.008; for speed 3, chi square = 13.92, p = 0.008), *p < 0.05, Tukey test. There is no significance between baseline and after 10 or 20 min of anodal tsDCS. c, morphine caused steady increase in muscle resistance during a sham treatment (no tsDCS).
Effects of Direct Current Stimulation after MK-801 Injection

MK-801 is an N-Methyl-D-aspartate (NMDA) receptor uncompetitive antagonist. It is used as anticonvulsant and anesthetic (Zuo et al., 2007). This study aimed to investigate tsDCS immediate effect on MK-801’s ability to reduce stretch resistance in mice with SCI. Two groups of mice with SCI were used for this study: 1) an experimental group (n=6), received anodal tsDCS, 2) a non-stimulated, control group (n=2). Data show that, compared to baseline, MK-801 caused significant reduction in peak muscle resistance 55 min after injection. While cathodal tsDCS had no effects on peak muscle resistance at all speeds, short term (1 min) application of anodal tsDCS reduced peak resistance at all speeds, significantly (figure 3-9a). Control group shows stable reduction of muscle peak resistance (figure 3-9b). These findings indicate that in SCI mice, anodal tsDCS further depresses spasticity in the presence of MK-801.
Figure 3-9  Anodal tsDCS further depresses Spasticity in the presence of MK801 in animals with SCI

a, peak muscle resistance expressed as percent of baseline (n = 5). MK801 causes significant reduction in peak muscle resistance 55 min after injection. Furthermore, short term (1 min) application of anodal tsDCS reduces peak resistance at all speeds. However, cathodal tsDCS has no effects on peak muscle resistance at all speeds. The difference in the mean values among the groups was tested using RM ANOVA, for speed 1, F = 25.5, p < 0.001; for speed 2, F = 8.3, p < 0.001; for speed 3, F = 10.5, p < 0.001). To isolate the different groups, Holm-Sidak method was used; *p <0.001 compared to baseline; **p <0.002 compared to MK801. b, control group (n = 2) shows stable reduction of muscle peak resistance.
Chapter 4 – Discussion

Implantable vs. Surface Electrodes

Characterized as an upper motor neuron and muscle tone disorder, spasticity is defined as a velocity-dependent increase in muscle resistance in response to passive stretching (Lance, 1980). Management of muscle tone pathology is a crucial component of the rehabilitation process following SCI. This study investigated the immediate and long-term effects of tsDCS on spasticity, and the interaction of tsDCS with certain pharmacological agents, in order to understand the combined effects with tsDCS and possible tsDCS mechanisms of action. In this study, the immediate effects of tsDCS were examined using two different current-delivery settings: one utilized an implanted electrode and the other utilized a surface electrode. Initially, the electrode systems were implanted over the spinal column and the sciatic nerve of SCI mice according to Ahmed, 2016 (figure 2-3). And the systems were successfully used to provide anodal (spinal-to-sciatic) or cathodal (sciatic-to-spinal) DCS. Anodal DCS treatment for 20 minutes using the implanted electrode system caused significant reduction in spasticity. This reduction was noticeable as soon as the anodal DCS started, and continued for an hour after the current stopped (figure 3-1). Although the implantable electrode system provided precise DCS to the spinal cord and sciatic nerve, implanting it was a tedious surgical procedure that involved possible risks of infections and post-surgical complications. Additionally, the procedure was time consuming and required extensive preparations before, during and after the surgery. Therefore, the effectiveness of surface electrodes, fabricated in our laboratory, in providing tsDCS was investigated. The surface electrodes were designed to be permanent components of the mouse holder (figure 2-1). Additionally, provided that anodal current possessed potential modulatory effect on spasticity, delivering the current noninvasively would hold far more clinical importance due to its non-
invasiveness. For the experiments in this study, not only the noninvasive setup was effective in reproducing data comparable to those obtained using the implanted system, but it eliminated the risks and complications associated with surgery, and the time needed for preparation and recovery. Additional important consideration in choosing over-skin electrodes over implantable electrodes is the practicality of their use in clinical settings.

**Expected Anodal Direct Current Stimulation Responses**

The concurrent reduction of spasticity in response to anodal tsDCS was anticipated in both setups of the immediate effect studies (figures 3-1 and 3-2): Eccles et al., 1962 found DC current to be an effective modulator of synaptic activity between the motor neurons and sensory afferents neurons within the spinal cord (Eccles et al., 1962). The anodal current on the spinal cord caused reduction in the amplitude of EPSP in motor neurons. Additionally, Ahmed, 2014 investigated the modulatory effects of DC stimulation, and concluded that spinal-to-sciatic current reduced the muscle and nerve responses induced by steady and transit stretches (Ahmed, 2014b).

**Justification of Stretch Parameters**

To quantify responses to tsDCS via the stretch apparatus, the following parameters were used: peak muscle resistance, rising slope, EMG amplitude, and EMG area. Peak muscle resistance (calculated in gram of muscle resistance) is a sensitive parameter, reached at the end of the dynamic phase of the muscle stretch. The rising slope (calculated in gram/second) is a parameter that takes into consideration the variability of the rate of change along the resistance curve. EMG amplitude is an indicator of muscle activity; the higher the EMG amplitude, the higher the muscle resistance. EMG area is a parameter that takes into consideration the aggregated EMG activities in a specific period of time.
Neuronal vs. Passive Resistive Components

Data analyzed for all parameters are the raw data (data collected while the animal is awake) minus those collected during isoflurane anesthesia. This method of data analyses was chosen to isolate values that represent the neuronal component of the stretch responses from passive resistance. The data collected during isoflurane, while the animal is fully anesthetized, represent muscle resistances that are attributed to passive forces only. The neuronal component of stretch resistance is greatly eliminated during isoflurane application. Therefore, when isoflurane values are deducted from the raw values, the difference will represent the neuronal component of the stretch responses only. The elimination of the passive muscle resistance is crucial to obtaining accurate data, since passive components can be altered due to SCI and repetitive stretching during testing. This method also makes it possible to quantify the portion of the stretch resistance that can be attributed to neuronal activity.

Reliability of the Stretch Apparatus

The SCI mouse model is ideal because of its availability, turnover speeds, ease of handling, and genetic closeness to humans. To investigate the effects of tsDCS on spasticity using this model, a tool to quantify stretch responses in mice was needed. However, there was no tool on the market made for this purpose. Therefore, a stretch apparatus was designed to accommodate the SCI mouse model, and then assembled in our laboratory. The sensitivity and validity of the apparatus have been verified through a number of uses: it detected differences between stretch responses in awake vs. isoflurane-anesthetized animals (figure 2-1). Additionally, control groups have been tested multiple times with no or negligible differences in subsequent recordings. The apparatus had also detected the differences between normal muscle stretch responses and those elevated due to morphine rigidity (figure 3-8), or decreased due to the influence of GABAp (figure...
One feature of the apparatus proved to be of great importance to this study is its ability to detect stretch responses at different speeds. This feature was crucial for this study since spasticity is a velocity-dependent response.

**Rate-Dependent Depression is reduced in Spastic Mice**

Rate-Dependent Depression (RDD) is the attenuation of the h-reflex amplitude following repeated-sessions stimulations (Ishikawa et al., 1966; Lloyd and Wilson, 1957; Meinck, 1976). RDD is reduced in spastic humans (Aymard et al., 2000; Nielsen et al., 1993), and animals (Hedegaard et al., 2015; Lee et al., 2014). In our RDD study, there were no significant differences in RDD reduction between the different frequencies in the sham group. There was significant difference between the control and sham groups at 5 Hz (figure 3-6 F). These findings support prior work correlating decreased RDD with increased spasticity after CNS injury (Lee et al., 2014).

**Effects of Ketamine on Rate-Dependent Depression**

In order to obtain RDD recordings, mice were anesthetized with a ketamine/xylazine cocktail (90/10 mg/kg, i.p). However, RDD recordings showed dependence on the level of ketamine anesthesia for the same animal, with increases in RDD in deeply anesthetized mice, and decreases in RDD when the same animal was under light anesthesia. These observations indicated that RDD was affected by ketamine concentrations in the body (figure 3-6). To eliminate the effects of deep ketamine anesthesia, the RDD protocol was performed while the animals were lightly anesthetized. The state of light anesthesia was accomplished when the animals reached precise values of heart and breathing rates. These values were reached approximately 45 minutes after anesthetic injection, where heart and breathing rates were 2 Hz and 0.5 Hz, respectively (figure 3-6 B, C, and D). It was crucial to standardize the RDD protocol initiation point for all
animals. This testing setup ensured that any RDD changes were caused by the tested variables, not the differences in ketamine levels. For future investigations, it is our recommendation to control for the effects of ketamine when used as anesthetic during RDD protocols. The process through which ketamine exerts its modulating effects on RDD has not been investigated. And the exact mechanism of its anesthetic activity is not well understood (Tyler et al., 2017). However, ketamine’s role as a nonspecific N-Methyl-D-aspartate receptor (NMDAR) antagonist has been well supported by research (Anis et al., 1983; Sleigh et al., 2014).

**MK-801**

Therefore, this experiment hypothesized that NMDAR may play a role in RDD in spastic mice. To test this hypothesis, MK-801 was used, which is an NMDAR specific antagonist, to block ketamine activity. Our results show significant reduction in stretch amplitudes following MK-801 injection (figure 3-9 A). These results are in agreement with prior work that explored the role and the potential therapeutic use of MK-801 in the treatment of spasticity (Brunson et al., 2001; Li and Tator, 1999; Simpson et al., 1995). Notably, MK-801 blocked cathodal but not anodal stimulation effect on the stretch resistance in all three speeds. And it seems that anodal stimulation has an additive effect that is probably mediated by a different mechanism.

**Repeated Sessions of Anodal Stimulation Restore Rate-Dependent Depression in Spastic Mice**

The monosynaptic circuitry mediating the stretch reflex includes two types of neurons: The Ia afferent sensory neurons from the muscle spindle, and the alpha motor neuron that innervates the same muscle. The Ia afferent projects on the motor neuron to form an excitatory synapse. In addition to stretching, the circuit can be activated with electrical stimulation to produce
the H-reflex. The reduction of the H-reflex amplitudes associated with repeated stimulation of the stretch reflex pathway is the result of post-activation depression (Crone and Nielsen, 1989; Hultborn et al., 1996).

The mechanisms that underlie post-activation depression involve a decrease in the probability of neurotransmitter quantal release from the Ia afferent due to its prior activation (Hirst et al., 1981; Kuno, 1964a; Kuno, 1964b; Lev-Tov and Pinco, 1992). Post-activation depression is functionally beneficial during voluntary movement, as it lowers the Ia afferent’s synaptic efficacy; therefore, prevent clonus and oscillations (Hultborn and Nielsen, 1998). Decreased post-activation depression; however, would increase the efficacy of the synapse, which in turn may lead to the hyper-excitability commonly associated with spasticity (Hultborn and Nielsen, 1998).

RDD data were obtained from mice that underwent two types of stimulations, during tsDCS and after a week of repeated tsDCS (long-term) sessions. The results show significant RDD increases in the anode group compared to the sham group in both, the short-term tsDCS (data not shown) and long-term tsDCS (figure 3-6). These results can be explained in light of the modulating effects of DC current at the synaptic level. Anodal DC current, applied on the dorsum of the spinal cord, depolarizes the presynaptic terminals of Ia afferents, decreasing motor neurons’ EPSP. However, cathodal DC current hyperpolarizes the presynaptic terminals of the Ia afferents, increasing motor neurons’ EPSP (Eccles et al., 1962). Additionally, anodal current increases the frequency of miniature end-plate potentials due to enhanced spontaneous release (Del Castillo and Katz, 1954), likely causing depletion of presynaptic neurotransmitter.

In summary, the stretch reflex circuit is partially capable of modulation via embedded mechanisms, such as the adjustment of spontaneous neurotransmitter release and post-activation depression. These mechanisms, which are independent of higher CNS control, contribute to the
functional decrease in the stretch reflex activity during voluntary movements. After SCI, the mechanisms are altered and the reflex pathway becomes hyperactive (Dietz and Sinkjaer, 2007; Little and Halar, 1985; Xu et al., 2015). However, using dorsal anodal stimulation after SCI, normal activity within the stretch reflex pathway was restored. Worth noting, the effect of anodal stimulation reported by Del Castillo & Katz, 1954 continued for few minutes after the stimulation was terminated. Our repeated stimulation study results show a carryover effect that lasted for weeks after the repeated-sessions anodal tsDCS was terminated. These findings have great implications for future studies aiming to investigate the mechanisms of the carryover effect at the cellular and molecular levels.

Repeated Sessions of Direct Current Stimulation Result in Long-Term Effects on Spasticity

This study was conducted to investigate the long-term effects of repeated-sessions tsDCS (7 days of 20-minute daily treatment) on spasticity in mice with SCI. Because spasticity is velocity-dependent, the TS was stretched at three different speeds: 18, 180, and 1800˚/s. Four data parameters thought to be important in assessing spasticity were measured. These parameters are as follows:

1- The peak of active resistance
2- The active slope
3- The amplitude of EMG.
4- The area of EMG

For each of these components, on the active portion was including in the data analysis. The active component of each parameter represents the portion caused by neuronal activities, whereas the passive component represents the portion attributed to other elements, such as connective tissue. Each active component was calculated by subtracting the raw amplitude value
obtained while the animal was anesthetized with isoflurane from the raw amplitude value while the animal was awake, as explained in the methods section. Calculating the active values proved important when analyzing active peak and slope due to the inherent passive characteristics of the tissues involved in muscle contraction and joint stabilization, such as tendons and ligaments. Calculating active values was less important for EMG peak and area, because EMG activities are muscle responses to neuronal inputs, and only traces of EMG activities were present during isoflurane anesthesia (figure 2-1b, right –blue and green trances). The long-term tsDCS study data show that mice treated with anodal tsDCS had significant reductions in all four parameters compared to the other groups (figure 3-3). A plausible explanation to this finding involves the modulating effects of tsDCS on three components that directly affect the stretch reflex amplitude. These components are gamma motor neuron activity, Ia afferent-motor neuron synapse, and interneurons activity.

The reduction of active muscle resistance with anodal tsDCS (figure 3-3a) can, at least partially, be attributed to the current’s ability to modify the activation threshold of muscle spindles. Matthews in 1972 found that the dynamic and static activation threshold of the muscle spindle could be controlled by two different types of motor neurons, the dynamic and static gamma efferents, respectively (Matthews, 1972). In this context, any activity modification of the gamma motor neuron caused by DC can have a direct impact on the activation threshold of the muscle spindle. This notion was confirmed when gamma efferents were found to exhibit decreased evoked activity with anodal current, and increased evoked activity with cathodal current (Ahmed, 2016).

There are two defined mechanisms through which tsDCS can modulate gamma motor neurons activities: 1- direct modification of their membrane potential, 2- indirect modification of their presynaptic transmission (Ahmed, 2011; Ahmed, 2013a; Ahmed, 2013b; Ahmed, 2014b;
Ahmed, 2016; Bolzoni and Jankowska, 2015; Eccles et al., 1962). As explained by Ahmed 2016, gamma motor neurons have high input resistance due to their small size, which makes their membrane potentials less responsive to the electrotonic spread of tsDCS, possibly favoring the indirect presynaptic mechanism pathway.

**Direct Current-Induced Calcium Accumulation and Neuronal Plasticity**

Calcium is responsible for initiating and modulating a variety of activities within the cell, such as control of neurotransmitter release (Borst and Sakmann, 1996), long-term changes in synaptic transmission associated with memory (Dunwiddie and Lynch, 1979; Nicoll et al., 1989; Turner et al., 1982; Wang and Stelzer, 1996; Yasuda and Tsumoto, 1996), axonal regeneration (Hannila and Filbin, 2008; Henley et al., 2004), and activation of kinases that mediate gene expression (Bading, 1999; Li et al., 2016). One intriguing effect of tsDCS on neural tissue is its ability to redistribute calcium ions. DC current was found to increase calcium accumulations in axons, synaptosomes and segments of the spinal cord, where anodal current was more effective in the axons and cathodal current was more effective in synaptosomes (Wieraszko and Ahmed, 2016). Early work investigating the effect of DC current on the monosynaptic stretch reflex junction shows that anodal current decreased the amounts of neurotransmitter release (Hubbard and Willis, 1962b), and cathodal current produced the opposite effect (Hubbard and Willis, 1962a). It is important to note that our tsDCS spinal electrode, located over the injured segment, should target different types of neurons and neuronal structures. These structures may include local interneurons and terminals of descending axons from higher spinal segments and the brain. However, importantly, the anodal current will have a direct effect on the Ia-motor neuron synapses, whether on those that survived the contusion injury, or the intact ones in the surrounding area. Therefore, the decrease in stretch responses found with anodal tsDCS (figure 3-3) can be
explained as a direct result of calcium influx, modulating the spontaneous quantal neurotransmitter release at the Ia-motor neuron synapses. The increased spontaneous transmitter release possibly depletes its presynaptic concentrations, causing depressed evoked potentials seen as reduced EMG activities during anodal tsDCS.

Interestingly, the intracellular increased calcium mobilization may not only explain the short-term decreased spasticity found during anodal tsDCS, but can also explain the continuation of spasticity reduction in the long-term study. DC was found to continue modulating intracellular calcium concentrations for hours after its termination, possibly by increasing the activity of membrane-bound calcium channels, or activating and relocating calcium channels into the membrane (Wierszko and Ahmed, 2016). Additionally, DC was found to exert synaptic modulations, where anodal current increased, and cathodal current decreased long-term potentiation (LTP) (Podda et al., 2016; Ranieri et al., 2012). Therefore, the mechanism by which DC exerts its long-term effect can alternatively be explained in light of synaptic plasticity (Monte-Silva et al., 2013).

**Gabapentin**

Initially emerged as an antiepileptic drug (Johannessen and Ben-Menachem, 2006) and later used to treat neuropathic pain (Rosner et al., 1996), GABAp has a structure that is closely related to GABA (Rose and Kam, 2002). The mechanism through which GABAp exerts its action is not fully understood (Pfizer, 2014; Sills, 2006), and has been a subject of considerable debate for a number of reasons: despite early notion, GABAp does not bind GABA_A (Rose and Kam, 2002) nor GABA_B (Lanneau et al., 2001) receptors, and does not seem to affect the concentration, synthesis, or synaptic clearance of GABA (Pfizer, 2014). However, a strong body of evidence indicates that GABAp has an affinity for αδ-1, (Gee et al., 1996; Marais et al., 2001; Patel and
Dickenson, 2016), a subunit component of the voltage-gated calcium channel, responsible for its trafficking (Dolphin, 2012), membrane localization (Dolphin, 2013), and more importantly, modulation of calcium current density (De Waard and Campbell, 1995). GABA\(\alpha_2\delta-1\) functions as \(\alpha_2\delta-1\) ligand, causing disruption of calcium channel trafficking (Hendrich et al., 2008) and reduction of calcium current. \(\alpha_2\delta-1\) subunits are upregulated in the sensory neurons of the dorsal root ganglion (DRG) after peripheral nerve injury (Bauer et al., 2009; Lana et al., 2014), and upregulated in the dorsal, but not the ventral, spinal cord, 30-40 days post-SCI (Boroujerdi et al., 2011). Overexpression of \(\alpha_2\delta-1\) has been associated with the increased surface expression of calcium channels (Gurnett et al., 1996), and increased excitability of the dorsal spinal cord (Li et al., 2006; Nguyen et al., 2009). And in the presence of the astrocyte-secreted protein thrombospondin, in a process independent of the calcium channel current, \(\alpha_2\delta-1\) increases excitatory synaptogenesis (Eroglu et al., 2009). These facts underline the important function of \(\alpha_2\delta-1\) in modulating calcium current and neuronal plasticity. Prior work attributes spasticity, in part, to increases in presynaptic glutamatergic signaling, leading to motor neuron hyper-excitability (Kitzman, 2006). Therefore, the decreased peak resistance encountered after GABA\(\alpha_2\delta-1\) injection (figure 3-7a) can be explained as a reduction in presynaptic calcium current due to GABA\(\alpha_2\delta-1\) binding its \(\alpha_2\delta-1\) receptor, leading to decreased quantal glutamate release. The enhanced reduction of peak resistance induced by anodal tsDCS after GABA\(\alpha_2\delta-1\) injection (figure 3-7b) is probably due to depletion of the presynaptic glutamate reserves as a result of enhanced spontaneous transmitter release (See Chapter 4 on DC-induced calcium accumulation and neuronal plasticity).

**Morphine**

Systemically administered opiates, such as morphine, cause dose-dependent muscular rigidity (Havemann et al., 1980; Vučković et al.; Wand et al., 1973); however, the exact mode of
action is not clear. A number of brain structures have been implicated in opioid-induced rigidity, such as the basal ganglia (Slater and Starkie, 1987), locus coeruleus (Lui et al., 1989), thalamus (Ossowska et al., 1986), periaqueductal gray, and nucleus raphe pontis (Weinger et al., 1991). It has been suggested that morphine interacts with the dopaminergic receptors in the striatum, reducing the amounts of released dopamine (Kuschinsky and Hornykiewicz, 1972), through a mechanism similar to that of Parkinson’s disease (PD). Though the reduction in the levels of dopamine in PD are caused by loss of dopaminergic nigrostriatal neurons, (Korchounov et al., 2010), morphine rigidity is likely due to inhibition of tyrosine hydroxylase, the enzyme that catalyzes the rate-limiting step in dopamine synthesis (Freye, 1976). Opioid rigidity was also explained as a result of the interaction with the opiate binding receptors in the striatum, particularly the mu (μ) type (Goldblum and Loew, 1991; Vankova et al., 1996) located in the head of the caudate nucleus (Havemann et al., 1980). Opioid (μ) receptors are not exclusive to the brain. They are abundant in the spinal cord and DRG where they are implicated in the local nociceptive depressive effects of opioids, segmentally and peripherally (Besse et al., 1990; Stein, 2003). It seems that the opioid-induced anti-nociceptive spinal depression is independent of the opioid effect on the brain, and independent of the influence of the descending connections (Chen and Pan, 2006). However, this may not be the case for opioid-induced rigidity, where supraspinal centers plays a major role (Kuschinsky et al., 1977; Vankova et al., 1996). Supraspinal involvement had been confirmed via spinalization experiments that abolished morphine-induced rigidity, where descending influence excited the extensor alpha motor neurons (Kuschinsky et al., 1977) and inhibited the flexor alpha motor neurons (Seeber et al., 1978).

The results show palpable morphine-induced extensor muscle rigidity, 35 minutes after morphine injection (figure 3-8a), and significant peak resistance responses in all three passive
stretch speeds compared to baseline (figure 3-8b). Although the immediate response to anodal tsDCS was a reduction in active peak resistance, the resistance was still significant compared to baseline. However, 10 minutes after anodal tsDCS, the morphine-induced resistance was no longer significant, and the reduction continued for 20 minutes after the initial application of anodal tsDCS (figure 3-8b).

The increased peak resistance of the TS (an ankle extensor) following morphine injection (figure 3-8b) confirms the findings reported by Seeber et al. (1978). And although the anticipated reduction in the tibialis anterior muscle tone (ankle flexor) could not be assessed due to stretch apparatus limitations, it is assumed to be present. Since the tibialis anterior muscle is a TS antagonist, its morphine-induced tone reduction can be viewed as an added force that can enhance the peak resistance of the TS stretch. So, two forces are contributing to the increased TS stretch peak resistance: 1- its morphine-induced rigidity, and 2- the force added by the morphine-induced inhibition of the tibialis anterior muscle. The results show the effectiveness of anodal tsDCS as modulator of morphine-induced rigidity by overcoming these combined forces. And although the exact mechanisms of morphine-induced rigidity are not yet fully understood, it seems that anodal tsDCS reduces morphine-induced extensor muscle rigidity, possibly via decreasing synaptic activity (See Chapter 4 on DC-induced calcium accumulation and neuronal plasticity).

**Locomotion Recovery**

Impaired locomotion is one of the most debilitating consequences of SCI. Great body of research has been dedicated to finding methods to increase the motor function of patients after SCI. In this study, the long-term effects of tsDCS on spasticity, recovery of gait and skilled locomotion were assessed. Specific gait parameters that reflect possible tsDCS-induced changes in spasticity were investigated. The direct relationship between spasticity and locomotion
abnormalities is well documented in research (Adams and Hicks, 2005; Bravo-Esteban et al., 2013; Faist et al., 1999; Lamontagne et al., 2001). For example, in healthy humans, continuous modulation of the stretch reflex is essential for maintaining the proper progression of the gait cycle (Capaday and Stein, 1986; Crenna and Frigo, 1987). However, after CNS injury, this modulation suffers different levels of impairment, depending on the extent of spasticity, and the origin of injury, cerebral or spinal (Faist et al., 1999). Additionally, the level of SCI and the severity of spasticity have a major impact on the effectiveness of walking, and can predict patterns of gait abnormalities (Krawetz and Nance, 1996). Moreover, spasticity slows locomotion and interferes with skilled movements due to co-contraction (Dyer et al., 2011), and associative movements (Mayer, 2002).

In this study, the data clearly show that repeated anodal tsDCS resulted in Long-term amelioration of spasticity, as indicated by the significant decreases in active peak resistance, active resistance slope, and EMG activities (figure 3-3c). However, it was important to investigate whether the anodal-induced reduction in spasticity can be linked to improved functionality. As discussed earlier, after SCI, spasticity interferes with the components of the gait cycle; therefore, significant reductions in spasticity should have normalizing effects on at least some of these components. This investigation concentrated on parts of the cycle that involve stretches of the TS during various parts within the stance phase – when the foot is in contact with the ground. In stance, it is ideal to assess the effects of spasticity on gait, as the TS stretch at different velocities, which is on par with the definition of spasticity coined by Lance (Lance, 1980).

To assess the effects of tsDCS on gait (figure 3-4), three locomotion-related parameters were investigated: rising slope, peak paw area, and falling slope. These parameters were chosen due to their sensitivity to changes in the stance phase of the gait cycle. For example, the beginning
of the rising slope marks the initiation of the stance phase, when the animal’s heel strikes the ground. The rest of the rising slope corresponds to the progressive lowering of the foot to the ground, as the animal shifts body weight (the loading response) to that foot. The shifting continues till midstance – a point at the middle of the stance phase with the foot flat on the ground. At midstance, the second parameter, the peak paw area, was measured. The falling slope, however, follows the end of midstance, and begins as heel starts to elevate (heel off), and continues as the foot gradually prepares for push off to propel the animal forward.

It is important to note that spastic animals show tip-toeing paw placement during the heel strike and will have a decreased peak paw area. They will also have a lower rising and falling slopes due to the increased transition time caused by spasticity. Consequently, changes in the peak paw area, rising slope, or falling slope can be directly attributed to modulation of the severity of spasticity. In order to appreciate this relationship, one must remember that the slopes provide important temporal information regarding the lengthening and contractibility of the TS during different points of stance. For example, an increased falling slope after anodal tsDCS signifies a faster transition from midstance to push-off. During most of the stance phase, the TS undergo progressive lengthening, as the ankle flexes, and the body moves forward. In spastic animals, this lengthening triggers locomotion-interfering reflexive contractions due to the impaired reflex modulation associated with spasticity (Faist et al., 1999). Therefore, the significant increases in the falling and rising slopes following anodal treatment shown by the data (figure 3-4d, middle and lower graphs), indicate enhanced transition, and decreased transition time. Additionally, the peak paw area for the same animals also increased during midstance for both slow and fast speeds (figure 3-4d, top), compared to sham-treated animals (figure 3-4c top).
These data collaborate with our observations during locomotion testing, as the anodal-treated animals gradually changed their tip-toeing paw placements during midstance to foot-flat placements. Considering that the anodal-treated animals didn’t receive any additional locomotion training compared to the animals of the other groups, these findings show genuine intrinsic plastic changes. Therefore, it is safe to deduce that increased paw area signifies improved modulation of the stretch reflex of the foot and toe flexors in response to passive stretches from the ground. And that the increased rising and falling slopes signify improved modulation of the stretch reflex in response to stretches of the TS during the stance phase.

Our data show consistency with respect to the effect of anodal tsDCS as a stretch reflex circuit modulator. As explained earlier, long-term anodal tsDCS restored RDD (figure 3-6e and f). It also reduced stretch reflex-induced peak resistance, resistance slope and EMG activity, acutely and chronically, at all different tested speeds, after SCI (figure 3-3). These findings point to a modulated stretch reflex circuit model that is capable of receiving and executing input signals from multiple CNS sources. For example, the new modulatory properties of the stretch reflex circuit are not only capable of adjusting to inputs received from the locomotion-mediating central pattern generators, but are also capable of adjusting to the skilled-movement commands from higher CNS centers. This was confirmed by the improvement in skilled locomotion scores exhibited by the anode-treated animals (figure 3-5b and c).

**Study Weaknesses**

The animals of the immediate effect study were evaluated for only one hour after treatment, with no subsequent assessments. Follow up evaluations should have been conducted to find a threshold in the treatment protocol (the least number of treatment session) where the immediate effect becomes long-term.
Future Studies

1- Follow up investigations would allow us to quantify the amount of time it takes for the electrophysiological effect of tsDCS to dissipate, and assess the amount of time needed for the initiation and completion of protein synthesis necessary for the long-term effect changes. More evaluations at different set points during the treatment protocol can reveal the long-term effect threshold.

2- Assuming that LTP-, LTD-like mechanisms are behind the long-term changes encountered post-tsDCS, a subsequent study will be needed to assess protein changes and map their pathway(s). The application of a protein synthesis inhibitor, anisomycin, within three hours of stimulation may also allow us to investigate treatment threshold.
Bibliography


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