

NEUROMODULATION IMPROVES MOTOR AND
COGNITIVE PERFORMANCE IN ANIMAL MODELS

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Biomedical Engineering – Doctor of Philosophy

2022

ABSTRACT

Neurons change the way they respond to a specific stimulus by functional and structural changes, known as neuroplasticity. Neuroplasticity can be modified via different stimuli as electrical, chemical, and mechanical interventions, causing alterations to central and peripheral nervous system functions. Past neuroimaging studies related to chronic pain showed changes associated with altered cortical balance between excitation-inhibition and maladaptive plasticity. However, the mechanisms behind neuroplasticity and the optimal parameters which induce long-term, and sustainably enhanced performance remain unknown. Previous studies have shown that neuromodulation can induce beneficial changes through neuroplasticity. Therefore, in this study we focused on identifying the best strategies to induce neuroplasticity in the somatosensory cortex (S1). First, we tested if non-invasive repetitive transcranial magnetic stimulation (rTMS) induces neuronal excitability, and cell-specific magnetic activation via the Electromagnetic-Perceptive Gene (EPG). EPG is a novel gene that was identified and cloned from glass catfish (*Kryptopterus vitreolus*). In response to magnetic stimulation, this gene promotes neural activation, which could potentially restore cortical excitability. The results demonstrated that neuromodulation significantly improved long-term mobility, decreased anxiety, and enhanced neuroplasticity, reinforcing the growing amount of evidence from human and animal studies that are establishing neuromodulation as an effective strategy to promote plasticity and rehabilitation. Second, we identified the best protocol to facilitate the greatest changes in fMRI activation maps in the rat S1. The results showed that a single session of rTMS increased S1 activity, but induced changes that are absent three days after the session. Instead, forepaw

stimulation of 10 Hz delivered synchronized with 10 Hz rTMS for five consecutive days demonstrated the greatest increase in the extent of the evoked fMRI responses. These results provide direct indication that pairing peripheral stimulation with rTMS induces long-term plasticity, and this phenomenon appears to follow a time- dependent plasticity mechanism. Given these results, we can conclude that neuromodulation induced by changes on S1 can improve cortical balance, and this therapy could be used in the future to treat different types of disorders.

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This thesis is dedicated to my family and all my friends; my parents Lya Neuberger and Herschel Cywiak, my sister Vanessa and my brother Mendy, and my friend that is like my stepmother Gabriela Saldana, who support and inspiration for my academic pursuits. And, not forgetting my little dog Lulu Cywiak, which was hours and days setting near to me to write this entire thesis.

ACKNOWLEDGMENTS

First, I would like to express my sincere gratitude to the Biomedical Engineer Program, specially to Dr. Chistopher Contag, Dr. Adam Alessio, Dr. Katy Colbry, Dr. Dana Spence, and Tiffany Owen, for the support throughout my entire PhD program and the opportunity to prove myself. I am very grateful for all the patient, understanding and encouragements through a hard time in my life.

Second, I want to thank my committee members: Dr. Aitor Aguirre, Dr. Adam Alessio, Dr. Zhen Qui, for all the effort, time, help and suggestions to finish my PhD in such a short timeline. Also, to Dr. Pelled, my Principal Advisor, for teaching me that not all the mentors wan their students to succeed.

Furthermore, I would like to thanks to all my friends and colleagues: Alesa Hugshon, Abigael Metto, Ryan Hunt, Evran Ural, Harvey Lee, Connor Grady, Yonathan Israeli, Ron Israeli, Petra Telgkamp, Rita Martin, Gabriela Saldana, Nisachon Chaiwang, David Filipovic, Marco Lopez, Kylie Smith, Ti'ar Riggins, Monica Setein, Ayarith Guittens, Ligia Hernandez, Norka Guedez, Ayala Aharon, Orly Farjon, Carol Waldman, Yanelis Kahn, Norka Guedez, Devi Romero, Albiris Alvarez, Diana Flores, Sharon Waich, Hector David Graterol, Caridad Farimich, Elyssa Cox, Lauren Wed, Mariana Saldana, Cristina Moreau, Johana Gonzalez, Chelsea Boodoo, Alexander Farnum and everybody else that where part in my life and currently I do not recall the names (sorry for that), thanks for been part of my growing process and for the small or big contribution you made to help me to finish my degree, and support in all this year's specially on difficult moments.

In addition, I would like to thank the writing center for taking the time to read my entire thesis with me and fixing my grammar errors, to present the best writing thesis part as I was able to do so.

Finally, and the most importantly, I would like to sincerely thank my family, without them I was not able to get to this point.

I would like to end these words with a famous quote phrase used by a special Spanish Argentina Singer Gustavo Cerati: “*Gracias Totales*”, which means “*Thanks for everything*”

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Chapter 1: Introduction

1.1 Dynamic network communication in the brain

The brain operates through a complex interaction that combines flow of information and signaling processes within neural networks [2, 3]. Neurons communicate via a combination of electrical and chemical signals. This communication occurs in cellular gaps called neuronal synapses, facilitated by a chemical transmission. The pre-synaptic neuron releases a chemical called a neurotransmitter that is received by the post-synaptic neurons via the neurotransmitter receptors. Once the neurotransmitter binds to the receptor, it alters the conformation of the post- synaptic neurons, leading to excitation or inhibition depending on what is required[4].

Network neuroscience in the past two decades in animal models and in humans has helped us to understand underlying mechanisms of network communication. Physiological and anatomical changes are driven by natural sensory stimulation, skill acquisition, peripheral injury, central injury, exogenous growth promoting agents, exogenous neuromodulation drugs, and exogenous electrical/magnetic stimulation [5].

Rodents and humans are mammals with very similar brain structures at the general neural architecture, therefore rodents are widely used for neuroscience research. In addition, we can readily implement neural genetic manipulation techniques in rodent models, allowing for thorough assessment of changes in cellular structure and functional architecture. Many functional connectivity neural disorders have been successfully modeled in animals, including peripheral nerve injury, and phantom limb disorder.

Some of the many benefits of using rodents for preclinical research are the low cost, ease of handling, and extensive library of existing neurological and genetic literature. Using

rodents, researchers can easily conduct scientific studies such as *in-vivo* imaging, electrophysiological recording, and post-mortem histology. One key benefit of using rodents in the sciences is the ease of implementing genetic engineering strategies. As an example, studies have used the transgenic rodents allowing to visualize how treatments induce positive or negative changes to specific pathways [6].

In addition, studies have proven that PNI injured rats have alterations in cortical representational maps of the somatosensory cortex (S1), and these changes most likely bring discomfort and neuropathic pain [5]. Functional neuroimaging and thorough investigation of neural connectivity networks can help us to better understand how the brain changes after an injury. These approaches are crucial to the development of novel therapies to relieve pain after injury.

12 Understanding the Somatosensory Cortex Area

Considering that previous studies have shown the importance of the primary somatosensory cortex (S1) in neuropathic pain[7], in this section I will provide a brief explanation of the location and function of this brain region. S1 is located in the parietal lobe of the brain on the postcentral gyrus, posterior to the central sulcus. In early 1909 [8], Wilder Penfield categorized this area as part of Brodmann's areas 3a,3b, 1 and 2; but more recent work done by Kaas [9, 10] suggests that only area 3 should be referred as the "primary somatosensory cortex". Previous animal studies showed that area 3a receives inputs from muscles and joints, 3b from the skin and 2B/A can combine skin and proprioceptive information [11, 12].

Figure 1 shows the “*Sensory homunculus*” [13], which is a topographic map of the body surface identified in the human brain. It is represented by the sensory distribution and usually illustrates the body parts at the surface of the postcentral gyrus of the parietal lobe. The density and space of each body part is dependent on the receptor’s density and differs on each body part.

This is the reason why the body parts are represented in different sizes as it extends over the cortex. The topography always corresponds to the contralateral side of the body.

The similarity between nervous system anatomy, physiology, and biochemistry between rodents and other mammals permits common use of rodents as a model for neurological function and disease. At the microscopic level, major cells are very similar across species. Although the brain structure is similar between rodents and humans, there is a difference in sensory and motor modalities. Therefore, the body proportion representation on the somatosensory cortex differs markedly between them; in rodents it is called the “*Sensory musunculus*”. The dominant structure is different between the two species; in humans face and hands are the dominant structures, and in rodents, the nose (olfaction) and the whisker barrels are the dominant structures [14].

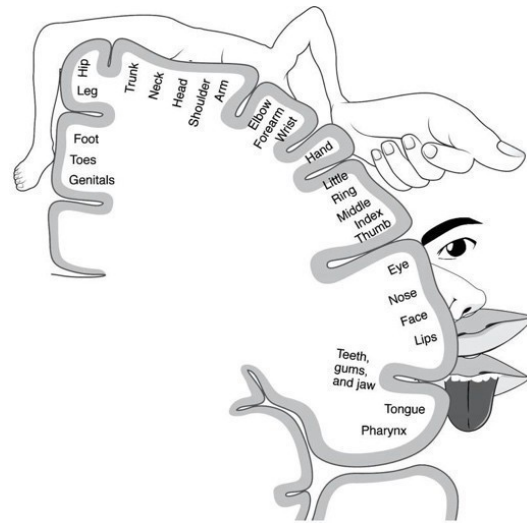


Figure 1. Sensory Homunculus of a human body illustrating how brain maps different sensory organs of the body according to their allocated proportions in the cortex[1].

Impulses from parts of the body are sent to the spinal cord and ascend to the brain to be processed. The trigeminal pathway carries the somatosensory information from the face, head, mouth, and nasal cavity. The sensory pathway can be divided in three successive nuclei neurons. First, axons from trigeminal ganglion enter to the brain stem at the pons level where it is then projected to three locations. The spinal trigeminal nucleus receives pain and temperature sensation. Consecutively, the axons are projected to the chief sensory nucleus at the pons or the mesencephalic nucleus on the midbrain, where the dorsal column system receives information such as touch, pressure, and vibration. Finally, the sensory impulses terminate into the postcentral gyrus, where the sensory map within the somatosensory cortex, is located[13].

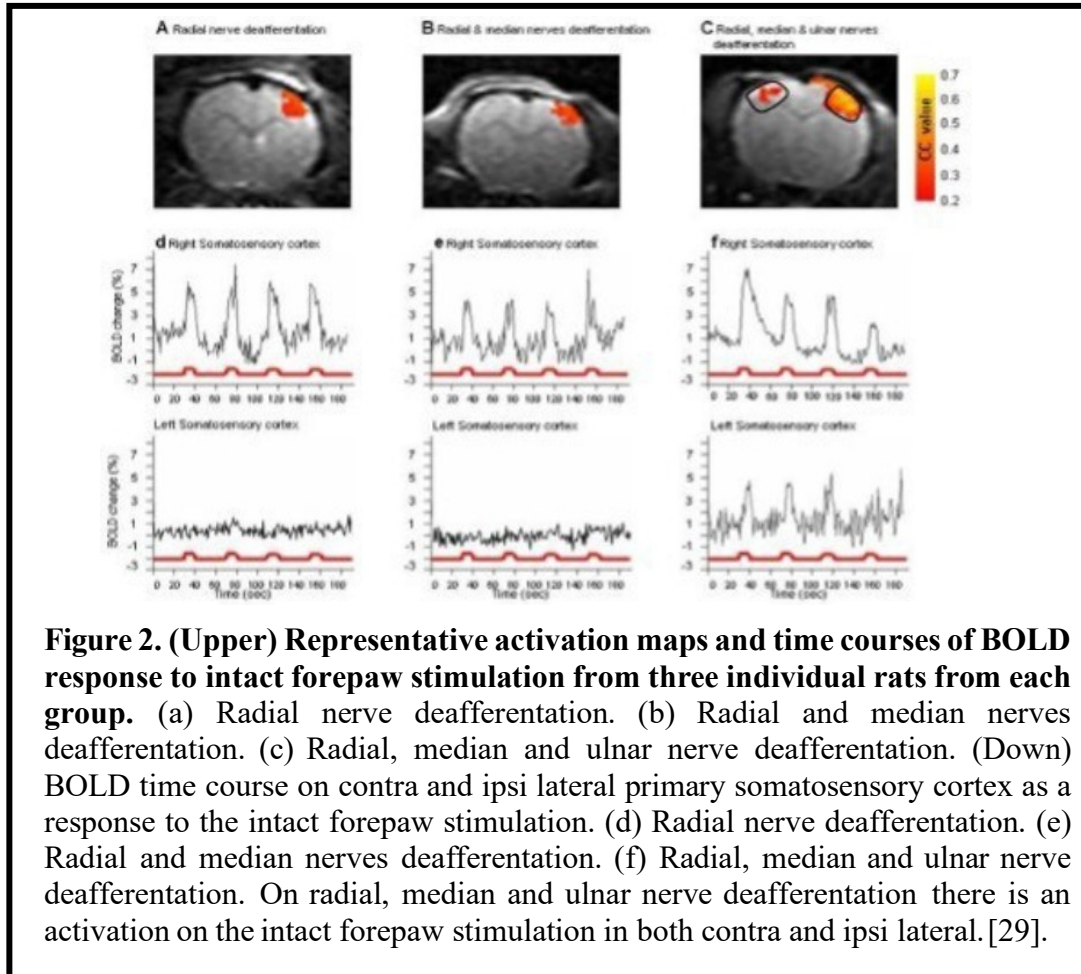
Talbot in 1991 [15], demonstrated the significant collateral somatosensory areas that are activated in response to arm stimulation and showed the relation between pain perception to S1. Other studies done on the human brain have helped to identify

significant pain receptor activation in the contralateral somatosensory cortex, S1. While a pain perception exists, those receptors have a significant role on nociceptive processing [16]. In this study, we will examine if the S1 cortex could be a good approach to relieve or modulate the perception of pain in humans.

13 Brain mapping by functional Magnetic resonance Imaging

The brain is capable of adapting in response to external and internal events. These changes in neuronal communication are known as plasticity. Studies have shown that changes in plasticity directly affect the brain's response to any stimulus, hence new techniques have been developed to understand those changes [17]. One such technique is Functional MRI (fMRI), a temporal form of Magnetic Resonance Imaging. This technique is non-invasive and provides a high spatial resolution, signal reliability, robustness, and reproducibility [18]. fMRI mapping works by utilizing a venous blood oxygenation level-dependent (BOLD) contrast technique. A BOLD signal is an indirect measurement of neuronal activity and is reflected by changes in the regional cerebral blood flow, volume, and oxygenation [19]. The visual results show local stimulation of neuronal activity, which requires higher energy consumption and an increase in oxygenated blood, oxyhemoglobin, and increased balance of oxygenated arterial blood to deoxygenated venous blood associated with deoxyhemoglobin. Changing the ratio between oxy/deoxy hemoglobin leads to an increase in the fMRI signaling compared to the surrounding tissue. Increase in neuronal activity provides regional vasodilation and flow increases. This is called hemodynamic response function (HRF), and BOLD signaling characterizes this HRF shape [20].

Hence, fMRI studies can be described as the technique that projects images of the differential neuronal response to a different activity or stimuli [21, 22]. fMRI can be used to detect plasticity changes in rodents, and this approach has been extensively used in our lab, as well as others. Results from these studies are shown in Figure 2 [17, 23-29].



To date, fMRI has been used within multiple cognitive neuroscience disciplines [30] such as sensory-motor functions [31], language [32], visuospatial orientation [33], attention [34], memory [35], affective processing [36], working memory [37], personality dimensions [38], decision-making [39] and executive function [40]. It has also been demonstrated to be useful to detect addiction behavior [41], neuromarketing [42], politics [43], etc. Moreover, fMRI has the potential to improve clinical neuroimaging due to the tremendous possibility of brain mapping pre- surgical and post-surgical differences. Additionally, fMRI has shown changes in neuronal plasticity from drug addiction or any other disease that leads to a maladaptive change in brain function.

Hence, fMRI, to this day, has been an excellent tool to recognize spontaneous and intrinsic brain activity, and the most accurate technique to explain how connectivity patterns work in both healthy and diseased brains [44, 45].

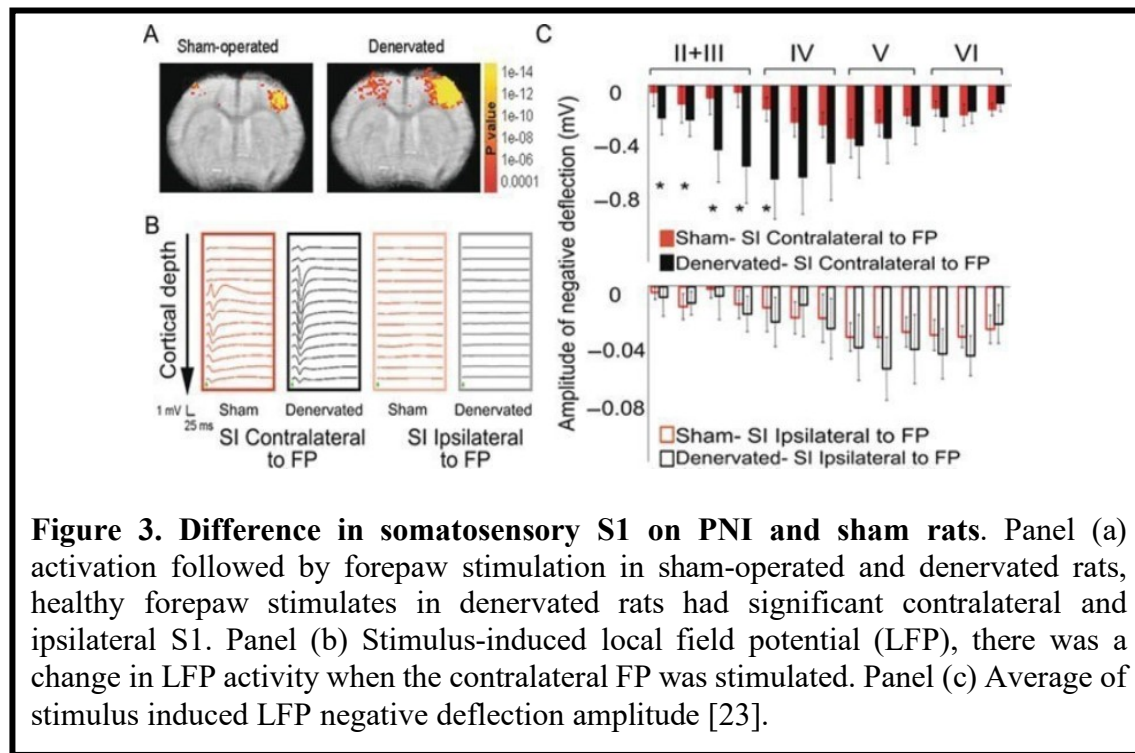
14 Peripheral Nerve Injury can induce maladaptive changes in the brain

As described in the previous sections, S1 is involved in neuropathic pain. This section will focus on Peripheral Nerve Injury (PNI). PNI leads to significant changes in cortical and subcortical neuronal activity. These changes frequently result in maladaptive activity, causing potentiating neuropathic pain, dystonia or muscle atrophy, and profound weakness. As a result, at least 80% of amputee patients suffer phantom limb pain [29, 46]. Most of the time, these injuries are caused by an accident, trauma, or other causes. PNI can constitute total loss of the limb or incomplete recovery of motor

and or sensory function [47, 48].

Nerve injury causes changes that can be divided into two categories: immediate changes after the injury, and changes that come over days to weeks. New neural connections are often observed [49]; to date, the mechanisms underlying the transition from short term to longer term changes after a PNI remain unknown. In addition, plasticity mechanisms can alter neuronal layer communication, interhemispheric communication, and subcortical activity, which sometimes affects recovery [50].

Figure 3 demonstrates studies done in our lab on healthy rodents, where stimulation on the limbs leads to persistent activation in the S1 contralateral to the stimulated arm, with minimal activity in the ipsilateral S1. After PNI is induced in the rodents, a consistent pathway changes in magnitude, and there are also changes in the location of fMRI responses [17].



As shown in previous studies conducted in our lab, neuronal activity changes 60 minutes after PNI [51]. Specifically, after sensory deprivation, we observed increased excitability in layer V in the pyramidal neurons of the S1 barrel cortex on to the ipsilateral and collateral hemispheric brain and a small appreciation increased on layer IV [52]. Thus, layer V could also be a potential target to guide plasticity after a limb injury in adults.

15 Neuromodulation as a therapeutic intervention

When neurons respond to a stimulus with functional and structural changes, these changes are called neuroplasticity. The stimulus provides neuronal excitability, increasing the strength of synaptic connections, resulting in functional modification. Neuroplasticity manipulation can be done via electrical, chemical, and mechanical

interventions, causing alterations to central and peripheral nervous system function [53, 54].

Previous studies have shown structure neuronal changes persist in long-lasting and non- neuronal activity after the cessation of the stimulation [55, 56]. Neuromodulation can be invasive, minimally invasive, or non-invasive. The stimulus, which can be electrical or chemical, to a specific nerve, can improve the quality of life for humans suffering from neurological disorders [57]. Neuromodulation as a therapeutic option varies for each individual due to multiple factors, such as age, sex, psychological and genetic factors, pain pathology, and the timing of neuromodulatory treatment [58].

The clinical concept for neuromodulation is described as the process through which electrical, chemical, and mechanical intervention facilitates changes in peripheral nervous system function. This is done by electrical or pharmacological intervention, with either implanted or non- implanted devices [59]. The neuromodulation technology is classified as reversible, because inhibited or stimulated neurons produce therapeutic effects on different chronic pain, epilepsy, ischemia, cardiac, bowel, bladder dysfunction, nervous system injury, and movement, visual, auditory, or psychiatric disorders [60].

Neuromodulation can be defined also as:

- Neuroaugmentation: enhancing the nervous system and the activity by implantation of the devices [61].
- Neurostimulation: providing electrical currents with implanted electrodes to stimulate or inhibit specific neuronal groups [62].
- Functional electrical stimulation (FES): selective stimulation of

motor fibers generates functional muscle contraction, whether transcutaneous (non- invasive) or subcutaneous (invasive) [63].

- Assistive technologies: equipment used to increase, maintain, or improve the functional capabilities of an individual with disabilities [64].
- Neuroprosthetics: biotechnology that constructs and implants artificial devices that generate electrical stimulation, replacing the function of the damaged limb [65,66].
- Neural engineering: integration of electronic interface to the brain, spinal cord, and nerves [67].

The research into this novel technique of neuromodulation is providing many research groups a tremendous opportunity to help to improve the quality of human life [68].

1.6 Tracking neuromodulation improvement with behavior tests in rodents

Tracking changes in behavior after a PNI and therapy delivery is a critical tool. Usually, the scientific use of animal models in laboratories are used to estimate if the therapeutic improvement can also improve the quality of human life. This is because of the existing homology between rodents and humans [69, 70].

Animals cannot describe or report pain, therefore scientific studies have performed battery of behavioral tests in order to observe changes in welfare and mood once pain is present [71]. Rodents with chronic pain have been reported to exhibit behavior indicative of depression and anxiety. Therefore, there exists several preclinical

behavior tests to observe chronic pain conditions. Those tests address basic locomotor and sensory functions, which can assess cognition and emotional behavior [72].

Laboratory rodent behavior test samples:

- Home cage activity: measure changes on normal activity,
- Rotarod: measure balance on the stationary rod,
- Hot place (nociception): measure discomfort related to the nociception (pain),
- Morris water maze: study learning and memory,
- T maze: measure spatial and working memory,
- Objective recognition: measure memory task,
- Fear condition: paradigm learning where animal is trained to recognize aversive stimulus,
- Condition place preference: most common test, the rodent receives a reward in a place preference, etc.

However, sometimes the reproducibility and validity are difficult to attain with different variables due to animal-dependent factors. Hence, to avoid these variables, it is recommended to increase one's sample size in order to improve statistical power. [73].

In this study, we used “*beam walking*” known also “*beam balance test*” to measure motor-coordination and balance. The goal is for the rat to stay upright walking across an elevated narrow beam to a safe platform, and to quantify the time required to cross the beam. This same principle is used for the “*challenge ladder*” test with the primary difference being that the beam is replaced with a ladder, allowing for the metric

of counting the number of paw slips during the transverse time [74].

In addition to the above metrics, “*grooming*” or “*self-grooming*” which is an innate behavior in rodents involving hygiene maintenance, thermoregulation, and social communication was quantified. Self-grooming in rodents is very similar in humans and usually is altered in response to a stressful condition [75].

Lastly, “*open field maze*” and “*novel object maze*” were used. These are common tests used to measure overall locomotor activity and anxiety related emotional behavior; variation performed on these tests makes it more difficult compared to other studies. For open field, the total distance the rat traveled during the entire experiment portion, and the time the remained in a corner allowing us to understand anxiety-like behavior was calculated. In case of novel object, novel objects are placed on the open field arena and the interaction time with the novel object is counted [76].

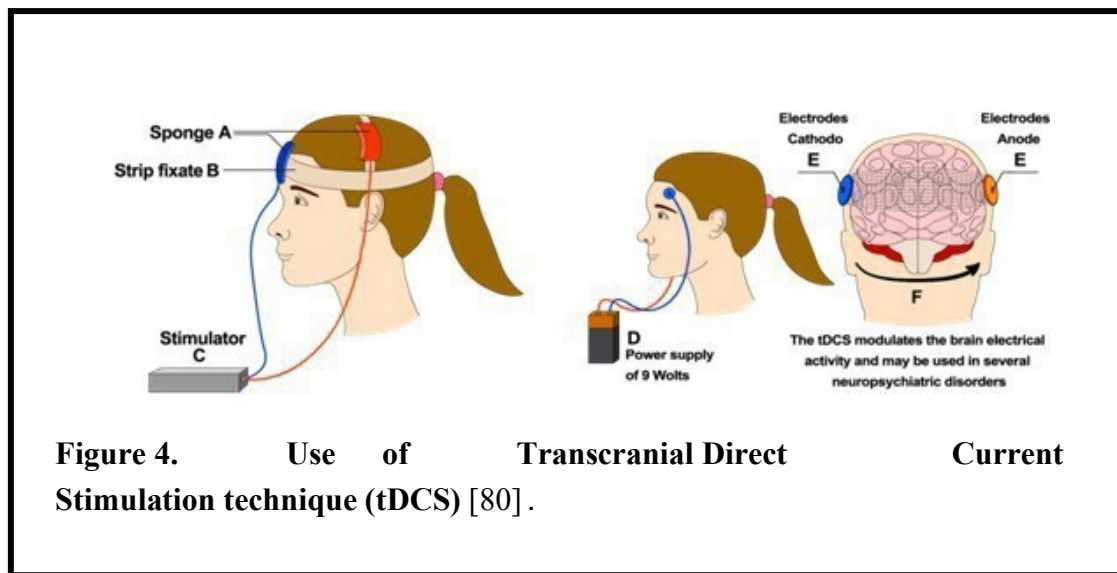
Currently, scientific studies also are working with large animal models due to the similarity to the human brain and will allow us to better study the behavioral changes once a therapy is delivered, and to be able to implement in clinical trials and improve quality of human life.

1.7 Neuromodulation by Transcranial Direct Current Stimulation (tDCS)

One of neuromodulation techniques used to improve the quality of human life is known as Transcranial Direct Current Stimulation (tDCS), a non-invasive neuromodulatory technique that works by stimulating the brain. To date, tDCS has been extensively investigated and used in clinical and cognitive neuroscience, and also is used

to improve the relief of chronic pain [77].

This technique involves a weak direct electrical current delivered through the scalp to specific areas in the brain with two or more electrodes [78] and induces changes in cortical excitability with a subthreshold level shift, modulating the neural firing from resting membrane potentials towards depolarization or hyperpolarization. Figure 4 shows the use of tDCS where the anodal tDCS increases the excitability of the underlying cortex, which in turn increases the amplitude of motor evoked potential (MEP), and the cathodal will produce the opposite effect, decreasing MEP [79].



Previous studies done in humans and in rodents have shown that stimulation for a few seconds induces excitability changes, however, the outcome is not significant. Therefore, a prolonged stimulation time is required to induce long-term changes at the synaptic level through mechanism responsible for long-term potentiation (LTP) and depression (LTD) to produce changes in neuroplasticity [81, 82].

Also, pharmacological studies proved that calcium-dependent synaptic plasticity of glutamatergic neurons plays a key role in neuroplastic mechanisms from

tDCS, because they blockade of N-methyl d-aspartate (NMDA) receptors can diminish the tDCS effects.

Likewise, tDCS reduces the effects on gamma-aminobutyric acid (GABA) neurotransmission, independent of stimulation polarity (anodal or cathodal), and modulating changes on glutamatergic plasticity [83].

In addition, tDCS has been proven to modulate membrane resting potentials at the synaptic level, but it presents a higher-level effect along the whole axons contributing the long- lasting after-effects called “non-synaptic effects,” where there are conformation changes of function of various axonal molecules [84]. The main issue of tDCS is that almost all tissue and cells are sensitive to electric fields, therefore providing a current to the field can also produce changes in a non-neuronal level, such as changes to brain tissue, endothelial cells, lymphocytes, or glial cells. Current hypotheses suggest that those effects improve the tDCS effects, but so far, there is not sufficient evidence to definitively prove this [85].

Multi-parameters lead to different efficacy results for each patient. For example, intensity, as well as session repetition, increased timing, strength, and duration of stimulation, could enhance the efficacy, but not always. In some cases, increasing the electrical current strength could lead to harmful results because the electrical current gets deeper into the brain and produces the wrong modification [86].

tDCS has an analgesic effect in experimental and clinical pain models, but its effects, magnitude and duration are low. Therefore, scientists are still investigating how to enhance and improve neuropathic pain. In 2021, Segal et al. [87] combined mirror therapy, which has neuroplasticity effects, with tDCS to modulate

neuroplasticity. The results were significant, and the group with combined treatment had a robust analgesic effect. Also, Soler et al. in 2010, using the same theory, in which the combination between tDCS and visual stimulus on neuropathic spinal cord patients combined with a visual illusion, improved the results [88].

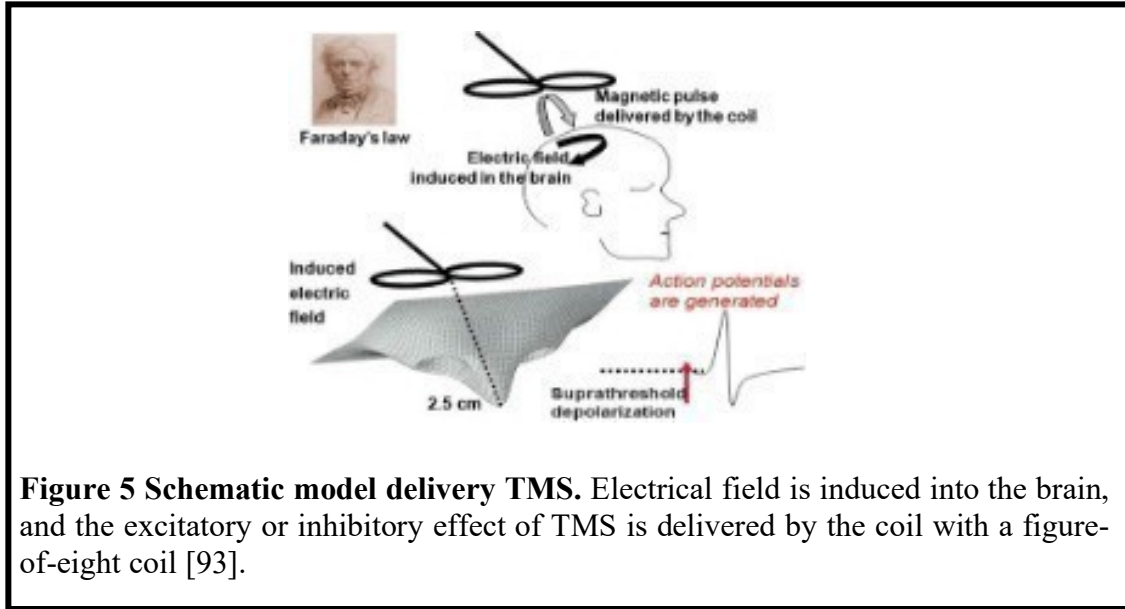
Unfortunately, to date tDCS has not been the best approach to relieve neuropathic pain that comes from PNI or any other disorder, and so scientists are still looking for the best way to improve quality of life for these patients.

18 Neuromodulation by Transcranial Magnetic Stimulation

In recent years, Transcranial Magnetic Stimulation (TMS) has gained a lot of interest and has been used for multiple studies within neuroscience and clinical research, including behavioral research and rehabilitation after a brain surgery [89]. It is widely used in humans, primates, and rodents.

TMS is a non-invasive technique that delivers magnetic stimulation directly to the scalp via a wire coil and crosses the skull bone without being attenuated, generating an electrical field once it enters the brain [90] and stimulating specific areas in the brain [68], using the Faraday law discovered in 1831 by Faraday (Figure 5) [91].

Pulses can be delivered as a single pulse, used to explore the brain. This pulse depolarizes neurons transiently and is the most commonly used method for repetitive TMS (rTMS). This induces changes in the brain where the effects stay for a longer period, and the excitability can be increased or decreased depending on the parameters of stimulation [92].



Due to its non-invasive advantages, this technique is frequently used without risk of complications. Studies done in humans, primates, and rodents demonstrate that different variables in the rTMS parameters can create a wide range of outcomes in different patients. These three variables are:

- “Stimulation intensity”: low frequency (~ 1 Hz) induces a suppression of excitatory synaptic transmission reducing cortical excitability, whereas high frequency (5-20 Hz) does the opposite [94, 95],
- “Coil geometry”: circular coils, figure eight coils (the most common) or double- coils [94, 95],
- “Duration or train lengths”: the number of pulses that the magnetic field will deliver to the area [94, 95]

The main disadvantage of TMS is that it requires time and commitment for better results, and most patients do not continue treatment as recommended.

The mechanisms underlying TMS still remain unknown, but recent studies suggest that the current sent via the coil to the brain leads to a spike in time-dependent

plasticity [96], and the stimulus depolarization of corticospinal tract neurons directly change at the axons or indirectly via depolarization of interneurons [92]. rTMS actively initiates action potentials in neurons and alters the neuronal excitability during and after stimulation. Also, the induced modification of membrane resting potentials and thresholds, channels properties with subsequent alterations in spontaneous activity, synaptic connectivity, and the timing dynamic of cellular gating components [97]. In addition, TMS has been believed to have an association with neuromodulators (for example, dopamine) and growth factors (for example, the brain derived neurotrophic factor BDNF) [98].

Changes in cortical excitability induced by rTMS are different from the classic forms of LTP or LTD synaptic transmission described in previous studies. This difference occurs due to the different stimulation conditions: TMS activates a huge number of axons, presynaptic terminals, and postsynaptic sites simultaneously, producing a massive synaptic bombardment of excitatory and inhibitory cells [92].

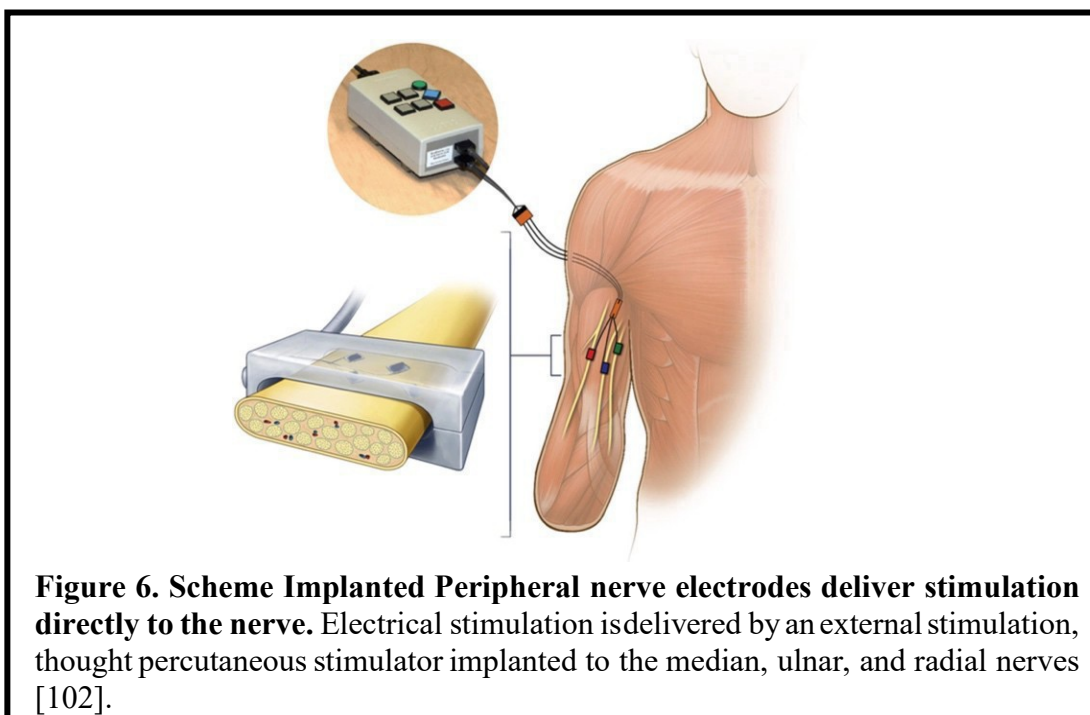
rTMS is starting to be used by scientists for neuropathic pain. A TMS coil with a figure- of-eight coil, delivered biophasic pulses when placed over the precentral gyrus (M1) [99], or postcentral gyrus (S1) [7] at the contralateral side brain. It is recommended to use a high frequency (10-20Hz) to project the axons and the local interneurons, but a lower threshold to avoid triggering the muscle contraction. Yet, the capability of rTMS to improve pain remains unknown.

19 Neuromodulation by Peripheral Nerve Stimulation

In recent years, interest in the treatment of chronic pain has developed and

increased electronic stimulation of the peripheral nerve has been widely used for medical purposes. The neuromodulatory effect of controlling peripheral nerve stimulation (PNS) for the control of pain was first explored in 1965. This process shares similarities with acupuncture and transcutaneous electrical nerve stimulation (TENS). In 1967, Wall and Sweet demonstrated that there is pain-free stimulation once the electrodes are percutaneous. Today, PNS is very commonly used in many cases of chronic pain, including PNI, complex regional pain syndrome, phantom limb pain, and fibromyalgia [100].

PNS is an invasive technique that involves the implantation of subcutaneous electrodes targeting a nerve (Figure 6). Once the electrical current from the electrode stimulus is applied to the skin, factors such as the pressure exerted on the skin or the amplitude of a skin vibration, create two consequences on the evoked neuronal response: (i) increase the firing rate of nerve fibers that are involved in the mechanical stimulation, and (ii) an activation of the fibers near the nerves involved in mechanical stimulation [101].



Studies done in humans have suggested that PNS may directly inhibit pain neurotransmission, by altering the local inflammatory mediators. Stimulation at a low threshold alter non-nociceptive A β fibers, causing excitation of inhibitory dorsal horn interneurons. These are involved in the processing and transmission of nociceptive information from the A δ nerves ~~for~~ leading to inhibition of pain signal transmission from the spinal cord to the central nervous system (CNS). Low threshold changes on A β and high threshold changes on A δ and C fibers, ~~both contribute~~ to the generation of pain [100]. The main issue is that to date, the exact mechanism of this is unknown. However, nonpainful stimulation of the peripheral nerve territory decreases pain [100].

PNS has been a good approach to relieve pain, but it still remains an invasive technique due to the need to perform surgery to implant the electrode. The future goal is to develop a non-invasive therapy that will improve quality of life.

1.10 Neuromodulation by Optogenetics

Understanding the neural circuit, genetic anatomical experimental interventions and temporal precisions has led to the development of a new technique known as Optogenetics. Optogenetics is a minimally invasive neuromodulation therapy where we combine genetic and optical methods of excitation or inhibition, with the introduction of a single gene. This gene encodes light-sensitive and ion-conductance regulators or biochemical signaling proteins into the target cells. The cellular behavior [103] can be changed by different membrane voltages within potentially excitable cells.

Optogenetics activators are: channelrhodopsin (ChR), halorhodopsin, and archaerhodopsin (Arch), which attach to calcium or voltage membrane indicators [104]. This system has an advantage due to the light high spatial and temporal resolution through the multiple wavelengths and location.

In neurons, the depolarization can lead to an activation of transient electrical signaling, which is the basis of neuronal communication. By exogenously expressing light- activated proteins, we can change the membrane potential in neurons, creating an ON/OFF switch [104].

In Figure 7 we can see the wavelength of each membrane modulator where ChR is mostly used to activate the neurons with blue sensitive light, as the opposite of NpHR and Arch inhibiting the signaling and blocking and response to yellow light [105].

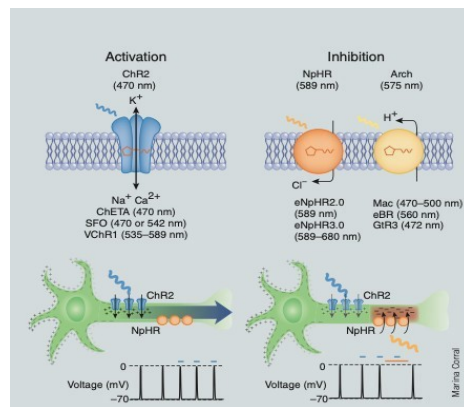


Figure 7. Optogenetics tools for modulating membrane voltage potential [105].

With optogenetics we could potentially increase the activity of injured neurons to avoid potential barriers inherent in clinical implementation of activity-dependent therapies as needed. As an example, in our lab, Li et al. (2011), developed a strategy to manipulate and control transcallosal activity facilitating appropriate plasticity, mediated with changes on the deprivation on the somatosensory cortex. Excitatory pyramidal neurons are present on lamina III and V on transcallosal fibers. Therefore, this group engineered excitatory neurons on the S1 that expressed halorhodopsin (eNpHR), a light sensitive pump, where hyperpolarization of specific wavelength neurons is a trigger. Forepaw denervation was also performed to observe the deprivation and changes on neuroplasticity on the S1 and to follow changes induced by this light pump. As a result, with electrophysiology, optical imaging, and fMRI, we observed an increase in activity in the intact forepaw once the neurons were illuminated.

This means that optogenetic manipulation led to the inhibition of the deprived cortex, and resulted in better transcallosal projection, creating better neuroplasticity. Therefore, this technique could be a potential strategy to improve rehabilitation and restore the cortical function in the near future [27]. Also, different group in 2021, English et al. [106], used this basic technique to relieve pain in PNI. Using bioluminescent optogenetics (BL-OG) and expressing luminopsin molecules on the excitatory neurons via bioluminescence, they observed an increase in excitability of motoneurons over a similar time period, promoting motor axon regeneration and muscle reinnervation of injured neurons and enhanced regeneration.

The BL-OG used by this scientist was a fusion of proteins of light-generating luciferase and channel-rhodopsin, with the light sensitive cation channel. Where once the light was generated by the enzymatic action from the luciferase, a neuronal excitation was produced, and the axon regeneration was promoted [106].

Therefore, treatment of PNI with optogenetics is feasible, and a future line of study to be developed becomes apparent with the potential to move toward addressing a major public health issue. The challenge of this scenario is to fit this therapy into human life.

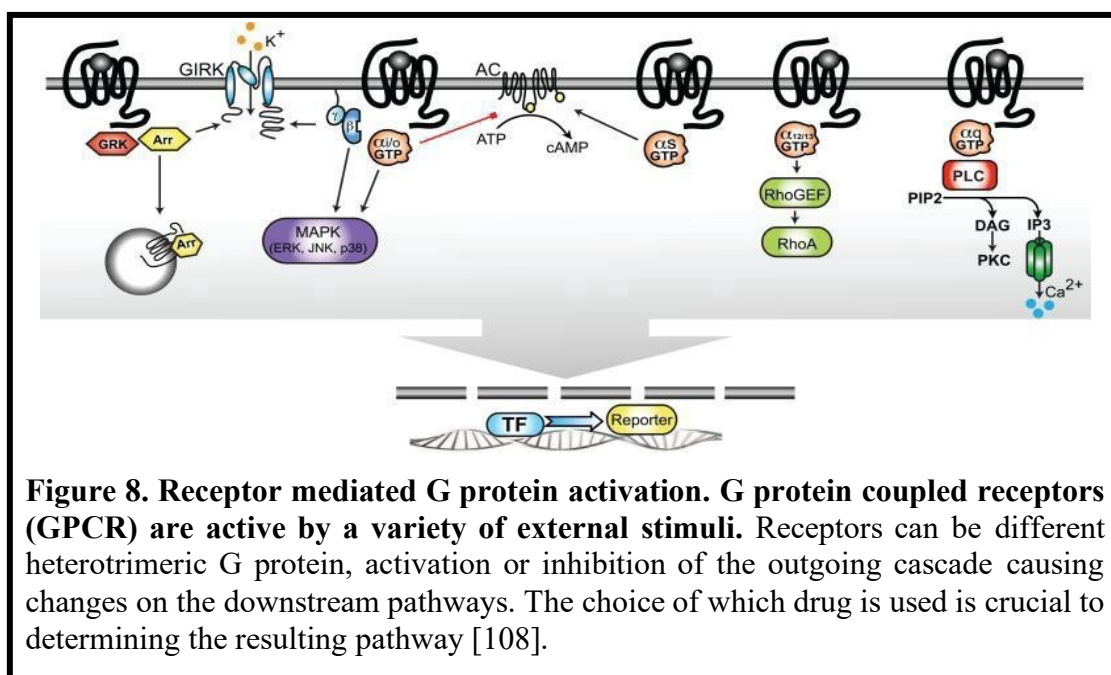
1.11 Neuromodulation by Designer Receptors Exclusively Activated by Designer Drugs (DREADD)

A powerful neuromodulation technique that can selectively manipulate a specific cell has been used in research as a chemogenetic tool, which allows us to manipulate awake animals without tools or instruments. This technique, designer receptor exclusively activated by designer drugs (DREADD), has the potential to be

applicable in humans as well. DREADD uses the biological “lock-and-key” system for the selective manipulation of cells’ activity through G- protein signaling pathways. Since the discovery of this approach in 2007, it has been adopted by many investigators, and in neuroscience this technique has been enhanced for use in a cell-type specific and non-invasive manner to excite or inhibit the neurons [107].

Although the temporal precision and control of chemogenetics in DREADD is lower than in optogenetics, it has been proven to be an invaluable tool in neuroscience because it allows for cell-type-specific modulation, bidirectional control of cells, and the mapping of function networks [108].

The first class of DREADDS is the muscarinic GPCRs that was engineered to be insensitive to their endogenous agonist. In Figure 8, we can see that there is a battery of different muscarinic-based DREADDS, named as Gq, Gi and Gs [109]. Gq increased the neuronal activity via any stimulation of Phosphoinositide-specific phospholipase C (PLC) and released intracellular calcium [110]. Gi mediated neuronal silencing via inhibition of adenylate cyclase activity, decreasing the production of cAMP, inhibiting the voltage gated calcium channels, and hyperpolarizing the neuronal membrane. Activation of the less commonly used Gs induced production of cAMP and led to an increase in cell activity [108, 110].



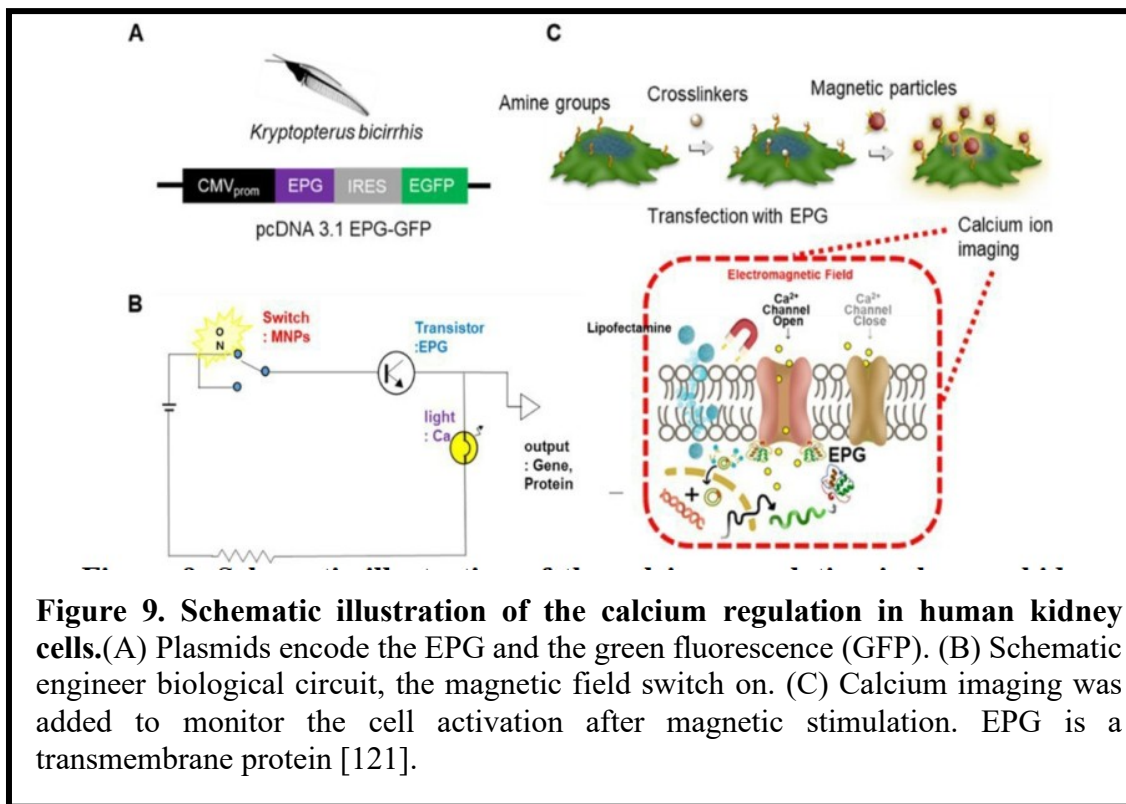
Further, by stimulating or inhibiting specific neuromodulator circuits, we could potentially relieve the pain of patients that suffer PNI or any other brain disease.

Once the desired receptors are expressed, the activation is created by the administration of a drug, which in this case is the clozapine-N-oxide (CNO) [109], The drug can be delivered in multiple forms including systemic injection [111], intracerebral injection [112], intracerebroventricular injection [113], in food or drinking water [114] or in-eye injection [115]. Multiple studies have shown that the effects start to appear after 5-10 min and can last for 1-6 hours. Therefore, DREADDS has begun to be used instead of optogenetics, due to the ability to manipulate the neurons for a longer period [116]. Another advantage of DREADD is that it does not require the light exposure of optogenetics, which can damage the neurons if they are overexposed.

DREADD has been shown to have a great potential for neural manipulation and control. When complemented with neuroimaging, such as MRI and positron emission tomography (PET), a better and faster approach to labeling and mapping brain changes can be instilled. With drugs that are specific for neurons, scientists have been able to detect in PET, with radioactivity, and with fMRI, changes to the BOLD signal once the neurons are active [116].

1.12 Neuromodulation by the novel Electromagnetic perceptive gene (EPG)

Most of the technologies described in this chapter that are used for PNI patients are non-specific, and can potentially cause secondary effects. Therefore, scientists are working toward finding a specific neuronal target. In our lab, we have been able to discover a novel protein called “Electromagnetic Perceptive Gene” (EPG) [117]. This protein comes from the glass catfish (*Kryptopterus vitreolus*) [118, 119]. It is a membrane anchored protein with an N-terminal extracellular domain [120]. In Figure 9, we can see a scheme illustration that shows the effect of the EPG protein on mammalian cells: a remarkable change due to electromagnetic fields (EMF) at 50- 150mT range. This magnetic field creates conformational changes in the target cells, increasing the intracellular calcium, and allowing the expression of the desired target protein/genes, which is represented as the circuit output [121].



Our lab is currently working on exploring the immense potential from this protein. So far, we have learned that EPG increases calcium imaging in mammalian cells and cultured neurons. This is done remotely, via magnetic field. As a result, it increased the intracellular calcium concentrations. This mechanism could activate calcium-sensitive receptors, such as c- fos, BDNF, or NFAT [121]. Moreover, magnetic activation of EPG in rat motor cortex induced motor-evoked responses on the contralateral forelimb *in vivo* [117].

Thus, EPG is a potential new approach for specific target neurons that changes during any disorders or neuropathic pain. As an example, we could potentially target neurons that are related to neuropathic pain coming from PNI and improve the quality of human life, but so far, further research must be performed.

1.13 Dissertation's structure

This dissertation has five chapters, including the introduction, where I discuss the main topics and the historical background behind my research. My first-authored paper that was published on Brain stimulation in 2020 is the second chapter. Next, my equal first-authored contribution paper that was published on Brain Stimulation in 2021 is the third chapter. Chapter four discusses preliminary work I have done to establish a cell culture from octopus' neurons to be used as a platform to test new genetic constructs. Chapter five summarizes the results, conclusion, and closing remarks including future work.

1.14 Research and Objectives

Aim 1: We developed a novel non-invasive neuromodulation-based technique to increase performance after PNI in rats. We hypothesized that excitation of S1 contralateral to the injury will be effective in increasing performance after injury. We tested two non-invasive neuromodulation approaches: Aim 1 Repetitive transcranial magnetic stimulation (rTMS). We delivered an excitatory stimulus over the brain regions contralateral to the injury. Improvement was validated via a battery of behavioral testing of the sensory dysfunction. Long-term cortical plasticity was tested with immunochemistry and cortical representation maps using fMRI. Aim

1.b. The Electromagnetic-perceptive gene (EPG): a novel gene that was discovered and

cloned from *Kryptopterus* (Glass Catfish), and was demonstrated to respond to electromagnetic fields [117, 119] and induce neural activity.

We injected the EPG in the somatosensory cortex and activated these proteins by applying electromagnetic currents non-invasively. The outcome was assessed by behavioral testing and immunohistochemistry.

Aim 2: We sought to determine which delivery protocol of rTMS is optimal to produce sustainable changes in the activity of the S1. We tested two groups of rodents: short-term group which only received a single pulse, and long-term group received multiple pulses. We also tested whether synchronous or asynchronous electrical sensory stimulation pulses produced a more sustainable change. Changes in neuroplasticity were observed with fMRI and neuron specific antibodies, such as CamKII and ARC.

Chapter 2: Paper Published 2020

Title

Non-invasive neuromodulation using rTMS and the Electromagnetic-Perceptive Gene (EPG) facilitates plasticity after nerve injury

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Abstract

Background: Twenty million Americans suffer from peripheral nerve injury. These patients often develop chronic pain and sensory dysfunctions. In the past decade, neuroimaging studies showed that these changes are associated with altered cortical excitation-inhibition balance and maladaptive plasticity. We tested if neuromodulation of the deprived sensory cortex could restore the cortical balance, and whether it would be effective in alleviating sensory complications.

Objective: We tested if non-invasive repetitive transcranial magnetic stimulation (rTMS) which induces neuronal excitability, and cell-specific magnetic activation via the Electromagnetic- perceptive gene (EPG) which is a novel gene that was identified and cloned from glass catfish and demonstrated to evoke neural responses when magnetically stimulated, can restore cortical excitability.

Methods: A rat model of forepaw denervation was used. rTMS was delivered every other day for 30 days, starting at the acute or at the chronic post-injury phase. A minimally- invasive neuromodulation via EPG was performed every day for 30 starting at the chronic phase. A battery of behavioral tests was performed in the days and weeks following limb denervation in EPG- treated rats, and behavioral tests, fMRI and immunochemistry were performed in rTMS- treated rats.

Results: The results demonstrate that neuromodulation significantly improved long- term mobility, decreased anxiety and enhanced neuroplasticity. The results identify that both acute and delayed rTMS intervention facilitated rehabilitation. Moreover, the results implicate EPG as an effective cell-specific neuromodulation approach.

Conclusion: Together, these results reinforce the growing amount of evidence from human and animal studies that are establishing neuromodulation as an effective strategy to promote plasticity and rehabilitation.

Significance Statement

We developed state-of-the-art neuromodulation strategies to augment recovery from injury, including repetitive transcranial magnetic stimulation (rTMS) and a novel, neural-specific technology based on a novel magnetic-sensitive gene (EPG). This work impacts basic, translational and clinical research on several levels, including:

- Neuromodulation by TMS promoted brain plasticity as was demonstrated by a comprehensive battery of behavioral tests, functional MRI and immunohistochemistry; Genetic-based neuromodulation also promoted brain plasticity as was demonstrated by a comprehensive battery of behavioral tests.
- The development of a novel minimally-invasive and wireless technology to control neural firing rate in a cell-specific, region-specific, and temporal-specific manner without the ongoing need for implanted electrodes, optic fibers and stimulation devices.
- Both the rTMS and the EPG-based neuromodulation strategies demonstrate that the excitation of the affected cortex facilitates neuroplasticity. These conceptual strategies and rTMS treatment protocols could be readily translated into clinical practice.

Introduction

Twenty million individuals in the United States are suffering from peripheral nerve injury. Current strategies to facilitate recovery following peripheral nerve injury mainly focus on manipulating the activity of the injured and the non-injured limbs. However, in spite of refined surgical techniques and the available rehabilitation strategies, the clinical outcome in adults is generally poor with persisting sensory dysfunction and pain complications [122, 123].

Over the last 35 years, studies have shown that acute or chronic disturbance to sensory afferents is reflected in distorted cortical representations. These anatomical and functional changes are evident by electrophysiology and fMRI methods and may impact clinical outcome. For example, human studies suggest a strong correlation between abnormal post-injury cortical responses that are often observed with fMRI to the degree of sensory dysfunctions and phantom limb pain [124-126]. The neural mechanisms implicated in the post-injury cortical changes have been extensively studied in animal models; Studies indicate that peripheral injury evokes cellular mechanisms effecting immediate [51] and long-term [23, 25, 27, 29, 127] function of the primary somatosensory cortex (S1) contralateral and ipsilateral to the injured limb. These mechanisms include alteration in the excitation-inhibition balance [128], changes in GABAergic function [129], and increases in the activity of inhibitory interneurons in cortical layer 5 (L5) in the affected (deprived) cortex [23, 27, 51]. Therefore, it is conceivable that post-injury cellular changes affect neurorehabilitation and may dictate the degree of recovery.

Evidence from human studies support that modulation of cortical function,

and specifically, increasing cortical excitation, have clinical implications. For example, removing the afferents of the “good hand” via tourniquet-induced anesthesia, anesthetic block, and constraint induced therapy led to improved hand function [130, 131].

Harnessing the brain’s innate plasticity mechanisms through non-invasive methods such as transcranial magnetic stimulation (TMS) has recently gained interest for use in functional and behavioral research as well as rehabilitation research after brain injury [132-134] and neurodegenerative diseases [68]. Various studies showed promising results using TMS in humans [135, 136], primates [137], and rodents [133, 138-141]. Importantly, TMS has shown effectiveness in manipulating transcallosal communication in patients suffering from peripheral injury [142] and alleviating pain associated with injury [143]. TMS has also been shown to increase neuronal excitability and markers associated with plasticity such as brain-derived neurotrophic factor (BDNF)[144], *c-fos* [145] and Ca^{2+} /calmodulin- dependent protein kinase II (CaMKII) [133]. However, it is still not clear when is the optimal and most effective time for TMS intervention (acute, subacute or chronic phase) to take place after injury. Identifying the most effective stimulation protocols in an animal model could impact moving this treatment intervention forward. Furthermore, evidence suggest that TMS increases the activity of a wide type of neural population [146] and currently there is no protocol that enables targeting a specific neural population. Thus, developing neuromodulation strategies to restore normal neural excitability levels with cell- specific precision could lay the groundwork for transforming current clinical practice.

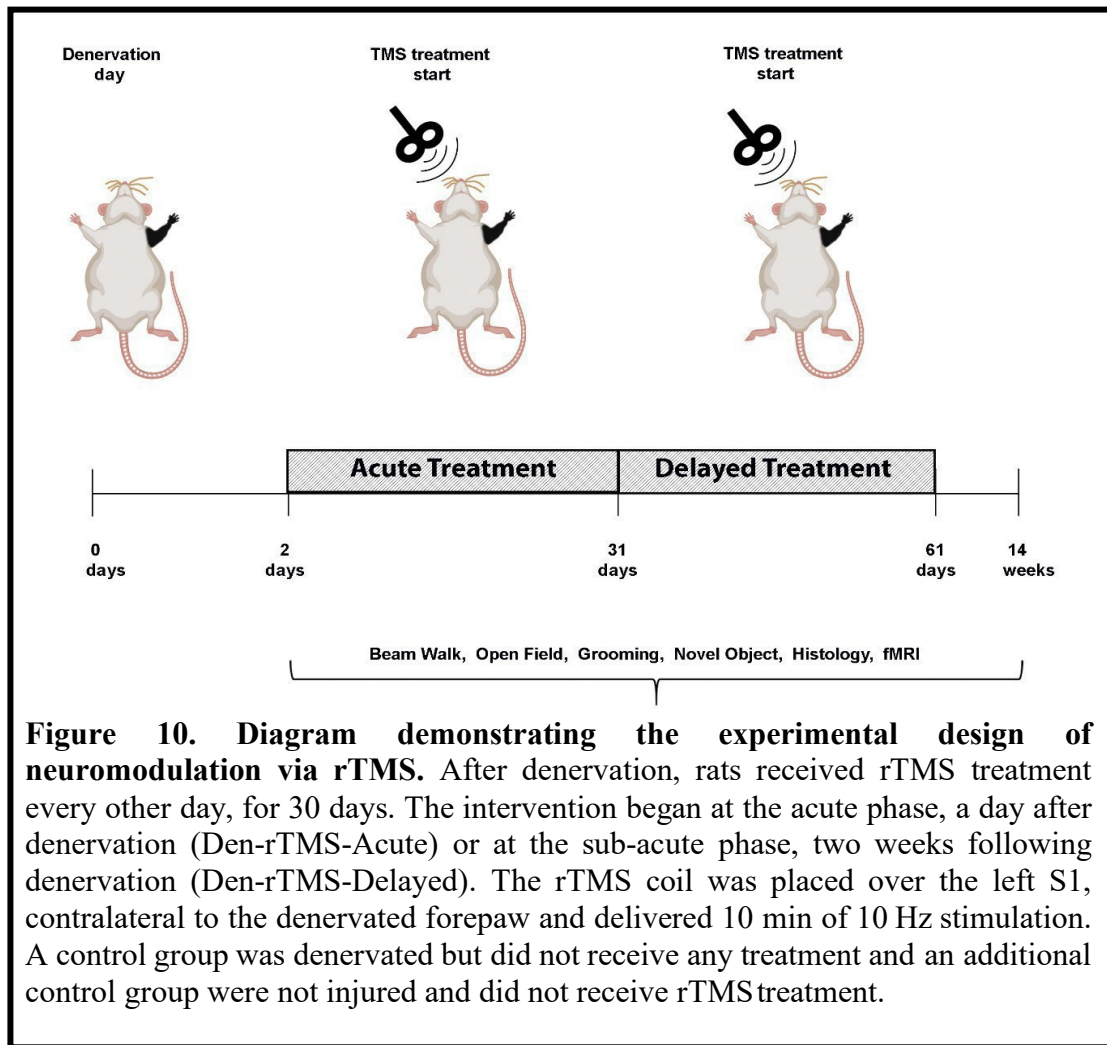
Major advances in molecular and synthetic biology have revolutionized the capability to control cell excitability in living organisms. One of these technologies, magnetic manipulation by the electromagnetic preceptive gene (EPG), allows minimally invasive and cell-specific neuromodulation using external magnetic fields. EPG is a protein that is sensitive to electromagnetic fields which was recently identified in the fish glass catfish [147-149]. Recent work had demonstrated that calcium imaging in mammalian cells and cultured neurons expressing EPG activated remotely by magnetic fields led to increases in intracellular calcium concentrations, indicative of cellular excitability. Moreover, wireless magnetic activation of EPG in rat motor cortex induced motor evoked responses of the contralateral forelimb *in vivo*. Expressing EPG in S1 contralateral to the injury in rats may provide a way to increase excitation by specifically targeting the excitatory cortical neurons and minimizing off-target effects.

Here we capitalized on a battery of behavioral tests, functional MRI (fMRI) and immunohistochemistry to test the effectivity of rTMS intervention, and behavioral tests and immunohistochemistry to test the effectivity of EPG-based neuromodulation in improving short- and long-term sensory, motor, and cognitive outcomes in a rodent model of peripheral nerve injury.

Results

TMS enhances sensorimotor functions

We first tested if non-invasive brain stimulation via rTMS focused on the deprived S1 (contralateral to the denervated forelimb) is an effective strategy to facilitate plasticity and rehabilitation. We performed a battery of behavioral tests to characterize sensorimotor and cognitive functions associated with denervation injury. An illustration depicting the animal model and the different modulation strategies is shown in **Figure 10**.



A beam walk test with two different width settings was performed every other week to evaluate sensorimotor functions. The results show that denervated rats that received rTMS treatment every other day for 30 days, starting the day after injury (Den-rTMS-Acute, $n=6$), showed significantly shorter traverse times and enhanced mobility compared to rats that received rTMS treatment starting 3-weeks after injury (Den-rTMS- Delayed, $n=6$) and injured rats that received no treatment (Den-No rTMS, $n=6$). This was true for both the 6.3 cm width (Den-rTMS- Acute, 5.64 ± 0.4 s; Den-rTMS-Delayed, 6.35 ± 0.4 s; Den-NoNS) and the more challenging, 3.9 cm width beam

(Den-rTMS-Acute, 6.29 ± 0.4 s; Den-rTMS- Delayed, 7.02 ± 0.3 s; Den-No rTMS, 26.4 ± 3.4 s; Control, 5.95 ± 0.2 s; $F(3,11) = 13.57$, $p < 0.05$) (**Figure 11(A)**).

The results show a shortening in the traverse time reflecting an improvement in sensorimotor functions and mobility throughout the course of rTMS treatment. The results demonstrate that at the end of the 4-week rTMS treatment, both the Acute and the Delayed group's traverse times were shortened and similar to the Control group times; The Den-rTMS-Acute group demonstrated an improvement of 61.3% on the 6.3 cm width challenge beam walk, and a 64.8% on the 3.9 cm challenge beam walk after 4 weeks of treatment. The Den-rTMS-Delayed demonstrated an improvement of 54.7% on the 6.3 cm challenge, and a 55.1% on the 3.9 cm challenge, and the Den-No rTMS showed only a 19% and 37.8% improvement on the 6.3 cm and 3.9 cm challenge, respectively, over the same time frame.

An open field test where rats were placed in a 109 cm x 35.56 cm arena and their movement videotaped with a ceiling camera was performed every other week to assess locomotion and anxiety. During the 10 min test, the denervated rats that received rTMS treatment starting the day after injury showed significant increases in the averaged speed (values at week-4: Den-rTMS- Acute, 0.047 ± 0.0016 m/s; Den-rTMS-Delayed, 0.038 ± 0.0028 m/s; Den-No rTMS, 0.023 ± 0.0014 m/s; Control, 0.041 ± 0.003 m/s; $F(3,6) = 7.028$, $p < 0.05$; Acute vs. No rTMS: $p < 10^{-5}$ for each time point; Delay vs. No rTMS: $p < 0.02$ at each time point; Acute vs. Delay: NS), and traveled a greater distance compared to the other groups (Den-rTMS-Acute, 28.75 ± 1.75 m; Den-rTMS- Delayed, 23.01 ± 0.8 m; Den-No rTMS, 14.11 ± 0.9 m; Control, 24.88 ± 2.31 m; $F(3,6) = 30.64$, $p < 0.05$; Acute TMS vs. No rTMS: $p < 10^{-4}$ for each time point; Delay vs. No rTMS: $p < 0.05$ at

each time point; Acute vs. Delay: NS). The results demonstrate that these improvements lasted throughout the rTMS treatment sessions and weeks following the completion of treatment.

After 4 weeks of treatment the Den-rTMS-Acute group increased their speed by 56.9%, while the Den- rTMS-Delayed and the Den-No rTMS increased by only 10.9% and 19.6%, respectively (**Figure 11(B)**).

A novel object recognition test was carried out to evaluate the rats' emotional and cognitive functions reflected by the rats' interest in new objects placed in the open field arena, as was indicated by the number of times the rats approached the object. This test is known to evaluate anxiety and depression levels which are often increased in patients suffering from chronic pain. The results indicated that denervated rats that received rTMS treatment starting the day after injury spent significantly greater time exploring both the familiar (Den-rTMS-Acute, 7.16 ± 0.7 approaches; Den-rTMS-Delayed, 3 ± 1.5 approaches; Den-No rTMS, 3.8 ± 1.7 approaches; Control, 7.6 ± 1.2 approaches; $F(3,9) = 21.07$, $p < 0.05$) and the novel objects (Den- rTMS-Acute, 9.8 ± 1.5 approaches; Den-rTMS-Delayed, 7.6 ± 2.6 approaches; Den-No rTMS, 2 ± 0.5 approaches; Control, 9.1 ± 0.4 approaches; $F(3,6) = 12.82$, $p < 0.05$; Acute vs No rTMS: $P < 0.05$ at all time points; Delay vs No rTMS: $P < 0.05$ at time points 1 & 2;). Rats that received the rTMS treatment immediately after injury have shown the greatest gradual increase in the time they spent exploring the novel object over the course of the treatment regime (Den-rTMS-Acute, 84%, Den-rTMS- Delayed, 66.6%, Den-No rTMS, 33.3%) (**Figure 11(C)**).

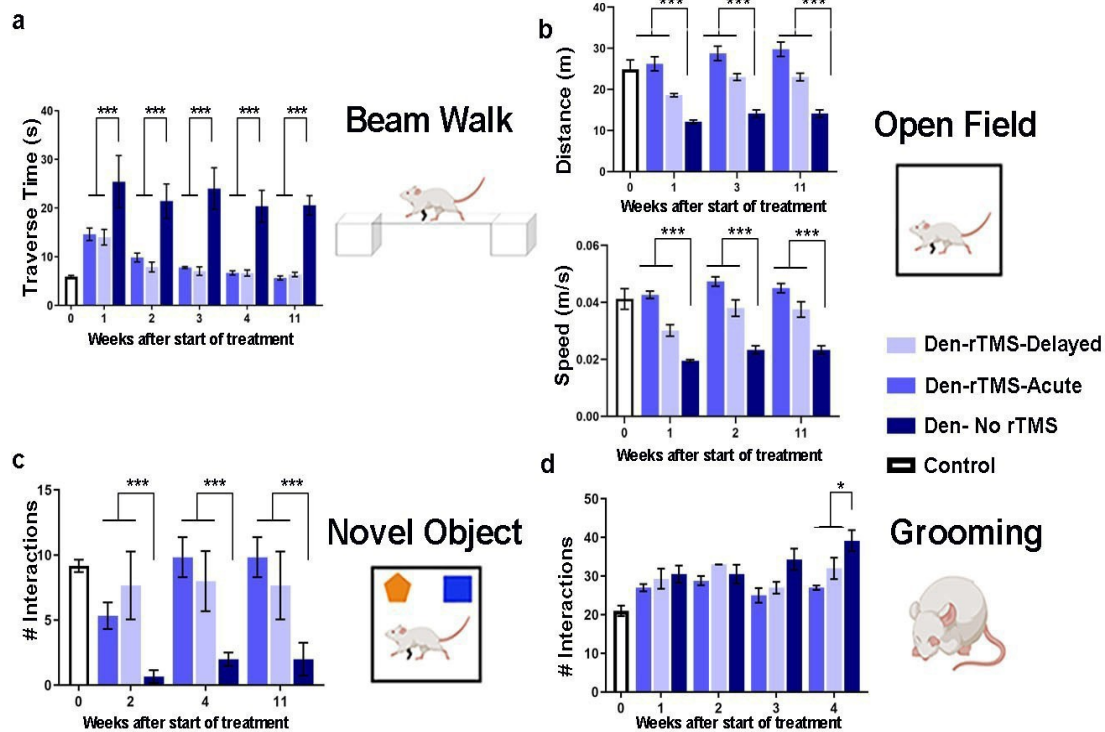


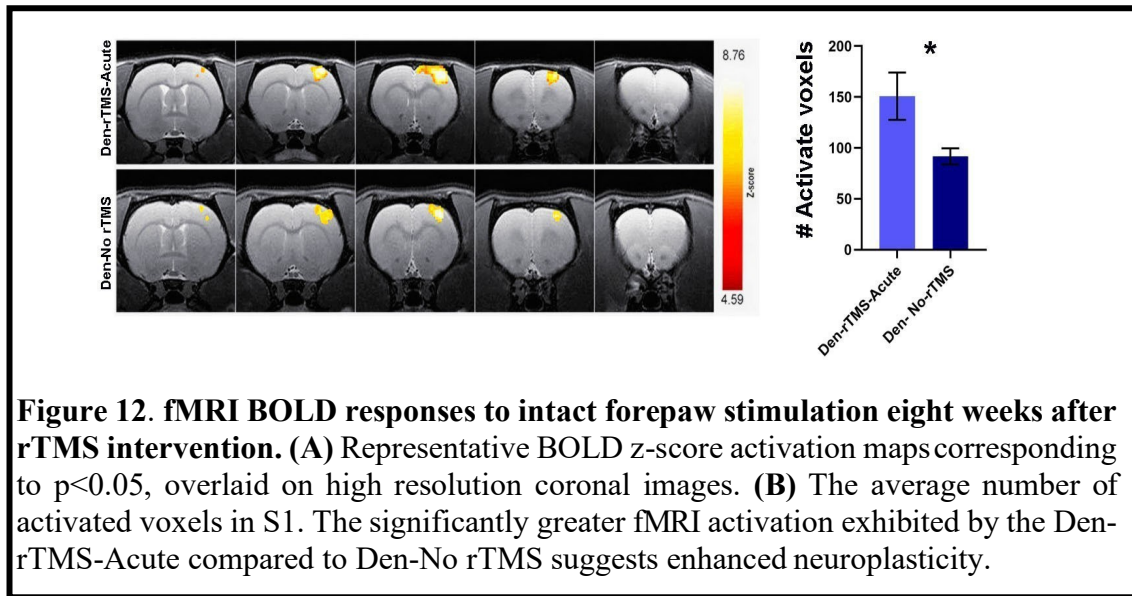
Figure 11. A battery of behavioral tests to assess sensorimotor and cognitive functions was performed before, throughout, and after the rTMS intervention. (A) Sensorimotor functions were evaluated by the traverse time on a 6.3 cm beam walk. **(B)** Sensorimotor and cognitive functions were evaluated by the time and the velocity of movement in the open field arena. **(C)** Emotional and cognitive function were evaluated by the time the rats spent exploring new objects in their arena. **(D)** Sensory dysfunction and pain associated with denervation was monitored by the number of strokes rats made in the self- grooming test. Results show that rTMS intervention leads to improved long-term mobility and decreased anxiety. Furthermore, rats that received rTMS treatment immediately after denervation (Den- rTMS-Acute) exhibited the greatest improvement compared to Den-rTMS-Delayed and Den- No rTMS (*, $p<0.05$; ***, $p<0.001$).

An additional method to assess sensory dysfunctions and pain associated with injury is monitoring the self-grooming behavior. Over-grooming such as compulsive licking, scratching, and biting on the limbs are often observed in animals suffering from nociceptive pain [75, 150- 152]. Rats were placed in a clean cage and videotaped for 20 min, and the number of strokes the rats made during that time was counted. The results show that self-grooming had gradually increased over the weeks after denervation in all denervated rats.

Analysis of variance showed modest evidence ($p < 0.05$) for variation among groups, but no significance for comparisons of either rTMS group against no rTMS (HSD $p > 0.05$). However, denervated rats that received rTMS treatment showed a marginally significant slower increase in self-grooming compared to denervated rats that did not receive rTMS treatment ($P = 0.05$ for comparison of slopes of linear regression on time) (**Figure 11(D)**).

We then sought to determine if the rTMS treatment induced improvements in sensorimotor and cognitive functions that were observed in the behavioral tests also had physiological correlates. We measured whether rTMS treatment led to long-term plasticity and sensorimotor function. Measurements of Blood-Oxygenation-Level-Dependent (BOLD) fMRI responses evoked by tactile stimulation of the non-injured forelimb were performed 12 weeks after denervation, and 8 weeks after the end of rTMS treatment. **Fig. 12** shows BOLD fMRI activation Z maps of individual Denervated-rTMS-Acute and Denervated- No rTMS rats overlaid on high- resolution anatomical MRI images across S1, as well as the statistics for the groups. The number of activated voxels (General Linear Model statistics with a Z score > 2.3 , corresponding to $p < 0.05$) in

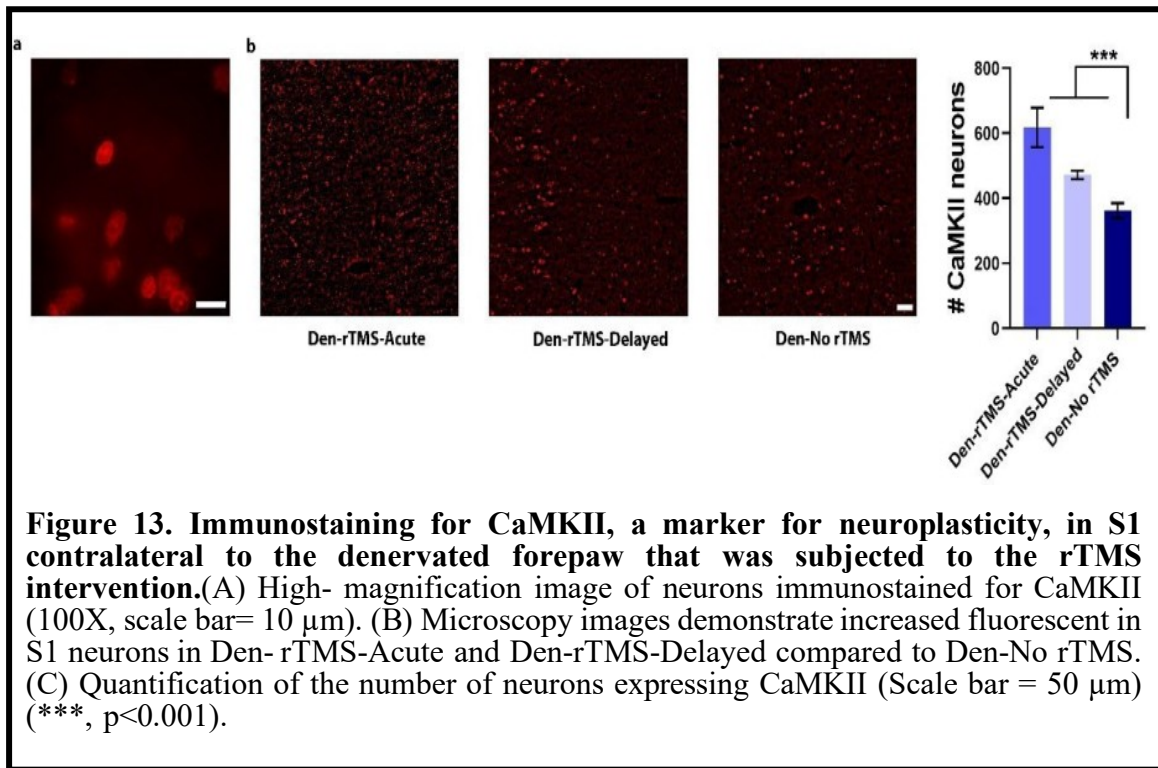
S1 induced by tactile stimulation was calculated for Den-rTMS-Acute (n=5) and Den- No rTMS (n=5) groups. The results demonstrate that rTMS treatment led to significant increase in the number of activated voxels across S1 (Den-rTMS-Acute, 150.8 ± 23.3 voxels; Den-No rTMS, 91.6 ± 7.9 ; $p < 0.05$) suggesting an increase in neuroplasticity in S1 contralateral to the injured forelimb.



Further immunostaining to identify biomarkers associated with neuroplasticity were performed on 25- μ m thick brain slices obtained from rats that were sacrificed 16 weeks after the denervation procedure. We calculated the number of cells and the expression levels of CaMKII, a gene known to be involved in long-term potentiation (LTP). The results showed that rats that received rTMS treatment starting the day after injury exhibited a significantly greater fluorescence intensity of CaMKII (Den-rTMS-Acute, 617.5 ± 60 cells), compared to both rats that received delayed rTMS treatment (Den-rTMS-Delayed, 472 ± 13 cells) and denervated rats that did not receive rTMS treatment (Den- No rTMS, 362 ± 23 cells; $F(2,3) = 22.61$, ($p < 0.05$)). **Fig. 13** shows the normalized CaMKII intensity across the deprived S1 (contralateral to denervated

forelimb). The non-invasive fMRI and the immunostaining results are consistent with the behavioral tests, and together the results show that rTMS treatment that started at the acute phase after injury led to neuroplasticity and rehabilitation.

Furthermore, the results suggest that the rTMS treatment induced long-term neuroplasticity changes that were evident in the behavioral, system, and cellular levels, lasting for months after the treatment has ceased.

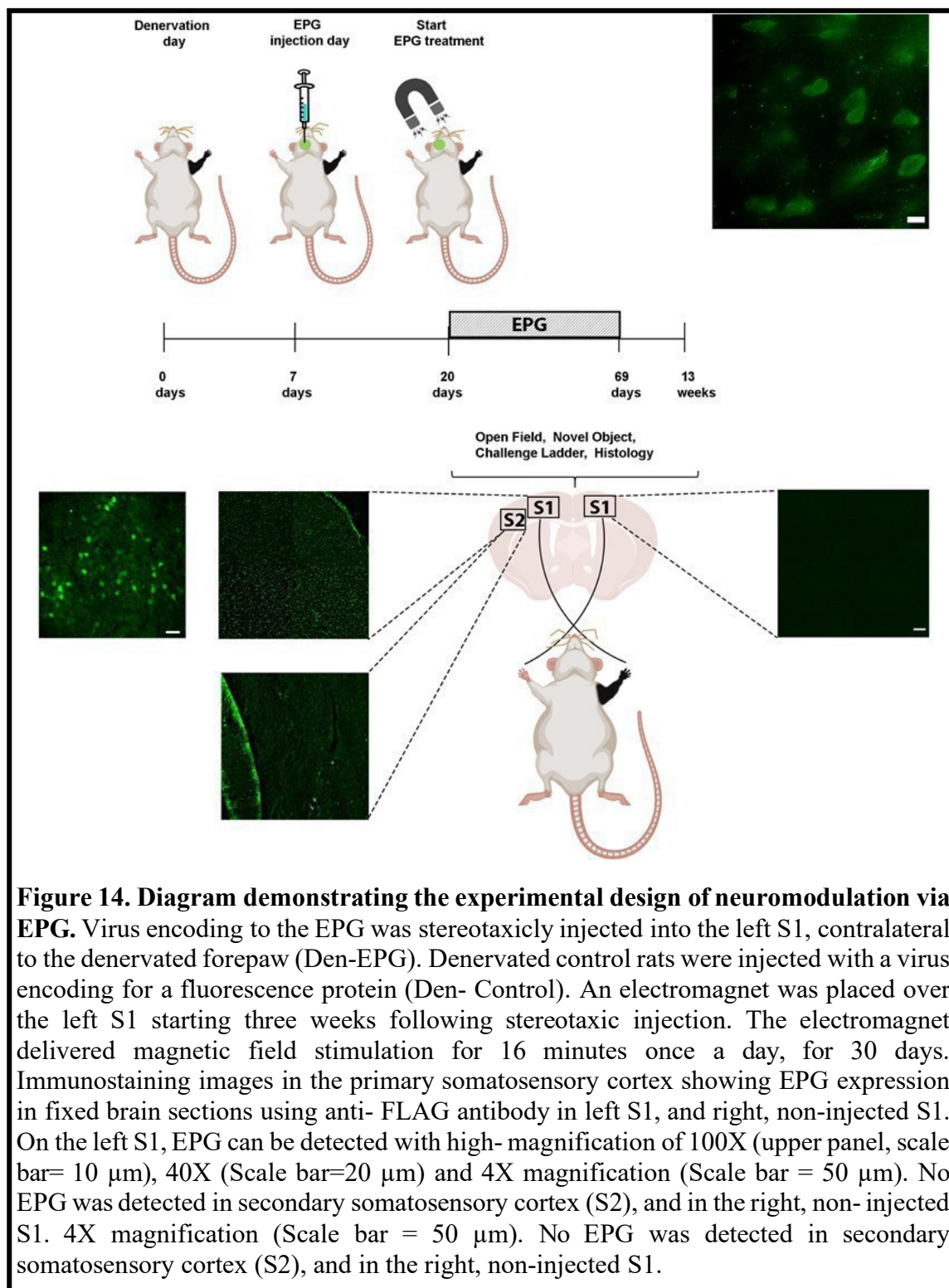


Cell-specific neuromodulation via EPG

EPG is a protein that is sensitive to magnetic fields that, upon magnetic activation, increases neural excitability. We tested if expression of EPG in excitatory cortical neurons would restore normal excitation-inhibition balance in deprived S1, which could lead to increased plasticity and rehabilitation.

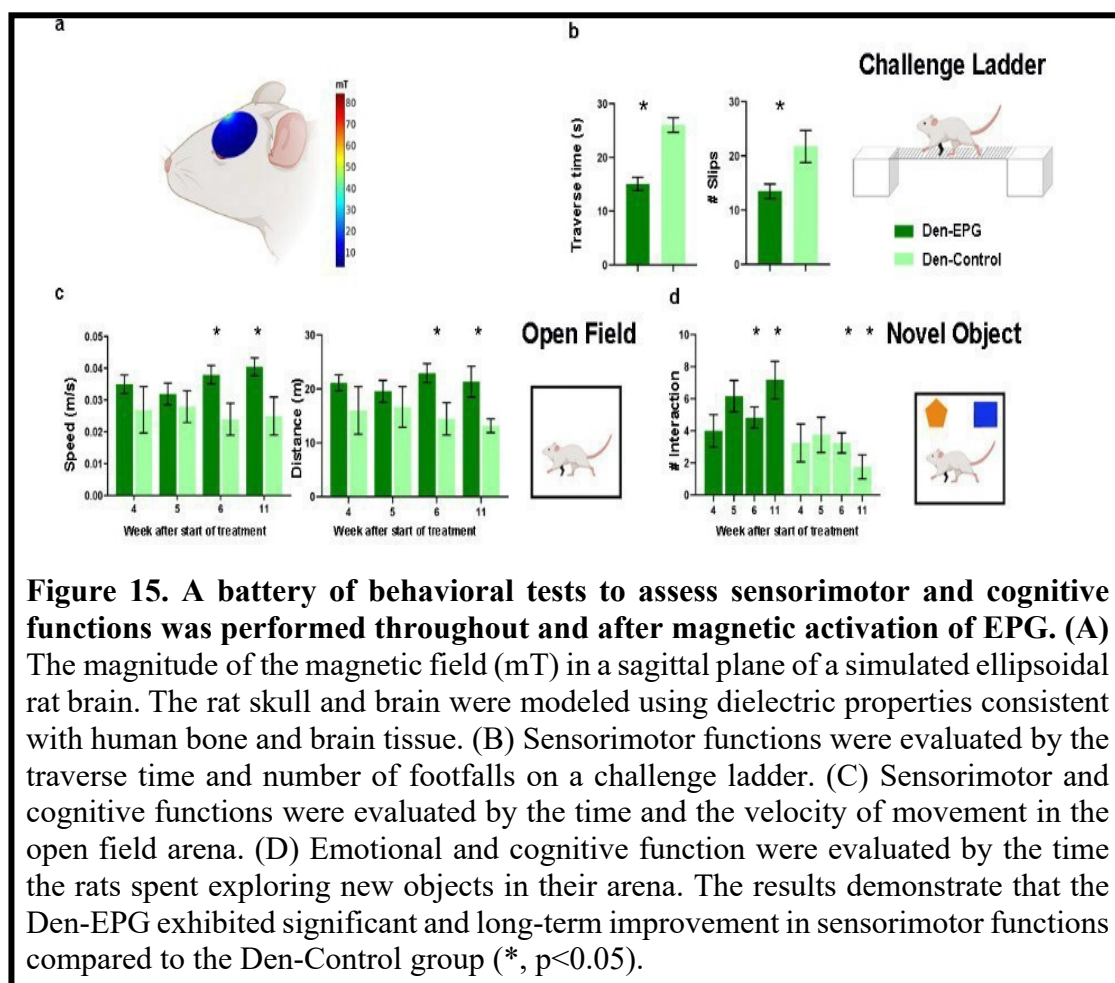
Right forepaw denervation was performed in 11 rats. One week after the

denervation procedure, rats were stereotaxically injected with a virus encoding for EPG under the CaMKII promotor (AAV-CaMKII:EPG-GFP). Virus was injected into four different locations covering S1 (layers 4 and 5) contralateral to the denervated limb (Den-EPG, n=6). Control rats went through a similar procedure but were injected with virus containing only a GFP marker (Den-Control, n=5). Three weeks after virus injection, and four weeks after denervation, we placed an electromagnet generating a field of 41 mT inside the rat's skull, directly over S1 expressing EPG, which was contralateral to the denervated limb. A diagram of the experimental paradigm is shown in **Figure 14**. The electromagnet consisted of a ferromagnetic core wound with 2,000 turns of magnetwire, and a simulation demonstrating the magnitude of the magnetic fields experienced by the rat based on the dielectric properties of brain tissue and bone[153] are shown in **Figure 14(A)**. The magnetic stimulation was performed for 16 min once a day, for 30 days, while rats were anesthetized with 2% isoflurane. Immunohistochemistry was performed on brain slices obtained from all of the rats in this study to confirm EPG expression in S1 (**Figure 14**).



A battery of behavioral tests to characterize sensorimotor and cognitive function associated with denervation injury and EPG treatment, was performed throughout the course of the stimulation. Long-term improvement in sensorimotor functions and mobility was evaluated using the challenge ladder test, whereas the travers time and the number of slips were determined by laser sensors. This test was performed 12 weeks following denervation, 8 weeks after EPG injection, and 4 weeks after the EPG magnetic stimulation treatment ended. The results show that Den-EPG rats had crossed the ladder in a significantly shorter time (Den-EPG, 15.08 ± 1.2 s; Den- Control, 26.05 ± 1.3 s; $p < 0.05$) and exhibited fewer slips (Den-EPG, 13.5 ± 1.3 s; Den-Control, 21.75 ± 2.9 s; $p < 0.05$) (**Figure 15(B)**).

Open field was performed once a week throughout the EPG magnetic treatment. The results demonstrated that within three weeks after starting the magnetic stimulation, the denervated-EPG rats showed significant increases in speed (values at week-4: Den-EPG, 0.04 ± 0.002 m/s; Den- Control, 0.02 ± 0.006 m/s; $p < 0.05$), and traveled a greater distance compared to the control group (Den-EPG, 22.94 ± 1.7 m; Den-Control, 14.45 ± 3 m; $p < 0.05$) (**Figure 15(C)**).



These improvements lasted for a month after the magnetic stimulation treatments ended, suggesting that the EPG manipulation induced long-term neuroplasticity changes in S1 circuitry. Den-EPG rats also demonstrated increased interest in new objects during the novel object recognition test. Significant increase of the time they spent exploring the new object, compared to controls, was observed four weeks after the magnetic stimulation treatment ended (Den-EPG, 7.16 ± 1.1 approaches, Den-Control, 1.75 ± 0.7 approaches; $p < 0.05$) (**Figure 15 (D)**). Overall, the results show that EPG neuromodulation in denervated rats led to substantial improvement in sensorimotor function and rehabilitation.

Discussion

Evidence from human studies suggest that rTMS application over the affected cortex can significantly decrease phantom limb pain, anxiety and depression in amputees [154, 155]. In agreement with these reports, our study demonstrates that neuromodulation in the days and the weeks following peripheral nerve injury leads to short-term and long-term plasticity and neurorehabilitation. This is the first study to test the effectivity of rTMS in a rat model of peripheral nerve injury and test different stimulation protocols. Daily neuromodulation regimes with rTMS have shown to improve sensory, motor, and an overall well-being of the injured rats in a battery of behavioral and imaging tests that were performed up to 8 weeks after the rTMS treatment ended. The results suggest that both an immediate and delay rTMS intervention are effective. This builds on a growing bulk of evidence demonstrating that peripheral nerve injury leads to immediate changes in neural function that may dictate the degree of future rehabilitation [23, 51, 156-158]. Immediate changes in both spontaneous and evoked neural activity have been also demonstrated in models of spinal cord injury [159]. Indeed, and early intervention of rTMS therapy in a rodent model of spinal cord injury has also shown to be more effective compared to later-stage intervention [139]. Nevertheless, delayed rTMS stimulation also led to behavioral improvement compared to rats that did not receive any treatment. Thus, post-injury rTMS treatments may be tailored to benefit patients in the acute, sub-acute, and even chronic phases.

Neuromodulation by rTMS may provide an effective, accessible, relatively inexpensive and completely non-invasive approach to attenuate pain associated with peripheral nerve injury and improve sensorimotor outcomes.

Nevertheless, there are ongoing efforts to develop minimally invasive therapeutic strategies that will diminish non-specific activation but will allow temporal precision. Tools such as optogenetics and chemogenetics have the advantages of cell type specificity and superior spatial and temporal resolution compared to prior neuromodulation methods. Indeed, we have previously shown that neuromodulation via optogenetics approaches was successful in restoring cortical excitation-inhibition balance in the weeks following the peripheral nerve injury [27]. Specifically, light activation of halorhodopsin in the healthy cortex combined with forepaw stimulation lead to increase of excitatory neuronal activity in the deprived somatosensory cortex of peripheral nerve injured rats. However, one of the drawbacks of this technology is the requirement to deliver the light directly into the target neural population. Here we tested if neuromodulation via the magnetic sensitive protein EPG, which provides cell and temporal specificity while being activated remotely via non-invasive electromagnetic fields [147], can be utilized to restore cortical excitability and achieve similar sensorimotor outcomes compared to rTMS. The results demonstrate that daily magnetic activation of EPG improved sensory, motor, and an overall well-being of the injured rats in a battery of behavioral tests that were performed up to 4 weeks after the EPG treatment ended.

Growing amounts of evidence from human and animal studies are establishing neuromodulation as an effective mechanism to strengthen and promote cortical functions [68]. The behavioral results indicate that both rTMS and EPG treatment have led to considerable improvement in sensorimotor functions. Overall, both acute and sub-acute rTMS treatment alleviated pain as evident by the behavior assays performed.

In addition, neuromodulation via EPG is a new and upcoming technology and efforts are being made towards discovering the molecular structure and the signal transduction basis of this phenomenon.

It is also anticipated that utilizing synthetic and molecular biology approaches as well as improving in the hardware will make the EPG function more robust. The EPG technology complements other neuromodulation methods and expands the current toolbox for basic and translational research. Since rTMS intervention is already FDA approved for other conditions, and can be readily translated to clinical practice for patients with peripheral nerve injury and amputation, this study was design to provide a comprehensive characterization of how this intervention affect neural functions the cellular, network and behavior levels. Nonetheless, this is the first application of EPG-intervention in an animal model of injury. Thus, the experiments were design to build the hardware required for magnetic stimulation, validate EPG expression, and test if this approach could also lead to behavioral modifications. Future studies will further characterize EPG-based neuromodulation on cellular and network levels.

Materials and Methods

Animals: All animal procedures were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and approved by the Michigan State University Animal Care and Use Committee. Thirty-five Sprague-Dawley rats (19 male and 16 female) were provided with food and water *ad libitum* and housed in a room with a reverse cycle.

Surgeries and Stimulation: Forepaw denervation was performed on 29 eight weeks old rats weighing 80-90 g. Rats were anesthetized with 2% isoflurane which was delivered through a nose cone. Skin incision was made on the right forepaw, and the radial, median and ulnar nerves were cut, and a 5 mm gap was made in each one. The incision was closed with silk sutures and tissue glue. Tramadol (0.1 mg/300 mg) was administered orally for 5 days after the injury. The TMS system was equipped with a figure eight, 25 mm custom rodent coil (Magstim, Rapid2) that was secured by a metal frame over the left hemisphere directly on the head such that the center of the coil was on top of the left S1 (bregma 0). This coil design has been shown to induce focal stimulation in rats [160]. TMS was delivered once a day, for 31 days with the following settings: 4 s cycles of 10 Hz stimuli, 26 s interval, and 7 cycles (total of 280 pulses per day, 1680 total stimuli). This stimulation frequency has been found to have long-term effects in rats [133, 139, 140, 161]. During the stimulation, rats were anesthetized with 2% isoflurane. Denervated control rats that did not receive the TMS treatments were subjected to the same daily anesthesia protocol for the same length of time.

The EPG was identified and cloned from the glass catfish [147]. Until recently, the transparent glass catfish was commonly identified as *Kryptopterus bicirrhus* but is now known to be *Kryptopterus vitreolus*. [162]. Eleven rats received stereotaxic injection a week following the denervation surgery: 6 of them were injected with virus encoding for EPG under CaMKII promoter (pAAV2-CaMKII::EPG-IRES-hrGFP), and 5 with virus encoding only to GFP (pAAV2- CaMKII::IRES-hrGFP). Rats were anesthetized with 2% isoflurane which was delivered through a nose cone and secured in a stereotaxic frame. The microinjection needle was placed in four locations in the left primary somatosensory cortex (S1) area: AP: +0.2 mm and +0.3, ML:-3.8 mm and -3.2. A volume of 1 μ L of virus was injected in each location starting at a depth of 1.2 mm and retracting the needle up to a depth of 0.8 mm. EPG expression was limited to excitatory neurons in layers 4 and 5.

Rats were divided into the following six groups: **1.** Denervated rats that started receiving rTMS 48 hours following denervation (Den-rTMS-Acute, n=6). **2.** Denervated rats that started receiving TMS 3 weeks following denervation (Den-rTMS-Delayed, n=6). **3.** Denervated rats not receiving TMS (Den-No rTMS, n=6). **4.** Non-denervated not receiving TMS (Control, n=6). **5.** Denervated rats injected with virus containing EPG in S1 contralateral to denervated limb (Den- EPG, n=6). **6.** Denervated rats injected with virus containing only GFP in S1 contralateral to denervated limb (Den-Control, n=5).

Behavioral Assessments: A comprehensive battery of behavioral tests to assess sensory, motor, and cognitive functions was performed over 30 days since the beginning of TMS therapy. Grooming: Rats were placed separately in a clean cage

(43.62cm (L) x 22.86cm (W) x 20.32cm (H)) with food and water. Grooming was recorded for 20 min. The first minute was considered habituation period, and the rest of the 19 min were analyzed. The number of interactions on each part of the chain grooming actions was counted for each individual. This test was performed once a week.

Open Field: The open field was carried out in an arena with the following dimensions (L) 109 cm x (W) 35.56 cm x 142.24 cm (H) (San Diego Instruments). During the session, the open field was isolated from the observer, and the light intensity was maintained stable. Movements were recorded by a ceiling mounted camera for 10 min. The freezing time, total distance and averaged velocity were analyzed by an automated tracking system (ANY-Maze software, San Diego, USA). After each session the arena was cleaned with 70% ethanol. This test was performed every two weeks (for TMS treated rats), and once a week (EPG rats).

Novel Object Recognition: Rats were placed in the open field arena. In the first stage, rats were acclimating to the environment (5 min). In the second stage two identical objects were placed in the arena and the rat got familiarized with them (5 min). In the third stage we replaced one of the objects for a new and unfamiliar object (5 min). The time spent exploring the novel object was analyzed by automated tracking (ANY-maze software). This test was performed every two weeks (for TMS treated rats), and once a week (EPG rats).

Beam Walk Test: TMS-treated rats were placed on one end of 114.3 cm-long suspended, narrow wooden beam. Two different widths were tested: 6.3 cm and 3 cm. The traverse time from one end to the other was measured. Three training sessions were performed for each animal once a week. For the denervated-TMS group, the rats

started to walk on the 6.3 cm width beam and then were challenged on the 3 cm wide beam.

Challenge Ladder: Den-EPG rats crossed a 114 cm-long horizontal suspended ladder with rungs spaced 1.3 cm apart (San Diego Instruments, USA). The traverse time and the number of failures to place the paw correctly on the ladder were observed. Two training sessions were performed for each animal on test days, with this test being performed only once.

Functional MRI: fMRI activity was assessed in denervated rats that received TMS (TMS acute, n=5) and rats that did not receive the TMS (denervated no-TMS, n=5). Rats were anesthetized with dexmedetomidine (0.1 mg/kg/h, SC) which is known to preserve neurovascular coupling [17, 25, 163]. Rats were then placed in a 7 T/16 cm horizontal bore small-animal scanner (Bruker BioSpin, Rheinstetten, Germany). A 72-mm quadrature volume coil and a 15-mm-diameter surface coil were used to transmit and receive magnetic resonance signals, respectively. Respiration rate, heart rate, and partial pressure of oxygen were continuously monitored throughout fMRI measurements (Starr Life Sciences, Pennsylvania, USA). For fMRI, FID-EPI was used with a resolution of $150 \times 150 \times 1000 \mu\text{m}$. Five, 1 mm thick coronal slices covering the primary somatosensory cortex (S1) were acquired (effective echo time (TE), 16 ms; repetition time (TR), 1000 ms; bandwidth, 333 KHz; field of view (FOV), $3.5 \times 3.5 \text{ cm}$; matrix size, 128×128). A T2-weighted TurboRARE sequence was used to acquire high-resolution anatomical images (TE, 33 ms; TR, 2500 ms; bandwidth, 250 KHz; FOV, $3.5 \times 3.5 \text{ cm}$; matrix size, 256×256) corresponding to the fMRI measurements. Two needle electrodes were inserted into the left and right forepaws to deliver electrical

stimulation. Electrical stimulation was applied in two 40 s trains (3 Hz, 0.4 mA, and 0.4 ms). fMRI analysis was performed using SPM fMRat software (SPM, University College London, UK). Activation maps were obtained using the general linear model. The experimental design was rest <stimulate, T-contrast Thresholded:FWE (Family Wise Error) and $P < 0.05$, Extent threshold $k=2$, Z-score statistics were used with a threshold of $Z > 2.3$.

Electromagnetic stimulation: The electromagnet used to deliver the magnetic stimulation to the rats' brains consisted of a ferromagnetic Iron-Nickel core wound with 2,000 turns of 30 AWG magnet wire. Iron core had dimensions of 16.29 cm in length and a diameter of 1.05 cm. A 45 Degree angle was cut into each end, so that both tips of the core came to a point. During stimulation, 5 V was applied to across the connections of the magnet and a current of about 390 mA flowed through the coil to generate the magnetic field. An external digital signal was used to turn the magnet on and off. Measurements of the magnet at varying distances from the core demonstrated that a magnetic field value of 41 mT was generated just in front of the core. Finite element analysis was performed using ComSol Multiphysics to simulate the magnetic field stimulation delivered to the rat brain. An electromagnet with 2,000 turns wound around a ferromagnetic Iron-Nickel core with a length of 16.29 cm, a diameter of 1.05 cm, and a relative permeability of 100,000 was used to stimulate the rat brain. A current of 390 mA was passed through the simulated coils and the tip of the core was placed 0.5 mm from the surface of the skull. The rat skull and brain were modeled by concentric ellipses, the larger of which having dimensions 21 mm x 11 mm x 16 mm, with a skull thickness of 0.7 mm used [153]. Dielectric properties for human bone and brain tissue were used

to represent bone and brain tissue in the rat, specifically a relative permittivity of 1.53×10^3 and 6.10×10^4 , a relative permeability of 1 and 1, and a conductivity of 2.03×10^{-2} S/m and 1.06×10^{-1} S/m respectively [153].

Immunohistochemistry of Brain slices: Rats were perfused with 0.1 M phosphate buffer saline solution (PBS) in pH 7.4 followed by ice cold 4% paraformaldehyde solution and the brains were removed. Brains were sliced on a cryostat to obtain 20 μ m thick sections. Sections were incubated overnight with primary antibodies to detect CaMKII (anti-CaMKII rabbit, Abcam #ab52476); FLAG (anti-flag mouse antibody, Abcam #ab49763), and GFP (anti-GFP chicken polyclonal antibody, Abcam #ab13970). Sections were incubated for 3 h at room temperature with secondary antibodies, processed with ProLong Gold antifade reagent with DAPI (Thermo Fischer Scientific 2078923) and then imaged on the DeltaVision microscope. ImageJ was used for analysis. *Statistics:* The number of rats in each group was determined to achieve a power of 0.93 assuming an effect of 1 standard deviation while minimizing the number of rats in each group.

All the results and figures show mean $\pm$ standard error of mean (SEM). Each analysis compared outcomes across several treatment groups; analysis of variance (ANOVA) was used to flag comparisons with at least one significant difference. Within each ANOVA, there are several comparisons of interest, and to address multiple comparisons issues, we used the studentized range statistic. The studentized range statistic (a.k.a. Tukey's Honest Significant Difference (HSD)) is an exact test for each of the possible comparisons between two groups embedded in a multi-way ANOVA, where significance is adjusted to control the false positive probability for all pairwise

comparisons at the specified level.

Conclusion:

Together, these results reinforce the growing amount of evidence from human and animal studies that are establishing neuromodulation as an effective strategy to promote plasticity.

Chapter 3: Paper Published 2021

Title

Multi-session delivery of synchronous rTMS and sensory stimulation induces long-term plasticity

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Key words: fMRI, plasticity, time-dependent plasticity, multimodal stimulation

Abstract

Background: Combining training or sensory stimulation with non-invasive brain stimulation has shown to improve performance in healthy subjects and improve brain function in patients after brain injury. However, the plasticity mechanisms and the optimal parameters to induce long-term and sustainable enhanced performance remain unknown.

Objective: This work was designed to identify the protocols of which combining sensory stimulation with repetitive transcranial magnetic stimulation (rTMS) will facilitate the greatest changes in fMRI activation maps in the rat's primary somatosensory cortex (S1).

Methods: Several protocols of combining forepaw electrical stimulation with rTMS were tested, including a single stimulation session compared to multiple, daily stimulation sessions, as well as synchronous and asynchronous delivery of both modalities. High-resolution fMRI was used to determine how pairing sensory stimulation with rTMS induced short and long-term plasticity in the rat S1.

Results: All groups that received a single session of rTMS showed short-term increases in S1 activity, but these increases did not last three days after the session. The group that received a stimulation protocol of 10 Hz forepaw stimulation that was delivered simultaneously with 10 Hz rTMS for five consecutive days demonstrated the greatest increases in the extent of the evoked fMRI responses compared to groups that received other stimulation protocols.

Conclusions: Our results provide direct indication that pairing peripheral stimulation with rTMS induces long-term plasticity, and this phenomenon appears to

follow a time- dependent plasticity mechanism. These results will be important to lead the design of new training and rehabilitation paradigms and training towards achieving maximal performance in healthy subjects.

Highlights

- A single rTMS session induced short-term changes but they were not sustainable.
- Multi-session delivery of rTMS paired with sensory stimulation induced long-term plasticity.
- rTMS paired with sensory stimulation induced plasticity via time-dependent mechanism.
- Delivery of only sensory stimulation did not induce long-term plasticity.

Introduction

Throughout history humans have been pursuing new regimes to augment and maximize motor and cognitive performance. Intense physiological training is known to increase endurance and enhance motor performance in athletes; purposeful physical therapy is instrumental to acquire and rebuild sensorimotor abilities in patients with impaired brain function; and cognitive skills training via traditional learning methods, and more recently by virtual reality and gaming- based methods have shown to increase mental endurance, maximize academic abilities [164, 165] and improve brain function in stroke patients [166].

The advent of non-invasive brain stimulation technologies had opened a new frontier in achieving motor and cognitive functions in levels and speed comparable and even exceeding traditional training methods. Repetitive transcranial magnetic stimulation (rTMS) is known to increase neural activity, and its application over a period of time have been shown to induce long- term and sustainable effects in healthy [141, 144, 167] and in disease conditions, in human [68, 168] and in animal models [140, 169-171]. These approaches may be particularly valuable to patients who may be unable to fully participate in a traditional training routine due to disability.

New paradigm in human performance now seeks to capitalize on benefits achieved via traditional training and non-invasive brain stimulation technologies by combining them and reaching peak performance. Pairing of peripheral and central nervous system stimulations has shown to improve endurance and athletic performance in healthy individuals [172], improve motor functions in stroke patients [173], and increase the cognitive processing speed in adults[174]. These changes are believed to occur through

associative, Hebbian-like plasticity mechanisms. Indeed, new evidence using optical imaging in an animal model shows that a visual stimulation delivered during TMS can change cortical maps [175]. Nevertheless, the exact mechanisms and the optimal parameters to induce long-term and sustainable enhanced performance remain unknown; If indeed the mechanism is time-dependent plasticity, then it is likely that the exact timing of which the tactile, sensory or cognitive stimulation is presented during the brain stimulation protocol will determine the effectivity of this approach. Elucidating the plasticity mechanism associated with these protocols would greatly impact performance of healthy individuals and their adaptation in clinical practice.

This work was designed to identify the protocols of which combining sensory stimulation with rTMS will facilitate the greatest changes in activation maps in the rat's primary sensory cortex (S1). We quantified the spatial functional MRI (fMRI) activity and the expression of molecular markers associated with plasticity. The results demonstrate that rTMS significantly increases short- and long-term plasticity, and that synchronous delivery of the peripheral stimulation and rTMS have led to significant and sustainable increase in S1 performance.

Methods

All animal procedures were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and approved by the Michigan State University Animal Care and Use Committee. Forty-two adult Sprague-Dawley rats (14 males and 28 females, 250 g) were provided with food and water *ad libitum*.

Rats were anesthetized with 1.5% isoflurane followed by an initial s.c. injection of dexmedetomidine (0.1 mg/kg) which is known to preserve neurovascular coupling [17]. Then the isoflurane was discontinued and dexmedetomidine (0.1 mg/kg/h) was delivered SC. Rats were imaged in a 7 T/16 cm aperture bore small-animal scanner (Bruker BioSpin). A 72-mm quadrature volume coil and a ^1H receive-only 2x2 rat brain surface array coil (RF ARR 300 1H R.BR. 2x2 RO AD) were used to transmit and receive magnetic resonance signals, respectively. An MRI oximeter (Starr Life Sciences, Pennsylvania, USA) was used to measure the respiration rate, heart rate, and partial pressure of oxygen saturation throughout the experiment. For fMRI, Free Induction Decay -echo-planner images (FID-EPI) was used with a resolution of $150 \times 150 \times 1000 \mu\text{m}$. Five coronal slices covering the somatosensory cortex were acquired with TR/TE 1000/16.5 ms, FOV 3.5 cm, Flip angle 75° , matrix size 128×128 and slice thickness 1.0 mm. A T2-weighted TurboRARE sequence was used to acquire high-resolution anatomical images with TR/TE 3000/33 ms, FOV 3.5 cm and matrix size 256×256 . Two needle electrodes were inserted into the left forepaw to deliver two 40-second, 3 mA of tactile-electrical stimulation.

The rTMS system (Magstim, Rapid2) was equipped with a figure eight, 25 mm custom rodent coil that was placed over the center of the head at bregma 0. This coil

design has been shown to induce focal stimulation in rats [160]. rTMS was delivered with the following parameters: 20 seconds cycles, 20 seconds interval, and 2 periods (total of 400 pulses per day). This stimulation frequency has been found to have long-term effects in rats [133, 139, 140, 161]. Rats were randomly assigned into seven groups for short-term (ST) and long-term (LT) plasticity studies: ST Group 1 received 10 Hz rTMS stimulation (n=6); ST Group 2, received 10 Hz rTMS stimulation with 3 Hz forepaw stimulation (n=6); ST Group 3, received 3 Hz forepaw stimulation (n=6); For long-term studies rats received stimulation for five continuous days. They did not receive rTMS on the fMRI scanning days (i.e., Day 1 and Day 7). LT Group 1, received 10 Hz forepaw stimulation synchronized with 10 Hz rTMS stimulation (n=6); LT Group 2, received only 10 Hz rTMS stimulation (n=6); LT Group 3, received 10 Hz rTMS stimulation with 3 Hz forepaw stimulation w (n=6); and LT Group 4, received only 10 Hz forepaw stimulation(n=6).

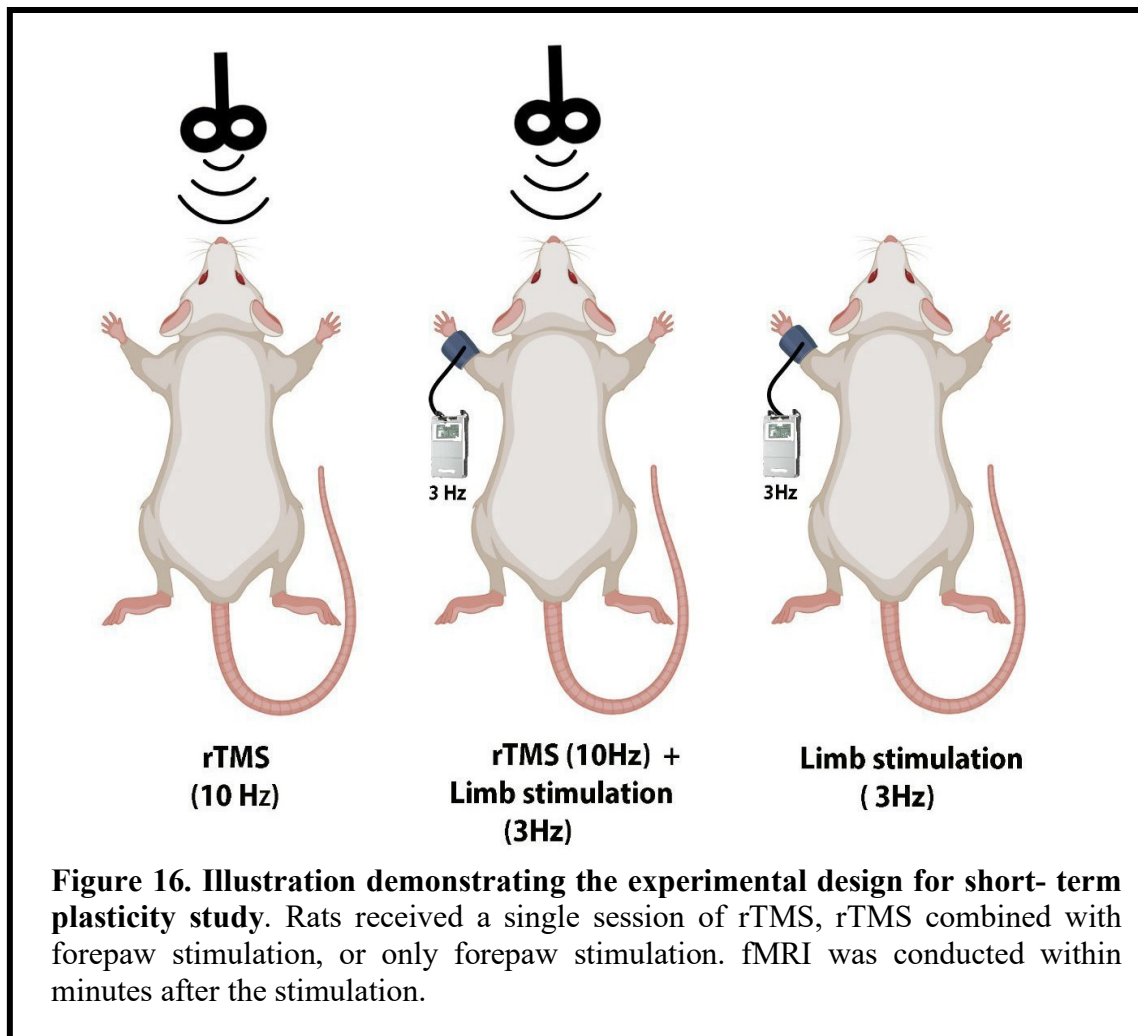
Analysis: Functional images were processed with SPM fMRat software (SPM, University College London, UK). For each subjects the functional images were realigned to T2- weighted high-resolution images. In addition, head motion correction was done in three translational and three rotational directions (X,Y,Z). For each subject, EPI images were re- oriented, averaged and smoothed with full width half maximum (FWHM) = 1.25 mm in the coronal direction spatial in order to reduce randomly generated noise. Finally, an fMRI block design was used, and activation maps were obtained using the general linear model. For each individual, the Z-score statistics was cluster-size threshold for an effective significance of $P < 0.05$. Statistics was conducted with a threshold of $Z > 4.58$. For group analysis, the anatomical images from the rats brain

atlas [176] were used for reference frames. All the images were coregistered and normalized to this template using SPM software. Every Z-score map was clustered into a new mask. The overlap between the masks is shown as the t-value.

Histology: Rats were perfused with 0.1 M phosphate buffer saline solution (PBS) in pH 7.4 followed by ice cold 4% paraformaldehyde solution and the brains were removed and immersed in sucrose solution. Brains were sliced on a cryostat to obtain 25 μm thick sections. Sections were incubated overnight with primary antibodies to detect CaMKII (anti-CaMKII rabbit, Abcam #ab52476); Arc (anti-Arc rabbit antibody, SYSY #156003). After three washes with PBS, sections were incubated for three hours at room temperature with secondary antibodies for CaMKII (Alexa Fluor 647, Jackson #711605152) and Arc (Alexa Fluor 488, Abcam #ab150073), processed with ProLong Gold antifade reagent with DAPI (Thermo Fischer Scientific 2078923) and then imaged with a DeltaVision microscope. ImageJ was used for cell counting and analysis. The number of cells were counted for an ROI of 1024 X 25 μm .

Results

High-resolution fMRI with spatial resolution of $150 \times 150 \times 1000 \mu\text{m}$ and temporal resolution of 1000 ms was used to test how pairing sensory stimulation with rTMS induces changes in somatosensory responses in the primary somatosensory cortex (S1). First, we tested if rTMS, or combining rTMS with sensory stimulation or sensory stimulation alone, will lead to short-term plasticity. **Figure 16** illustrates the experimental paradigm.



Rats received 3 Hz tactile stimulation to the forepaw (40 s OFF, 40 s ON, repeated twice) and the extent of the fMRI responses at S1 was measured by the number of activated pixels. Then, rats were removed from the scanner with the stimulation electrodes remaining in the same location. After 15 min, rats received 10 Hz rTMS stimulation (ST Group 1) for 260 s (2 trains of 20 s repeated twice with one-minute break in between, for a total of 260 s), or 10 Hz rTMS paired with 3 Hz sensory stimulation (ST Group 2) for 260 s, or 3 Hz sensory stimulation (ST Group 3) for 260 s. Once the stimulations were completed, the rats were positioned again in the scanner and sensory stimulation was delivered. Thus, the second fMRI measurement was performed 30 min after the first one. The results indicate that both groups that received rTMS stimulation demonstrated significant increase in the extent of the fMRI response in the second fMRI scan (ST Group 1, $222 \pm 171\%$, $F = 29.2 > F_{\text{critical}} = 6.6$; ST Group 2, $104 \pm 138\%$, $F = 28.3 >$, two-way ANOVA analysis without replication) (**Figure 17**).

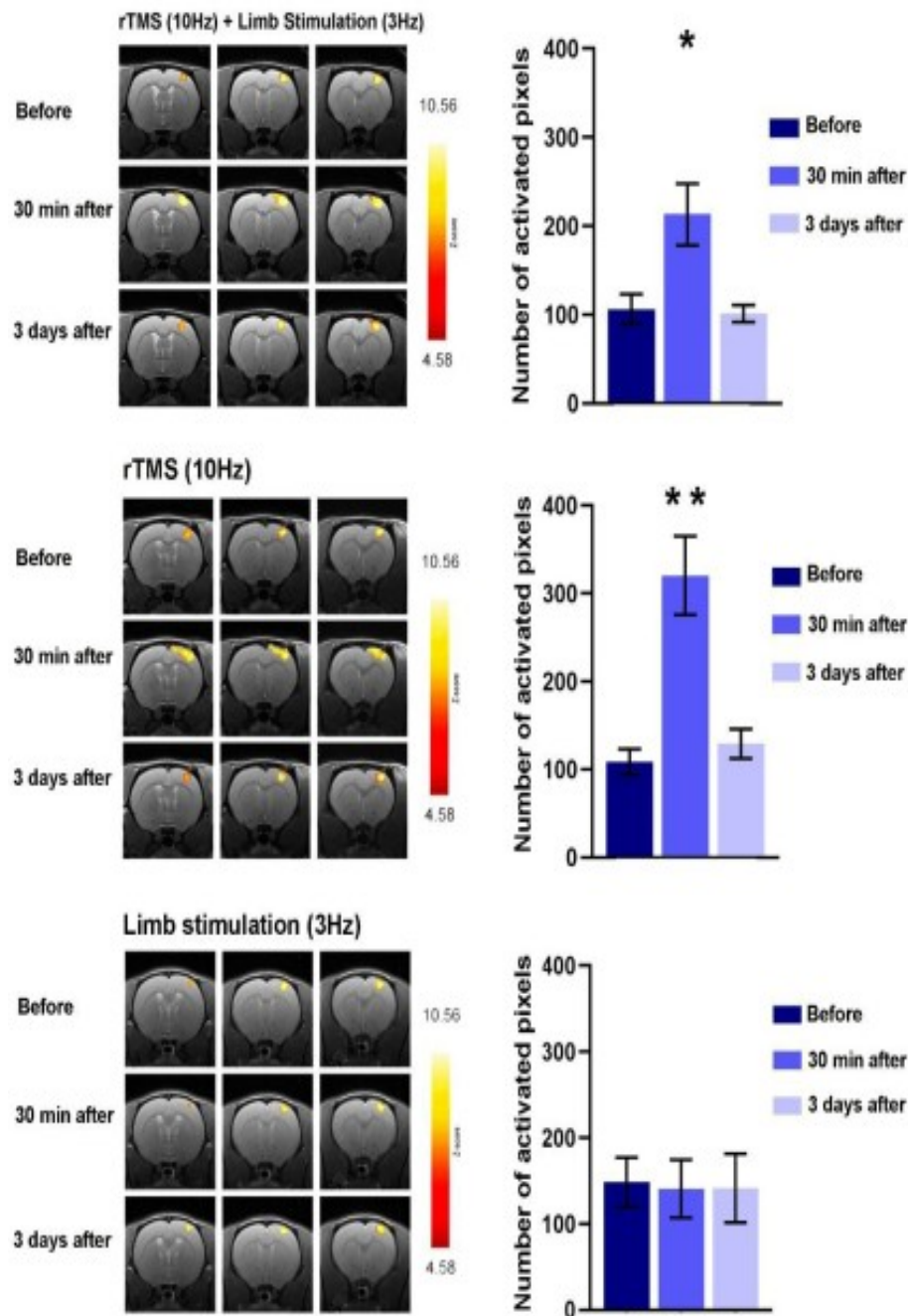
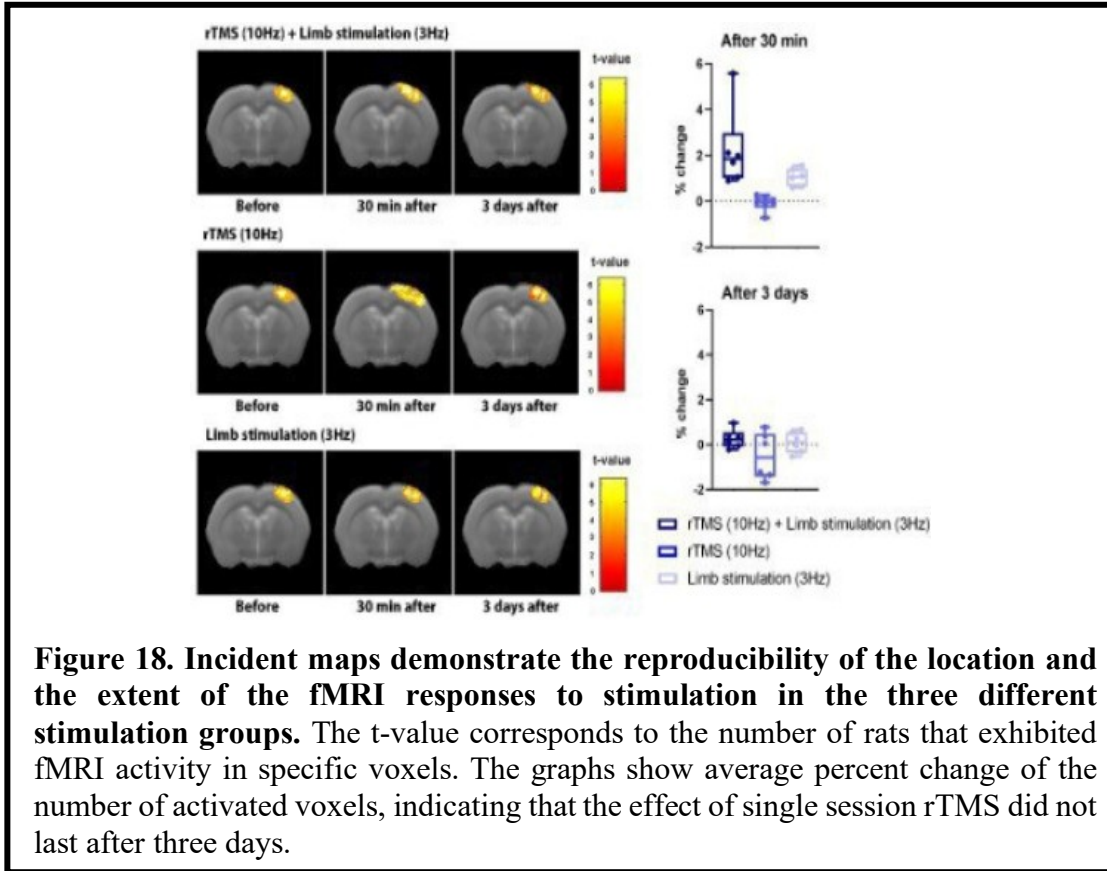


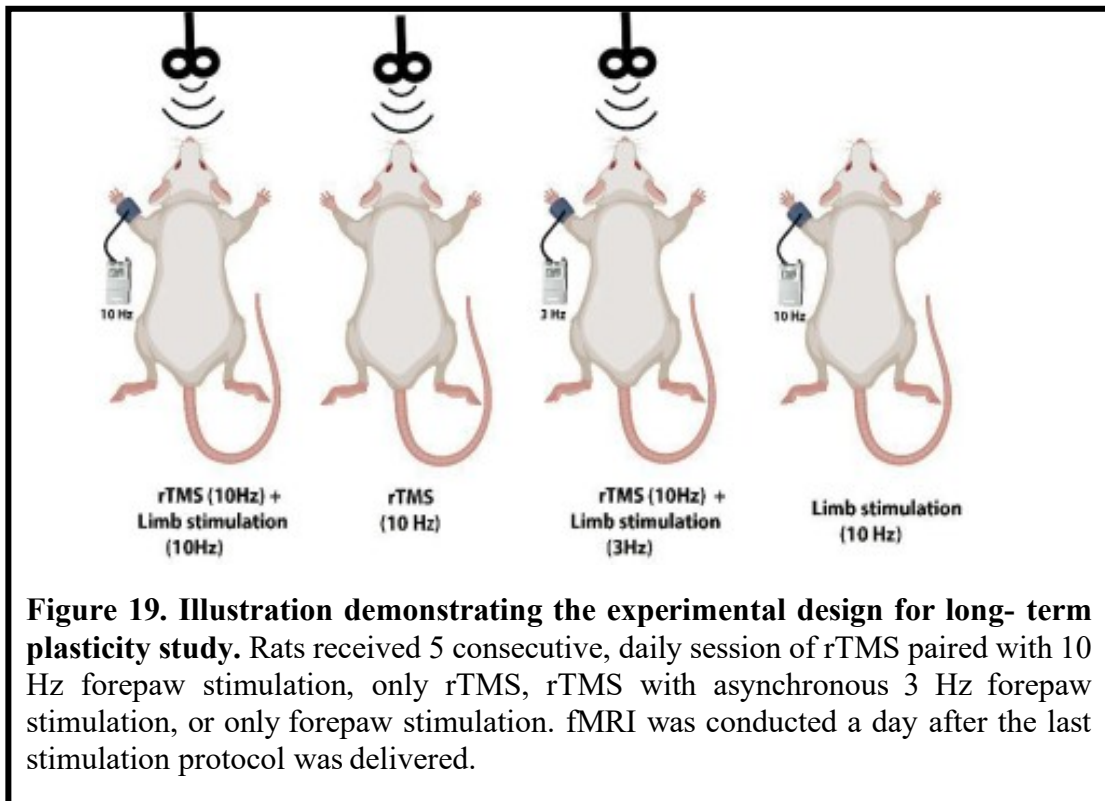
Figure 17. Evoked fMRI responses to forepaw stimulation were measured before and after a single- session stimulation protocol. Representative BOLD z-score activation maps corresponding to $p < 0.05$, overlaid on high resolution coronal images across three brain slices, are shown for each group. The average number of activated voxels in S1 was significantly greater minutes after the stimulation in both groups that received rTMS, but these increases did not last three days after the session. (results are shown average $\pm$ SEM, *, $p < 0.05$; **, $p < 0.005$).

Statistical analysis showed that both these relations justify the rejection of null hypothesis, indicating that the fMRI responses do not remain the same before and after stimulation. Moreover, 10 Hz rTMS alone in ST group 1 was slightly more effective than combining application of 10 Hz rTMS and 3 Hz forepaw stimulation in ST group 2. In contrast, ST Group 3, that received only sensory stimulation but no rTMS, did not show any significant change in the extent of the fMRI response ($-8\pm34\%$, $F = 0.22 < F_{\text{critical}} = 6.6$, two-way ANOVA analysis without replication).

The extent of the fMRI responses to sensory stimulation was tested again three days after the initial fMRI measurement. Even though that ST Group 1 and ST Group 2 have shown a short-term increase in the extent of fMRI activation, this phenomenon did not last. All three groups have exhibited fMRI responses similar to the initial ones (ST group 1, $F = 2.1 < F_{\text{critical}} = 6.6$; ST group 2, $F=0.06 < F_{\text{critical}} = 6.6$; ST group 3, $F=0.01 < F_{\text{critical}} = 6.6$, two-way ANOVA analysis without replication). The location and the extent of the fMRI responses to stimulation in the different groups and across the measurements were consistent, as demonstrated in the Incident maps in **Figure 18**.



While short term plasticity acts on a timescale of milliseconds up to minutes, long term plasticity lasts for minutes and days. To facilitate long-term changes, each group of rats received the exact same stimulation that lasted 260 s, for 5 consecutive days as illustrated in **Figure 19**.



The results indicate that all three groups that received rTMS stimulation showed increases in S1 activity (**Figure 20**).

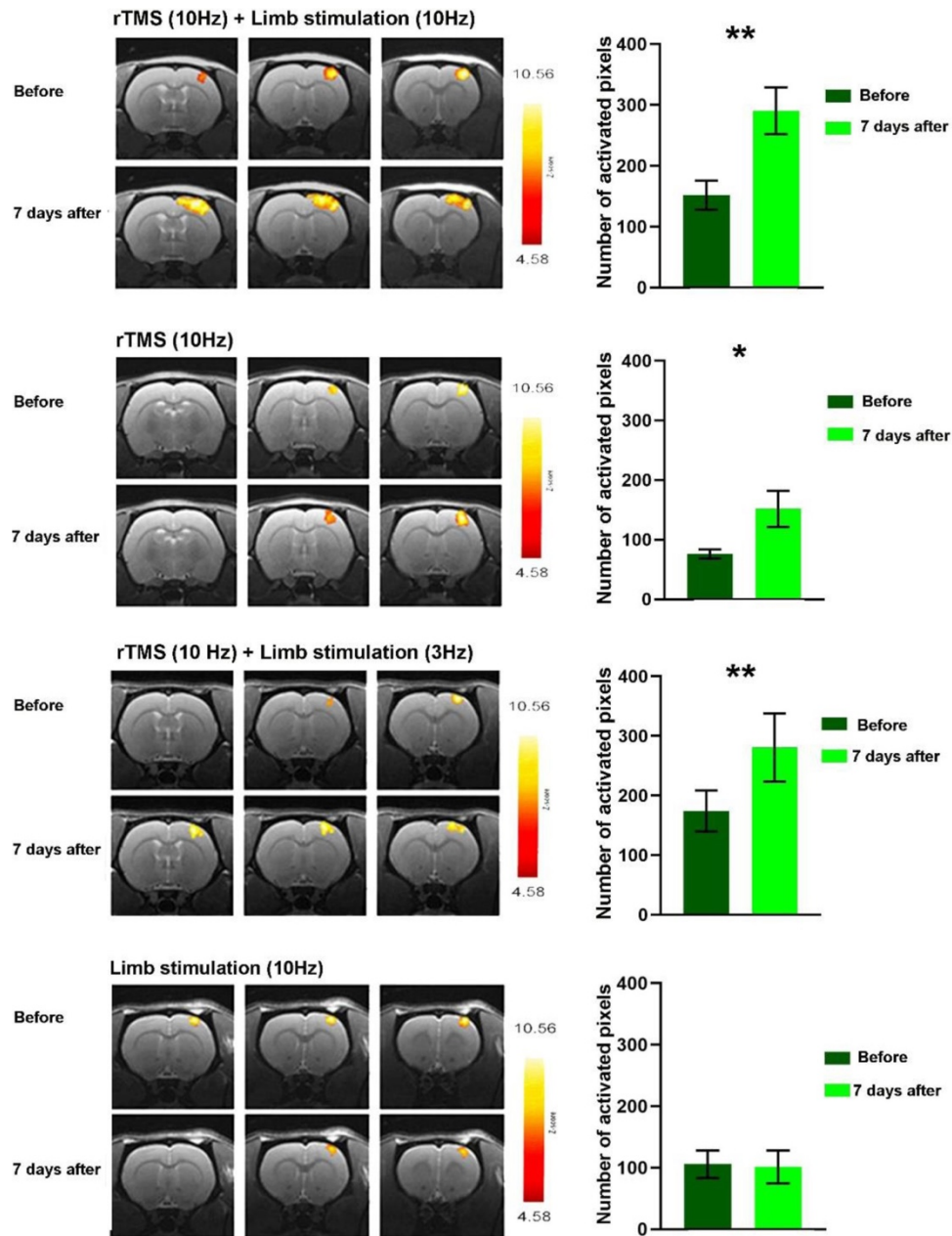


Figure 20. Evoked fMRI responses to forepaw stimulation were measured at day 1 and day 7. Between fMRI measurements the rats were subjected to daily stimulation protocol. Representative BOLD z-score activation maps corresponding to $p < 0.05$, overlaid on high resolution coronal images across three brain slices, are shown for each group. The average number of activated voxels in S1 was significantly greater in the groups that received rTMS, but the group that received a stimulation protocol of 10 Hz rTMS paired with 10 Hz forepaw stimulation showed the greatest fMRI responses (results are shown average $\pm$ SEM, *, $p < 0.05$; **, $p < 0.005$).

However, the rats that were subjected to paired 10 Hz forepaw stimulation that was delivered simultaneously with 10 Hz rTMS (LT Group 1) demonstrated the greatest and most significant increase in the extent of the evoked responses compared to the other three groups (LT Group 1, 10 Hz Forepaw stim + 10 Hz rTMS, $103 \pm 51\%$, $F = 37.2 > F_{\text{critical}} = 6.6$; LT Group 2, 10 Hz rTMS, $98 \pm 71\%$, $F = 8.3 > F_{\text{critical}} = 6.6$; LT Group 3, 10 Hz rTMS+ 3 Hz Forepaw, $73 \pm 62\%$, $F = 7.7 > F_{\text{critical}} = 6.6$, two-way ANOVA analysis without replication). All these relations justify the rejection of null hypothesis, indicating that the fMRI responses do not remain the same before and after 7 days of stimulation. The largest F/F_{critical} in LT group 1 indicates this group has the largest change. Consistent with the results of the short-term plasticity tests, 10 Hz rTMS alone in LT group 2 was slightly more effective than combining application of 10 Hz rTMS and 3 Hz forepaw in LT group 3. In addition, LT Group 4, that were subjected to daily forepaw stimulation but without rTMS, did not show any change in the extent of fMRI responses ($-6 \pm 25\%$; $F = 56.3 > F_{\text{critical}} = 6.6$).

Figure 21 shows incidents maps of the fMRI responses in the center of S1 (bregma 0) demonstrating the consistent distribution of the activated pixels for each condition and within each group. After the final fMRI measurement, rats were perfused and immunohistology was performed to measure cellular markers associated with plasticity. Twenty μm thick brain sections were stained for CaMKII, a gene known to be involved in long-term potentiation (LTP) and Arc, an immediate-early gene known to play a role in synaptic plasticity [177]. Both the right and the left S1, contralateral and ipsilateral to the limb stimulation, respectively, were imaged. The number of CaMKII-positive

and Arc-positive cells were counted in each region and averaged for n=4 in each of the four groups.

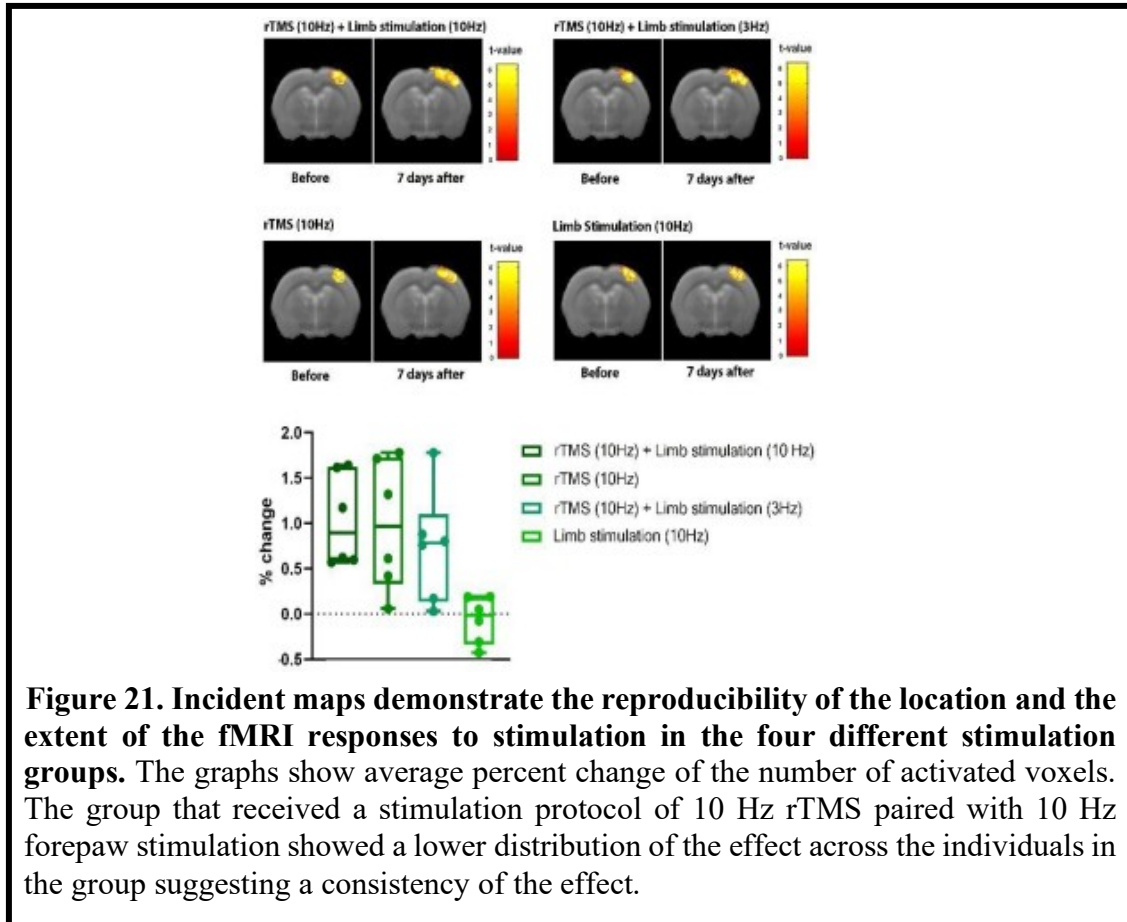
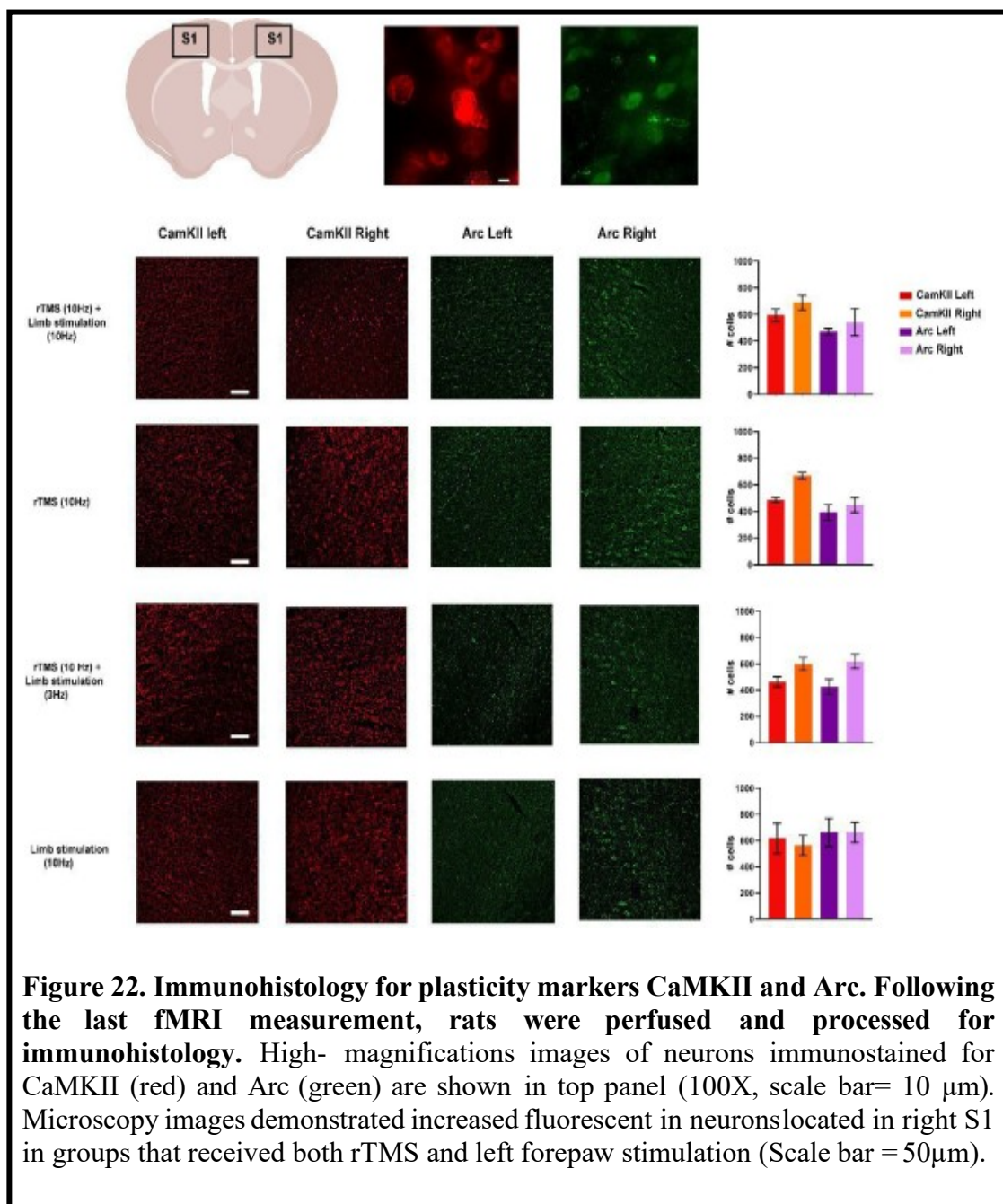


Figure 22 shows representative immunohistology results as well as the quantitative measurements. It is manifested that all three groups that received rTMS over 7 days exhibit increased expression of both CaMKII and Arc in S1 contralateral to stimulation, and only LT Group 1 and LT Group 3 that received both rTMS and forepaw stimulation showed differential expression in both plasticity markers between right and left S1. The group that received only the forepaw stimulation (LT Group 4) did not show any difference between right and left S1. These results demonstrate that the pairing of

rTMS with peripheral stimulation induce plasticity that can be detected in the cellular and the network levels.



Discussion

Short term plasticity is important for neurons to produce appropriate responses to acute changes in activity [178, 179]. Short term plasticity lasts for milliseconds to minutes and is known to work through mechanisms of depression due to vesicle depletion or facilitation due to elevated calcium levels [180]. The results demonstrate that a single rTMS application immediately increased neural activity in S1 as was evident by the fMRI results. These results are consistent to what have been previously demonstrated in human and animal models [167]. However, this short- term increase failed to lead to long-term changes; Three days after rTMS application, the extent of the fMRI responses was identical to the pre- rTMS stimulation ones. It is plausible that the one- time rTMS application was not long enough to induce long-term plasticity, which is also supported by human studies [181]. Thus, the main implication is that for sustainable and long-term changes in cortical function, multiple rTMS sessions are required. In addition, these results suggest that rTMS application opens a time window where the individual may be susceptible to therapy; this has significant clinical implications for rehabilitation strategies.

Long term plasticity is fundamental for learning, memory, and recovering function after injury, and have been shown to last for minutes and days. There are several forms of long- term plasticity that induce rapid and long-lasting changes including long-term potentiation (LTP), long- term depression (LTD), and Hebbian synaptic plasticity [182]. A critical factor for these changes is the temporal sequence and interval between the pre- and post-synaptic spikes, known as spike timing-dependent-plasticity (STDP) [183, 184]. The results indicate that a protocol consisting of daily rTMS stimulation is

effective in inducing long-term changes in cortical function. Notably, the greatest and most significant change in fMRI responses was evoked when the rTMS was delivered exactly at the same frequency as the sensory stimulation. This suggests, that rTMS combined with an additional stimulation considerably augment brain response, and that this long-term effect is via a time-dependent mechanism such as STPD. The latter is also supported by the observation that in both the short-term and long-term studies, when the rTMS was combined with sensory stimulation, but each stimulation was delivered asynchronously (10 Hz rTMS and 3 Hz forepaw sensory stimulation) then the changes in fMRI responses were less than when both modes of stimulation were delivered synchronously. These results have an immediate and translational impact. Synchronous rTMS and other modes of stimulation are required to reach peak performance. Subsequently, it is plausible that delivering rTMS and another mode of stimulation in an asynchronous manner, may diminish effectivity.

An interesting result was that sensory stimulation by itself, did not lead to short-term and long-term plasticity. This builds on a great amount of evidence suggesting that learning and memory is best achieved with multimodal forms of stimulation and experiences. For example, non-invasive brain stimulation paired with current stimulation increased LTP in brain slices [185], and TMS combined with visual stimuli led to remodeling of maps in cat's primary visual cortex [175]. The combination of TMS with another modality of stimulation has also shown to improve post-stroke function in humans [186-189] and in animal models of injury [140].

The nature of this study required the rodents to be lightly anesthetized. TMS has been shown to improve brain function in patients with consciousness disorders [190, 191]. It

is conceivable that rTMS combined with another form of stimulation that is applied in a conscious, participating individual, may lead to even greater changes in brain function. This might be especially significant if the individual receives tactile feedback on top of the rTMS and sensory stimulation for individuals engaged in rehabilitative therapy, or visual and auditory feedback if the rTMS is delivered to enhance mental and cognitive learning. Nevertheless, our results demonstrate that combining rTMS and sensory stimulation has the potential to induce long-term plasticity in unconscious, disabled patients.

Finally, cortical responses associated with short-term and long-term plasticity have been observed with high-field fMRI and supported by traditional immunohistology markers. This builds on a growing amount of work showing that preclinical fMRI is becoming an instrumental tool in basic and translational neuroscience to non-invasively detect changes associated with neural activity in health and disease [127, 192-198].

Our results provide direct indication that pairing peripheral stimulation with non-invasive brain stimulation induces long-term plasticity, and this phenomenon appears to follow Hebbian mechanisms. These results will be important to lead the design of new training and rehabilitation paradigms and training towards achieving maximal performance.

Chapter 4: Current Work

Working towards understanding motor performance in octopus

4.1 Introduction

Neurological disease, sensory processing disorders, and injuries such as Peripheral Nerve Injury (PNI) disturb sensorimotor networks and change normal motor control and behavior. Understanding the organization and function of the sensorimotor circuit is crucial for developing technologies and pharmacological therapies. Neurorehabilitation is an old technique used to improve the quality of life for patients living with chronic disease. The field of neurorehabilitation has been advancing since the beginning of the 2000's. The goal of neuromodulatory approaches is to improve neurologic disabilities in a shorter period by understanding the effects of the brain after an injury [199].

Neuromodulation derives from neurorehabilitation therapy and includes different treatments:

- Deep Brain Stimulation: (DBS)- electrode implanted in the brain that produce electrical impulses which adjust chemical imbalances [200],
- Transcranial Magnetic Stimulation (rTMS) – non-invasive procedure that uses magnetic fields to stimulate or inhibit neurons improving chronic pain [201],
- Transcranial Direct Current Stimulation (tDCS) – non-invasive technique that decreases or increases polarity stimulation that provides changes in neural excitability [202], and Brain-machine interface (BMI) [203].
- BMI is a potential technology used as a treatment for several dysfunctions by

providing communication, environmental control, exoskeleton, restorative therapies, and neuro-prostheses [204- 206].

Artificial limbs and neuro-prostheses have been used for several years, helping improve mobility and ability to manage daily performance. Prostheses can be divided by: lower leg and foot, leg with knee, arm and hand. The following list describes the different kinds of prosthesis:

- Passive Prostheses- look like natural arm, hand and fingers, they are lightweight and do not have the active movement.
- Multi-positional- presence of joints allowing movements as a specific angle assisting on holding or carrying an object.
- Body-powered- less appearance more function, electrically- powered prosthetic arm- use motors and batteries to make the movement desire.
- Hybrid Prosthesis- combine the electrically powered and body powered, allowing to have a greater function and.
- Activity specific prosthesis- are designed for activities as sport, hobbies, work, they are designed to be durable and with particular function [207, 208].

Unfortunately, these technologies are not independently controlled [209]. We suggest developing a smart, adaptive prosthesis that will move and make decisions similar to a neural network mechanism behind different movements.

To achieve this, we have chosen the octopus as an animal model. The octopus' nervous system is highly developed with 45 million neurons in the central brain, 180 million neurons in the optic lobes, and an additional 350 million neurons in the eight axial cords and peripheral ganglia [210, 211]; where just 32K efferent and 140K afferent fibers are connected the brain from millions of neurons in the brain to the millions of neurons in

the axial cord.

This suggests that most of the motor execution comes from the axial cord itself. The octopus also has extraordinary arms with fine motor control which allow for manipulation skills such as opening the lids of jars; this has also been recently demonstrated in VanBuren et al. 2021 [66]. This motor capability is specific to the octopus [212] as each of the eight arms contains an axial nerve that functions like the vertebrate's spinal cord. Together, this hierarchical organization resembles the vertebrate systems and results in a very capable and versatile gripping ability [213].

Grasping movement are divided into the grasping motor act and the action of grasping [214]. We can define grasping, a motor act, as the series of joint movements. The grasping action is needed for feeding, exploring the environment, or interacting with other individuals. Previous studies done using electrophysiological recordings in primates showed changes in neuronal firing rate before grasping movement, meaning that neurons in motor cortex (M1) might play a key role in planning the movement in visuo-motor integration[215]. Also, a specific circuit linking parietal area (AIP) and premotor area F5 have shown interaction [216].

This area contains motor and sensory-motor neurons: canonical and mirror neurons, and a recent classification called “canonical-mirror” which is a hybrid class [217]. But it is still unclear how the information is delivered to the central nervous system and the brain. Therefore, we suggest that understanding the neural ensembles encoded to grasping movements in the octopus will make it possible to mimic the neural network and to encode adaptive grasping movement in a human patient's prostheses in the future. Hence, it is essential to be able to determine the anatomical locations of sensory and

motor neurons in the axial nerve cord (ANC) and understand the difference between the activity of sensory and motor neurons. Once the type of neurons present is determined, it will be easier to target specific cells with fluorescence neuron markers and visualize the pathways whether via ascending or descending from the brain to the periphery with an optical microscope.

To accomplish this goal, we worked with the species *Octopus Bimaculoides*, the genome of which has been recently sequenced [218], allowing us to create genetic manipulation in the future. We were able to build a platform protocol for ANC neuron cell culture and kept them alive for four days. We anticipate these results are a starting point that, in the future, will be a crucial tool for gene-editing because isolated neurons on a dish facilitate manipulation and can help in the future to create a transgenic octopus.

For gene-encoding, we will use a transformation technique often used in microbiology called “electroporation”, using a high electrical intensity field pulse at the cell surface which causes conformation changes, allowing the introduction of any vector into the cells [219]. In this study, we will use specific neurons as calcium indicators (GECIs), which detect intracellular concentration of free calcium ions in the neuronal cytosol [220]. For this purpose, we will use specifically GcaMP [221, 222] which contain: a circular permuted green fluorescence protein (GFP), calmodulin (CaM) and calcium [223]. GcaMP has been detected in previous studies’ activity in neurons on the motor cortex [224], barrel cortex [225] and hippocampus [226]. As another specific indicator for neurons, we will use voltage indicators, (GEVIs) [222] a very successful technique used on neural circuit activity *in vitro* in transparent invertebrate models, with

samples such as *Hydra*, *C. elegans*, *Drosophila larva*, or *larva zebrafish* [227]. This indicator reports changes on membrane potential with an optical reporter; previous studies have used ArcLight in mouse brains with successful results [228]. Combining both indicator GEVIs and GECIs has been used in several studies and represents a new strategy that overcomes the limitations of using each technique alone and provides optimal results [222].

In summary, we hypothesize that this study is a starting line to understand in the future how neural activity in the ANC of the octopus works.

4.2 Protocol Cell Culture Octopus

4.2.1 Material, Methods, and Experimental Procedure

4.2.1.1 Animal Model:

Adult octopus were anesthetized with 3% magnesium chloride in a saltwater tank for arm removal and were later injected with 0.25% lidocaine to minimize pain. All our procedures have been approved by MSU Institutional Animal Care and Use Committee.

4.2.1.2 Tissue Preparation:

The day before the experiment, surgical tools were sterilized, the solutions were prepared, and glass plates were coated with a minimum volume of 1 ml mixed with Matrigel (10 mg/ml) and L15 (2mg/ml) and incubated at 37 °C overnight prior to use.

On the day of the experiment, the octopi were moved from their aquarium to the surgical area. The octopi were weighed and placed into the new tank where the respiratory rate was observed to establish a baseline. The anesthesia solution (0.25% EtOH) was placed in this new tank, and we carefully paid attention to the following parameters for 5 minutes: changes in respiratory rate, skin color, and response to mantle pinch. The concentration EtOH was increased by 0.25% every 5 minutes until the octopi were unresponsive to any stimulus and the breathing dropped to half from the baseline rate. If the octopus had more than one surgery, the animal was euthanized following the guidelines provided by the university.

For the survival experiment, one arm was removed, and 0.25% lidocaine was injected into the area to minimize pain; the animal was placed in the original aquarium and observed for 48hours. For non-survival experiments, the animal was

moved to the euthanasia tank (4% MgCl) and observed until the breathing ceased. The remaining body was sealed and placed inside an -80°C freezer for further use.

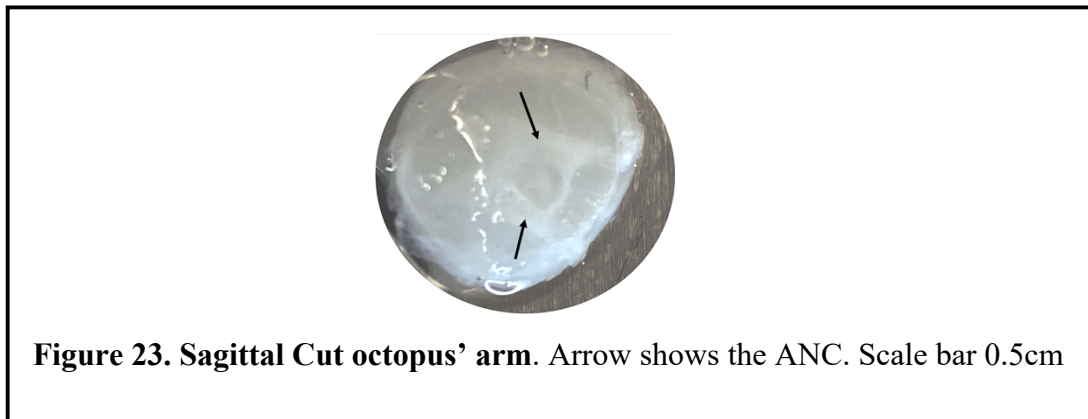
4.1.1.1 Neural Cell Culture:

To establish an optimal cell culture, dissociation enzymes were used. Changes were made to previously performed primary neuronal cell culture of the octopus' optic lobe based on improving and prolonging cell life on isolated cells, and describe on Table 1 [229]. The octopus's nervous system is rich in collagen in the connective tissues and adhesive proteins surrounding the neurons, therefore we used a mixture of natural enzymes that contain proteolytic and collagenolytic enzyme activity. First, we dissected the ANC (**Fig. 23**) and separated it into small pieces (tissue dimensions: 1 mm x 1 mm). Then we incubated the tissue with Collagenase P (4 mg/ml) in 50% L15 and 50% sea water from the tank for 40 min at room temperature. Afterward, we added 0.25% Trypsin and incubated it for 35 minutes at room temperature, and as a last step, we added Accutase (a non- mammalian source enzyme that derives from invertebrate species) 0.1% for 40 minutes. We rinsed the plate three times with 50% L15 and 50% sea water (SW) from the tank to stop the enzyme reaction and we plated the cells with the media (50% L15, 50% SW, Amphotericin B1%, PenStrip1%, 0.1 mL B27, 0.1M HEPES, 0.05M Glucose at pH=8.2) and PenStrip1% and Amphotericin B1% in a 24 wells plate with Matrigel (Matrix Corning 356234). We maintained a sterile environment and verified each day that no contamination was introduced. We checked each plate under a microscope and if media changed color to indicate contamination, the well-plate was immediately disposed of. We used Trypan Blue for 10 minutes, which is recommended to be used to evaluate cell viability, to test the health condition of our

culture, and to count neurons with the use of a Hemocytometer. Viability of the cells was evaluated in percentages, using the following equation:

$$\frac{\% \text{ viable of cells} \times 100}{\text{total of cells}}$$

All results and figures show mean $\pm$ standard error of mean (SEM). The media was changed once a day for four days.



4.1.1.1 Histology:

To verify the presence of neurons, we used antibodies specific to neurons. We used two antibodies: anti- β tubulin III, because the cytoskeletal protein isotype is present in human central nervous system development and neoplasia, and is also abundant in the brain during fetal and postnatal development, therefore it has been associated to the neuron [230], and CaMKII, which is the central coordinator and executor of calcium signals. It is also important as a mediator for learning and memory [231]. Previous studies have shown its presence in rat brains (1% in the forebrain and 2% in the hippocampus) [232] and in invertebrates such as the octopus, *Aplysia Californica* [233]. We compared the cell culture *in vitro* structure with the structure from the neurons in previous literature. We fixed the cell culture with 4% PFA for 20 min and

placed it with sucrose 30% overnight and washed three times with 1% PBS for anti- β tubulin III, and for CaMKII. We used a blocking solution (0.1% GS with 1% PBS with triton for anti- β tubulin III and 3% NDS with PBS for CaMKII,) at room temperature for 1 hour. We mounted the first antibody (1:250 Abcam for CaMKII in PBS, 1:250 for anti- β tubulin III Santa Cruz Biotech Inc in blocking solution) overnight at 4°C. Then, we washed the primary antibodies with 1X PBS and incubated them for 3 hours with the second antibody, Alexa Fluor 647 Anti-CaMKII (1:200 Abcam ab#196165 and FITC-conjugated goat anti-mouse IgG (1:200, Thermo Fisher Scientific). Cells were washed three times with 1X PBS and mounted with mounting media with ProLong Gold antifade reagent and with DAPI (Thermo Fisher Scientific 2078923), and then imaged on the DeltaVision microscope. ImageJ was used for analysis.

4.1.1.1 Live Imaging Cell Culture:

To validate if the structure and size from cell culture plates *in vitro* are neurons at day four, we proceeded with Live Imaging cell culture, where the cells grown on the cover glass with Matrigel matrix were mounted in the Leica DM6 FS. We screened over the entire glass cover with objective 50x in a brightfield neuron presence. The cells structured similar to neurons were pictured with a Hamamatsu ORCA-Fusion sCMOS camera and processed through the software LAS X. ImageJ was used for the analysis afterwards.

4.1.1.2 Multi-Electrode Array Recordings:

On day four, the cell culture with the media were placed on the Maxwell

Biosystem single well microelectrode array (MEA). This technique has been used to characterize cultured neuronal networks due to the efficacy of the microelectrode arrays to sense neural electrical signals. [35, 36]. MEAs can also provide spatiotemporal information about the network activity, helping to quantify the cells present on the dish [234]. The electrodes detect signals that originate from the extracellular ionic current flow when action potential is present [235]. Previous studies showed that this technique has been used on brain slices [236, 237]. In this experiment, we used a special MaxOne plate designed for cultured neurons where we tracked and characterized the presence of any spontaneous activity or an evoked neural response. First, we recorded an activity scan that selected the most active electrodes. After, we performed a network scan that allowed recording activity from a large number of cells simultaneously, and we determined where the active electrodes were located. We then stimulated the neurons and recorded evoked responses. The stimulation paradigm consisted of 100 pulses for each set of stimulations at a single voltage, 50 ms inter-pulse intervals (IPI), and 1000 ms interburst intervals (IBI). The amplitude was increased from 25 mV to 600 mV to determine the best stimulation range to use. The chip surface was rinsed with ethanol and left to dry for about 2-3 hours prior to culture.

4.2 Results

4.2.1 Tracking action potential with Multi-Electrode Array

The data used for this experiment was collected from 6 octopus'; and for each octopus, at least 4-5 well-plates were used. As discussed earlier in the methods, the changes made to the previous protocol improved on previous cell culture studies. We were able to observe the presence of isolated neurons from the ANC and maintain them for 4 days in a dish. As a first step, we determined the activity of the isolated cells by tracking the action potential [238]. The recording was performed using the MEA.

In **Figure 24, Panel A**, we observed a temporal distribution of the recorded events, demonstrated in the raster plot, the x-axis shows time frame 0s – 300s and the y-axis shows the 1048 channels that were analyzed. As we can observe, the previously described stimulus evoked a significant response. The extracellular amplitude depends on transmembrane current on neural compartments, but it also depends on the spatial arrangement of the compartment. Therefore, quantification cannot be solely determined by the transmembrane currents from the extracellular recording. We saw the voltage traces of Action Potential (AP) on the cells from the cell culture and represented as a footprint. Where, on **Panel B**, an AP from 1.8 ms up to 3 ms is represented at a spike threshold of 4.5. These results suggest that the cells present in the cell culture are alive.

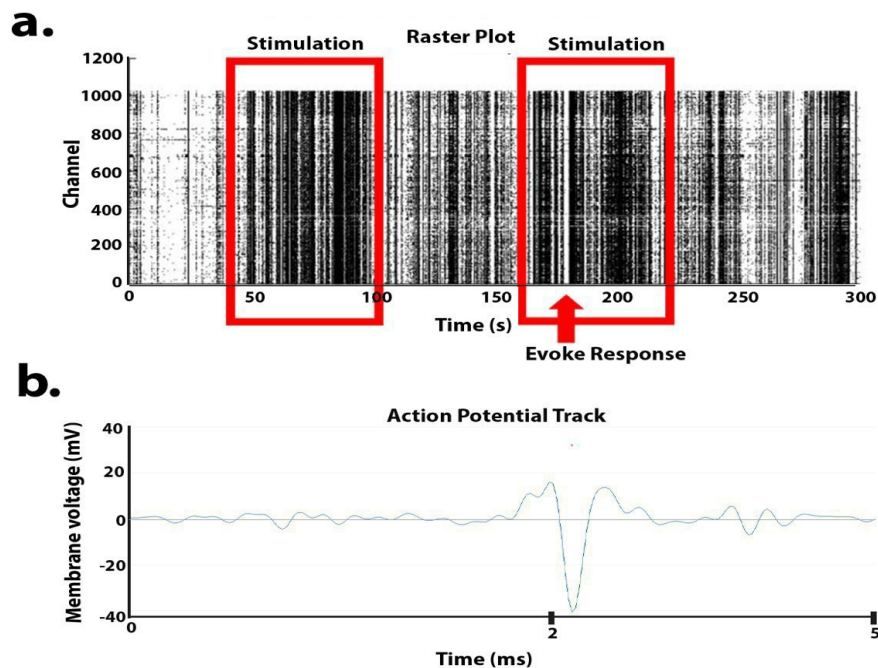
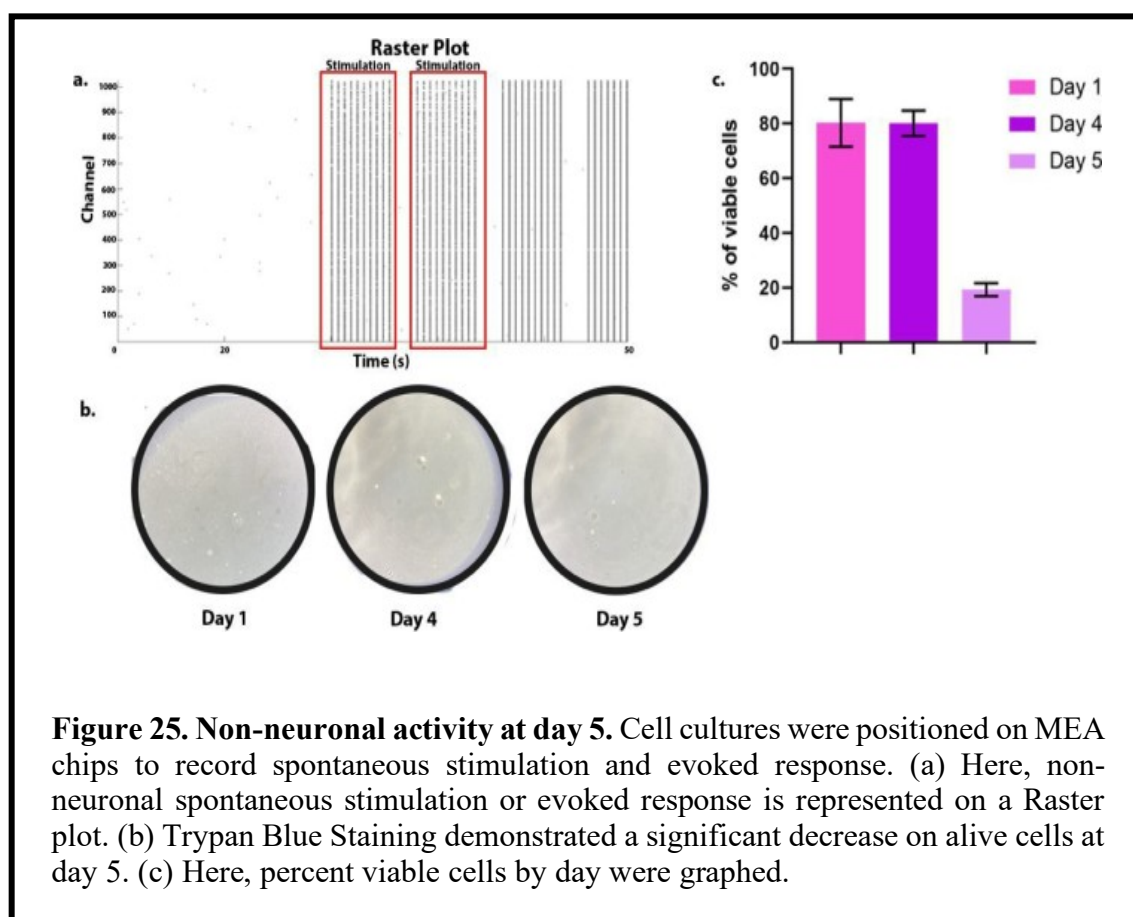


Figure 24. Spontaneous and evoked responses of octopus' primary neuronal cell culture. We maintained a viable primary cell culture for several days and were able to record action potentials from these neurons up to 4 days post- dissection. The cell cultures were positioned on MEA chips and spontaneous activity was recorded, suggesting that the octopus' cells were viable and functional, and are likely to be neurons. Results are demonstrated on Raster plot and show spontaneous action potential and evoked responses (a). Here, the Action Potential Track is represented as a footprint(b).

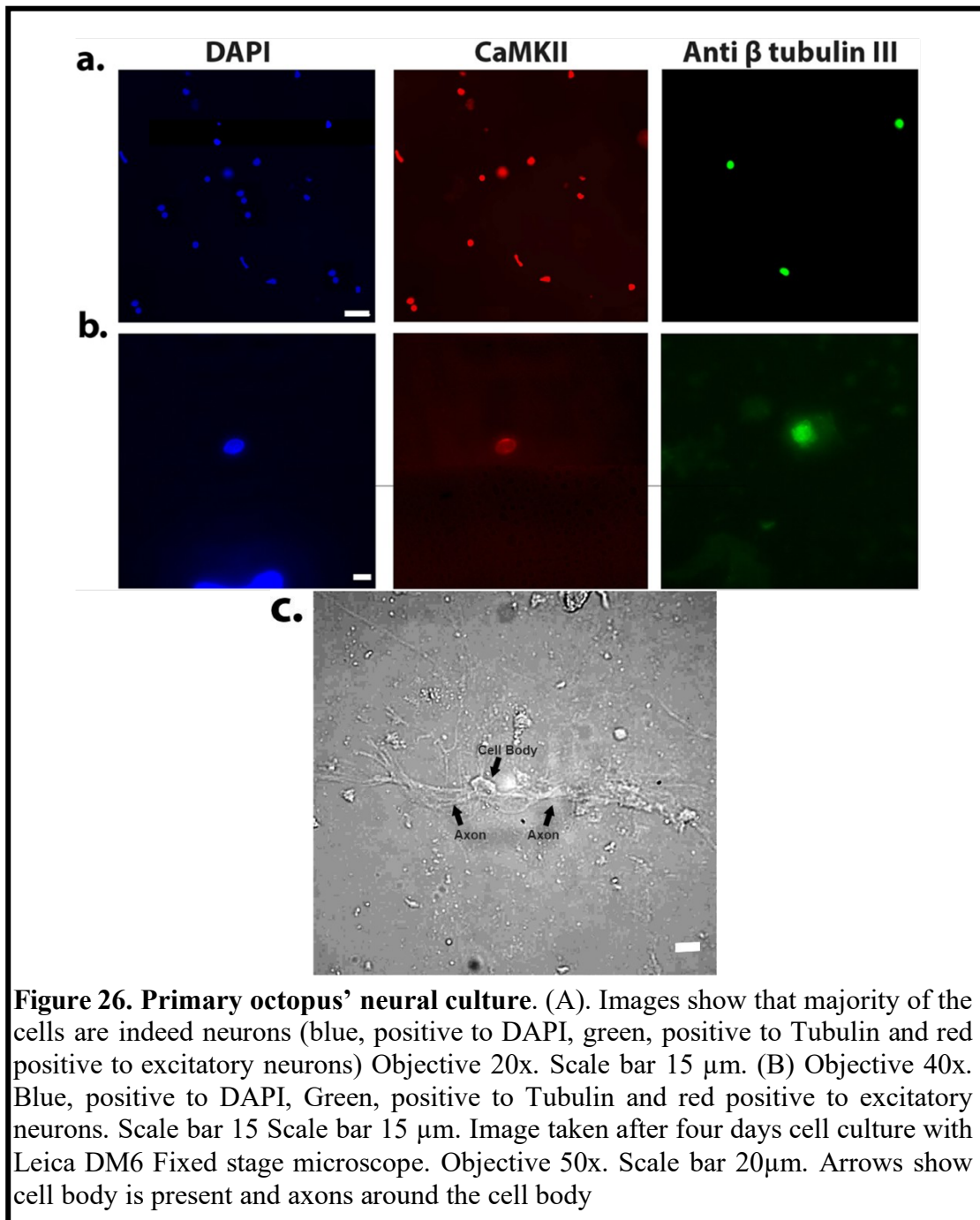
The challenge of this experiment was to keep the cells alive for longer than four days. **Figure 24, Panel A** is a temporal distribution, where the x-axis shows time, which goes from 0- 50s, and the y-axis shows the 1049 channels that were recorded. We could not see any present APs nor an evoked response at Day 5. **Panel B** shows cells stained with Trypan Blue, which allow us to count the number of cells alive. In this experiment there were similar results at Day 1 ($n=24 \pm 2.16$) and at Day 4 ($n=21 \pm 3.27$). However, at Day 5 ($n=4.75 \pm 0.96$) there was a significant decrease in living cells. Results are verified on **Panel C**, with the percent viable cells being the following: Day 1 = $80.25\% \pm 8.71$; Day 4 = $80.07\% \pm 4.63$; and Day 5 = $19.25\% \pm 2.40$.

As described in the literature, at least 7 to 14 days is recommended to keep regular cells alive on the dish. In the case of stem cells, however, the cell cultures can stay alive for at least 4 weeks. This time frame is due the time it takes for the cell to reproduce with the vector inserted, sometimes lasting from 5-7 days [239, 240]. On the fifth day, we proceeded with the same protocol as before and we could not record any action potential from spontaneous stimulation or evoke response from any stimulus. This suggested the need for further experimenting to improve the maintenance level on this protocol to avoid morbidity by apoptosis from the cells [241]. Keeping the cells alive longer will make the genetic manipulation possible.



4.2.2 Determining the presence of the cells in the preparation using immunostaining and live imaging

To further determine the type of cells, we used immunohistology. Cells were fixed with 4% PFA and incubated with mouse polyclonal Anti β III Tubulin antibody (neuronal indicator= green color) and CaMKII (excitatory neuron indicator= red color). Results are shown in **Fig. 25**, Panel A and B where we used two different magnifications: Smaller objective 20x, where we evaluated several cells positive to each indicator, and the bigger objective, 40X, where we focused on a single cell to estimate the cell size (scale bar= 15 μ m). These results are very similar to previous publications where β III Tubulin was localized in the cytosol [229].



Also, we applied live imaging methods to the cell growth to correlate extracellular activity to the morphology of an individual neuron for this purpose and used a Leica DM6 Fixed microscope. Through the 50x objective and brightfield stage, we were able to observe the cell's body surrounded by long axons that are shown with arrows in Figure 25 (B) (size $\sim 15 \mu\text{m}$). This result indicates that this experiment was the first attempt to use this protocol, but there is still more work to be done.

4.3 Discussion

Neurorehabilitation as Brain-machine interface (BMI) is used today as a treatment for several dysfunctions [204, 205] but at present, patients cannot independently operate BMI without an external control. Identifying the neurons involved in grasping movements will allow us in the future to mimic those pathways to create a prosthesis that will work independently.

As described above, the octopus has a magnificent arm with fine motor control allowing for complex manipulation skills such as opening lids of jars. Thus, by identifying the neurons involved in delivering information from and to ANC will open new strategies to improve the existing BMI.

So far, previous studies [229] showed the presence of neurons in the optical lobes. However, these studies were only able to keep the neurons alive for a few hours. Additionally, their main focus was on structural aspects, and they did not perform any assays to improve viability of those neurons. Therefore, the present study is the first done on primary cell culture for ANC where the cells survived four days *in vitro*. These results were made possible by improving the published protocol, where we changed incubation time for Collagenase P and Trypsin, increased pH ~ 8.2 , and added Accutase as a new

enzyme that is a non-mammalian or bacterial component that previous studies showed efficacy on neuronal dissociation [238, 242].

We also modified the solution by adding Neurobasal/B27, which is used in neuron growth processes in mammals, instead of using Hemolyph (octopus' blood) [243, 244], adding antibiotic [245], and by replacing conventional culture coating (PoliD) with Matrigel coating because it is known in the literature [242] that neurons thrive in this environment as it closely mimics the *in vivo* environment and improves the attachment [246].

Table 1. Comparison Old Protocol vs. New Protocol

Old Protocol	This Protocol
Collagenase P 30 min	Collagenase P 40 min
pH= 7.8	pH ~ 8.2
Temp 21-22 °C	Temp 19-20 °C
Trypsin 20 min	Trypsin 35 min
PenStrip 1%	PenStrip 1% + Amphotericin B 1%
Poly – D and Poly - L media coat	Matrigel Cornix media coat
Hemolyph (10% HEMO)	Neurobasal/B27
	Accutase 0.1 mg 40 min

The octopus's nervous system is divided in two parts; the optic lobe, and the nervous system of the arm. The peripheral nervous system of the arm is distributed along the arm and contains at least 300 ganglia. The arm nervous system has millions of tactile and chemical sensory attached to the skin and the suckers [213]. The ventricle lobe (VL) is one of the specific areas in the octopus brain dedicated to learning and memory. When the VL is removed, there are no changes in behavior, but we can demonstrate changes on the long-term memory. Two types of unipolar neurons are present in octopus: small amacrine cells, which range in size from 6-10 μm , and the large amacrine cells, which can be up to $\sim 17 \mu\text{m}$.

In summary, we were able to demonstrate the presence of neurons on the ANC by isolating them on a dish. We identified spontaneous and evoked responses of the cells at day four with the MEA chip system. Also, with immunochemistry using specific antibodies for neurons, we detected positive fluorescence and the expected size, and those results were also confirmed with live imaging.

To prolong the lifetimes of the cultured neurons, future research could focus on cultivating the cells in smaller well plates. This would allow the cells to be closer to nutrients, improving the proteostasis network to maintain balance in the cellular pathways that are responsible for protein synthesis, folding, processing, assembly, trafficking, localization, and degradation [247]. Also, recent work done in 2020 has shown improvement: changes from old protocols in murine primary cell culture were modified by reducing how often media solutions were exchanged [248], and the neurons cells were able to stay alive for several weeks. The changes made in this work were to completely replace the media after 48 h and remove just half the volume of the media on

days 5 and 8, and subsequently whenever the color changed in the cell culture. Also, previous work on zebrafish has added to the media fetal bovine serum (FBS), improving the quality of life of the cells on a dish [249].

Usually, *primary cell cultures* do not proliferate, therefore it is very common on cell culture protocols to remove apoptotic cells and subculture adherent cells with a dissociation reactive, eliminating all the toxins and promoting cell proliferation to a large number generating *secondary cultures*, which allow them to reproduce for week or months [250]. This technique is controversial because it uses a chemical that can cause damage to the cell.

In summary, we were able to create a platform *primary cell neuron from the octopus ANC*. Once we are able to improve and prolong the cell life period, the gene-editing techniques will employ electroporation, where we will use high-voltage electric shocks to introduce a vector into the neurons. After, we will identify with specific neuron markers whether they are the sensor, for example Br3a-Pou4f1 anti-body, POU homeodomain transcription factor that regulates genes expression and differentiation of sensory neurons [251] or motor with MO-1 which bind on somatic motor neurons in the adult rat brain [252]. First, we will try to introduce to the specific neurons the adeno-associated virus (AAV) vector with an optical report such as calcium indicators (GECIs) [9- 11] or voltage indicators (GEVIs) [12]. We will try this vector first because it is stable and provides long-term expression of transgene in non-dividing cells [253]. In case we are not able to introduce this vector, or we do not see any fluorescence, we will try introducing this vector via the expression of baculovirus system (Bac-Bac), which mediates the efficient production protein expression [254]. After the expression, the

vector will be introduced, and the protein will be expressed. We will observe with an optical microscope how the information is delivered to the central nervous system, whether via ascending or descending neural pathways from the brain to the periphery.

Chapter 5: Conclusion and future direction

Neuromodulation can induce changes in neuroplasticity. This can be achieved via electrical, chemical, and mechanical interventions, causing alterations to the central and peripheral nervous system functions. This dissertation focused on identifying strategies to induce neuroplasticity in the somatosensory cortex (S1) that can improve one's quality of life.

Past research has shown that neuromodulation can be a good approach to decrease phantom limb pain, anxiety, and depression in amputees. In this report, we were able to prove that activation of the somatosensory cortex can improve neuropathic pain by restoring cortical equilibrium. Results shown in chapter 2 demonstrated the efficacy of rTMS and EPG (novel proteins discovered in our lab) on peripheral nerve injury in a rat model. Rodents with PNI that received rTMS treatments every other day for 30 days showed improvement in long-term mobility, decreased anxiety, and neuroplasticity compared to rats without treatment. The treatment was followed by a battery of behavior tests. These outcomes showed us that post-rTMS treatment can lead to benefits in acute, sub-acute, and chronic phase patients. The challenge with rTMS is that the changes induced are not cell specific, which can cause activation or inhibition of cells that are not meant to be involved in the treatment.

Hence, scientists are trying to develop cell-specific neuromodulation techniques that are minimally invasive. Optogenetics is a cell-specific neuromodulation technique shown to improve neuropathic pain and several disorders, which requires light to induce neuronal changes in the specific target cells. Therefore, in this study, we tested if daily electromagnetic stimulation of S1 in rodents with EPG could be a potential

minimally invasive and cell-specific neuromodulation technique. Leading and restoring cortical balance and achieving similar or better results than rTMS.

The results showed an amazing improvement on sensory and motor behavior in a battery of behavioral tests performed during the 4 weeks of treatment with an enhancement of neuroplasticity on S1, showing that EPG could be a new therapy to use for several disorders. Nevertheless, this is the first application any study has conducted for the EPG in an animal injury model, and characterizing this protein is a crucial key to improve this neuromodulation method in order to address and improve relief in chronic disease.

Changes induced by neuromodulation lead to immediate changes called short-term plasticity, which are very important to the acute response. However, to date, the mechanism that switches from short-term to long-term changes remains unknown. Therefore, in this study, we focused on discovering the best sensory combination with rTMS that mediated the greatest neuroplasticity. These changes were visualized using fMRI map activation on S1 via immunofluorescence. Results and methods are explained in chapter 3, but to summarize, we divided the groups into short-term and long-term treatment. In each group, we combined a synchronous and asynchronous electrical stimulation on the forepaw with rTMS. We included single-stimulation sessions and compared them with multiple-stimulation and daily sessions. The best pairing combination was determined with a fMRI high resolution. In the short-term group, the changes induced by the rTMS did not last for three days. The animals' brain returned to the baseline (pre-rTMS), meaning more than a single stimulation is required to induce long-term plasticity. However, daily delivery of rTMS induced long-term changes in cortical

function; the greatest and most significant was when we combined forepaw stimulation synchronously with rTMS, showing us that the combination augmented brain response. Neuroplasticity changes were also verified with specific neuron markers via immunochemistry. The results obtained in these studies are an important key to designing new therapy and rehabilitation paradigms with optimal results.

Overall, this dissertation has shown that inducing changes in S1 via neuromodulation can improve the cortical balance, reducing the neuropathic chronic pain coming from the PNI. In addition, multi-session rTMS is required to evoke changes in long-term plasticity, and also combining synchronous sensory stimulation with rTMS can enhance the neuroplasticity in the S1. Further studies are required in order to translate this therapy in humans, thus we suggest working with large animals, such as pigs and monkeys. The brains of these higher-order species are very similar to the human brain and will allow us to better understand any similar response that humans could have once the therapy will be provided. This therapy could also be used to study different types of disorders that lead to changes in the S1.

Lastly, this dissertation has also shown that the EPG neuromodulation technique is a potential minimally invasive therapy that can specifically target neurons and induce changes on S1. This neuromodulation technique could be a potential therapy in the near future, and will expand the therapies that currently exist. This study was the first to use EPG with animals, and therefore, future studies are needed to characterize EPG on the cellular and network level.

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