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Development of a skilled forelimb reach task in mice and the effects of C-8 projecting cortical spinal neuron ablation in motor learning by photothermal Au nanoparticles

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### Publication Date

2015

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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Development of a skilled forelimb reach task in mice and the effects of C-8  
projecting cortical spinal neuron ablation in motor learning by photothermal Au  
nanoparticles

A thesis submitted in partial satisfaction of the  
requirements for the degree Master of Science

in

Biology

by

Justin R. Montenegro

Committee in charge:

Mark H. Tuszynski, Chair  
Stefan Leutgeb, Co-Chair  
Takaki Komiyama

2015



The Thesis of Justin R. Montenegro is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2015

## Dedication

To my unconditionally loving mother, Vangie, my fiercely loving father, Farolito, my endearing sister, Ashten, my role-model brother, Tristan, and my beloved dog Wrinkles, without whom, I would have never have accomplished what I have today. You are forever in my thoughts, in my endeavors, and in my heart.

## **Epigraph**

“Science and art tend to coalesce in aesthetics, plasticity, and form. The greatest scientists are artists as well.”

Albert Einstein

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## Acknowledgments

For the past two years, I have had the amazing opportunity of working in the Tuszynski neurosciences lab. My sincere gratitude goes out to everyone in the lab who has made my experience as a developing researcher a most memorable and integral part of my life. My deepest appreciation goes to Dr. Erna Van Niekerk whose guidance, direction, and inspiring work ethic motivated me for all of my time here and continues to motivate me to this day. Thank you for laying the groundwork of this research project and your flexible understanding as my mentor. My utmost gratitude goes to Dr. Mark Tuszynski whose firm direction and articulate demeanor has brought out the best of my capabilities.

I would also like to acknowledge Drs. James O'Conner, Jeremy Biane, Yoshio Takashima, and Sergei Svarovsky who had equipped me with the knowledge, skills, and tools to proceed with research in neural plasticity and retrograde tracing. Thank you for taking the time to pass on the knowledge and skills to me. I would be remiss to leave out Lori, Eileen, and Sara, without whom, I would always be in trouble with Buster at the VA. To Myrna, Kitty, and An who continue to brighten up my all days, good and bad, at the lab with meaningful conversations.

I would also like to acknowledge Drs. Takaki Komiyama and Stephan Leutgeb, my committee members. Thank you for taking the time and serving on my committee. I am deeply honored. Thank you to all my friends, both inside and outside of the lab, who had helped me all along my journey heretofore.

## ABSTRACT OF THE THESIS

Development of a skilled forelimb reach task in mice and the effects of C-8  
projecting cortical spinal neuron ablation in motor learning by photothermal Au  
nanoparticles

by

Justin R. Montenegro

Master of Science in Biology

University of California, San Diego, 2015

Professor Mark H. Tuszynski, Chair

Professor Stefan Leutgeb, Co-Chair

Motor learning is measured quantitatively through many behavioral tests. Behavioral models for motor learning observe skill acquisition and performance over a period of time within rodents. One such behavioral test is the skilled

forelimb reach-to-grasp test. This skilled forelimb reach-to-grasp test has been extensively used to observe motor learning in behavioral studies and is an appropriate metric that can be used to assess experiments of the motor cortex. In this study, the skilled forelimb reach-to-grasp task was developed in young adult mice to measure motor skills as a function of learning. The skilled forelimb reach task was developed to consistently tease out the rapid learning phase of learning in adult mice and was further characterized for future studies. In this study, the skilled forelimb reach task was corroborated that age and gender do not significantly affect the degree of learning between groups. However, the amount of time spent during handling and conditioning mice play a significant role in capturing the steep learning curve characterized as the rapid learning phase. In this study, 50 trials per training session was found to be a significant number of trials that allowed motor learning to occur whereas 30 trials of skilled forelimb reaching was not.

Layer V cortical spinal neurons project to the eighth cervical segment of the spinal cord (C8) and synapse onto interneurons that control the movements of the digits. Multifunctional gold rod nanoparticles (nanorods) possess the ability to transform photoenergy into thermal energy. Using CTB peptides conjugated to the gold nanorods for retrograde transport, these C8 projecting neurons were tagged with these photothermal golden nanorods and ablated by the heat generated by the golden nanoparticles. Ablation of these C8 projecting layer V cortical spinal neurons after seven days of training did not result in a significant deficit of motor performance skilled forelimb reach task the day after the neurons

were ablated. After more time had passed after the ablation, motor performance returned to levels prior to the ablation and post training day 5. We could not draw any conclusions in this study if ablation of layer V cortical spinal neurons prior to training in the skilled forelimb reach task resulted in a decreased degree of motor learning. The learning curve of mice exposed to the 760nm laser did not experience as much of steep learning curve that was observed in mice that did not receive 760nm light exposure. Other types of neural plasticity are at play that may compensate for the elimination of neurons that have been suggested to play a key role in motor learning.

## Introduction

The brain has the remarkable ability to change as it learns. To adapt information from experience and organize that new information entails the strengthening, weakening, or forming of new connections between neurons. Dynamic changes that result in strengthened communication between neurons give way to neural plasticity. Intrinsic, structural, and synaptic changes all play key roles in how effectively neurons communicate between each other and establish memories, learning, and ultimately behaviors. Neural plasticity allows the brain to make appropriate adaptations through changes such as increasing dendritic complexity and spines. In the motor cortex, we see extensive increases in dendritic complexity and density as a function of motor learning within a specific subset of cortical spinal neurons of the motor cortex (1). Connections between specific and functionally important cortical spinal neurons within the motor cortex confers the brain's capacity to undergo a large degree of structural change upon motor learning resulting in adaptive and skillful motor functions (2). Acquisition and performance of a motor skill involve the motor cortex, which is necessary for valuable fine motor skills that are important for everyday life. Using the skilled forelimb reach-to-grasp task in mice, studies of motor learning can be performed to assess changes on the molecular, cellular, and functional levels. In an effort to understand further how the brain learns and forms motor memories, the plasticity field aims to characterize the innate changes of specific

neuronal populations within the motor cortex and expand the pool of knowledge in regard to learning in the adult brain.

### **Synaptic, intrinsic, and structural plasticity**

Studies of neural plasticity have broken down how the excitability of neurons changes into three different types: synaptic plasticity, intrinsic plasticity, and structural plasticity (3). All forms of plasticity are important in storing, and organizing new information obtained from one's surroundings and experiences. Synaptic plasticity involves the synapse which occupies an important position where neurotransmitters are exchanged and long and short-term modifications take place depending on how constant the activity between two neurons occurs. Repetition or longer periods of training result in lower thresholds to trigger an action potential as measured by intracortical microstimulation in the motor cortex. This is suggestive of increased synaptic efficacy after repetition (4). This repetitive activity between two neurons, through the synapse, strengthens or weakens their connection and thus filters and funnel important signals in the brain via subpopulations of cortical spinal neurons important for motor memory. Biane et al, 2015, however, showed that motor cortex maturation is associated with reductions in recurrent connectivity among functional subpopulations, but increases in intrinsic excitability (5). Essentially, repetitively activated cortical spinal networks eventually lose general connectivity at the expense of increasing intrinsic excitability and efficacy. Intrinsic plasticity involves the increase or decrease of ionic channels in both the pre-synaptic or post-synaptic neurons.

$\text{Ca}^{+2}$ ,  $\text{K}^{+}$ , and voltage-gated channels of neurons become up-regulated or down-regulated, thus altering the excitability of individual neurons. Action potentials either become more prone to occur or less likely to fire depending on if the connection is potentiated or depressed by the synaptic activity between the two neurons (3). The quality of a connection is enhanced through the addition of more neurotransmitter release sites. The synaptic strength of motor neurons is facilitated by short form response enhancement mediated by increase in  $\text{Ca}^{+2}$  at or near release sites (6). An increase in intrinsic excitability allows for a higher quality connection as thresholds for firing action potentials are lessened and pathways between functionally relevant motor neurons in the motor cortex are better accessed.

Previous studies in rats have shown that alterations in neuronal structure occur as a function of experience and learning (7, 8). Structural plasticity involves the formation of dendritic spines and these dendritic spines stem off of neurons, branching out into dense arbors and allowing the neuron to be more susceptible to electrochemical signals from axons of other neurons, resulting in an interconnected cortical network of neurons with differing sensitivities to activation. Moreover, it has been reported that new spines are created and stabilized right after training (9, 10). This increase in synaptic efficacy to other neurons further strengthens pathways relevant to acquiring a motor skill. Wang and colleagues had discovered that, specifically, dendritic spines of layer V cortical spinal neurons within the motor cortex increase in number and density following motor training. They had found that the motor cortex does not undergo a global change

in neuronal structure upon learning, but learning is parceled out to a subset population of neurons that are intermingled with other motor neurons in the motor cortex. Structural refinement was confined to C8 cortical motor neurons in layer V of the motor cortex after training in the skilled forelimb reach-to-grasp task in rats. Experiential representation when learning a motor task is reflected by the increase in dendritic spines and density of specifically C8 cortical motor neurons.

### **Learning and memory**

Learning and memory causes an individual neuron's excitability to change and is part of the process in memory formation. Structural plasticity has been associated with memory formation upon learning a novel task (1). To study memory formation as a function of learning, studies look into motor learning where small rodents learn how to perform a skilled task in order to reach a goal. Motor learning involves exposure to a novel experience, acquisition of a new skill, adaptation to motor movement, and selecting the correct movements to reach a movement goal. It is the process of taking in information from the environment that requires one to initiate and follow up an action with a physical motion that results in a reward or accomplishing an objective. Motor learning allows researchers to gauge quantitatively the gradual process of learning by using motor skills as an output to the function of learning. When forming new memories of the novel task, structural plasticity within the brain develops and strengthens as a result from constant practice. Lesion-induced structural plasticity in the form of dendritic spines branch arborize and increase in synaptic



number when animals learn a new motor skill (11). As previously mentioned, dendrites also demonstrate structural plasticity when their spines increase in density and complexity in a stabilized manner (9). Learning and memory studies utilize motor learning as a model to study the effect of how memory formation is physically represented in the brain, meaning which populations of neurons are growing new dendrites, undergoing up-regulation of ion channels, and synapsing to other neurons that in turn forms a strengthened network. By observing motor learning in rodent models, researchers are able to further explore how memories are ultimately formed for long-term use after a unique skill has been learned (12).

### **Single pellet skilled forelimb reach to grasp task in mice**

Many behavioral tasks exist to assess motor learning. Tasks involving digits of the mouse paw include the Capellini Pasta test, staircase test, and skilled forelimb reach test. In the present study, the skilled forelimb reach to grasp task was selected as the main behavioral test of focus because of its specific dexterous engagement of the digits of the mouse paw which in turn engages the C8 cortical motor neurons in the motor cortex. The Capellini pasta test was omitted from this study as previous studies utilized it for kinematic studies where they observed the amount of times mice manipulate the pasta with their digits (13). Moreover, Capellini pasta pieces were very large and easy objects to grasp. In an effort to ensure skill was involved in this motor learning task, we needed a small and consistently sized object that mice would be able to reach and grasp. The staircase tests were omitted from this study as it did not

represent an ideal skill that we aimed to measure. The skilled forelimb reach to grasp test represented the best test to measure learning. It involves dexterous utilization of the digits, thus fully inducing learning and engaging the digits of mice.

Previous research has shown that differences in gender did not have a significant effect on motor movements in rats (14). Moreover, the same group found that although rats had individual differences in performance, these individual differences between rat subjects were not related to training, movement ability, and cortical anatomy (15). In mice, motor learning via the single pellet reach task was studied in young and adolescent mice, during postnatal weeks 4, 5, 7, 9. At all ages, mice were able to learn the task and improve performance with training. Tennant et al., 2012 corroborated that young (3-5 months old) and aged (18-20 months old) did not significantly differ in their behavioral performance in reaching (16). However, using intracortical microstimulation, they found that young mice underwent drastic changes in movement representations and movement thresholds in the motor cortex, whereas aged mice failed to exhibit any of these changes seen in the motor cortices of young mice. Experience driven topographical reorganization of the motor cortex varies with age and time, with younger mice displaying more forms of plasticity in comparison to aged mice. This narrowed the window of age of mice that we utilized in the current study as we aimed to observe any deficits in learning due to decreased plasticity or lack thereof within the motor cortex.

The motor cortex is organized into six different layers wherein each layer contains diverse populations of neurons where subcortical regions also contribute significantly to motor output. Impulses from cortical motor neurons travel to different segments of the spinal cord to provide movement to particular muscles. For the present experiment, the complex skillset chosen to be acquired by mice involves the use of muscles that control shoulders, arms, and fingers. Previous studies have established that cortical spinal neurons that project to the cervical section of the spinal cord, number eight (C8) are responsible for the active flexion of one's fingers and cortical spinal neurons that project to C4 are responsible for shoulder and arm movement (17). With this information, one can trace axons back to the cell bodies of specific population of neurons within the motor cortex that are responsible for voluntary movement of fingers. This is important for this particular study because the use of digits elicits structural plasticity in the C8 projecting cortical motor neurons in the motor cortex.

We aimed to fully engage the digits of mice while learning a skilled motor task to evaluate the cortical motor neurons that project axons into distinct spinal segments that serve as effectors of forelimb musculature. Wang et al, 2011 corroborated that cortical motor neurons from layer V in the motor cortex project their axons to the cervical 8 section of the spinal cord and innervate the musculature that specifically control the digits of the mouse paw by observing increases in structural plasticity in this population. Moreover, the lower cervical cord contains more motor neurons connecting to distal forelimb muscles which is important for grasping (18). Using this information, the skilled forelimb reach-to-

grasp task was deemed the best assessment for the purpose of observing motor learning of a skilled task.

Wishaw et al. (1995) had previously described and compared the forelimb reach to grasp task in mice and rats (19). They had found that the skilled forelimb reach task was a viable assessment that could be used for rats and mice and that rats consistently performed better than mice. Both species of animals locate the single food source using olfactory senses. Both mice and rats posturize themselves diagonally from the food source contralateral with their reaching forelimb and support their reaching posture with the ipsilateral to reaching hind limb. Mice and rats both undergo three components that describe the reach to grasp skilled forelimb task. The first component is the transport component where the reaching forelimb is raised, brought medially below the head of the animal, aimed, advanced to the food source, and turned 90 degrees over the food source. The second component entails the manipulation of the food source when grasping where the digits fall onto the food source in an arpeggio motion with the most lateral digits grasping the food source. The third component is the withdrawal component in which the paw is withdrawn as it is rotated via the wrist facing medially to the body and then presented to the mouth. Wishaw et al. also showed that the initial velocity of reaching was slower in mice than in rats but the shape of the velocity curve and trajectory between the two species were similar. Their movement profile of mice has laid the foundation that the use of the forelimb reach to grasp task could be applied to the analysis of motor systems of animals suspected of having motor irregularities.

In previous studies, the amount of training allotted to mice has been consistently two weeks. In the present study, we spend an extensive time initially handling the mice in order for the trainer and mice to become better acclimated to each other. The present study allots 21 days prior to the start of training mice, which allows for enough time for the mice to become acclimated to the trainer and enriched environment. This enriched environment is a reaching chamber made of clear Plexiglas with a vertical window in the center of the front. A raised pedestal with an indentation for a sucrose pellet is situated in front of this window where the mice will learn that this is the location of where to retrieve reward pellets. Using food restriction, mice are motivated by hunger to reach and grasp for a single sucrose pellet. The frequency of training, or training intensity, also plays a major role in motor skill rehabilitation after insult to the brain is performed. Depending on whether mice receive training twice a day, once a day or not at all will affect how fast motor performances rehabilitate (20).

The skilled forelimb reach to grasp test is a strong model that is able to provide a quantitative and qualitative assessment of deficits, recovery and compensation. Wishaw et al. 2002 describes five movements in sequence that are similar in both rats and mice: moving digits to midline and aiming, advancing and extending digits, pronation (rotating paw downward), grasp and supination I (gripping and rotating paw medially), supination II and release (rotating paw upward and presenting to the mouth) (21). Their study focused on mice for the purpose of utilizing this forelimb reach task in mouse models with genetic modifications or other insults and injuries to the motor cortex. Using a pial

stripping injury model, they quantitatively and qualitatively studied skilled reaching after a focal motor cortex stroke. Experimental mice that had the focal motor cortex stroke had significant impairments that resulted in a reduced number of successful reaches. Within successful reaches of mice with the motor cortex stroke, it was observed that digits were placed more laterally next to the reward pellet compared to control mice. These mice displayed a rotated movement of their entire body to bring the forelimb to an appropriate aiming position. In the study, stroke mice had advanced and extended their digits diagonally to the food pellet whereas control mice extended and advanced their digits directly over the food pellet. In grasping, stroke mice slapped their paws laterally to the food pellet whereas control mice gasped in an arpeggio movement where the outer digits contract first followed by the medial digits in sequence. In supination I, stroke animals rotated their wrists very little whereas control mice rotated their wrists fully facing the midline. In supination II and release, the stroke mice were able to present the food pellet to the mouth with the help of the non-grasping forepaw.

Using this qualitative and quantitative analysis of the skilled forelimb reach to grasp test, I aim to introduce a newly developed model of eliminating C8 cortical spinal neurons using a one-step delivery method in a non-genetically modified mouse. In studies of traumatic brain injury, the surgical injury model used is a cortical lesion of the motor cortex. Nakagawa et al., 2013 had shown that after mice were trained in single pellet reach to grasp task for 2 weeks prior to the surgical injury, the mice were able to improve recovery of this task (22).

However, the injury model removes a large chunk of the motor cortex (diameter 3.0mm, depth 1.0mm) whereas the injury model I hope to use will only eliminate a specific subset of neurons; the C8 projecting cortical spinal neurons. In Wishaw's 2002 study, an indiscriminate chunk of the motor cortex is removed and this serves as the injury model. In the present study, I aim to eliminate the specific cortical spinal motor neurons responsible for distal forelimb musculature using a one-step delivery system.

### **Topography of the motor map via intracortical microstimulation**

Motor maps are organized arrangements of cells that occupy a specific area in the cerebral cortex. They are comprised of discrete physical movements caused by specific populations of neurons that are responsible for movement of different parts of the body. Motor maps essentially are regions of cells that have been characterized by their particular location and function (4). Two motor maps exist within the motor cortex of mice and have been characterized to be responsible for movement of the forelimb and fingers. Forelimb representation is divided up into the rostral (towards the head) forelimb area and the caudal (towards the tail) forelimb area. Microstimulation within these areas of the cortex revealed that the caudal forelimb area represented the movement of digits (fingers) whereas the rostral forelimb area represented movements of the wrist and elbow (4). The location of neuronal populations that represent digits is important because this illuminates where the population of neurons that innervate the forelimbs and fingers are in the motor cortex (23). Additionally, Tennant and

colleagues showed that reorganization of the motor cortex is dependent on age after unilateral ischemic insult of the sensorimotor cortex (24). After inducing stroke like conditions in both young and aged mice, it was shown that only in young mice did changes in the rostral forelimb area increase whereas no such changes were seen in the aged mice. In order to evaluate the plasticity after learning the single pellet reach task, post brain injury, young mice from p21 to 6 weeks after birth were selected for this study.

### **Retroactive labeling of cortical spinal neurons using fluorogold and cholera toxin B - gold**

Studies involving retroactive tracing of axons from the spinal cord to the motor cortex have determined the specific population and locations of cortical spinal neurons responsible for digit movements. These specific neurons are located in layer five of the motor cortex (1). After learning a new motor behavior, the overall global motor maps do not change, but changes do occur in only these cortical spinal neuron populations located in layer V of the motor cortex. The C-8 projecting neurons from layer V undergo extensive structural plasticity and the learning that an animal does is funneled into this population of cell bodies rather than affecting a global change in neuronal structure of the entire motor map (1). The tracers used to label C8 and C4 cortical spinal neurons were Lumafleur beads (composed of latex microspheres). The signal to noise ratio using these lumafleur beads are close however which makes it difficult to discern C8 cortical spinal neurons from the rest of the intermingled neurons they are nested in. To



overcome this obstacle in the present study, fluorogold was used. Fluorogold undergoes retrograde axonal transport by endocytosis, particularly pinocytosis. It displays less fading than lumafLOUR beads, is non-toxic, extremely stable, and does not leak from axons when transported (25). Fluorogold has an excitation band from 350nm to 395 nm and can remain in retrogradely labeled neurons for several months and still produce signals in a cell with filled somas as well as dendrites (<http://www.fluorochrome.com/FGProtocol.htm>).

Llewellyn-Smith et al, 1990 had also shown that the B subunit of cholera toxin (CTB) conjugated to colloidal gold particles, sizes 7nm and 15nm, can be retrogradely transported in axons via terminals and fill the somas of neurons and persist for weeks (26). It has been shown that CTB-gold can persist in the medulla for up to 42 days and produce a strong signal. This long retention of CTB-gold allows for longer manipulations and studies to be performed and is ideal for the present study. Matsubara and colleagues had discovered that the peptide sequence motif that allows the CTB peptide to bind to GM1 receptors and trigger endocytosis is RxLPxxFxxxRxP (27). In the present study, this CTB peptide is conjugated to golden nanorods, serving as the vehicle for retrograde transport to the cell body. CTB binds to GM1 ganglioside receptors, a component of lipid microdomains, located on non-terminal sides of axons and mediate uptake of CTB-gold through a clathrin independent manner (28). Studies have shown that CTB is retrogradely transported along with p75 neurotrophic factor receptors attached to nerve growth factors and transferrin by an organelle called the signaling endosome. CTB in particular, is transported by GM1 positive

endosomes where ultimately it accumulates in the trans-Golgi network and the endoplasmic reticulum (29). An additional benefit of using CTB-gold retrograde tracers is that their injection sites are small, ranging for .5mm to 1.5mm, allowing for restricted diffusion of the CTB-gold and thus more accurate uptake into targeted axons. Using a 1:5 mixture of fluorogold and nanorod CTB-gold, delivery of the nanorods can occur and visualization by cell filling with the fluorogold can be achieved for histochemical analysis. Using fluorogold to fill the somas of C8 cortical spinal neurons, we hope to observe disfigurement of the organization of somas and dendrites when photothermal energy is passed through the golden nanorods.

### **Plasmon-resonant golden rod nanoparticles**

Surface plasmons are the collective excitations of electrons using light as a resonant frequency. A plasmon is a quantization of oscillations of free electron density within ions in a metal. In an electric field, electrons are both attracted and repelled by other electrons and positive ions within a metal, causing them to oscillate between the positive and negative forces. This oscillation is quantized as plasmonic energy. Gold metal particles of certain size and composition are able to amplify the scattering of energy by these surface plasmons. Plasmon resonance elicits a certain antennae effect where they increase the extinction of through particles so that they are many orders higher than that of molecular chromophores. The smaller the nanoparticle, the less absorption of light occurs because you increase the surface area resulting in more scattered electrons that are necessary to give rise to plasmon resonance. Extending golden nanospheres

into rods allows for longitudinal surface plasmon to occur which shifts the surface plasmon resonance from the visible range to the near infrared range. This is important because the gold rod shaped nanoparticles can be excited between 750nm to 1.3 microns, which is the ideal biological window to observe important proteins and tissues of interest. Golden nanoparticles are multifunctional in the sense that they can be utilized for enhancing fluorescence signals and can have peptides easily conjugated to the surface. One could target cell membrane receptors with functionalized Au nanorods. One such study had conjugated arginine-glycine-aspartic acid peptides to golden half-shell nanoparticles, which served as a targeting moiety for inflammatory cells in rheumatoid arthritis (30). In cancer imaging targeting, folate–oligoethyleneglycol ligands were conjugated to nanorods surfaces, allowing for the imaging and targeting of cells of interest (31). In essence, nanoparticles can be coated with functional ligands that allow it to target and be taken up by specific cellular populations.

Another useful function of nanorod technology is that they can convert 95% of absorbed photon to thermal energy. By exposing the correct wavelength over the nanoparticle, which is dictated by the shape to surface plasmon resonance properties, heat is generated locally around the nanoparticle. In the present study, golden nanorods of size 60nm X 15nm are used and coated with an amino acid sequence of CTB that allows it to bind to GM1 receptors. The size of this nanorod allows it to be excited near infrared at 760 nm where heat is generated locally around the center of the rod. The capability of nanoparticles to generate heat locally has allowed studies to show site-dependent

photothermolysis in tumor cells mediated by folate-conjugated Au nanorods. Tong et al., 2007 had shown that shining a 6mw laser on plasma membrane bound nanorods had produced necrosis within a healthy cell and transformed it into to a cell with severe membrane blebbing. For internalized nanorods, a laser power of 60mw was used to produce the same necrotic effect. Using live cell imaging Tong et al. had observed blebs forming distal and proximal to the membrane bound nanorods. The membrane bound nanorods had mediated an extracellular  $Ca^{+2}$  influx which resulted in the blebbing of the individual cell. Heating was kept localized and induced other biological responses within the cell but did not cause systemic damage to surrounding cells.

### **LED/Low level light therapy**

LED light therapy has become widely used to provide palliative care in the health professions fields. LED light therapy as well as low level laser therapy have been shown to excite the cytochrome oxidase C in the electron transport chain, inducing production of more ATP in mitochondria and resulting in increased levels of ATP thought to be conducive to healing conditions. Light penetration through the skull bone is greater than that of the epidermis when applying light to the brain using red or near infrared wavelengths. Wavelengths of 850nm have been shown to penetrate and become absorbed by cortical neurons twice as well as 660nm, penetrating deeper and better. In the present study, 760nm near infrared laser is chosen to excite the golden nanorods. This wavelength falls within the range of studied wavelengths and will penetrate the

very thin skulls of mice, making the procedure less invasive. Lee et al. had utilized near infrared light at  $1.69 \text{ W/cm}^2$  for 10 minutes and saw therapeutic effects in mice with rheumatoid arthritis in their paws (30). Similarly, we will use near infrared light to excite Au nanorods within the motor cortex with a 760nm laser that has been calculated to penetrate deeper in human skull bone than in human skin. It so follows that penetration within the mouse skull will be deeper due to mouse's inherent thinner skull plates.

### **Conclusions and Experimental Proposal**

With the recent availability of Au nanoparticles and its multifunctional implications, a new paradigm for eliminating specific motor neurons provides the potential to achieve a deeper understanding of specific populations of motor neurons. The use of Au nanorods provides several advantages over analyzing cortical spinal networks in the motor cortex. First, bioimaging of these nanorods can be achieved using darkfield microscopy, allowing visualization of the localization of the nanorods. Secondly, the gold surfaces of the nanorods can be conjugated to a plethora of targeting moieties. The specific moiety used here is a CTB peptides that serves as the vehicle for retrograde transport into neuronal population of interest. Injection into the C8 cervical spinal section allows it to be retrogradely transported to C8 projecting cortical spinal neurons that innervate the musculature of digits. Thirdly, the local photothermolysis can be achieved within the cell by shining light into the skull and inducing surface plasmon resonance that cause local generation of heat. This localized heating can induce

cell necrosis and apoptosis, thus eliminating the C8 projecting cortical spinal neuronal cell without collateral damage to surrounding neurons of different populations. These advantages point to a new single-step tool that can be used for ablating specific populations of neurons rather than a two-step process of viral vectors, which run a variety of risks such as cytotoxicity and it also alters cells on a molecular level.

With the use of the skilled forelimb reach-to-grasp training, one can observe whether functional changes occur after ablation of C8 cortical spinal networks using the Au nanorods conjugated to CTB peptide and injected at the C8 section of the mouse spinal cord. Based on the preceding observations, I hypothesize with the developed paradigm of behavioral training in the skilled forelimb reach task, that C8 projecting cortical spinal neurons in the motor cortex is necessary for acquiring and maintaining the skilled forelimb reach-to-grasp motor skill.

Studies of the motor cortex have shown that C8 projecting cortical spinal neurons undergo increased plasticity by displaying higher densities of dendrites (1). The structural plasticity seen in these cortical spinal neurons suggested that motor learning did not involve a global reorganization of the motor cortex, but reorganization was parceled out to a specific neuronal population. Furthermore connectivity of local immature cortical networks in the rat motor cortex was found to project to the same general spinal segment. However, as the motor cortices of rats developed, connectivity within the population of neurons projecting to the same spinal segment decreased and connectivity of within-population neurons as

well as across-population neurons increased to a similar connection frequency, giving rise to communication between different populations of diverse motor neurons and resulting in fine motor control (5). From these previous experiments, an understanding that diverse populations of neurons exist and play extensive and possibly multiple roles in motor learning. However, much remains to be learned about the importance of individual roles of specific neuronal populations and the importance of their contributions to motor learning. I aim to understand the role of the C-8 projecting cortical spinal neuronal population in the motor cortex in the context of learning a skilled dexterous motor task; the single pellet skilled forelimb reach task. In this endeavor, I aim to expand the pool of knowledge of learning in the adolescent and young adult mouse as well as assess the application of multifunctional gold nanoparticle with photothermal properties as a useful tool for delivery and means of ablation in neuronal cortical networks.

## Materials and Methods

### Animals

All procedures involving animals were carried out in strict adherence to guidelines provided by The Guide for the Care and Use of Laboratory Animals (The Institute of Laboratory Animal Resources, 2011), The Public Health Service Policy on Humane Care and Use of Laboratory Animals (NIH, 1986), The Animal Welfare Act/Regulations and subsequent amendments (PL 89-544), and The Veterans Health Administration Handbook 1200.07 "Use of Animals in Research" (2011); VA San Diego Healthcare System (VASDHS) Research Services Policy 01 section 151-04 (Institutional Animal Care and Use Committee, IACUC) and VASDHS IACUC Policy 03 (Pre and Post-procedural Care of Laboratory Rodents). The animal use protocol was approved by the VASDHS IACUC.

A total of 45 C57BL/6 mice of both genders from Jackson Laboratories (Bar Harbor, ME) were obtained 6 – 8 weeks olds for the first three experiments and the last experiment of 14 male and female mice were obtained at postnatal day 19. Mice were kept on a 12:12 light dark cycle and housed in groups of up to 5 mice per cage. They received standard enrichment nesting material and pieces of cardboard roll. Food restriction was implemented 5 days prior to training animals in the single pellet reaching test. Mice were fed 1.2 grams to 2.0 grams of standard rodent chow daily. Weights were monitored and food intake was adjusted to maintain a weight of  $\geq 85\%$  body weight prior to food restriction.



### **Single Pellet Reaching Test**

Young adult C57BL/6 (wildtype) mice were handled three weeks prior to shaping and training. Handling involved the acclimation of mice to trainer and trainer to mice with 15 minutes of leaving gloved hands in each cage followed up by regular lifting and labeling of tails in the first week of handling. The second week of handling involved the acclimation of mice to the enriched environment of a Plexiglas chamber with a wire mesh for the floor 0.5 cm from the floor so that missed rewards pellets could not be retrieved. The front of the chamber had an adjustable window in the center in which mice were able to reach out to a pedestal with an indentation for single 20 mg Noyes Precision reward pellets (sucrose pellets, Formula F, New Brunswick, NJ). Mice were allowed to explore the chamber with their cage mates for 20 minutes daily and then individually for 10 minutes. Sugar reward pellets were left at the front of the chamber during this week of handling. Mice were food restricted the last week of handling and two days prior to training, the mice were all shaped and conditioned to begin reaching for reward pellets. Mice were allowed to successfully reach and retrieve a pellet a maximum of two times in the first shaping day and a maximum of one time in the last shaping day. During this shaping procedure, preferred forelimb was identified.

Mice were then trained from as little as 5 to 14 consecutive days depending on the experiment. A successful reach was defined as extending a single forelimb through the reaching window such that the entire forepaw was outside the chamber walls. An unsuccessful hit or a miss was defined as the

extension of an entire forepaw outside of the chamber walls without retrieval of the sucrose reward pellet. Mice were initially allotted 30 trials to reach through the center aperture. After this initial experiment, mice were allotted 50 trials to reach through the center aperture in the experiments after. Forelimb reach success rate was measured by the total number of successful reaches divided by the total number of reaches. Each set of 50 reaches was recorded in bins of ten to help keep track of trials.

### **C8 spinal cord nanoparticle and fluorogold injections with 760nm laser exposure**

Wild type C57BL/6 mice were heavily sedated and anesthetized with 1.5% oxygen and 4% isoflurane prior to the C8 spinal injection surgery. After initial knockdown of the animal, 1.5% oxygen with 1.5% isoflurane gas was used to keep the mice sedated in a mouse stereotax. A laminectomy was performed on C7 to cut the dura of the spinal cord and access the spinal cord with pulled pipette glass needles. Each mouse received 0.5 ul of a mixture containing a 1:5 ratio of fluorogold (Denver, Colorado) to CTB-goldrod nanoparticles (Glycodots Inc, San Diego CA). Each lateral side of the spinal cord received one injection site at 0.3 mm lateral of the midline and 0.5 mm depth into the spinal cord. The pulled pipette glass needle was allowed to remain in the spinal cord after each injection for 60 seconds before removing it from the site. The mice were then sutured and given banamine, ampicillin, and ringers for the next three days. Mice

were given three weeks to recover from the surgery and then trained in the single pellet reaching task.

In the penultimate experiment, after mice were injected with the nanoparticles, three weeks of handling proceeded and then mice were trained for 7 consecutive days. After the seventh day of training, all 9 mice had received were sedated as mentioned previously and an incision was made to reveal the skull of the mouse brain. A 760nm, 300mW powered near infrared laser was shone on each hemisphere of the brain for 5 minutes on four of the mice. Each of the randomly selected 4 mice received a total of 10 minutes of 760nm laser exposure. Mice were then trained for three consecutive days afterwards and performance of the skilled forelimb reaching task was observed.

In the last experiment of CTB-nanorods injected mice, 14 mice received incisions of the scalp to reveal the skull of the mouse brain. Seven mice had received the previously mentioned treatment of 760nm laser exposure at 5 minutes for each hemisphere of the region lateral to the bregma before the start of the training period. After 7 days of training using the single pellet reaching task, the 14 mice were sacrificed and analyze via immunohistochemical analysis.

### **Immunohistochemical tissue processing**

After they were trained, the mice were sacrificed; perfused intracardially with 4% paraformaldehyde, and the whole brain tissue was harvested. The whole brain was then sliced at coordinates 2mm anterior and 2 mm posterior to bregma and sectioned using a cryostat and microtome into 35 micron sections. Sections

were then stained with Iba1 antibodies (Wako, cat#019-19741), NeuN (Abcam, cat#AB104224), Cleaved caspase-3 (Cell signal, cat#9661), and Nissl stained.

### **Live cell imaging**

Mice were heavily anesthetized with 0.5 ml of ketamine, xylazine, ace before perfusion with live cell imaging solution (71mM NaCl, 2.5mM KCl, 3.3mM MgSO<sub>4</sub>, 1.2mM NaH<sub>2</sub>PO<sub>4</sub>, 26.2mM NaHCO<sub>3</sub>, 22mM D-glucose, 2mM thiourea, 0.5mM pyruvate, 0.4mM L-ascorbic acid, 72mM sucrose, 10mM choline chloride). The mouse brain sliced 2mm posterior and anterior to bregma and subsequently sectioned into 200 micron sections using a vibratome with 5% CO<sub>2</sub> and 95% O<sub>2</sub>. Live slices were then recorded using a two photon microscope (Leica SP5) for a time period of 50 minutes.

### **Data and statistical analysis**

Statistical significance of the single pellet reaching test was determined using PRISM software. Analysis of variance (ANOVA) were run for all preoperative and postoperative experiments.

## Results

**Longer handling periods and increased number of single pellet skilled reaching trials in C57Bl/6 mice resulted in steeper learning curves representative of motor learning.**

In order to assess the paradigm for the skilled forelimb reach task in the young adult mouse, we first performed an experiment using protocols established by earlier studies in the skilled forelimb reach task. We subjected C57Bl/6 mice to one week of handling where mice were acclimated to the trainer and vice versa. Handling establishes familiarity and comfort between mice participants and trainer as well as acclimating mice to the enriched environment of the Plexiglas reaching chamber. Mice entered a five-day shaping period in which they were conditioned to reach for single rewards pellets prior to the beginning of training. During training, mice were allowed to reach either 50 times to retrieve a 20mg sucrose reward pellet (New Brunswick, New Jersey) or until 15 minutes had passed.

Mice handled for one week and conditioned for another week were subjected to 15 minutes of reaching in the reaching chamber and had undergone motor learning as represented by a 1.4-fold increase in successful reaches after five to seven days of training before mice reaching success reached a plateau as shown in Figure 1. Mice training under this paradigm had resulted in a high initial reaching success rate at 38%. Mice were able to increase their success rate to 55%, a 1.4 fold increase in an already elevated success rate of reaching.

Significant learning occurred but we did not observe an ideal slope of learning that directly correlates to the rapid phase of learning in which neuronal dendrites rapidly form. The rapid learning phase was too shallow and we wanted to see a greater degree of learning.

To assess the required conditions for teasing out a steeper learning curve and lower initial success rate of skilled forelimb reaching, mice were handled for three weeks and the shaping period was reduced from 5 days to 2 days, where mice were allowed to only successfully reach for an award pellet twice in the first shaping day and once in the last shaping day prior to training. Figure 2 displays a cohort of mice,  $n=3$ , receiving 30 trials of reaching per training day for five days. These mice did not undergo significant learning as indicated by deviation from zero of the slope ( $p<.00001$ ). A separate cohort of mice of  $n= 4$  was handled for 3 weeks, shaped for two days, and underwent training for five days and were allotted 50 trials per training day. When allowed 50 trials of reaching, mice underwent significant learning within five days of training ( $p<.0001$ ). There was a 3.5 fold improvement of performance with the initial success rate of day 1 starting out at 10% or lower. Success rate of single pellet reaching was statistically significant between training days 1 and 5, 2 and 5, and 3 and 5.

### **No gender differences in skilled motor learning in mice.**

Gender differences were not seen in previous studies involving behavioral tests with rats. Gender in mice, however, was not explored and is studied here. A cohort of 10 mice (5 females, and 5 males) had underwent three weeks of

handling, two days of shaping and seven days of training where each group attempted a skilled forelimb reach for a maximum 50 trials per training days. Figure 3 shows that there was no statistical significant difference in the slopes of successful reaches representing motor learning between males and females ( $p>0.05$ ). Both groups of male and female mice began with learning at similar initial success rates and plateaued near training day 5, ending with non-significant similar success rates at training day 7. From this study, it was determined that males and females can be used in subsequent experiments.

#### **Exposure of Au nanorods to 760nm laser causes cellular death in layer V motor-cortex.**

In order to assess the induction of cellular death in C8-projecting cortical spinal neurons, we first analyzed the cellular dispersion within the motor cortex using live cell imaging through two photon microscopy. Mice were injected with a 1:5 mixture fluorogold and Au CTB-nanorods in the C8 section of the spinal cord. Fluorogold was used to fill neuronal cell bodies allowing for visualization of neurons and imaged at 405nm. 760nm light was shone on 200micron sections at time zero and was observed for 50 minutes.

Figure 4 show C8 cortical spinal neurons labeled with fluorogold within layer V of the motor cortex underwent a significant decrease in area. At  $t=0$ , a substantial amount of C8 cortical spinal neurons are present in layer V of the motor cortex. At  $t=50$ , a considerable amount of fluorogold labeled C8 cortical

spinal neurons disappear, qualitatively showing dispersion of fluorogold within layer V possibly due to cellular apoptosis and necrosis.

To confirm cellular death of this neuronal population, 35 micron sections of the mouse motor cortex were Nissl stained. Figure 5 shows a comparison between two hemispheres in which once side was exposed to 760nm laser and the other hemisphere was not. The side exposed to the 760nm laser showed a qualitative disruption in the layer V and less pyramidal cells were observed.

**Ablation of C8 cortical spinal neurons does not affect maintenance of performance of skilled forelimb reach to grasp task.**

C8 cortical spinal neurons are responsible for innervating the distal forelimb, particularly the digits of the paw. When rats had undergone skilled forelimb reach training, increased dendritic density was seen within this specific population of neurons, indicating a possibility that C8 cortical spinal neurons are essential for motor learning requiring the use of fine motor digits (Wang et al., 2011). C8 cortical spinal neurons were ablated using Au golden nanorods coated with a CTB peptide that provides the Au nanorods a vehicle for retrograde transport through the nerve endings of C8 projecting cortical spinal neurons. Shining a 760nm through the skull plate of these mice causes the nanorods to generate localized heat and cause the cells they are in to undergo apoptosis. Mice of 6 to 8 weeks of age were injected with fluorogold and Au nanorods, handled, shaped, trained for 7 days until they acquired the skilled forelimb reach to grasp task, and then exposed to the 760nm laser for 5 minutes for each hemisphere of



the brain and trained for three days afterwards. Control groups consisted of n=4 and were not exposed to the 760nm laser but still received surgical incisions of the scalp.

Figure 6 shows that mice underwent significant motor learning represented by the 3.5-fold increase in success rate of reaching for single pellets for 7 days. After mice had plateaued between training day 5 and 7 in their success rates, half of the group was exposed to 760nm laser light at training day 7. After exposure to the 760nm laser, training continued for three more days. No significant changes were observed in the performance of the skilled forelimb reach to grasp task in the experimental group. Performance of this motor skill was not affected by the elimination of C8 cortical spinal neurons.

**Ablation of C8 cortical spinal neurons did not show conclusive results in reducing learning and acquisition of the fine motor skill - forelimb reach to grasp task.**

Previous studies have shown that using viral systems to eliminate cortical spinal neurons in motor cortex reduce the rate of acquisition of a fine motor skill (33). In this experiment, mice of 22 days of age were handled for 2 weeks, shaped for 2 days, and half of the cohort received exposure to 760nm laser the day before training began. Figure 7 shows that the experimental group that was exposed to 760nm did not learn or acquire the skilled forelimb reach task as well as the control group that was not exposed to the 760nm laser. From training day 2 to training day 7, mice that were exposed to 760nm light saw an increase in 3%

success rate from the initial success rate of 15%. In the same time frame, control mice that were not exposed to 760nm light displayed an increase from the initial success rate of 5% to 15%. The non-significant amount and degree of learning experience by the control group made this study inconclusive as we cannot draw any interpretations from this data.

## Discussion

The primary hypothesis of this study was that C8 cortical spinal neurons play an important role in acquiring and maintaining a fine motor skill. In pursuit of this endeavor, the skilled forelimb reach to grasp task was used to assess functional changes as a function of motor learning. Previous studies have shown that acquisition or learning of a motor skill is associated with neuronal correlates. In particular, C8 cortical spinal neurons undergo substantial plasticity with increased dendritic complexity and density (1). Although studies have been performed on synaptic reorganization after motor learning, it was only recently discovered that motor learning is parceled to diverse but specific neuronal populations. Fine motor movement in this case was correlated with C8 projecting cortical spinal neurons. Although it was discovered that C8 cortical spinal neurons underwent increased plasticity after motor learning, their role and significance of their contribution to fine motor movement, such as that of the digits, is still largely unknown. It is here that we investigate the contributions and role that C8 cortical spinal neurons play in motor learning and maintenance. We also had aimed to use a refined paradigm in the skilled forelimb reach to grasp task in order to tease out the rapid learning phase in mice and capture the window of learning in the young adult mouse. By way of means, a recently developed mode of eliminating neurons is explored in this study. Where viral retrograde tracers are used to silence or knock down functions of motor neurons, we use Au multifunctional nanoparticles in the shape of rods that are conjugated

with CTB peptides. The function of Au nanorods we are most interested in is the localized heat generation by transforming photoenergy into heat energy and exciting the Au nanorods to the point of inducing photothermolysis in neurons containing large amounts of these nanorods.

In this investigation, we have found that that handling mice for a longer period of time is critical to obtaining data for the skilled forelimb reach to grasp task. Moreover, in order to better record the rapid phase of learning, the amount of shaping is important. Over-shaping results in an elevated success rate of successful reaches and diminishes the fold increase indicating that learning has already occurred. Two days of shaping and capping successful reaches to a minimal amount allowed us to capture a 5-7 day window in which mice learned and acquired a skilled motor task. It was also found that there were no significant differences in learning between female and male mice. Both genders of mice had learned at the same rate and plateaued at between day 5 and day 7.

As a means to test functional changes in the acquisition of a skilled motor task involving the use of digits, Au nanorods are used to ablate a significant amount of C8 cortical motor neurons. We observe disorganization of layer V of the motor cortex using a Nissl stain and through observing live cell images. As seen in a study by Tong et al., cell death by localized heating was anticipated to occur in cells containing large amounts of Au nanorods. Further studies need to be performed in order to confirm whether this occurrence actually happens. Although we see disorganization of layer V and morphologically disfigured C8 projecting motor neurons we are still uncertain of the proportion of how many C8

motor neurons actually took up the CTB-nanorod conjugates. Igniting these Au nanorods with a 760nm laser showed a loss of signal in the live cell-imaging of the fluorogold filled cells. However, this is not hard evidence that tells us that this was due to cell apoptosis. The dispersion of fluorogold filled C8 motor neurons seen between  $t=0$  and  $t=50\text{min}$  could have very well be due photobleaching or death could have been induced since near UV light was used at 405nm. To address this issue, live cell imaging using differential interference contrast will be performed with appropriate amount of  $\text{Ca}^{+2}$  available in the extracellular fluid as as Tong et al. had shown that blebbing was caused an influx of this. Layer V disruption and the live cell imaging of C8 cortical spinal neurons did show some confidence that Au nanorods were effective in ablating C8 motor neurons, however, a caveat exists in that we are still uncertain whether this conjugate in taken up by C8 projecting motor neurons. These nanorods conjugated to CTB peptides as targeting moieties will need to be further tested if they enter C8 projecting cortical spinal neurons by multiple methods. Such methods are staining fixed sections with a primary antibody that recognizes the CTB peptide in layer five of the motor cortex or taking a western blot of the whole motor cortex and running the blot under the antibody for CTB. In order to visualize if the nanorods were taken up by cells, we used luciferase yellow to get away from using 405nm to visualize live sections at 428nm. However, the nearest laser wavelength available was still 405nm. In our efforts to remedy this, we had conjugated the nanorods to fluorsceine in order to have them visualized at 488nm. However, during live cell imaging the signal was not strong and we

hypothesize that adding fluoresceine to the nanorod may have quenched surface electrons, inhibiting its function to generate localized heat which resulted in no blebbing during live cell imaging using differential interference contrast or brightfield to visualize the live sections. Current plans are in play to visualize the nanorods in live sections using a darkfield microscope. Efforts to confirm whether C8 motor neurons uptake these CTB-nanorod conjugates are being performed.

With these Au nanorods tools, disruption on a cellular level to motor neurons in layer V of the motor cortex was achieved as seen in the Nissl stain. We again go back to the main hypothesis of this study and that is to see if C8 projecting cortical spinal neurons are vital to the acquisition and maintenance of a skilled motor task. We seek to eliminate the population of C8 motor neurons that innervate digits and observe any functional differences. Figure 6 shows us that after exposure to 760nm light, mice that had already learned the skilled forelimb reach to grasp task did not experience a loss of motor memory. With the methods used, no significant deficit to successful reachin were observed between mice exposed to the 760nm laser and mice who were not exposed. A caveat to this observation is that we are unaware of the proportion of C8 projecting corticospinal neurons that were actually ablated. Moreover, compensatory mechanism could have been at play within the population of X8 motor neurons or between other populations that are important in maintaining the cortical network for skilled forelimb reach to grasp task. To address this issue, stereological techniques could be performed to find the average number of fluorogold labeled neurons in a cohort of animals and then counted. Then, CTB-

nanorod conjugates can be injected along with a blanket label of fluorogold, exposed to 760nm, and perform stereological techniques to see how much of the pyramidal layer 5 C8 projecting motor neurons were reduced by to capture the percentage of ablation of this population. What could also be performed are intracortical microstimulation studies after exposing the supposed retrogradely transported nanorods to 760nm wavelength and see if there is an increase in the threshold to fire an action potential to the digits. This would provide evidence that cortical networks responsible for forelimb movement may have decreased due to ablation of C8 cortical spinal neurons. In essence, a functional difference was not seen in maintaining performance of a skilled motor task and more studies need to be done in order to clearly see if they play a defined role in maintaining motor memory of the skilled forelimb reach to grasp task.

Offlesky et al.'s study of viral elimination of motor neurons showed deficits to motor learning when motor neurons involving the digits are negatively altered (32). In our study of acquisition or learning where we shined the 760nm light before skilled forelimb reach training, we could not draw any conclusions as the control group did not learn. We hypothesize that perhaps the surgery may have impaired mouse learning but another possible caveat is that there may have been an issue with housing the mice. This experiment needs to be repeated in order to draw any conclusions from this study. However, if we did perhaps observe that motor learning still occurs after ablation of C8 motor neurons, one possible explanation to this scenario is that young mice utilize multiple and diverse neurons that project to section C8 of the spinal cord and a general

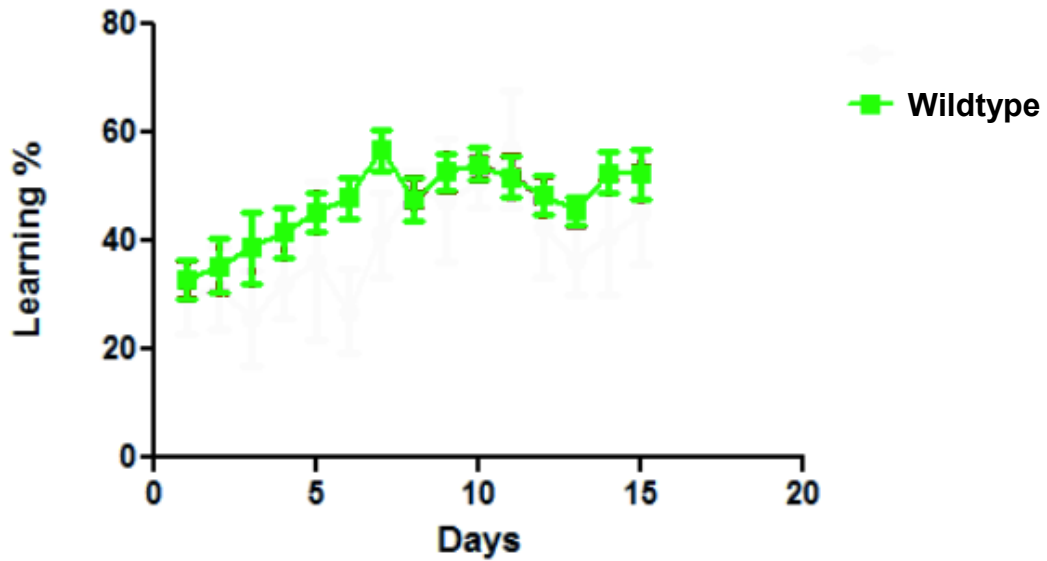
network is utilized in forming connections for fine motor movement. Only after maturation in elderly rodents do independent networks emerge to control fine motor movement (33). Perhaps reduction in general connectivity was present but maybe cortical networks for fine motor movement were not eliminated because the mice were not mature enough to have formed these specific connections between diverse functional neurons so there plasticity may have taken place to strengthen this cortical network (33). Compensation may have occurred after mice received 760nm light exposure prior to training and other motor neurons of the same population may have underwent larger increases in density and complexity to compensate for the cell bodies that potentially underwent apoptosis.

In summary, this study shows that handling and shaping are both critical to ascertaining data using the skilled forelimb reach to grasp task and that Au nanorods show strong potential to be used to induce apoptosis in motor neurons. Input from C8 cortical spinal neurons may be important in their contributions to learning fine motor movement but further studies need to be performed. In future studies, Au nanorods can also be used as a tool to deliver certain cargo to specific populations of motor neurons once we can definitively prove that when conjugated to CTB peptides, they are retrogradely transported. Au nanorods could then be used to cause cellular disruption in other populations of motor neurons that may have parceled roles in motor learning. The contributions and diverse functions of different populations of motor neurons largely remains unknown, but with the use of Au nanorods, targeting specific populations can be

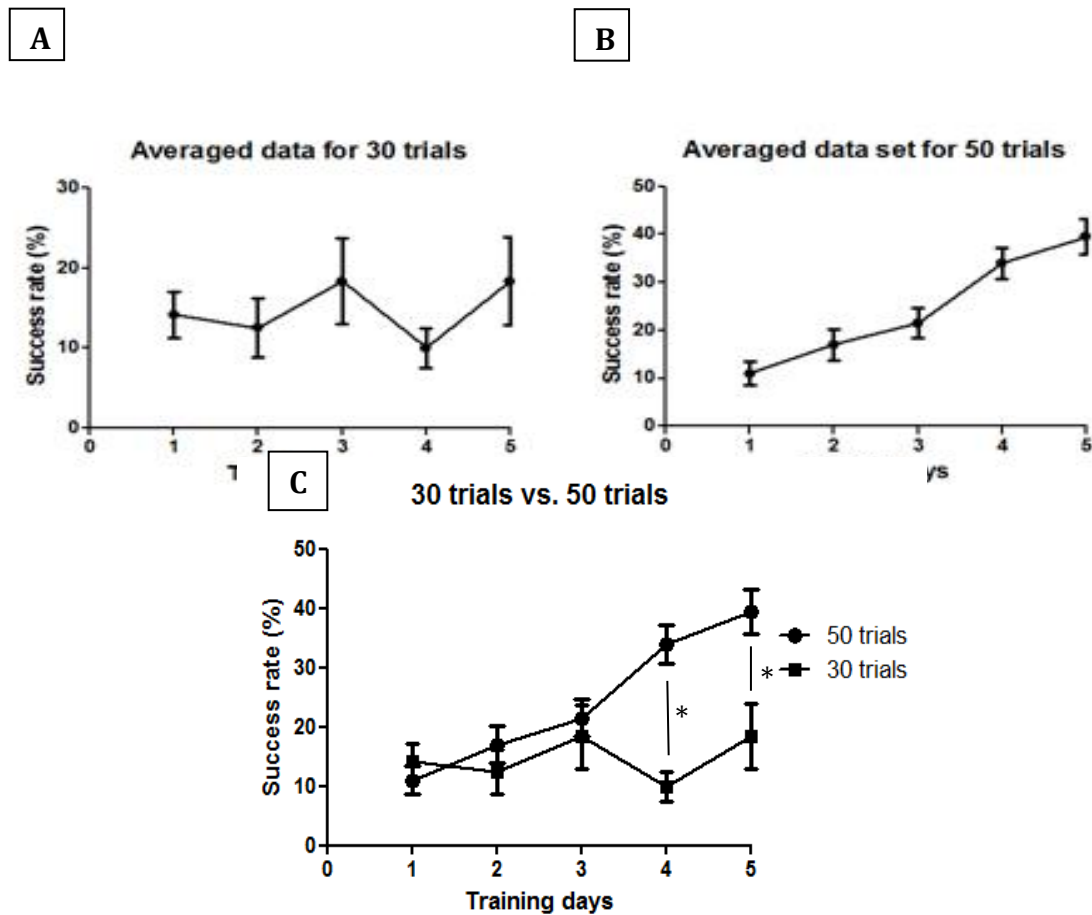


achieved and delivery of many different proteins or biomarkers can be achieved to further explore the cortical networks involved in motor learning.

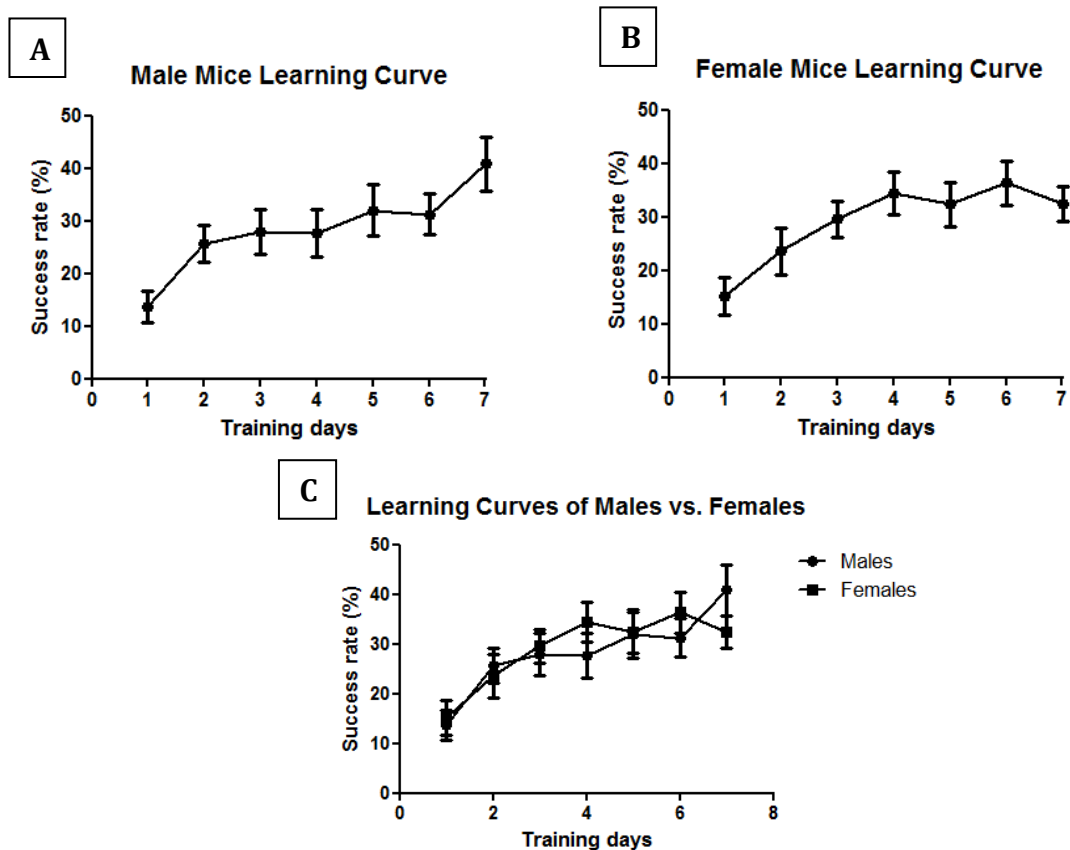
## Figures



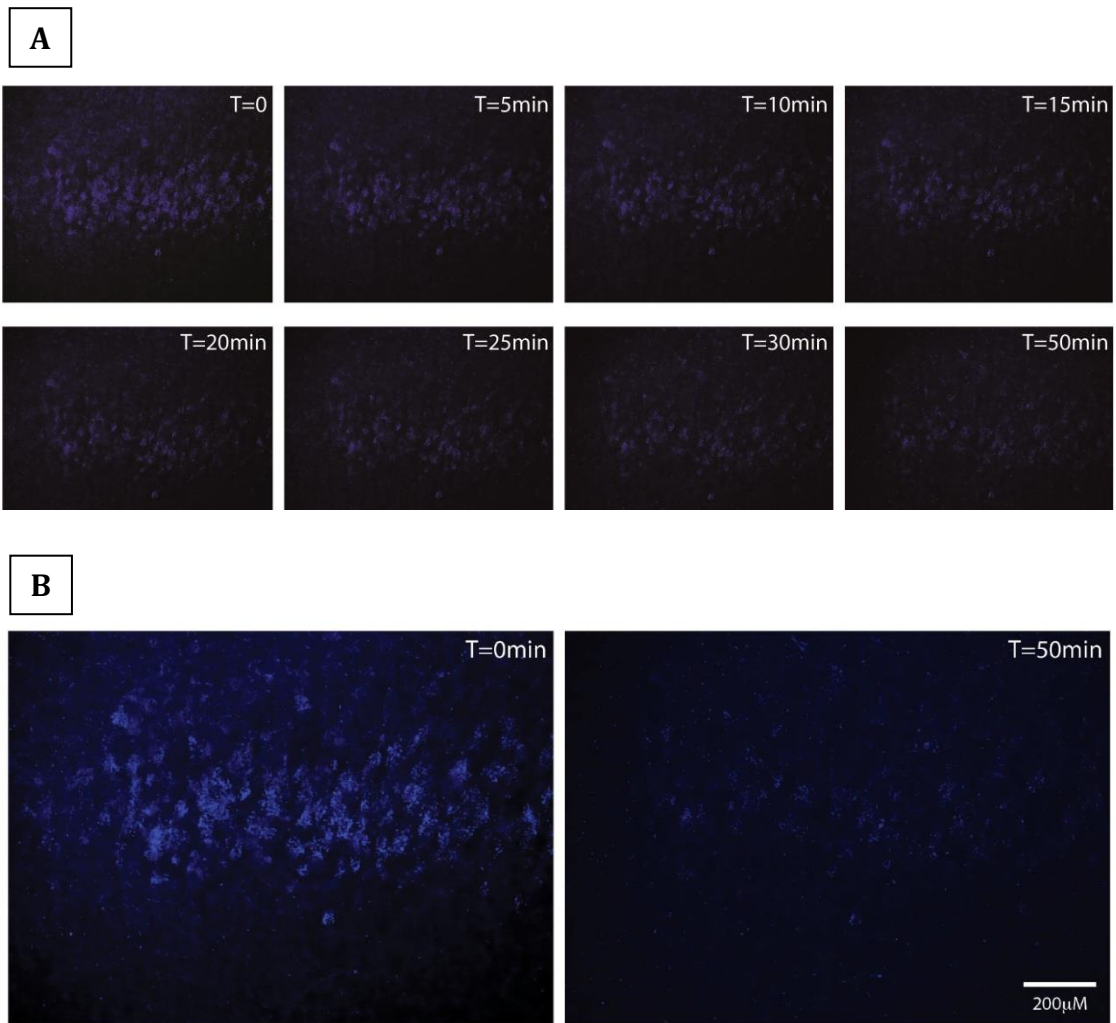
**Figure 1. Skilled forelimb reach to grasp task in under-handled and overshaped C57Bl/6 wildtype mice.** Mice handled for one week prior to training and conditioned to reach in excess of three days reveals a shallow learning curve with an elevated success rate in performance of the skilled forelimb reach to grasp task. A 1.4 fold increase is observed in success rate from the start of training at day 1 to the end at day 7. Mice begin to plateau in reaching successfully between the training day 5 and training day 7.



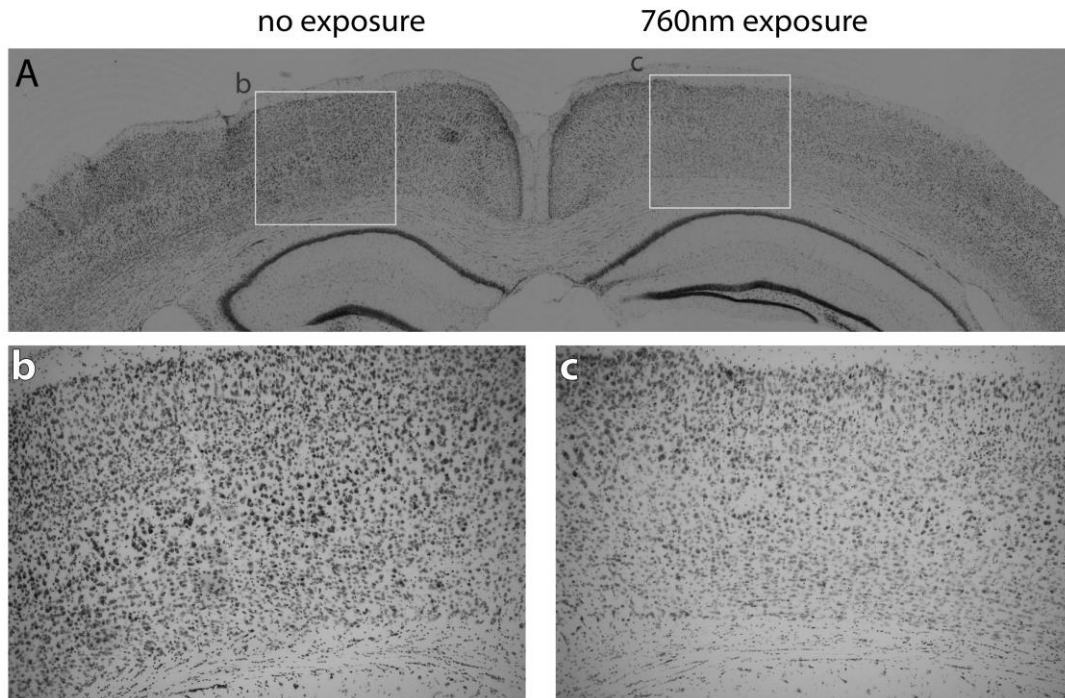
**Figure 2. Motor learning as a function of repeated skilled forelimb reach to grasp task trials.** Mice trained after 3 weeks of handling and 2 days of shaping in the skilled forelimb reach to grasp task given (A) 30 trials vs (B) 50 trials per training day. (C) Overlay of learning curves of mice given 30 trials versus 50 trials (N=3 for 30-trial mice and N=4 for 50 trial mice,  $p < 0.00001$ , one-way ANOVA, repeated measures). \* = significant.



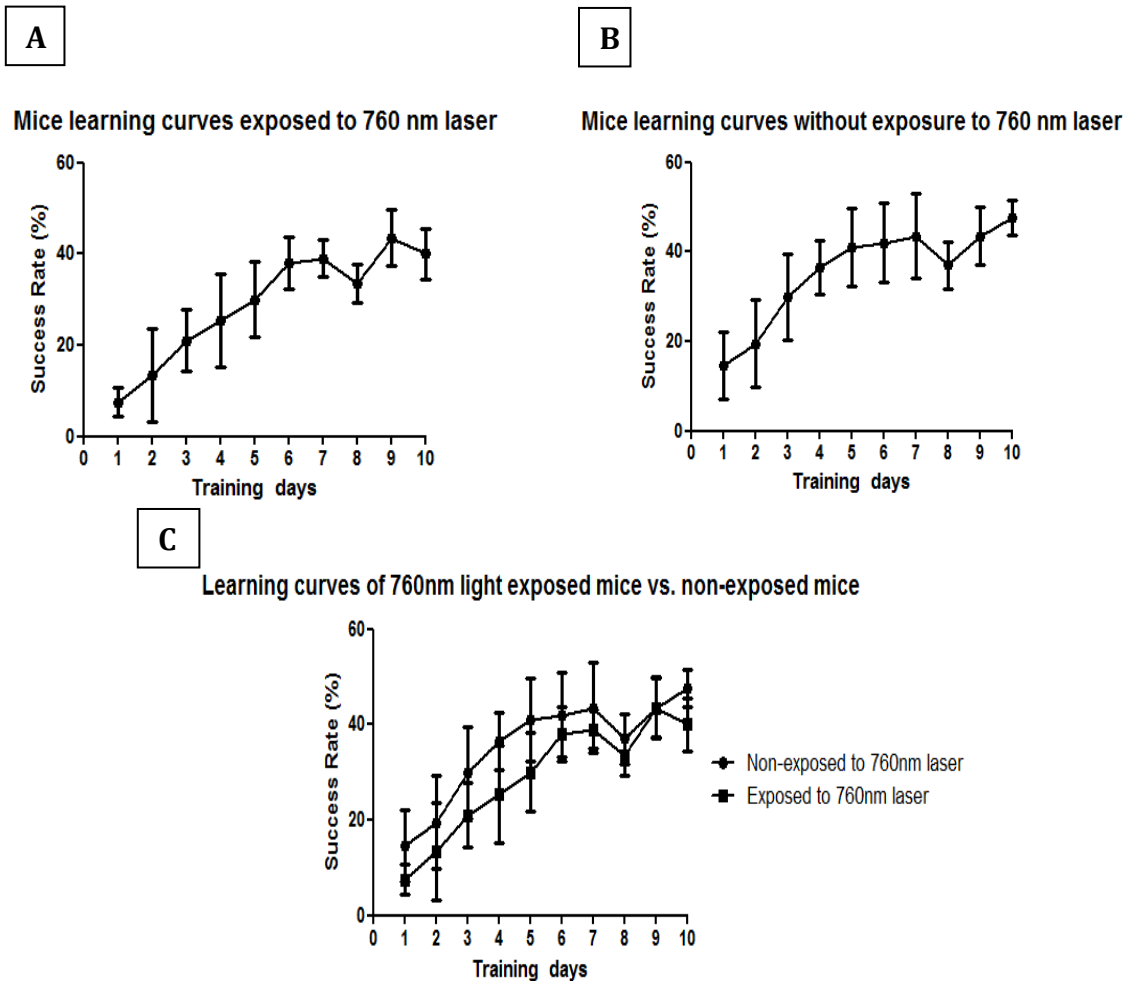
**Figure 3. Mice gender in training in the skilled forelimb reach to grasp task.** Both genders of mice were underwent training in the skilled forelimb reach to grasp task after three weeks of handling, two days of shaping, and five days of training. Learning curves were non-significant between both groups.  $N=4$  for males and  $N=4$  for females ( $p>0.05$ , one way ANOVA, repeated measures).



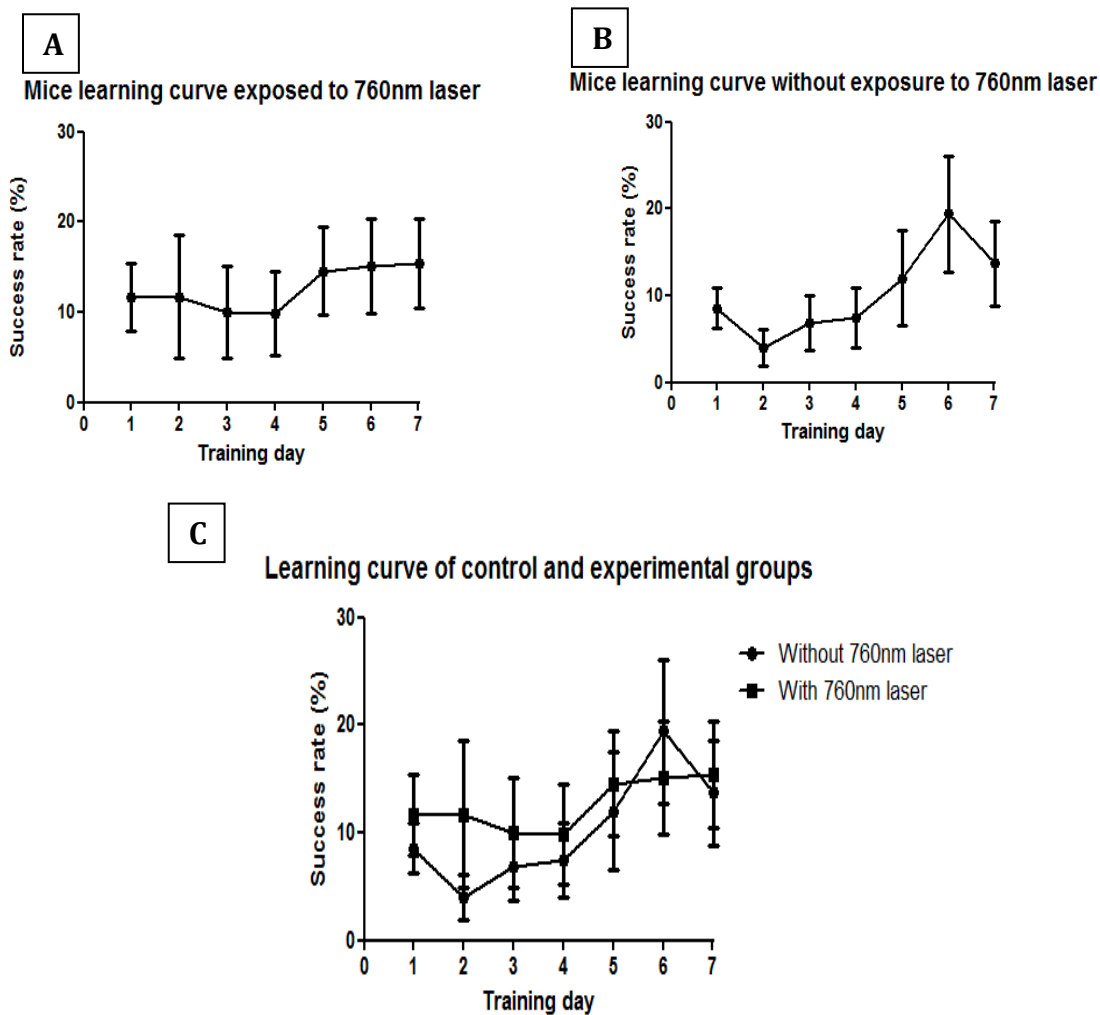
**Figure 4. Time-lapse imaging of cellular dispersion after exposure to 760nm laser.** Live sections of mouse motor cortex were observed after immediate exposure to a 760nm laser. Sections were exposed for 5 minutes to 760nm light and observed for 50 minutes. **(A)** Time-lapse of live cell imaging in increments of 5 minutes for the first seven panels and time at 50 min shown last. **(B)** A comparison of layer V motor neurons at T=0 shows robust fluorogold filled cells versus fewer neurons filled with fluorogold and less intensity at T=50.



**Figure 5. Nissle stain of layer V C8 cortical spinal neurons.** Layer V motor neurons show absence of pyramidal cells characteristic of layer V motor neurons after 760nm laser was shown three days prior to sacrifice. **(A)** Both hemispheres of the section are shown with the boxed areas magnified respectively in **(B)** and **(C)**.



**Figure 6. Performance of skilled forelimb reach to grasp task after exposure to 760nm laser.** Mice underwent training of the skilled forelimb reach to grasp task for 7 days. At training day 7, half of the group was exposed to the 760nm laser and half of the group was not. **(A)** Mice exposed to 760nm light did not experience a significant change in their performance of the skilled forelimb reach task. **(B)** Control mice that were not exposed to 760nm light did not experience a significant change in their performance. **(C)** Learning curves and performance of experimental and control mice overlaid shows no significance between the two groups (N=4 for 760nm non-exposed mice and N=4 for 760nm exposed mice).



**Figure 7. Acquisition of the skilled forelimb reach to grasp task with 760nm exposure prior to training.** Mice injected with Au nanorods at spinal cord section C8 received 760nm laser exposure one day before training. **(A)** Mice exposed to 760nm laser increased their success rate by 4% within 7 days of training. **(B)** Mice that did not receive exposure to 760nm light increased their success rate by 6%. **(C)** Overlay of learning curves between experimental and control mice.



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