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Probing the impact of cholinergic circuits in functional recovery after focal cerebral ischemia

Department of Neuroscience

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PROBING THE IMPACT OF CHOLINERGIC CIRCUITS IN FUNCTIONAL RECOVERY AFTER FOCAL CEREBRAL ISCHEMIA

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Abstract

The current thesis investigated the role of acetylcholine in recovery from diaschisis and skilled hand movement following ischemic stroke in a mouse model of photothrombotic stroke. Mice were trained in skilled reaching task in which they were taught to use their hand to reach for, grasp, and eat a food pellet. They were implanted with a fiber optic in their nucleus basalis, the main source of cholinergic projections to the sensorimotor cortex, for optogenetic stimulation and an electrode in their motor cortex for local field potential recordings. Then they received photothrombotic stroke in their primary forelimb somatosensory cortex. The general findings are that upregulation of acetylcholine reduced diaschisis following photothrombotic stroke to primary forelimb somatosensory area as measured by endpoint scores. However, upregulation of acetylcholine did not improve skilled hand movements as measured by movement component scores and fictive eating analysis.
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Chapter 1

Introduction
1. Introduction

Imagine that you awoke this morning with a pain in your head. You thought it might be a headache, so you carried on your morning routine. But you realize that your walking is slow and rigid. As you step into the shower, you lose your balance and fall into the wall. Your body is numb, and you realize you need help. You drag yourself to your phone, but you realize you cannot use your right hand to reach the phone. You manage to get hold of the phone with your other hand, but when you look at the numbers, they mean nothing. You want to say something, but nothing comes out of your mouth. This scenario sounds scary, but people who experience a stroke experience these symptoms and luckily you were not one of the 40 Canadians who experienced a stroke this morning. The mnemonic FAST is used in detecting and enhancing responsiveness to a person having a stroke. This acronym stands for Facial dropping, Arm weakness, Speech difficulties, and Time to call 911 ("Heart and Stroke Foundation of Canada: Stroke, ").

1.1. Stroke

Stroke is the third leading cause of death in Canada, with 50,000 Canadians suffering from a stroke annually. Most stroke victims survive but suffer lasting cognitive and motor disabilities. More than 405,000 Canadians live with long-term disability from stroke. The number will double in 20 years ("Canadian Partnership for Stroke Recovery,"). Any enhancement of recovery can enhance the quality of life of the stroke victims, and any enhancement in quality of life reduces medical costs for both the survivor and the Canadian Health System.
Stroke occurs when blood stops flowing in a region of brain causing brain cells to die. There are two types of stroke illustrated in Figure 1: Ischemic stroke, and Hemorrhagic stroke (Khaku & Dulebohn, Updated 2017 April 17).

Figure 1: Two different types of stroke. A: Ischemic stroke. In ischemic stroke the blood vessel is occluded and blood supply to the surrounding tissue decreases. B: Hemorrhagic stroke. In hemorrhagic stroke the blood vessel bursts and blood leaks to the surrounding tissue.
1.1.1. Ischemic stroke

Ischemic stroke, which encompasses eighty percent of all strokes, occurs when there is blockage of blood vessels resulting in lack of blood flow to the affected area. Ischemic stroke can be further categorized as thrombotic, embolic, and lacunar. In thrombosis, clots are formed in an artery of the brain; whereas in embolism, the clots are formed anywhere in the body and are carried through the blood flow to the brain. Lacunar strokes are caused by occlusion of small penetrating blood vessels in deep brain structures (Hui & Patti, Updated 2018 May 18).

1.1.2. Hemorrhagic stroke

Hemorrhagic stroke, which accounts for the remaining twenty percent of strokes, results from the rupture of blood vessels leading to leakage of blood in the affected area. Hemorrhagic stroke can be divided into four broad categories illustrated in Figure 2: epidural hematoma, subdural hematoma, subarachnoid hemorrhage, and intraparenchymal hemorrhage. Epidural hematoma is when bleeding occurs between the dura mater and the skull. Subdural hematoma is bleeding between inner layer of the dura mater and the arachnoid mater. Subarachnoid hemorrhage is bleeding between the arachnoid membrane and the pia mater, which is known as the arachnoid space. Intraparenchymal hemorrhage is bleeding into the brain tissue.
1.2. Modeling stroke in rodents

Due to ethical reasons, we cannot induce stroke in humans and manipulate their brains, so we use animal models. There are two methods of inducing stroke in rodents: global stroke, and focal stroke. Global stroke can be induced by occluding two or more of the main arteries that supply blood to the brain. Focal stroke can be induced by middle cerebral artery occlusion and photothrombosis that occludes the distal arteries. In my study, I chose photothrombosis because in this method blood flow in superficial blood vessels gets disrupted and not just in one artery; thus, it creates a selective lesion that is suitable for studying plasticity. Moreover, it is a procedure that can be performed in 40 minutes per animal. In this method, a photosensitive dye named Rose Bengal is injected intraperitoneally. After 5 minutes of diffusion, laser beam is illuminated over the intact skull at the site of the region of interest. Upon the illumination, the platelet aggregation and clot formation interrupt
the blood flow in the illuminated region leading to creation of a focal cerebral ischemic event (Labat-gest & Tomasi, 2013).

Depending on the location and severity of stroke, different impairments can manifest. Motor impairments affect activities of daily living such as reaching for and grasping objects, eating, dressing, etc. For a better understanding of the motor impairments that can arise from stroke, it is helpful to review the sensorimotor area in the next section.

1.3. Sensorimotor area

The sensorimotor area is composed of somatosensory and motor cortex. Because these two regions are connected to each other, the term sensorimotor is used. They involve peripheral sensory inputs, spinal cord, thalamus and cortex in generating movement. First, mechanoreceptors in the outer layer of skin sense fine touch and pressure. Then the dorsal root ganglions carry the sensory information ipsilaterally to the dorsal column of the spinal cord in lower medulla. There, the tracts cross to the contralateral lemniscus and from there on, they ascend contralaterally to the designated thalamic nucleus and to the somatosensory cortex (Figure 3).

Initiation of voluntary sequence of skilled movements is directed via the upper motor neuron pathway. Descending projections from primary motor cortex and premotor areas carry information to the spinal cord to activate lower motor neurons, which directly signals muscles to contract (Figure 4). Among the lower motor neurons, the \( \alpha \) motor neurons synapses via the neuromuscular junction to the extrafusal muscle fibers that generate the force needed for producing movement.
When a α motor neuron generates an action potential, it causes acetylcholine to be released in the space between the presynaptic terminal and the muscle fiber. The neurotransmitter acetylcholine is not only used in the neuromuscular junction but is also used in the autonomic nervous system and in the brain. Next section explains in detail about acetylcholine.
Figure 3: The ascending tracts. Mechanosensory information from the upper body is carried out through the dorsal column medial lemniscus pathway to the thalamus and then somatosensory cortex (Adapted from Purves et al (2004)).
Figure 4: The corticospinal tracts. Information from the motor cortex is carried out through the spinal cord and to the skeletal muscle (Adapted from Purves et al. (2004))
1.4. Acetylcholine

Acetylcholine was the first neurotransmitter discovered in the peripheral nervous system by Otto Loewi in 1921. In his experiment, Loewi placed two frog hearts in two chambers that were connected to each other and were filled with saline. When he electrically stimulated the vagus nerve on one of the hearts, it slowed down in beating, so did the other heart. Loewi concluded that electrical stimulation of the vagus nerve released a chemical into the fluid in that chamber that flowed into the other chamber (Loewi, 1921). When Loewi’s experiment suggested the theory of humoral transmission in the peripheral nervous system holds, it arose the possibility of humoral transmission in the central nervous system. As such since then many scientists have investigated the role of acetylcholine in central nervous system of rodents, mammals, and primates.

The early studies investigate the role of acetylcholine in treatment of epilepsy. McLaughlin (1933) administered acetylcholine bromide subcutaneously in epileptic patients for two weeks. For another two weeks, he gave the patients Pacyl, oral version of choline, two tablets three times a day. He reported that during Pacyl administration, no beneficial effect was observed in any of the patients. During acetylcholine bromide administration, no dramatic reduction in the number and severity of the seizures occurred. In a very similar experiment Lloyd (1933) administered acetylcholine to epileptic patients for two months. He reported that no significant change occurred in the number and the character of the seizures. Given the knowledge of the excitatory property of acetylcholine that we have today, there
was no wonder that acetylcholine had no therapeutic effect on the epileptic patients, since epilepsy is resulted from excessive neuronal activity.

Later studies characterized the action of acetylcholine on the brain. Dikshit (1934) administering acetylcholine into the ventricles of the anaesthetized cat brain while measuring the respiration and he noticed cessation of respiration. Macintosh (1941) obtained cat and dog brain, peripheral nerves and ganglia to measure the amount of acetylcholine in the periphery and different areas in the brain. He treated the animals with eserine, a reversible cholinesterase inhibitor that minimizes enzymatic destruction of acetylcholine, prior to extractions. He reported that acetylcholine is richer in some peripheral trunks and ganglia than in the brain or spinal cord. Among the brain areas, basal ganglia, midbrain, and cerebral cortex were richer in acetylcholine than in cerebellum or corpus callosum. His findings are compatible with known anatomy of cholinergic projections in the brain which includes the basal forebrain and the brainstem cholinergic systems (Woolf, 1991).

For the first time, Woody et al (1978) demonstrated that following acetylcholine application, neurons can undergo long-lasting excitability. They used iontophoresis, delivering a drug using a voltage gradient on skin, of acetylcholine with intracellular depolarization of cortical neurons in awake cats and measured the changes in the membrane resistance. Their findings indicate that sufficiently depolarized neurons that reach a certain threshold can undergo long lasting plastic changes.

When Wiesel and Hubel (1963) first demonstrated the changes that occur in the cat striate cortex following monocular deprivation, Kasamatsu and Pettigrew
proposed that noradrenalin is important for the shift in the ocular dominance columns after monocular occlusion in kittens. They used a catecholamine specific neurotoxin and injected it in the ventricles of the kittens whose eyelids were sutured. Sillito (1983) recognized that facilitatory action of noradrenaline that potentiates the postsynaptic response to a visual input, however he argued that acetylcholine influences both voltage and calcium dependent potassium channels in pyramidal cells that can result in synaptic modification. As such, he suggested that unless the involvement of acetylcholine or any other substance with similar actions is investigated, the role of noradrenaline in relation to visual cortical plasticity is open to question.

Bear and Singer (1986) examined the role of cholinergic system in the plasticity of the striate cortex for three reasons. First of all, biochemical and anatomical evidence suggests that the striate cortex receives dense cholinergic innervations. Second, facilitatory action of acetylcholine on excitatory transmission in striate cortex increases the probability of postsynaptic activation. Third, cholinergic projections are an important component of the ascending reticular activating system, which is necessary for ocular dominance plasticity. They showed that combined depletion of noradrenalin and acetylcholine is sufficient to halt synaptic modifications in the striate cortex, whereas basal forebrain or noradrenergic bundle lesions alone are ineffective (Bear & Singer, 1986).

Later on Rasmussen and Dykes (1988) reported increased responsiveness of somatosensory cortex to peripheral stimuli when paired with basal forebrain stimulation. They recorded cortical evoked potentials from somatosensory cortex of
anaesthetized cats while delivering electrical stimuli to the skin and basal forebrain. They discuss that acetylcholine release in the cortex could produce the long-term changes for the following reasons: one is that the stimulus sites in the basal forebrain contain cholinergic neurons that project to the somatosensory cortex. Second, application of atropine, muscarinic acetylcholine receptor antagonist, reduced the size of evoked potentials in somatosensory cortex when basal forebrain was stimulated which supports the involvement of acetylcholine.

A year later, Kilgard and Merzenich (1998) demonstrated that the primary auditory cortex of adult rats undergoes reorganization when electrical stimulation of the nucleus basalis is paired with auditory stimulus. They implanted electrodes in the nucleus basalis of the rats. After recovery, they played pure tones paired with nucleus basalis stimulation for twenty days. They anaesthetized the rats twenty-four hours after the last session of tone play and stimulation and mapped the primary auditory cortex using microelectrodes. The maps showed remodelling of the primary auditory cortex such that the region of auditory cortex that represented the frequency tone paired with nucleus basalis stimulation had a huge expansion.

Conner et al (2005) reported that cholinergic system in the basal forebrain is necessary for brain reorganization after lesioning the motor cortex of the rat. They pharmacologically lesioned the nucleus basalis after training rats in the single-pellet reaching task, and then induced lesions in the motor cortex by application of electric shock. Rats whose basal forebrain was lesioned had a lower rate of recovery after five weeks compared to the control rats. Functional mapping of the motor cortex using intracortical microstimulation also showed reorganization of motor
representation such that the vibrissae areas expanded into the caudal forelimb area. Behavioural and cortical mapping results suggest that basal forebrain cholinergic mechanisms play a critical role in process of functional recovery following motor cortex injury (Conner et al., 2005).

Looking at the anatomy and function of the basal forebrain cholinergic projections to the cortex, we can better understand why acetylcholine is proposed to be the candidate neuromodulator for brain plasticity after brain injury.

**1.5. Basal forebrain cholinergic system**

In primates and rodents, the major cholinergic inputs to the cortex and subcortical structures are provided by the nuclei in the basal forebrain (Ballinger, Ananth, Talmage, & Role, 2016). These nuclei in the basal forebrain have been divided into four groups: Medial septal nucleus cholinergic neurons (Ch1), vertical limb of the diagonal band (Ch2), horizontal limb of the diagonal band (Ch3), and nucleus basalis cholinergic neurons and substantia innominata (Ch4) (Obermayer, Verhoog, Luchicchi, & Mansvelder, 2017). Among these nuclei, nucleus basalis and substantia innominata send cholinergic projections to the cortex (Woolf, 1991). The cortical innervation by the basal forebrain cholinergic system is topographically organized in both primates and rodents, which suggests that the functional control of the cortical processes by the basal forebrain cholinergic system is very specific. For instance in Macaque, the anteromedial part of Ch4 innervates the medial cortical areas, such as the cingulate cortex; the anterolateral part of Ch4 innervates the frontoparietal cortex, such as insular cortex; and the intermediate part of Ch4 innervates laterodorsal frontoparietal, peristriate, and midtemporal regions, such as
temporal areas (Mesulam, Mufson, Levey, & Wainer, 1983). Moreover, retrograde tracing experiments in mice have shown that Basal forebrain projections to sensory cortices are discrete and selective such that neurons in the horizontal diagonal band project to the primary visual cortex, neurons in the anterior part of nucleus basalis project to the primary somatosensory areas, and neurons in the posterior part of the nucleus basalis project to the primary auditory cortex (Kim et al., 2016).

1.6. Theory

1.6.1. Diaschisis

The Diaschisis theory proposes that following brain injury the portion of the brain that is connected to the injured region but is distant from it undergoes a sudden change or loss of function. The injured brain region disrupts the function of the intact structures and causes physiological imbalance. Von Monakow coined the term diaschisis. He observed patients with neurological impairments manifested initially with symptoms that were not the same as later ones (Feeney & Baron, 1986). Among the early electrophysiological experiments that supports diaschisis theory was the work of Kolb and Whishaw (1977) on rats that showed following many kinds of cortical lesions, not only the cortical EEG is altered, but also theta rhythms in the hippocampus are affected, and that both cortical and hippocampal activities go back to normal after variable period of time.

1.6.2. Excitation/ inhibition as an explanation of diaschisis

Excitation/Inhibition balance means that cortex functions optimally when the neuronal excitatory and inhibitory inputs are balanced. With regard to stroke, not only excitation/inhibition balance is disrupted in the peri-infarct region, but also
excitation/inhibition is affected in distant brain regions that are connected to the infarct and peri infarct regions.

The term excitation/inhibition was first suggested theoretically (Shadlen & Newsome, 1994) and later experimentally in vitro (Shu, Hasenstaub, & McCormick, 2003) and then in vivo (Haider, Duque, Hasenstaub, & McCormick, 2006). This explanation is implied in a variety of neurological disorders such as epilepsy (Koyama et al., 1990), autism (Rubenstein & Merzenich, 2003), schizophrenia (Kehrer, Maziashvili, Dugladze, & Gloveli, 2008), and Alzheimer’s Disease (Rissman & Mobley, 2011).

Following ischemic stroke, two zones of injury occur: infarct region or the ischemic core, and peri-infarct region or penumbra area, which surrounds the infarct region. The peri-infarct region has the capability for reorganization and plastic changes; however, due to the excitation/inhibition imbalance, the peri-infarct region is inhibited so that the injury does not expand to further neighbouring regions. As such, the peri-infarct region is inhibited and presumably the neurons that synapse with the injured region undergo diaschisis.

1.7. Rationale

Following somatosensory forelimb ischemia, increased inhibition in the peri-infarct region on one hand prevents the chance for cortical reorganization, and on the other hand silences temporarily the cholinergic neurons in the basal forebrain that synapse with the cortical pyramidal neurons in the peri-infarct area. As such, optical stimulation of cholinergic neurons in the nucleus basalis could be used to restore tone and to overcome and normalize behaviour.
1.8. Hypotheses

Hypothesis 1: If photothrombotic damage to the primary forelimb somatosensory area interrupts the haptic sensation in the forelimb, then the mouse should be impaired in distinguishing successful grasps from misses in skilled reaching. That is, every time the mouse attempts to reach and grasps the food, it will bring the hand to the mouth for eating regardless of grasping or missing the food item (Experiment 1).

Hypothesis 2: According to the diachisis, after photothrombotic stroke in somatosensory forelimb area, not only will the cholinergic nuclei in the nucleus basalis experience diachisis, but cholinergic tone will decrease in the peri-infarct region. Thus, laser-induced upregulation of acetylcholine in the primary forelimb somatosensory after ischemic stroke in mice will reduce diachisis and improve skilled reaching (Experiment 2).
Chapter 2

Experiment 1
2. Experiment 1

2.1. Introduction to experiment 1

In order to ensure and characterize how photothrombotic stroke affects skilled forelimb movement in reaching task, photothrombotic stroke was induced in the somatosensory forelimb area of the mice to assess the deficits they exhibit three days and ten days after stroke in skilled reaching task.

2.2. Methods

2.2.1. Animals

Twelve adult male C57/BL6 mice (3-4 months of age), weighing 20-30 g, raised at the Canadian Centre for Behavioral Neuroscience vivarium at the University of Lethbridge, were used. The animals were housed in quads since being weaned under a 12h:12h light/dark cycle with light starting at 7:30am and temperature set at 22 °C. All testing and training were performed during the light phase of the cycle at the same time each day. The baseline weight of the mice prior to food restriction was recorded, and the mice were placed on food restriction 3 days prior to the beginning of training. The weight of the animals was monitored daily throughout the experiment to maintain body weight at 85% of baseline weight with additional food given in their home cage within two hours after completion of the training/testing. Two days before the stroke induction, animals were taken off of food restriction and were put back on food restriction two days after stroke induction. The animals received water ad libitum. All procedures were approved by
the University of Lethbridge Animal Care Committee in accordance with the guidelines of Canadian Council on Animal Care.

### 2.2.2. Surgical procedure

To induce photothrombotic stroke in the mice, animals were anesthetized with isoflurane (1-2%), and surgery was conducted using aseptic methods. The mice were placed in a stereotactic frame (Kopf Instruments) on a 37-38 °C heating pad. The animal's eyes were covered with a thin layer of lubricating ointment (Refresh, Alergan Inc.). Using a scalpel blade, an incision along the midline of the eye level down to the neck was made and the skull was exposed. To facilitate photoactivation, a round circle with 1.0mm in diameter on the skull was thinned to ~50% of its original thickness. The center of the circular region was AP=0.5mm and ML=2.5mm from the Bregma. Photothrombotic stroke was induced by injecting 10 mg/ml of Rose Bengal intraperitoneally 5 minutes prior to 20 minutes green laser (532nm wavelength at power 10mW) illumination at the thinned region. Then the skin of the scalp was closed using surgical suture. The control mice went through the same procedure except that they received intraperitoneal saline injection rather than Rose Bengal. After the surgery, the mice were kept in the recovery room for two days before being returned to the home cage and food restriction.

### 2.2.3. Video recording

Mice were filmed from a frontal view with a Panasonic HDC-SDT750 camera at 60 frames per second at an exposure rate of 1ms. Illumination for filming was obtained by using a two-arm cold light source (Nicon Inc.), with the arms positioned
to illuminate the reaching target area from a frontolateral location on each side of
the reaching apparatus.

2.2.4. Reaching apparatus, food reward, and skilled reaching task

The reaching box was made of clear plexiglass (20 cm long, 9 cm wide, and 20
cm high) with a slit (1 cm wide) located in the center of the front well (Farr &
Whishaw, 2002; Whishaw, 1996). On the outside of the front wall, a shelf 3 cm wide
was mounted 1 cm above the floor. Two divots were located on the shelf, one
centered on the edge of each side of the slit at a distance of 1 cm from the slit. Food
pellets were placed in the divots. At this location, it is difficult for the mice to obtain
food with their tongue, but food can readily be obtained with the contralateral hand
because mice pronate their hands with a lateral-to-medial movement that brings the
palmar surface of the hand over the divot.

Food items which the mice reached for were 14-mg food pellet (Bioserve Inc.,
Frenchtown, NJ).

Animals were habituated to the reaching apparatus by placing them in the
box for 10 minutes for ten days. Pellets were initially available on the reaching box
floor and within tongue distance on the shelf. Pellets were gradually removed from
the floor and placed further away on the shelf until the mice had to use their hands
to retrieve the food pellet. The pellets were placed in the right divot, so that the mice
were required to reach to obtain them with the left hand. After the mice learnt to
reach for food pellet from the divot, they received only twenty pellets in each
training session. Training was considered complete once each mouse’s success rates
reached asymptotic level on three consecutive days of training. On each
training/testing day, animals in each cage were placed in a clear plastic container containing sawdust bedding for transport to the testing room and then placed in the test apparatus. All mice were filmed in the reaching task two days prior the surgery day, three days and ten days after stroke induction, to assess the deficits the mice may exhibit during those timepoints.

2.2.5. **Behavioural scoring and analysis**

In each filming days, endpoint, component, and fictive eating measures were evaluated. The endpoint analysis includes total number of reaches, anticipates, misses, and success percentage. The component analysis includes reaching movement scores described previously (Farr & Whishaw, 2002). The fictive eating measure includes scoring of anticipate reaches, and misses (Whishaw et al., 2018).

1. **Anticipate reach.** An anticipate reach was any movement of the reaching hand that appeared directed to the divot in absence of the food pellet.

2. **Miss.** A miss was any movement of the reaching hand that appeared directed to the food pellet that did not result in purchasing or that knocked the food away.

The endpoint and component measures were subjected to Analysis of Variance (ANOVA) with repeated measures, and the fictive eating scores were subjected to Kruskal-Wallis H test using computer program SPSS (v.24.0.0.1). A p-value of <0.05 was considered significant.

2.2.6. **Histology**

On day 11th post-stroke, the mice were anesthetized and perfused through the heart with 1x phosphate buffered saline followed by 4% paraformaldehyde in 1x phosphate buffered saline. Brains were removed and fixed in 4% paraformaldehyde
overnight and then cryoprotected in 30% sucrose in 1x phosphate buffered saline and 0.02% sodium azide until the brains sank to the bottom of the jar. The brains were sectioned (40 μm) on Blockface. Every third brain section was mounted on Superfrost Plus Micro Slide (VWR), stained with Cresyl violet, and cover slipped using Permount (Fisher-Scientific).

2.3. Results

2.3.1. Histology

All brains had damage in primary motor cortex, primary somatosensory forelimb and hindlimb areas. Error! Reference source not found. represents coronal views of the stroke site. All strokes encompassed damage to cortical layers I to VI, just above the external capsule.
Figure 5: Representative Cresyl violet stained brain sections of photothrombotic stroke at three coronal levels. The intact left hemisphere indicates brain regions present (M2 – secondary motor cortex, M1 – primary motor cortex, S1 – primary somatosensory area, S1Upper limb – primary somatosensory forelimb area, S1 Mouth – primary somatosensory mouth area, S1 Nose – primary somatosensory nose area, S1Lower limb – primary somatosensory hindlimb area, S1Trunk – primary somatosensory Trunk area, S1Barrel field – primary somatosensory barrel area).

2.3.2. End point reaching analysis

Figure 6 A-B illustrate the average number of total reaches and spontaneous reaches made by mice in the two groups on pre-stroke day, three days post-stroke,
and ten days post-stroke chronic days. The number of reaches and spontaneous reaches by the Sham group and Stroke group were similar on all test days, although they varied across days. These results were confirmed by a repeated measure ANOVA on the average number of total reaches that gave no significant effect of Group, $F(1,10) = 0.088, p>0.05$, significant effect of Days, $F(1,10) = 9.672, p<0.05$, and no significant interaction of Day by Group, $F(1,10) = 0.093, p>0.05$. Similarly a repeated measure ANOVA on the average number of spontaneous reaches gave no significant effect of Group, $F(1,10) = 0.057, p>0.05$, a significant effect of Days, $F(1,10) = 9.339, p<0.05$, and no significant interaction of Day by Group, $F(1,10) = 0.004, p>0.05$.

Figure 6 C illustrates the average reaching success of the mice in the two groups on pre-stroke, post-stroke acute, and post-stroke chronic days. A repeated measure ANOVA on the average reaching success revealed no significant effect of Group, $F(1,10) = 4.908, p>0.05$, a significant effect of Days, $F(1,10) = 18.760, p<0.05$, and no significant interaction of Day by Group, $F(1,10) = 0.594, p>0.05$.

Figure 6 D illustrates the average number of misses made by the mice in the two groups on pre-stroke, post-stroke acute, and post-stroke chronic days. A repeated measure ANOVA on the average number of misses revealed no significant effect of Group, $F(1,10) = 4.789, p>0.05$, a significant effect of Days, $F(1,10) = 5.721, p<0.05$, and no significant interaction of Day by Group, $F(1,10) = 1.098, p>0.05$. 

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2.3.3. Component analysis

Figure 7 illustrates the mean score obtained for movement components sniff, lift, elbow in, advance, pronation, grasp, supination I, supination II, release, and replace by mice in the two groups on pre-stroke day, three days post-stroke, and ten
days post-stroke. Pre-stroke scores include scores from the mice in the Sham group before the sham surgery and the Stroke group before receiving stroke. A repeated measure ANOVA on the average movement scores revealed a significant main effect of Group, $F(1,10) = 93.175$, $p<0.05$, a significant main effect of Movement, $F(1,10) = 30.023$, $p<0.05$, and a significant interaction of Group by Movement, $F(1,10) = 11.996$, $p<0.05$. Follow-up pairwise comparisons indicated significant Group differences between pre-stroke day and three days post-stroke in Advance, supination I, supination II, release, and replace, and significant Group differences between pre-stroke day and ten days post-stroke in pronation, grasp, supination I, supination II, release, and replace.
Figure 7: Mean scores of the ten movement components of mice grasping 14mg food pellets for Sham and Stroke Groups on pre-stroke day, three days post-stroke (Day3), and ten days post-stroke (Day10). A score of 0 indicates normal movement, whereas a score of 1 indicates impairment. Scores on Day3 and Day10 post-stroke are compared with pre-stroke day. (*** p<0.001, ** p<0.01, * p<0.05)

2.3.4. Fictive eating analysis

Figure 8 and Figure 9 illustrate reach and hand withdrawal of the mice in anticipate and miss conditions before and three days after stroke. Before stroke in anticipate condition (Figure 8A), where there is no food and the mouse reaches to the approximate food location, the mouse advances the hand and since there is no
food in the divot for a hand contact, the mouse drags its paw back through the opening slot, whereas post-stroke (Figure 8B) after advancing to the approximate location of food, the mouse closes digits as if grasping the pellet (Grasp), supinates its hand as it withdraws it through the slot (Retract Closed), and sniffs the hand as if checking whether the pellet is in the hand (Hand to Mouth). The same behaviour was seen pre and post stroke in the miss condition, when the mouse misses or knocks away a present food pellet (Figure 9).

**Anticipate**

![Anticipate](image)

*Figure 8: Anticipate reach and hand withdrawal. A: Pre-stroke day. After reaching, the mouse does not close its digits and withdraws the hand without supinating. Once the hand gets to the end of opening slot, the mouse does not check the hand for food. B: Day3 post-stroke. After reaching, the mouse closes its digits and withdraws the hand while supinating. Once the hand gets to the end of opening slot, the mouse sniffs the hand and checks it for food*
Figure 9: Reach and hand withdrawal during a miss. A: Pre-stroke day. After reaching, the mouse does not close its digits and withdraws the hand without supinating. Once the hand gets to the end of opening, the mouse does not check the hand for food. B: Day 3 post-stroke. After reaching, the mouse closes its digits and withdraws the hand while supinating. Once the hand gets to the end of opening, the mouse sniffs the hand and checks it for food.

A Kruskal-Wallis H test showed that there was a statistically significant difference in Grasp and Retract Closed three days post-stroke ($\chi^2(2) = 17.104, p < 0.001; (\chi^2(2) = 18.326, p < 0.001)$ and ten days post-stroke ($\chi^2(2) = 15.751, p < 0.001; (\chi^2(2) = 15.520, p < 0.001)$, and Hand to Mouth three days post-stroke ($\chi^2(2) = 17.048, p < 0.001$) for Anticipate condition. In the Miss condition there was a statistically significant difference in Grasp, Retract Closed, and Hand to Mouth three days post-stroke ($\chi^2(2) = 14.323, p < 0.01; (\chi^2(2) = 16.984, p < 0.001; (\chi^2(2) = $
14.494, \( p < 0.01 \) and ten days post-stroke \( (\chi^2(2) = 13.386, \ p < 0.01; \ (\chi^2(2) = 15.038, \ p < 0.01; \ (\chi^2(2) = 12.537, \ p < 0.01). \)

Figure 10: Occurrence of movement components (Mean ± S.E.) seen in anticipate and miss conditions three days and ten days post-stroke in pre-stroke, stroke, and sham mice. Note that Grasp, Retract Closed, and Hand to Mouth behaviours were seen significantly higher in stroke group.

2.4. Summary

Photothrombotic stroke in primary somatosensory forelimb area impaired the mouse in reaching. The impairment was detectable via three different measures: endpoint measures, movement component measures, and fictive eating measures.
The endpoint measure includes success percentage of the mice. Three days post-stroke, stroke and sham mice did not have significantly different success percentage. In fact, the sham group showed a significantly lower success percentage three days after the sham surgery. An explanation for this result may be that mice were taken off of food restriction before the surgeries and were put back on food restriction on day two post-stroke. When taken off of food restriction, the mice gain weight and they continue gaining more weight during recovery period. By putting them back on food restriction, mice are not motivated to do the task. Ten days post-stroke the stroke mice had significantly lower success percentage than the sham group. In fact, the stroke group had quite the same success percentage on day three post-stroke, and that the sham group had regained their pre-stroke success percentage.

The movement components measure includes individual movements in reaching, grasping, and withdrawal movements in a single reach. Three days post-stroke, mice were significantly impaired compared to the sham mice in advancing their hand, supination I, which is a ninety degree rotation of the hand immediately after grasping the food item, supination II which is further rotation of the hand, releasing food to the mouth, and replacing their hand back on the floor. Ten days post-stroke, the mice were significantly impaired compared to the sham mice in pronation, which is positioning the digits over the food item in an arpeggio manner, grasping the food item, supination I, supination II, release, and replace.

Fictive eating measure include three behaviours; Grasp, Retract Closed, and Hand to Mouth. Stroke mice exhibited these three behaviours when reached with no
food present and when they missed the present food, while sham mice did not exhibit these three behaviours.
3. Experiment 2

3.1. Introduction to experiment 2

In the second experiment, photothrombotic stroke was induced in the primary forelimb somatosensory area following with daily optical stimulation of nucleus basalis to assess the effect of increased acetylcholine in stroke recovery/compensation.

3.2. Methods

3.2.1. Animals

Twenty three adult Chat-CreAi32 mice (3-4 months of age), weighing 20-30 g, raised at the Canadian Centre for Behavioral Neuroscience vivarium at the University of Lethbridge, were used. The animals were housed in quads since being weaned under a 12h:12h light/dark cycle with light starting at 7:30am and temperature set at 22 °C. All testing and training were performed during the light phase of the cycle at the same time each day. The baseline weight of the mice prior to food restriction was recorded, and mice were placed on food restriction 3 days prior to the beginning of training. The animals’ weight was monitored daily throughout the experiment to maintain the body weight at 85% of baseline weight with additional food given in the home cage within two hours after completion of the training/testing. All animals remained under food restriction until the end of the study. The animals received water ad libitum. All procedures were approved by the University of Lethbridge Animal Care Committee in accordance with the guidelines of Canadian Council on Animal Care.
3.2.2. Experimental procedure

All mice were food restricted and trained in skilled reaching. Once they reached asymptotic level of performance, they were implanted with an optrode in nucleus basalis and an electrode in the cortex. Once they recovered, they were habituated to the laser cable. Cortical LFP recording with stimulation was performed under isoflurane anesthesia for all mice to confirm cortical activation upon stimulation of nucleus basalis. Then they were randomly assigned in one of the three following groups: Stim group (n = 9), that received daily optical stimulation post stroke, No Stim Group (n = 8 mouse), that did not receive optical stimulation post stroke, and Sham Group (n = 6), that did not receive stroke neither optical stimulation post sham surgery. Animals in the Stim and No Stim Group received photothrombotic stroke and animals in the Sham Group had a sham surgery. After 4 days of recovery, all animals were filmed in the skilled reaching task. From Day 4 to Day 14 post-stroke, the animals in the Stim group received five one-minute 25-ms square pulses at 20Hz laser stimulation with three minutes of rest in between daily (Figure 11B). The animals in the No Stim and Sham Groups were brought to the stimulation room and were attached to the laser cable for the same duration as the Stim Group but did not receive laser stimulation. All mice were filmed on Day 7, 10, and 14 post-stroke. From day 14 to 28 mice were kept in their home cages. On day 28 post stroke, all mice were filmed in the reaching task. On day 29 post stroke all mice were perfused and brains were removed for slicing and Nissl staining. All mice were kept on food restriction from the beginning of the study until the day of perfusion.
Figure 11: Experimental and stimulation paradigm. A: Experimental paradigm. B: Stimulation paradigm. Mice in the Stimulation group received daily laser stimulation for 5 minutes with 4 minutes of interstimulus rest intervals from Day 4 till Day 7 post-stroke. The stimulation was a square pulse at 20Hz.

3.2.3. Fiber optic ferrule and electrode fabrication

The ferrules were prepared in house following guidance from Thor Labs (Manual FN96A, 2012). Fiber optic cores (Part # FT400UMT, Thor Labs) were initially cut to 16 mm lengths, inserted into ceramic ferrules (Part # CF440-10, Thor Labs) and cemented in place with epoxy (Part # T120-023-C2, Thor Labs). After allowing the epoxy to set for 24 hours, the “bare” end was cut to approximately 5.0 mm in length with a diamond cutter (Part # S90R, Thor Labs). Any ferrules with
improperly cut fiber optic cores were discarded. This “bare” end would be eventually inserted into the brain to a depth of ~4.5 mm. The other end of the fiber optic core was trimmed and polished until flush with the ceramic ferrule and showed an appearance of “polished glass” under a dissecting microscope at 10x power. Before implantation, the ferrules were tested for light transmittance with a light meter (Part # PM100D, Thor Labs) by attaching the polished end to a fiber optic cable of 473 nm. All ferrules implanted in nucleus basalis had an averaged measured output of ~5.5 mW (SD = 0.14 mW, N = 23 implants in 23 mice).

For recording the cortical LFP above the stroke area, a Teflon-coated stainless-steel wire (bare diameter 50.8 µm) was soldered to a golden pin (Part # 520200, A-M Systems). The end was cut to approximately 1.5 mm in length with a razor blade, and implanted into the cortex to a depth of ~200 µm.

Prior to the implantation, the fiber optic ferrules and electrode pins were disinfected with 70% isopropyl alcohol and allowed to air dry.

3.2.4. Surgical procedures

3.2.4.1. Optrode and electrode implant

Animals were anesthetized with isoflurane (1-2%), and surgery was conducted using aseptic methods. The mice were placed in a stereotactic frame (Kopf Instruments) on a 37-38 °C heating pad. The animal’s eyes were covered with a thin layer of lubricating ointment (Refresh, Alergan Inc.). Using scissors, a flap of skin about 1 cm² in area was retracted from the skull and the gelatinous periostium was removed with a small scissors. The skull was cleaned and dried with sterile cotton swab. Sites of optrode (AP = -0.2 mm, ML = +1.35 mm, DV = 4.0 mm) and
electrode (AP = 2.0 mm, ML = 1.0 mm, DV = 0.2 mm) implants were marked with a sharpie (Figure 12A) and then drilled. The optrode was lowered down with stereotaxic cannula holder (Part # XCF, Thor Labs) installed on one arm of the stereotaxic. The electrode was placed by hand. Optrode and the electrode were quickly glued on the skull using Krazy glue. A custom made headplate was directly affixed with a thin layer of C&B Metabond (Parkell) such that it covered the whole skull and the surrounding area of the optrode and electrode. A golden pin was glued on the surface of the skull on the cerebellum to serve as a reference for LFP recording. Then the skull and the head-plate were secured with first a thin layer of Metabond (C&B Metabond) and then with a layer of dental cement except the stroke area (Figure 12B).
Figure 12: The optrode implant, electrode implant, and stroke site. A: Top down view of the mouse skull under the first surgery. The scalp and gelatinous periostium is removed and the skull is cleaned with cotton swabs. Bregma, stroke site, electrode and optrode implant sites are marked with a black permanent sharpie. B: Implanted mice post-surgery. Each mouse was implanted with an optrode in the nucleus basalis, an electrode in the cortex, and a golden pin as a reference pin for local field potential recording.

3.2.4.2.  Photothrombotic stroke induction

Animals were anesthetized with isoflurane (1-2%) on a 37-38 °C heating pad, and the animal’s head-plate was secured with plastic forks on a custom-made surgical plate. The animal’s eyes were covered with a thin layer of lubricating ointment (Refresh, Alergan Inc.). To facilitate photoactivation, a round circle with 1.0mm in diameter on the skull was thinned to ~50% of its original thickness. The center of the circular region was AP=0.5mm and ML=2.5mm from the Bregma.
Photothrombotic stroke was induced by injecting 10 mg/ml of Rose Bengal intraperitoneally 5 minutes prior to 20 minutes green laser (532nm wavelength at power 10mW) illumination at the thinned region. The thinned area was covered with dental cement. The control mice went through the same procedure except that they received intraperitoneal saline injection rather than Rose Bengal. After the surgery, Mice were kept in the recovery room for two days before being returned to their home cage.

### 3.2.5. Video recording

Post-surgery one, two of the mice were filmed from a Birdseye view with a Panasonic HDC-SDT750 camera at 60 frames per second for the purpose of behavioural observation. Each mouse was filmed in two conditions: with and without their nest in the top left corner of the tub. In each condition, the mice were placed individually in a plastic transfer tub (12 inches long, 7 inches wide, and 5 inches high) with sawdust bedding while having laser cable attached to the optrodes for 10 to 15 minutes. They received 25ms square pulses at 20Hz laser stimulation every time they went to the top left corner of the tub as depicted in Figure 13.

Post-stroke, mice were filmed from a frontal view with a Panasonic HDC-SDT750 camera at 60 frames per second at an exposure rate of 1ms. Illumination for filming was obtained by using a two-arm cold light source (Nicon Inc.), with the arms positioned to illuminate the reaching target area from a frontolateral location on each side of the reaching apparatus.
Figure 13: Freely behaving mouse stimulation at the top left corner of the tub. A: Stimulation is delivered in the absence of nest at the top left corner. B: Stimulation is delivered when the nest is present at the top left corner.

3.2.6. Reaching apparatus, food reward, and skilled reaching task

The reaching box was made of clear plexiglass (19.5 cm long, 8 cm wide, and 20 cm high) with a slit (1 cm wide) located in the center of the front well (Farr &
Whishaw, 2002; Whishaw, 1996). On the outside of the front wall, a shelf 3.8 cm wide was mounted 1 cm above the floor. Two divots were located on the shelf, one centered on the edge of each side of the slit at a distance of 1 cm from the slit. Food items that the mice reached for were 10-mg food pellets (Catalogue # 1811213, TestDiet) placed in the divots. At this location, it is difficult for the mice to obtain food with their tongue, but food can readily be obtained with the contralateral hand because mice pronate their hands with a lateral-to-medial movement that brings the palmar surface of the hand over the divot.

For filming in the dark with stimulation, a gate was added to the reaching apparatus (Figure 14) that could be opened 90° and closed with a manual button via a servomotor (Part # MG995R, Digi-Key).

Animals were habituated to the reaching apparatus by placing them in the box for 10 minutes for ten days. Pellets were initially available on the reaching box floor and within tongue distance on the shelf. Pellets were gradually removed from the floor and placed further away on the shelf until the mice were forced to use their hands to retrieve the food pellet. The pellets were placed in the right divot, so that the mice were required to reach to obtain them with the left hand. After the mice learned to reach for food pellet from the divot, they received twenty pellets in each training/testing session. Training was considered complete once each mouse’s success rates reached asymptotic level on three consecutive days of training. On each training/testing day, animals in each cage were placed in a clear plastic container containing sawdust bedding for transport to the testing room and then placed in the test apparatus. Post recovery form surgery one, mice were habituated
to the laser cable by placing them in the reaching apparatus for 10 minutes for five days.
Figure 14: The single pellet reaching apparatus with a gate that opens and closes with a servo motor. Here the gate is open.
3.2.7. Local field potential recording

A week after optrode and electrode implants, cortical local field potential recordings of all mice were obtained. First, mice were anesthetized with isoflurane (0.6-0.8%) on a 37-38 °C heating pad. The animal’s headplate was secured with plastic forks on a custom-made plate. The animal’s eyes were covered with a thin layer of lubricating ointment (Refresh, Alergan Inc.). Each cortical field potential recording session consisted of 20 trials with each trial lasting 15 seconds. Each trial was composed of 2 seconds of optical stimulation, 25-ms square pulses at 20 Hz, delivered to the nucleus basalis via the implanted optrode and 13 seconds of inter-stimulus interval. The local field potential signal was amplified (x1000) and filtered (0.1-10,000 Hz) using a Grass P5 Series AC amplifier (Grass Instrument Co.) and was sampled at 2 kHz using a data acquisition system (Axon Instruments).

3.2.8. Statistical analysis

The behavioural results were subjected to Analysis of Variance (ANOVA) with repeated measures using computer program SPSS (v.24.0.0.1). A p-value of <0.05 was considered significant.

For local field potential analysis, spectrogram of each trial was calculated on the 3-sec long sliding window using custom written program in MATLAB (MathWorks). These spectrograms were averaged across the trials and divided by the baseline calculated from 6 seconds before the optogenetic stimulation.

For confirming the stroke size for each mouse, four of the coronal sections located at 0.5mm to 0.2mm from the Bregma which is the center of stroke were chosen and the area of stroke region was measured using the freehand tool in
NDP.view2 software. The average of stroke areas of the four sections were calculated for each mouse, and the mean area of stroke region for the Stim and No Stim groups was subjected to an independent t-test.

### 3.2.9. Histology

On Day 29th post-stroke, all mice were anesthetized and perfused through the heart with 1x phosphate buffered saline followed by 4% paraformaldehyde in 1x phosphate buffered saline. Brains were removed and post-fixed in 4% paraformaldehyde overnight and then cryoprotected in 30% sucrose in 1x phosphate buffered saline and 0.02% sodium azide until the brains sank to the bottom of the jar. The brains were sectioned (40μm) on Blockface. Every third brain section was mounted on Superfrost Plus Micro Slide (VWR), stained with Cresyl violet (nissl staining), and cover slipped using Permount (Fisher-Scientific). All the slides were imaged with Nanozoomer (2.0RS, Hamamatsu).

### 3.3. Results

#### 3.3.1. Histology

The location of fiber optic implant and stroke site was confirmed after brain sectioning and staining with Cresyl violet for all mice as shown in Figure 15. All stroke sites encompassed damage to the primary somatosensory forelimb area within cortical layers I to VI, above the external capsule. There was no statistically significant difference in the area of stroke for both Stim and No stim groups as confirmed by independent t-test, t(15) = .582, p > 0.05 (Figure 16).
Figure 15: Representative Cresyl violet stained coronal brain sections of photo thrombotic stroke and optrode implant t sites on the right hemisphere. A: Stroke site includes the primary somatosensory forelimb area. B: Optrode implant site in the nucleus basalis.
3.3.2. Endpoint analysis

Figure 17 illustrates the average reaching success of the mice in the three groups of Stim, No Stim, and Sham groups. A repeated measure ANOVA on the average reaching success on Days 4, 7, 10, 14, and 28 post-stroke revealed no
significant effect of Day, $F(4,80) = 1.327, p>0.05$, no significant effect of Group, $F(2,20) = 2.592, p>0.05$, but significant interaction of Day by Group, $F(8,160) = 5.418, p<0.001$.

**Figure 17**: Total success percentage (Mean ± standard error) for Stim, No Stim, and Sham Groups on Pre-stroke day (0), Day4 Post-stroke (4), Day7 Post-stroke (7), Day10 Post-stroke (10), Day14 Post-stroke (14), and Day28 Post-stroke (28). *$p<0.05$, **$p<0.01$, ***$p<0.001$ indicates a significant difference between the Stim and Sham Groups. #$p<0.05$, ##$p<0.01$, ###$p<0.001$ indicates a significant difference between the No Stim and Sham Groups. $p<0.05$, $$p<0.01$, $$$p<0.001$ indicates a significant difference between the Stim and No Stim Groups.
3.3.3. Component analysis

Figure 18 illustrates the averages mean scores obtained for movement components hindfeet, front feet, sniff, lift, elbow in, advance, pronation, grasp, supination I, supination II, release, and replace by mice in Stim, No Stim, and Sham groups on post-stroke days 4, 7, 10, 14, and 28. A repeated measure ANOVA on the average movement scores revealed a significant main effect of Group, $F(2,20) = 9.995$, $p<0.01$, significant main effect of Days, $F(4,80) = 10.253$, $p<0.001$, and no significant interaction of Group by Days, $F(8,160) = 1.005$, $p>0.05$. Post Hoc comparisons using the Tukey HSD test indicated significant Group differences between Stim and No Stim groups, $p=0.020$, and Sham and No Stim groups, $p=0.001$, but not Stim and Sham groups, $p=0.223$.

The twelve movement components were divided into three general categories of posture, advance, and withdraw as illustrated in Figure 19. Posture includes hindfeet, front feet, and sniff. Advance includes lift, elbow in, advance, pronation, and grasp. Withdraw includes supination I, supination II, release, and replace. A repeated measure ANOVA on the averaged movement categories revealed a significant main effect of Group, $F(2,20) = 10.766$, $p<0.01$, significant main effect of Days, $F(4,80) = 10.367$, $p<0.001$, significant main effect of Category, $F(2,40) = 24.095$, $p<0.001$, and significant interaction of Day by Category, $F(8,160) = 2.598$, $p<0.05$. Post Hoc comparisons using the Tukey HSD test indicated significant Group differences between Stim and No Stim groups, $p=0.016$, and Sham and No Stim groups, $p=0.001$, but not Stim and Sham groups, $p=0.190$. 
Figure 18: Averages mean scores (Mean ± S.E.M) of the twelve movement components of mice grasping 10mg food pellets for Stim, No Stim, and Sham Groups on post-stroke days 4, 7, 10, 14, and 28. A score of 0 indicates normal movement, whereas a score of 1 indicates impairment. (**p<0.01, *p<0.05)
Figure 19: Averaged mean score (Mean ± S.E.M) of the twelve movement components categorized as posture, advance, and withdraw for Stim, No Stim, and Sham Groups on post-stroke days 4, 7, 10, 14, and 28. A score of 0 indicates normal movement, whereas a score of 1 indicates impairment. (**p<0.01, * p<0.05)
3.3.4. Fictive eating analysis

Figure 20 illustrates the percentages of times the three behaviours of Grasp, Retract closed, and Hand to mouth is occurred in Stim, No Stim, and Sham groups on post-stroke days 4, 7, 10, 14, and 28 in a Miss condition, when the food is present and the mouse reaches but misses grasping it. A repeated measure ANOVA confirmed significant effect of Behaviour, $F(2,40) = 82.938$, $p<0.001$, significant effect of Group, $F(2,20) = 14.235$, $p<0.001$, significant interaction of Behaviour by Group, $F(4,80) = 5.171$, $p<0.001$, and significant interaction of Day by Behaviour, $F(8,160) = 8.717$, $p<0.001$. Post Hoc comparisons using the Tukey HSD test indicated significant Group differences between Stim and Sham groups, $p=0.001$, and No Stim and Sham groups, $p=0.002$, but not Stim and No Stim groups, $p=1.000$. 
Figure 20: Occurrence (Mean ± S.E.) of three behaviours Grasp, Retract Closed, and Hand to Mouth for Stim, No Stim, and Sham Groups on post-stroke days 4, 7, 10, 14, and 28 in Miss condition. (** p<0.01, * p<0.05)
3.3.5. Local field potential recording

In order to confirm the effect of nucleus basalis optical stimulation on the upregulation of acetylcholine in the cortex, local field potential from the cortex were recorded while delivering the laser pulses to the nucleus basalis (Figure 21A-B). Spectral analysis of cortical field potentials showed that nucleus basalis optical stimulation desynchronized the cortical local field potentials (Figure 21C). This desynchronization which is manifested in the reduction of cortical slow wave activity is due to the increase of acetylcholine level in the cortex.

![Optogenetic stimulation](image)

**Figure 21**: Changes in local field potential recording by acetylcholine release. A: Stimulation trace. B: Three examples of LFP recordings from M2. Area. Shaded blue area indicates illumination of nucleus basalis with 25-ms light pulses at 20Hz. C: Normalized spectrogram indicates changes in power at each frequency averaged for twenty trials.

3.3.6. Free behaving mouse stimulation

To determine whether the nucleus basalis stimulation is aversive, favorable, or neutral, two mice were filmed while freely moving in a tub and stimulated at a certain position (top left corner) with and without their nest presented in that
location. In both conditions, both mice exhibited similar behaviour in terms of exploring the tub, rearing, grooming, and nest searching within the 10 to 15 minutes of placement in the tub. In the absence of the nest, when the mice received stimulation at the top left corner, they did not stay in that corner more than three seconds. Also, after 8 or 9 minutes of being in the tub, the mice picked a corner other than the top left corner and sat in there for more than a minute. In the presence of the nest on the top left corner of the top, the mice more frequently went to that corner, and even stayed in the nest for more than three seconds. After 10 minutes of filming, both mice started exploring underneath the nest when the filming was stopped.

**3.4. Summary**

In this experiment, optical stimulation of nucleus basalis was used post stroke to investigate whether upregulation of acetylcholine close to the stroke area helps recovery/compensation by assessing the behavior of mice in skilled reaching task. The mice received the laser stimulation from day 4 to day 14 post stroke, and they were tested in skilled reaching task on days 4, 7, 10, 14, and 28 post-stroke.

The endpoint measure, which includes the success percentage of the mice, shows that on day 4 post stroke the mice that received stroke had a significant decrease in their success percentage compared to the sham mice as expected. After receiving three days of stimulation, the success percentage of the Stim group significantly increased compared to the sham and No Stim groups, such that the sham and Stim groups had very similar success percentage on day 7 post stroke.
The movement component analysis consists of ten components of the reaching, grasping, and withdrawal movements in a single reach. These components were grouped in three categories: posture, advance, and withdraw. Posture included orientation of hindfeet, front feet, and sniffing the food item. On day 4 post-stroke the posture of the mice that received stroke were significantly impaired compared to the sham mice. The posture score of mice in the stim and No Stim groups were similar to the Sham’s, but on day 28 post-stroke the posture of Stim group was significantly improved compared to the No Stim group. Advance included lifting of the hand, bringing elbow in, advancing towards the food, pronating over the food, and grasping the food item. On day 4 post-stroke the advance of the mice that received stroke were significantly impaired compared to the sham mice. On day 14 and 28 post-stroke, the advance of the Stim group significantly improved compared to the No Stim group. Withdraw included supination of hand to a ninety degree angle after grasping the food, further supination of the hand while withdrawing the hand through the slot, releasing the food to the mouth, and replacing the hand on the floor. Post-stroke, all mice had similar scores for the withdraw category except on day 10 and day 28 in which the No Stim group was significantly impaired than the Stim and Sham groups.

Since in this experiment, the mice did not have enough anticipatory reaches, reaching when no food is present, only misses were considered for fictive eating analysis. On day 4 and 7 post-stroke, the mice that received stroke exhibited Grasp, Retract closed, and Hand to mouth behaviors, while the sham mice did not show those behaviors. On day 10, 14, and 28 the all the mice exhibited Grasp, and Retract
closed behaviours, but only the mice that received stroke exhibited Hand to mouth behavior.
Chapter 4

Discussion
4. Discussion

This thesis investigated the effect of upregulated acetylcholine post-stroke in skilled reaching task in mice. In doing so, two experiments were performed. The first experiment measured the behavioural deficits in skilled reaching task that occurred three and ten days after induction of photothrombotic stroke in primary forelimb somatosensory area. The second experiment measured the behavioural outcome after photothrombotic stroke induction in primary forelimb somatosensory area in mice in skilled reaching task following optogenetic upregulation of acetylcholine adjacent to the stroke area.

The purpose of experiment one was to assess behavioural deficits post-stroke in skilled reaching. For that, sixteen mice were trained in skilled reaching task. When they reached asymptotic level of success, seven mice received photothrombotic stroke targeting primary forelimb somatosensory area and the remaining five mice received a sham surgery. Brain histology of the stroke mice revealed that the extent of stroke encompassed primary forelimb and hindlimb somatosensory areas and primary motor cortex. Three and ten days after stroke, mice were filmed in skilled reaching. The reaching videos were subjected to three types of analysis: endpoint analysis, movement component analysis, and fictive eating analysis. Endpoint analysis included the total number of reaches, anticipate, misses, and success percentage. The mice in both sham and stroke groups had the same number of total reaches (anticipate, miss, success) and anticipate reaches in both three and ten days post-stroke. The mice had no significant difference in success percentage and number of misses three days post-stroke. However, they significantly differed in.
success percentage and number of misses ten days post-stroke. Movement component analysis included ten components of the reaching, grasping, and withdrawal movements in a single reach. Three days post-stroke mice were significantly impaired compared to the sham mice in advancing their hand, supination one which is a ninety degree rotation of the hand immediately after grasping the food item, supination two which is further rotation of the hand, releasing food to the mouth, and replacing their hand back on the floor. Ten days post-stroke, the mice were significantly impaired compared to the sham mice in pronation which is positioning the digits over the food item in an arpeggio manner, grasping the food item, supination one, supination two, release, and replace. Fictive eating analysis included three behaviours of Grasp, Retract Closed, and Hand to Mouth. Stroke mice exhibited these three behaviours when reached with no food present and when missed the present food, while sham mice did not exhibit these three behaviours.

The first hypothesis that mice will be impaired in distinguishing successful grasps from misses in skilled reaching task following primary forelimb somatosensory ischemic stroke was supported by experiment one. Mice that received stroke exhibited fictive eating behaviours such as grasp, retract closed, and hand to mouth in anticipate reaches and misses, whereas sham mice showed normal behaviour in the same conditions. These behaviours account for the fact that the stroke mice exhibit sensory neglect. In other words, they do not know if they have the food item in their hand due to lack of sensory feedback, so every time they reach they behave as if they have grasped the food, which is grasp and hand supination,
but when they bring their hand to the mouth for food consumption they realize that they do not have the food in their hand.

The purpose of experiment two was to assess mice in skilled reaching when treated with optogenetic upregulation of acetylcholine adjacent to the stroke area. For that, twenty-two mice were trained in the skilled reaching task. When they reached an asymptotic level of success, they were implanted with an optrode in their nucleus basalis and an electrode in their M2 area. Cortical local field potential recordings confirmed desynchronization in the cortex at the time of stimulating nucleus basalis in all mice. After mice recovered from the implant surgery and habituated with the laser cable, seventeen mice received photothrombotic stroke in the primary forelimb somatosensory area and the remaining five mice had a sham surgery. Brain histology of the stroke mice revealed that all the strokes were induced exclusively in the targeted area, the primary forelimb somatosensory cortex. After three days of recovery, the stroke mice were divided into two groups: Stim group (n=9) and No Stim group (n=8). All mice were filmed in skilled reaching task on days 4, 7, 10, 14, and 28 post-stroke. Mice received daily stimulation of nucleus basalis on days 4 to 14 post-stroke. Success percentage of the mice showed a significant increase in the success score of the Stim group compared to the No Stim group on day 7 post-stroke. In other words, the Stim group had an improved success score by receiving three days of stimulation compared to the No Stim group. Movement component analysis across the filming days revealed that there was no significant improvement between Stim and No Stim groups. Similarly, fictive eating analysis revealed no improvement in sensory neglect in Stim group.
The second hypothesis that laser-induced upregulation of acetylcholine in the primary forelimb somatosensory after ischemic stroke in mice will reduce diaschisis and improve skilled reaching following primary forelimb somatosensory ischemic stroke was partially supported by experiment two. Increased acetylcholine reduced diaschisis as measured by the success percentage, but it did not improve skilled reaching in mice as measured by movement component analysis and fictive eating analysis. Mice that received optical stimulation had a significant increase in their success percent only after receiving three days of stimulation, whereas the mice that did not receive stimulation did not have an improvement in their success percent. Ten days post stroke, both Stim and No Stim groups had the same hit percent, which can indicate the period of diaschisis was over for the No Stim group. The trend of success percentage for the No Stim group is very similar to the success percentage of rats with small and medium motor cortex lesions (Whishaw, 2000). The component scores and fictive eating measures indicate no overall significant difference between the Stim and No Stim groups compared to sham mice.

Considering the cholinergic system and the obtained results in the two studies, three models can be proposed: The first model is that after stroke induction in the somatosensory area, the cholinergic terminals in the cortex get damaged resulting in reduction of acetylcholine in the peri-infarct region. Stimulation of the nucleus basalis makes the remaining terminals in the cortex release more acetylcholine, thus facilitating plasticity in the peri-infarct region. The second model is that after stroke induction in the somatosensory area, the cholinergic nuclei in the nucleus basalis that are remote from the stroke site yet have connections to it are
shut down and that is what diaschisis proposes. Neurons in the nucleus basalis go through a shock period resulting in reduction of acetylcholine release from the axonal terminals in the cortex. Stimulation of the nucleus basalis activates those neurons, bypasses the period of diaschisis, and increases acetylcholine in the peri-infarct region, thus promoting plasticity. The third model is that after stroke induction in the somatosensory area, not only cholinergic terminals in the cortex get damaged, but also the cholinergic nuclei in the nucleus basalis are shut down which results in reduction of acetylcholine in the peri-infarct region. Stimulation of the nucleus basalis reduces the diaschisis period and increases acetylcholine release from the remaining terminals in the peri-infarct region, thus promoting plasticity.

The results of previous studies (Gonzalez, Gharbawie, & Kolb, 2006; Lim, Alaverdashvili, & Whishaw, 2009) have shown that following daily administration of a cholinergic agonist, nicotine, after forelimb motor cortex stroke no improvement occurs in any measure of skilled reaching, despite affecting structure of collateral vessels and neurons, and behaviour in tasks such as cylinder and swimming tasks. Their finding suggests that any ameliorating effect from upregulation of acetylcholine such as decrease in diaschisis may be caused by muscarinic acetylcholine receptors and not nicotinic receptors.

Despite long lasting plastic changes that acetylcholine can induce (Woody et al., 1978), its necessity for brain reorganization after lesioning the motor cortex (Conner et al., 2005), desynchronizing cortical LFP and breaking the excitatory/inhibitory balance (Kalmbach, Hedrick, & Waters, 2012; Metherate &
Ashe, 1993), increased acetylcholine did not promote recovery of skilled forelimb use after ischemic stroke in mice.

A number of caveats can be pointed out in both experiments, absence of automatic scoring, absence of direct measurements for acetylcholine release in the cortex, use of one single laser power, and assessment of one single behaviour. First, the analysis of the movement components and the fictive eating measures are observational and scored using rating scales and not subjected to automated scoring nor averaging large number of trials. Nevertheless, the movement scores are based on frame by frame analysis of body movements described by Eshkol Wachman movement notation (Eshkol & Wachman, 1958). This analysis is sensitive to elbow and arm movements, hand pronation or supination used for reach and withdraw, movement scores used in the present study. Second, activation of cortical local field potential following optical stimulation of nucleus basalis was used to confirm acetylcholine release in the cortex which is an indirect measure. As such, further experiments need to directly confirm the amount of acetylcholine release following optical of stimulation of nucleus basalis. Third, a single laser power (5mW) was delivered from the tip of optrode into the brain for all mice, thus the second experiment did not investigate the relationship between laser power and recovery rate post-stroke, similar to dose response in pharmacological studies. Nevertheless, in a pilot local field potential recording study, in which a combination of various laser powers and various duration of stimulation was used, it was confirmed that stimulating for at least 2 seconds with 5mW laser power results in desynchronization of cortical local field potential. Fourth, the assay of functional
recovery post-stroke was the single pellet reaching task. Previous studies (Alaverdashvili, Lim, & Whishaw, 2007; Dunnett, Whishaw, Rogers, & Jones, 1987; Lim et al., 2009; Windle & Corbett, 2005) have reported on the resistance of single pellet reaching task to pharmacotherapy. However, experiment two utilizes optogenetic stimulation and not pharmacotherapy.

Future experiments can employ local field potential recording together with various behavioural tests in order to investigate at depth the mechanism of reduced diaschisis following increased acetylcholine post-stroke. Moreover, examining the size of cholinergic nuclei in the nucleus basalis via golgi staining and examining the patterns of activity of those cells via methods of in situ hybridization may delineate the changes that occur after stroke with regards to diaschisis. Furthermore, combined optical and pharmacological studies can dissect the role of nicotinic and muscarinic acetylcholine receptors in the process of reduced diaschisis.

In conclusion, optical stimulation of nucleus basalis following focal ischemic stroke to the primary forelimb somatosensory area restored some but not all functions. Upregulation of acetylcholine reduced diaschisis as measured by endpoint scores. However, upregulation of acetylcholine did not improve skilled hand movements as measured by movement component scores and sensory neglect as measured by fictive eating measures.
5. References


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