OPTOGENETICS STIMULATION IN NON-INVASIVE SKELETAL MUSCLE ACTIVATION: BIOMARKER IDENTIFICATION VIA KEY GENE EXPRESSIONS IN CALCIUM SIGNALING PATHWAY

by

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ABSTRACT

Optogenetic stimulation which enables spatially sensitive, cell-type-specific, pain-free stimulation, is an emerging alternative to electrical stimulation. Optogenetics directly activates the nerves distal to the paralyzed/injured skeletal muscle via expression of light-sensitive Channelrhodopsin-2 (ChR2) on cell membranes, Skeletal muscle contracts in response to optogenetic stimulation by triggering an action potential that propagates and induces a global cellular calcium response. Rises in cytosolic calcium then stimulate downstream calcium-dependent signaling pathways to regulate skeletal muscle contractions. Recognizing that calcium signaling pathways plays a crucial role in skeletal muscle contractions, my thesis aims to identify the key genes/transcription factors in the calcium signaling pathway that mediate skeletal muscle contractions. I achieved this aim by comparing differentially expressed genes between optogenetically stimulated skeletal muscle (triceps-surae) to contralateral, unstimulated skeletal muscle of young mice. My results show that pro-inflammatory cytokines (*Tnf, Il6*) and growth factors (*Egr1, Egr2*), are up regulated in the optogenetic stimulated skeletal muscle compared with the contralateral unstimulated control. These targets can potentially be utilized to regulate the production of a biological drug *in-situ*, by repeatedly applying light to the tissue and inducing expression of therapeutic transgenes in skeletal muscle paralysis.

Chapter 1

INTRODUCTION

1.1 Muscle generates force for skeletal movement and stability

Muscle is a soft connective tissue, which contains protein filaments of actin and myosin that produce force and motion. Muscles are classified (by appearances and location of cells) into three main types; skeletal/striated muscle, smooth/non-striated muscles, and cardiac muscle. Skeletal muscles are almost exclusively attached to the skeleton by tendons to produce all the movements of body parts in relation to each other (Mukund and Subramaniam; Swenarchuk). Skeletal muscle is a collection of muscles cells or muscle fibers which consists of sarcolemma (the cell membrane), sarcoplasm (the cytoplasm), myofibrils (the main intracellular structures, highly organized bundles of contractile and elastic proteins that carry out the work of contractions, composed of actin and myosin) and sarcoplasmic reticulum (a form of endoplasmic reticulum that wraps around each myofibril, consists of terminal cisternae) (Figure 1 and Figure 2). The sarcoplasmic reticulum concentrates and sequesters calcium (Ca^{2+}) with the help Ca^{2+}-ATPase (Adenosine triphosphatase) in the sarcoplasmic reticulum membrane. Ca^{2+} release from the sarcoplasmic reticulum initiates calcium signaling that play a key role in the contraction in the skeletal muscles(Swenarchuk; Tu et al.). The contraction of muscle fibers is a remarkable process that produces force to move or to resist a load, with the force created by

contracting muscle called muscle tension. Muscle contraction is an active process that requires energy input from ATP. Every skeletal muscle fiber in each skeletal muscle is innervated by a motor neuron at the neuro-muscular junction (which is the site where a motor neuron's terminal, meets the muscle fiber) (Hinsey; Jolesz and Sreter; Boncompagni et al.)

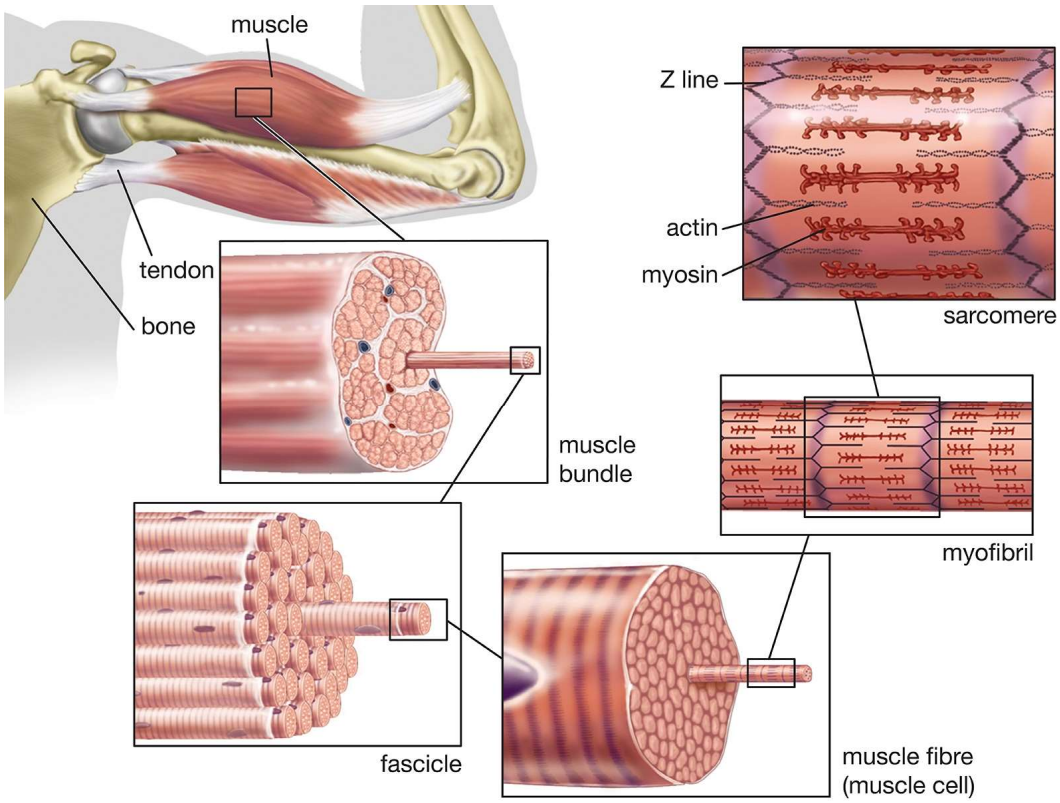

Figure 1 The structure of skeletal muscle (shown here as the human biceps muscle), consists of long, fine fibres, each of which is composed of A bundle of finer myofibrils. Within each myofibril are proteins, myosin and actin filaments; these filaments slide past one another as the muscle contracts and expands. Within each myofibril, regularly spaced dark bands, called Z lines, can be seen where actin and myosin filaments overlap. The region between two Z lines is called a sarcomere; sarcomeres are considered the primary structural and functional unit of muscle tissue (Encyclopedia Britannica 2015).

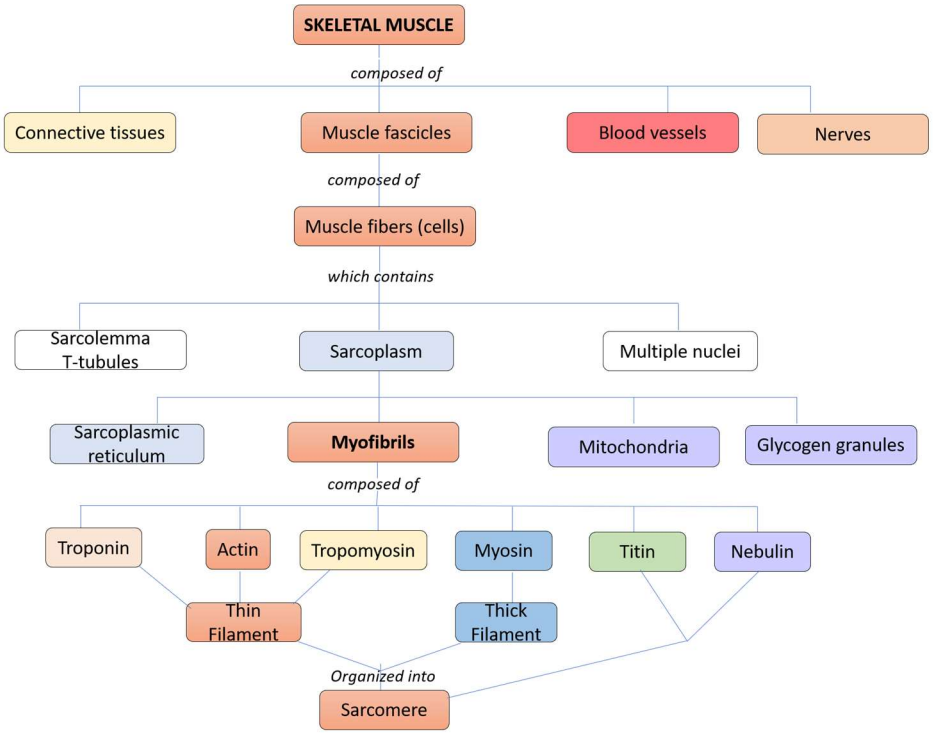

Figure 2 Skeletal Muscle Anatomy Summary. Skeletal muscle consists of sarcolemma (the cell membrane), sarcoplasm (the cytoplasm), myofibrils (the main intracellular structure, composed of actin and myosin) and sarcoplasmic reticulum (a form of endoplasmic reticulum that wraps around each myofibril, consists of terminal cisternae) (William Perreault).

Excitation signals from the neuron are the primary way to activate the skeletal muscle fiber to contract. In summary, the major steps lead to skeletal muscle contractions can be divided into three events i). Events at neuromuscular junction: Events at the neuromuscular junction convert and acetylcholine signal from a somatic motor neuron into an electrical signal in the muscle fiber. ii). Excitation-contraction coupling: the process of coupling signals (electrical, chemical) at the cell surface to the intracellular

release of calcium from the sarcoplasmic reticulum leads to contraction. iii). Contraction-relaxation cycle: Relaxation occurs when ATP attaches to and releases the myosin head from actin filament. In all events Ca^{2+} is then pumped back into the sarcoplasmic reticulum breaking the link between actin and myosin. Actin and myosin return to their unbound state causing the muscle to relax. Alternatively, relaxation will also occur when ATP is no longer available. In intact muscles, the contraction-relaxation cycle is called a muscle twitch(Calderón et al.; Shishmarev; Catterall).

1.2 Calcium signaling plays a major role in skeletal muscle contractions.

In skeletal muscle, contraction requires an increase in the sarcoplasmic levels of calcium, and the concentration of calcium in the sarcoplasm exerts control over the initiation, time course and the force of contraction. Calcium signaling has the power to turn on and off the contraction (Catterall). Skeletal muscle fibers use Ca2+ as their main signaling and regulatory molecule, and contractile properties of muscle fibers are dependent on the variable expression of proteins involved in Ca2+ signaling (Berchtold et al.)**.** The actomyosin fibers responsible for contraction requires an increase in the cytosolic levels of Ca2+, which signaling pathways induce by promoting influx from extracellular sources or release from intracellular stores. Rises in cytosolic calcium stimulate multiple downstream calcium-mediated signaling pathways, which can also regulate skeletal muscle contraction. In the skeletal muscles the Ca2+ signaling includes (Figure 3) the increase in Ca2+ levels due to its release from the sarcoplasmic reticulum stores via ryanodine receptor (Berchtold et al.; Kuo and Ehrlich). Nicotinic acetylcholine is a type of neurotransmitter that binds to receptors on the muscle surface(Galzi et al.; Evans et al.) and elicits a depolarization by causing sodium/calcium ions to enter through associated channels. This process

shifts the resting membrane potential to a more positive value, which in turn activates voltage-gated ion channels. Activation of voltage-gated ion channel result in an action potential. The action potential is initiated at the neuromuscular junction by release of acetylcholine because of the in the motor neuron. The action potential (discussed in section 1.1.b – 1.1.d) stimulates L-type calcium channels, which are mechanically coupled to the sarcoplasmic reticulum ryanodine receptors and open them directly (Allen et al.; Kuo and Ehrlich; Hill).

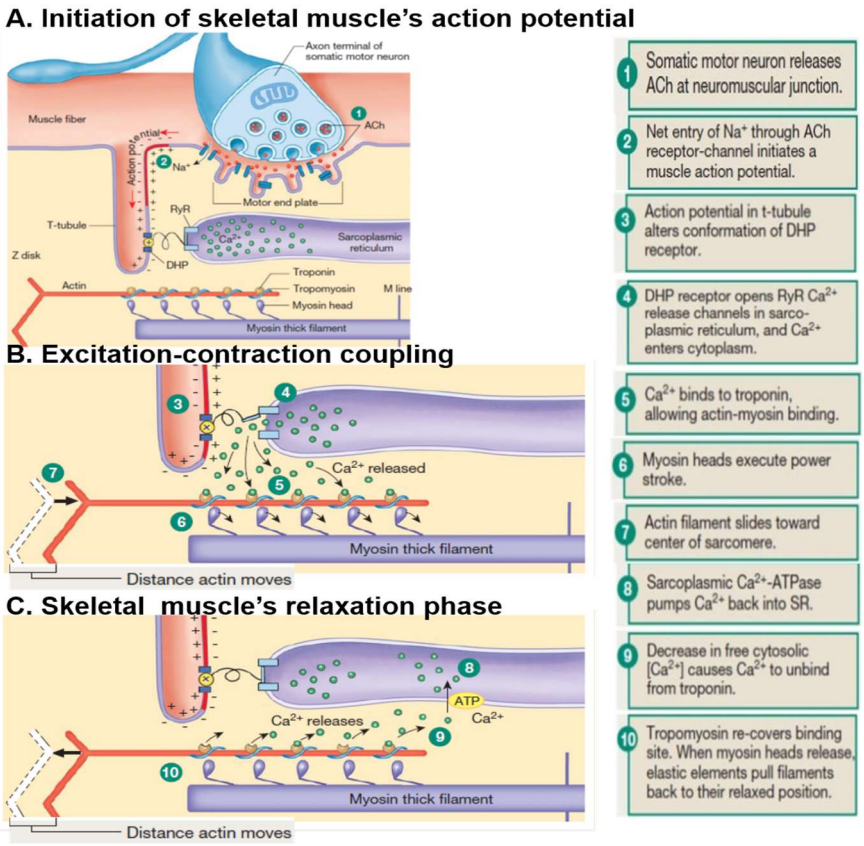

Figure 3 Overview of Ca2+ mediated contractions in skeletal muscles: Acetylcholine is released from the somatic neuron; acetylcholine initiates an action potential in the skeletal muscle fiber. The skeletal muscle action potential triggers Ca2+ release from the sarcoplasmic reticulum. Ca2+ combines with troponin & initiates contraction. (Silverthorn et al.).

Once the concentrations of cytosolic Ca2+ increases, Ca2+ binds to the troponin (Troponin is a calcium-binding complex of three proteins. Troponin controls the positioning of an elongated protein polymer, tropomyosin). The newly formed troponin-Ca2+ complex pulls tropomyosin away from actin's myosin binding site. Once the binding site is available in actin, myosin binds strongly to actin and completes the powers strokes resulting the movement in actin filament, that cause skeletal muscle contractions (Figure 4).

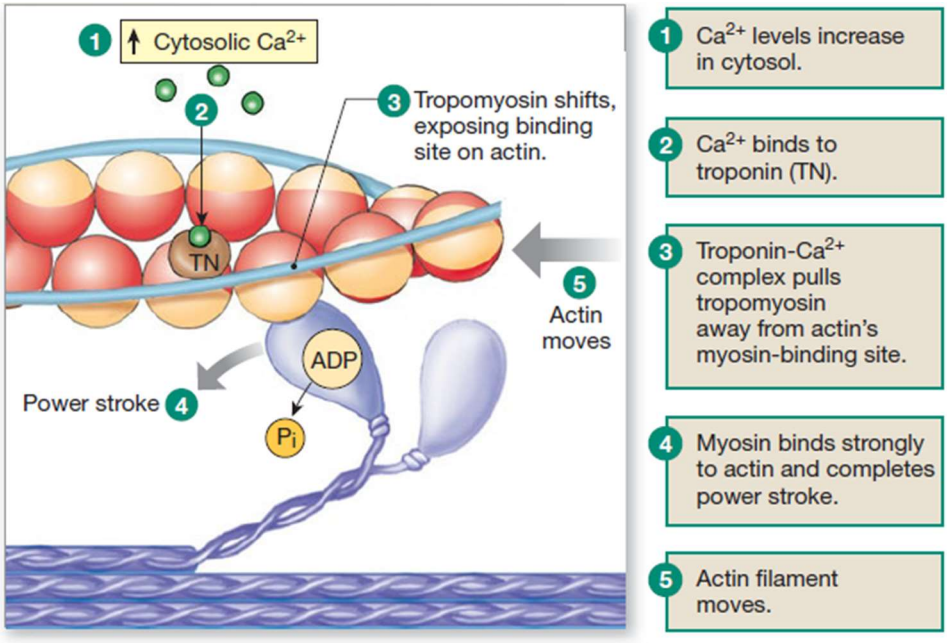

Figure 4 Initiation of Ca2 + mediated skeletal muscle contractions. When contraction begins in response to a calcium signal, one protein of the complex: troponin-C binds reversibly to Ca2+. The calcium-troponin C complex pulls tropomyosin completely away from the myosin-binding sites of actin.. This "on" position enables the myosin heads to form strong, high-force cross-bridges and carry out their power strokes, moving the actin filament. Contractile cycles repeat as long as the binding sites are uncovered. (Silverthorn et al.).

1.3 Electrical stimulation in skeletal muscle activation and paralysis.

Skeletal muscle paralysis is the loss of strength in and control over a muscle or group of muscles in a part of the body. In most cases, skeletal muscle paralysis occurs not due to a problem with the muscles themselves, but due to diminished neuronal input. This occurs somewhere along the chain of nerve cells from the motoric cortex to the neuromuscular junction. (Gundelach et al.; Vajtay et al.). The damage of peripheral nerves can lead to skeletal muscle paralysis (Gundelach et al). Aside from muscle damage, nerve damage, and metabolic functions, skeletal muscle paralysis also caused by stroke, spinal cord injury, and, sports injury (Armour et al). Skeletal muscle paralysis is common in the United States and globally, nearly 1 in 50 Americans are living with some form of paralysis which is about 5.4 - 6 million people (Armour et al). Currently, there are no permanent cure for skeletal muscle paralysis. However, depending on the cause and type of paralysis, some people experience a partial or complete recovery.(Cannon; Armour et al.; Matsumura et al.) The only treatment option to restore the function of paralyzed muscle is the use of electrical stimulation to the innervating nerve or muscle belly (van Bremen et al). Common stimulation used in the treatment option is transcutaneous electrical nerve stimulation (TENS) which is a painfull procedure and comes with side effects like lower blood pressure and muscle spasm (Vance et al).

Electrical stimulation can only be used for short depolarizations that initiate action potentials, and long-lasting depolarizations are technically not feasible (Table 1) (Bruegmann, Malan, et al). Electrical stimulation requires intact motoric nerve function and cannot be used in cases of diseases affecting the neuromuscular junctions or peripheral nerve dysfunction (Gundelach et al). Strong electrical stimulation activates sensible and pain neurons (Hultman and Sjöholm) preventing the therapeutic

application of direct skeletal muscle stimulation, in addition, the indirect electrical stimulation through motor neurons is not suited in cases of peripheral nerve injuries or dysfunction and diseases affecting the neuromuscular junctions (van Bremen et al). Furthermore, electrical stimulation produces inhomogeneous areas of depolarization and hyperpolarization using unipolar point stimulation or global field stimulation (Bruegmann, Malan, et al.).

1.4 Optogenetics as a tool for noninvasive skeletal muscle activation

Optogenetics is an emerging contact-less alternative to initiate action potentials in activatable cells both ex vivo and in vivo (Deisseroth, "Optogenetics"; Boyle et al.; Ganji et al.). In contrast toeElectrical stimulation optogenetic stimulation enables spatially sensitive, cell-type-specific, pain-free stimulation (van Bremen et al.). Previous studies demonstrated that optogenetic stimulation directly stimulates the nerves distal to the paralyzed/injured skeletal muscle and relies on the expression of light-sensitive Channelrhodopsin-2 (ChR2) proteins on cell membranes that provide precise control over the membrane potential by inducing depolarization and action potentials(Bruegmann, Malan, et al.; van Bremen et al.; Britt et al.). For example, exposure of pulsed blue light (455nm wavelength) to ChR2-expressing muscle cells (myotubes) results in depolarization of the membrane potential of a call which then evokes an action potential (Sebille et al.). ChR2 is the most widely used seven layer transmembrane optogenetic tool, and acts as a non-specific inward cation channel (depolarizing) with an intrinsic light sensitivity(Nagel et al.; Deisseroth, "Optogenetics").

An action potential occurs when the membrane potential of a specific cell location rapidly rises and falls, this depolarization then causes adjacent locations to

similarly depolarize. Action potentials occur in several types of excitable cells, such as, neurons, muscle cells, endocrine cells etc. (Hodgkin and Huxley).The blue light (455nm wavelength) also concurrently induces a twitch-like contraction pattern, generated with a given pattern of light emitting diode (LED) pulses. We and others have shown that direct optogenetic stimulation of skeletal muscle can generate a force equivalent to the electrical stimulation of the nerve (van Bremen et al.; Bruegmann, van Bremen, et al.) Additionally, optogenetic stimulation of skeletal muscle cells provides a distinct advantage over electrical stimulation because it is localized, cell-type-specific and pain free. This technique has been commonly used in mammalian neurons and more recently, it has been adapted for the activation of cardiomyocytes and skeletal muscle (Ganji et al.). It is reported that optogenetic stimulation to induce 84% of the maximal force from electrical stimulation (Bruegmann, van Bremen, et al.). Although electrical and optogenetic stimulation produces similar action potential morphology in different cell types (Williams and Entcheva), there are quite a few areas where optogenetics stimulations stand out.

Table 1: Functional comparison between electrical and optogenetics stimulation (Williams and Entcheva; Deisseroth, "Optogenetics"; Cogan et al.; Bruegmann, Malan, et al.; Nagel et al.; Ganji et al.; Ferenczi et al.; Deisseroth, "Optogenetics and Psychiatry").

Features	Electrical Stimulation	Optogenetics Stimulation
Function	Indirect stimulation. Elicit muscle contraction via nerve/NMJ.	Direct stimulation. Elicits muscle contraction directly without NMJ.
Action Potential	Electrical stimulation involves the injection of an external current with a predefined waveform and without a specific ionic identity, which brings the membrane potential above threshold to trigger an action potential	When the channel pore is opened, that are initiated by Optogenetics stimulation, the resulting net inward flow of positive ions results in a depolarizing photocurrent. This elicits an action potential when the excitable cell membrane reaches its firing threshold.
Specificity	Non-specific, does not uses any specific cell type.	Utilizes specific cell type (ChR2)
Side effects	Tissue-damage caused by repeated needle electrode placement.	Optogenetics stimulation is non-invasive, it has much lesser side effects than electrical stimulation (based on the current optogenetics model).
Duration	Electrical stimulation is Usually applied as brief pulses only, due to electro-chemical limitations	Optogenetic stimulation permits longer stimuli without undesirable side effects

The emergence of optogenetics to control the activation of excitable cells (Nagel et al. 2003), such as skeletal muscle (Bruegmann, van Bremen, et al.; Asano et al.; Magown et al.; Neal et al.; van Bremen et al.), may help in improving recovery of the denervated and injured skeletal muscle. To activate excitable cells, we and others have developed tools to express a light-sensitive ion channel, ChR2, specifically in

skeletal muscle in mice (Bruegmann, van Bremen, et al.; Magown et al.). With blue light exposure, ChR2 cells depolarize by opening non-specific ion channels on the cell membrane (Nagel et al.) and can generate contractile forces in adult skeletal muscle (Towne et al.; Bruegmann, van Bremen, et al.; Bruegmann, Malan, et al.; Steinbeck et al.; Magown et al.) and increased neural activity in the brain (Madisen et al). The use of optogenetics to control muscle activation is relatively new (S et al.; Nagel et al.; van Bremen et al.); to date, this tool has not been reportedly explored for inducing light-mediated skeletal muscle contraction during skeletal growth for therapeutic purposes.

1.5 Mechanism of skeletal muscle contraction by calcium signaling in response to optogenetics stimulation

Depolarization of skeletal muscle cells by blue light stimulation induces the release of calcium from internal stores (Valdés et al.), which is mediated by ChR2 (Figure 5 and Figure 6). The release of intracellular calcium elicits elusive molecular changes through a signaling cascade (Berdeaux and Stewart). The calcium signaling pathway regulates the contraction of skeletal muscle. Skeletal muscle contracts in response to optogenetic stimulation (which depolarizes the membrane potential by triggering an action potential that propagates and induces a global cellular calcium response) (S et al.). The actomyosin fibers responsible for contraction require an increase in cytosolic levels of calcium, which is induced by promoting influx from extracellular sources or release from intracellular stores (Kuo and Ehrlich). Rises in cytosolic calcium stimulate numerous downstream calcium-dependent signaling pathways that regulate contraction.

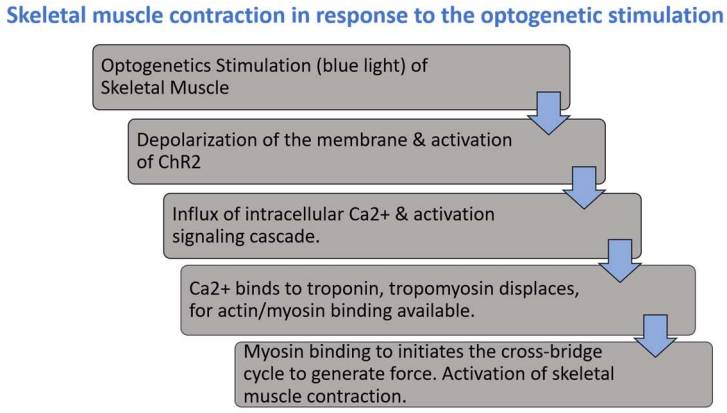

Figure 5 Stepwise process of skeletal muscle contraction in response to the optogenetic stimulation via activation of calcium signaling pathway(Tu et al.; Carrasco et al.). Blue light (Optogenetic-stimulation) activates the ChR2. Once ChR2 is activated, action potential cycle is initiated and depolarization of the membrane of the skeletal muscle, which create the influx of intracellular calcium & activate calcium signaling cascade. Once calcium ions reach inside the membrane & available to its binding site troponin. Once Ca2+ binds to troponin, tropomyosin displaces and making the place available for actin/myosin binding site for contraction. The contraction begins once myosin binds to actin and initiates the cross-bridge cycle to generate force.

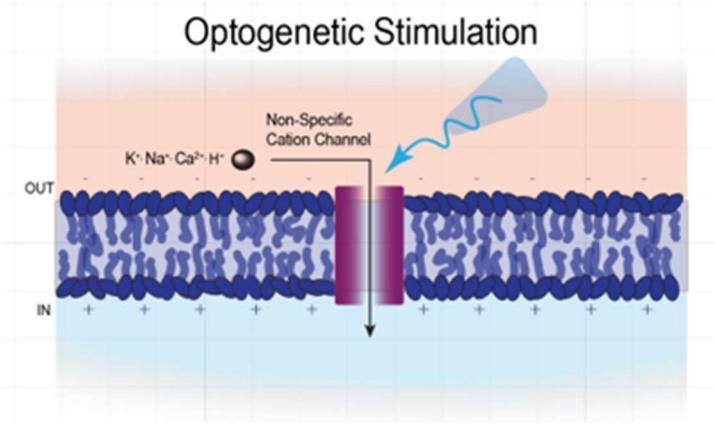

Figure 6 Optogenetic stimulation in nonspecific cation channel (eg: Ca2+), blue arrow indicates the blue light exposure (figure drawn by E.Ganji: Killian Lab).

1.6 Therapeutic implications of Optogenetic Stimulation

Optogenetic stimulation is useful for therapeutic purposes, such as improving the recovery of denervated and injured skeletal muscle (Madisen et al.). It stimulates numerous downstream calcium-dependent signaling pathways, which can also regulate muscle contraction(Ma et al.). Optogenetic approaches have been used in pre-clinical studies to improve nerve regeneration (Ward et al.) and to improve gut motility (Mickle and Gereau). Optogenetic regulation of transcription could potentially be used to regulate the production of a biological drug *in situ*, by repeatedly applying light to the tissue, and inducing expression of therapeutic transgenes when needed(Polesskaya et al.). By combining the light-sensing module and transcription factor module optogenetic tools can be used to regulate gene transcription (Polesskaya et al.). The advantage of light is that light-based treatment can be focused on a particular small area, is easy to deliver, and allows for the control of dosage and timing.

In gene therapy, optogenetics stimulation can also be employed to control localization, timing, and dosage in the expression of a therapeutic transgene to activate skeletal muscle contractions. Regulation of calcium level in the skeletal muscles by identifying the gene targets via calcium signaling pathways may help identify novel therapeutic avenues for restoring the mobility of skeletal muscles (Delbeke et al.). The optogenetic stimulation is especially attractive as it steps away from predominantly pill-based approaches towards much more targeted therapy by providing control over timing, location, and delivered dosage of biologic drugs (Polesskaya et al.). Optogenetics offers promise to examine the complex skeletal muscle and peripheral nervous system and has therapeutic potential for addressing unmet clinical needs (Montgomery et al.).

1.7 Motivation

In this study, I hypothesized that the expression of key genes in the calcium signaling pathway would be induces following optogenetic stimulation in skeletal muscle compared to contralateral, unstimulated skeletal muscle of young mice. This study aimed to define the transcriptional events and identify potential downstream signals involved in optogenetic stimulation. Based on a previous study and literature (Vajtay et al.; Magown et al.; Polesskaya et al.), I have observed that the transcription factors (TF) activity of the skeletal muscle is induced 30 mins after stimulation and sustained up to 3 days. I analyzed two key time points 3 hours and 24 hours post optogenetic-stimulation to identify unique differentially expressed genes and transcription factor activity of common biomarkers. These genes are potential future targets for modulating skeletal muscle contractions in denervated skeletal muscles.

Chapter 2

MATERIALS & METHODS

2.1 Animal model:

All procedures and protocols were approved by Institutional Animal Care and Use Committee (IACUC) at the University of Delaware (Appendix A). We have used *in vivo* cre-lox recombination for targeted expression of channelrhodopsin-2/YFP (ChR2(H134R)-EYFP) fusion protein (Ai32) in the muscle. To generate these mice, we crossed Acta1-Cre male mice (C57BL6) with Ai32 (RCL-ChR2(H134R)/EYFP) reporter female mice (C57BL6 background) (Figure 7) (Tanaka).

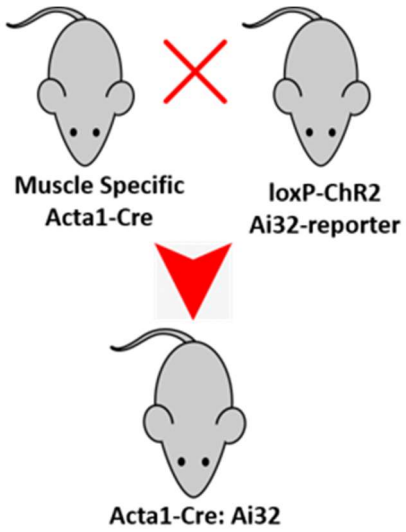

Figure 7 Schematic showing the breeding scheme used for generation of Acta1-Cre: Ai32 experimental mice.

We targeted the expression of Ai32 to Acta1-Cre lineage cells, limited to adult striated muscle fibers and embryonic striated muscle cells of the somites and heart (Rao and Monks). Doxycycline chow was provided ad libitium during gestation to dams and pups until weaned (postnatal day 28). Offspring were genotyped using PCR (Transnetyx, Cordova, TN, USA). Acta1-Cre; Ai32 homozygous mice (experimental) were used for light stimulation and Cre-negative; Ai32 heterozygous/homozygous offspring were used as controls and for optogenetics stimulation of the triceps surae muscles. To verify Acta1 Cre lineage, we crossed Ai14(tdTomato) reporters with Acta1-Cre-mice to generate Acta1-Cre Ai14 reporters, using a similar breeding scheme described in Figure 7. We have used N=3 samples per treatment (3 contralateral control and 3 optogenetically stimulated samples from the left and right leg of the same mouse respectively.).

2.2 Optogenetic stimulation protocol:

A 455nm, 900mW mounted LED (max current 1000mA; M455L3, Thorlabs, NJ, USA) and High-power 1-Channel LED Driver (DC2200, Thorlabs, NJ, USA) was used for pulse modulation. The driver controller was adjusted to allow for repeated pulsatile stimulation in user-defined intervals (Labview, National Instruments, TX, USA). A collimator (SM2F32-A; 350-700nm, Thorlabs, NJ, USA) was used to reduce energy dissipation from LED light divergence.(Ganji et al.)

Before each stimulation, mice were anesthetized using isoflurane. Hair was removed over the triceps surae muscle using chemical hair remover (Nair, Church & Dwight Co., NJ, USA). Animals were placed on a heating pad (Stoelting, IL, USA) at 37°C in the prone position and their hindlimb was placed in the Aurora (3-in-1) muscle stimulation apparatus (Aurora Scientific, ON, Canada) and clamped to

stabilize the knee joint construct during stimulation. Ankle torque measurements were collected using a foot pedal attached to a 1N force transducer (Aurora Scientific, ON, Canada). A 20mins duration of 240 reps (Single stimulation (1sec) profile:10Hz (ON: 70ms; OFF: 30ms; Rep: 10x), Rest (4sec)] used for each mouse to stimulate the triceps surae muscle of right leg. Each mouse was 23-25 days old at the time of the optogenetic stimulation protocol were performed.

2.3 Time-Points:

We have used N=6 samples (3 Contralateral control and 3 optogenetically-stimulated) for each time point (3 hours and 24 hours). The right leg from each mouse was optogenetically-stimulated and the left leg from each mouse was not stimulated and used as contralateral control from the same mice. Two different time points were used with each batch of the mouse. Batch 1 includes the mice which are euthanized 3 hours after the optogenetic-stimulation and Batch 2 includes the mice which are euthanized 24 hours after the optogenetic stimulation. The gastrocnemius muscle is collected from each leg under RNAse free condition, length of the muscle was recorded, and tissue flash-frozen in liquid nitrogen and stored in-80$^{\circ C}$ for downstream processing.

2.4 Euthanasia and dissection:

Mice were euthanized following the animal protocol from the Institutional Animal Care and Use Committee (IACUC) of University of Delaware**.** Tissues were pulverized for 30 seconds in 2 mL tubes with steel balls in liquid nitrogen-cooled blocks (MM400; Retsch, Verder Scientific).

2.5 RNA isolation and cDNA preparation

RNA was extracted using TRIzol/chloroform and column purified using a commercially available kit (PureLink RNA Mini Kit; Invitrogen). RNA quantity and integrity were confirmed by UV/VIS spectrophotometer. The 260/20 ratio of all samples were > 2.0. Integrity of RNA also confirmed using gel electrophoresis to confirm the presence of 18s and 28s sub-units of ribosomal RNA via fragment analysis (Table: 2). Total RNA was reverse transcribed to cDNA using Superscript III VILO (Invitrogen). cDNA was the used in reactions at 5ng/microliter.

2.6 qRTPCR (PCR Array Analysis):

We used the RT^2 PCR Array Profiler (mouse Calcium Signaling Pathway Finder, PAMM-066Z, Qiagen, Frederick, Maryland, USA) for quantitative reverse transcriptase polymerase chain reaction (q-RTPCR) using PowerUp SYBR Green (Applied Biosystems) on a LightCycler 96 System (Roche). The reference genes and genes of interests are listed in the following table (Table 2):

Table 2 List of reference genes and gene of interest (in the calcium signaling pathway).

Reference Genes	Calcium Signal Related Genes
Actb, B2m , Gapdh ,Gusb , Hsp90ab1.	*Adrb1,Cga,Chga,Gcg,Inhba,Kcna5,Krtap14,Nos2,Penk,Prl,S100a8,S100a9,S100g,Scg2,Slc18a1,Sst,Sstr2,Tacr1,Th,Vip, Bcl2,Brca1,Ccna1,Ccnd1,Cdk5,Cdkn2b,Gem,Nf1,Pcna,Pmaip1,Ppp1r15a,Rb1, Areg,Bdnf,Crh,Fgf6,Tgfb3,Tnf, Dusp1, Hspa5,Pln,Ppp2ca,Prkar1a,Sgk1,Ahr,Amd1,Eno2,Hk2,Ldha,Pck2,Sod2, Il2,Il6,Mif,Ptgs2, Atf3,Creb1,Crem,Egr1,Egr2,Fos,Jund,Maf,Per1,Pou1f1,Pou2af1,Stat3, Calb1,Calb2,Calcrl,Calm1,Calr,Ddit3,Ncam1,Npy,Plat.*

GeneGlobe analysis web service (Qiagen, Frederick, Maryland, USA) was used to analyze the statistical data analysis and differential gene expression. ΔCT (CT target gene – Average CT reference genes) values were calculated for each gene using the average CT value of the reference genes *Actb* and *Hsp90ab* for 3hour interval and 24hour interval experiment from the panel of 5 reference genes (Figure 8). *Actb* and *Hsp90ab* were the most stable genes as reference genes for 3hour interval (Average CT value for no-stim contralateral control was 25.47 & optogenetically stimulated samples were 25.85) and for 24hour interval (Average CT value for no-stim contralateral control was 25.42 and opto-stim samples were 24.50) Table 3. The reference gene was selected as the gene that changed the least due to optogenetics

stimulation treatment from a panel of five reference genes (*Actb, B2m, Gapdh, Gusb, and Hsp90ab1*).

Table 3: CT value of the reference genes from 3 hour & 24 hour time point.

	3 hour					
Reference Genes	**Unstimulated-Control**			**Optogenetically Stimulated**		
	1368-4L	**1368-5L**	**1368-7L**	**1368-4R**	**1368-5R**	**1368-7R**
Actb	24.86	25.98	25.66	25.66	26.02	24.95
B2m	25.13	26.06	25.84	26.78	26.66	25.79
Gapdh	19.93	21.29	20.62	23.53	23.51	21.97
Gusb	30.63	31.72	30.87	31.84	31.7	31.02
Hsp90ab1	24.78	26.27	25.25	26	26.89	25.58
Average CT value of *Actb* & *Hsp90ab1*		**25.47**			**25.85**	

	24 hour					
Reference Genes	**Unstimulated-Control**			**Optogenetically Stimulated**		
	1373-5L	**1373-6L**	**1373-7L**	**1373-5R**	**1373-6R**	**1373-7R**
Actb	24.53	25.1	26.12	23.01	23.57	24.86
B2m	25.45	26.02	27.05	24.14	25.59	25.56
Gapdh	21.24	21.92	21.72	21.25	22.63	22.28
Gusb	30.78	31.06	31.43	29.35	30.04	30.74
Hsp90ab1	25.4	25.51	25.89	24.4	25.3	25.93
Average CT value of *Actb* & *Hsp90ab1*		**25.42**			**24.5**	

2.7 Differential gene expression analysis:

All statistical comparisons were made using the Qiagen Gene Globe RT² Profiler PCR Data Analysis (https://www.qiagen.com/us/geneglobe/) suite. Student's t-test was used to compare the ΔCT of each gene when comparing optogenetically stimulated samples with no-stim contralateral control. The fold change and P.value for each gene were log2 and −log10 transformed, respectively, to visualize results in volcano plots using Prism (v8.4.3-686; GraphPad, LaJolla, CA). For genes that were statistically different between optogenetically stimulated samples with no-stim contralateral control, the data were linearized to $\log2(2^{-\Delta CT})$ and plotted as mean ± standard deviation. Differential gene expressions with a fold change of less than −1.5 or greater than 1.5 were plotted for paired comparisons with P value cutoff equal or less than 0.05.

2.8 Gene Ontology (GO) functional enrichment analysis of DE genes:

The R package (version 3.6.0, 2019-04-26) "clusterProfiler"(Yu et al.) and EnrichGO function were used to evaluate the Gene Ontology (GO) annotations of the differentially expressed RNA's to understand the enriched biological process (BP). P.value cutoff on enrichment tests to report were pvalueCutoff = 0.05 and p adjusted method were Benjamini-Hochberg, padjusted = "BH".

Chapter 3

RESULTS

3.1 Sample Quality verification:

RNA samples were quality passed; by a 260/280 ratio was greater than 2.0 (Table:2). The 3hour interval samples were taken for fragment analysis, even though RQN values observed for each sample were less than 8.0, each sample (18s and 28 rRNA fragment) were visually inspected and proceeded with qRT PCR experiment. The sample quality verification details are listed in the Table 4.

Table 4 RNA quantity (nanogram/ microliter) and integrity by UV/VIS spectrophotometer, 260/280 ratio. Quality Check by fragment analysis.

Mouse ID	Stimulation/Control	Time/Interval	Sex	Age (days)	Conc ng/ul	260/280	RQN
1368-4	Optogenetic (RL)	3hour	Female	25	657.53	2.09	3.5
1368-5	Optogenetic (RL)	3hour	Male	25	564.37	2.09	3.9
1368-7	Optogenetic (RL)	3hour	Male	25	1318.53	2.16	5.2
1368-4	Control (LL)	3hour	Female	25	1111.17	2.16	7.4
1368-5	Control (LL)	3hour	Male	25	527.57	2.02	5.4
1368-7	Control (LL)	3hour	Male	25	567.17	2.13	6.4
1373-5	Optogenetic (RL)	24hour	Female	23	1631.44	2.19	N/A
1373-6	Optogenetic (RL)	24hour	Male	23	647.44	2.01	N/A
1373-7	Optogenetic (RL)	24hour	Male	23	1102.3	2.18	N/A
1373-5	Control (LL)	24hour	Female	23	328.4	2.16	N/A
1373-6	Control (LL)	24hour	Male	23	303.76	2.17	N/A
1373-7	Control (LL)	24hour	Male	23	546.8	2.17	N/A

3.2 Ankle torque:

Ankle torque (mN.m) from each sample from each optogenetically stimulated samples (3hour and 24hour interval) plotted against time (Figure 8A, drawn by Elahe Ganji). When compared the ankle torque value of each optogenetically stimulated samples (Figure 8B), we did not see a significant difference between the groups (based on unpaired T-test). The individual ankle torque value of each sample from both the groups are also comparable. This quality check ensures that that the differences in gene expression between groups at 3hr and 24hr interval not because of the effect of ankle torque profile of each sample.

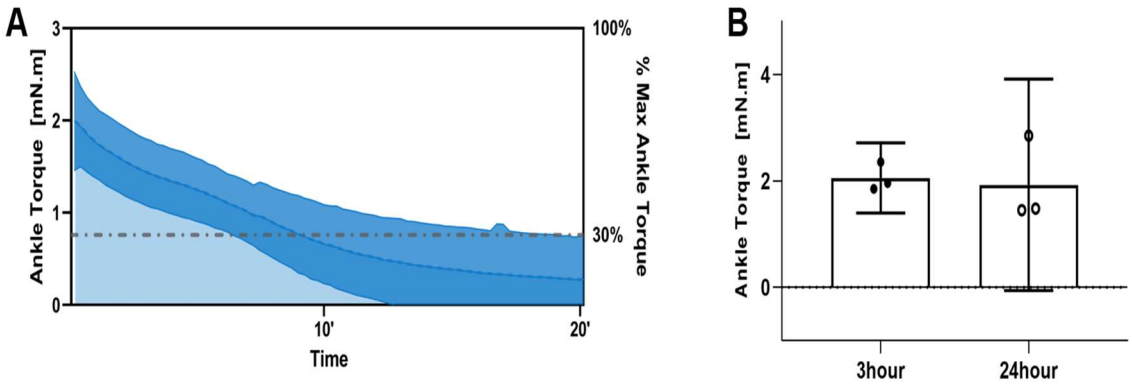

Figure 8 Average ankle torque of the optogenetically stimulated samples. A. Average ankle torque is plotted against total duration of the stimulation (time, minutes). B. Maximum ankle torque of 3hour and 24hour post optogenetically stimulated samples. The graph shows no significant difference between both the groups (based on unpaired T-test). The result helps in understanding that the differences in the gene in gene expression between groups at 3hr and 24hr interval not because of the effect of ankle torque profile of each sample.

3.3 Principal Component Analysis (PCA):

Principal components analysis (PCA) is a common unsupervised method for the analysis of gene expression microarray data, has been used for providing information

on the overall structure of the analyzed dataset for each time point(Lenz et al.). qRTPCR data is high dimensional because each gene whose expression was measured contributes a dimension. If we measured 84 genes, each sample could be represented by a point in 84-dimensional space--with the value in each dimension equal to the expression level of a different gene, PCA analysis helps in dimension reduction. The PCA plot (Figure 9) suggests that: a great variance observed between the sample groups (PC1 is 52.4%) and (no-stim control vs optogenetic stimulated samples in 3 hour and 24 hour time point) but not that much variance found (PC2 21%). Ideally, we would expect the clusters of optogenetically-stimulated samples from 3 hour and 24 hour interval to be much tighter what we observe in no-stim control samples in both the time point.

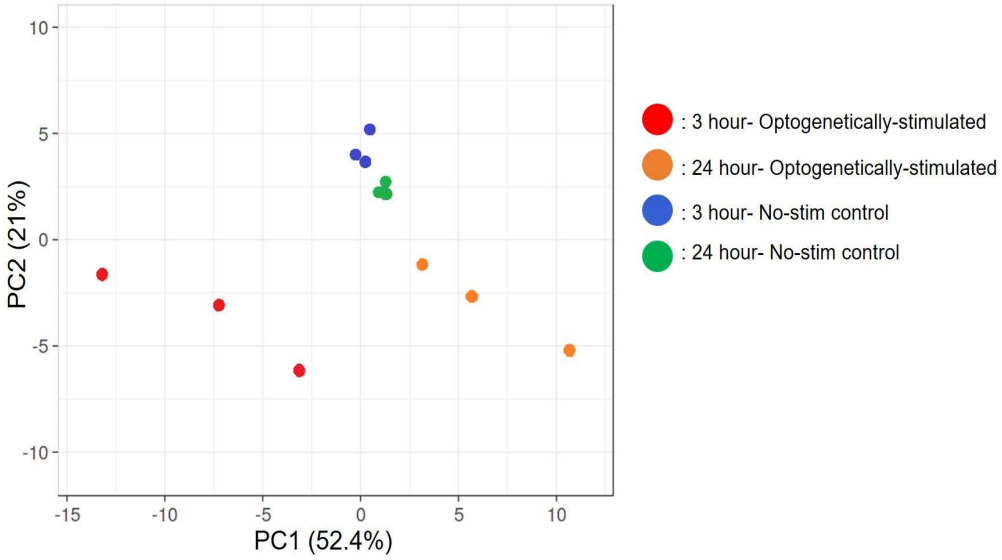

Figure 9 Principal Component Analysis (PCA) of no-stim control and optogenetically stimulated samples from 3-hour and 24-hour interval. Red and orange color denotes the optogenetically-stimulated samples from 3- & 24-hour interval respectively whereas blue and green color denotes the no-stim control samples from 3 & 24 hour.

3.4 Identification of Differentially Expressed Genes:

Differential gene expression analysis was used to determine the significantly different genes between optogenetically stimulated and contralateral control samples from each time-point (3hours and 24 hours interval). Gene expression was compared comparison between the experimental groups (i.e: Optogenetically stimulated samples and no-stim control samples) of the 3 experimental replicates using Student's T test. To determine the differentially expressed genes between the two groups, a fold change of 1.5/-1.5 and p-value of 0.05 applied as statistical significance thresholds (Figure 10).

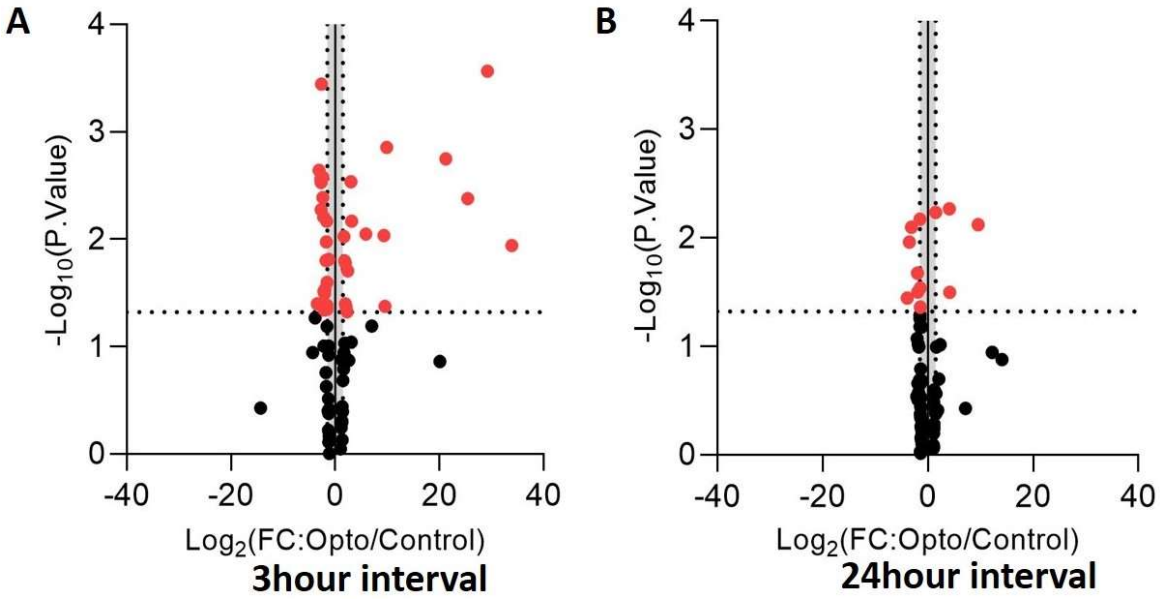

Figure 10 Volcano plots for visualization of the DE genes at 3hour & 24hour intervals. The volcano plot shows the fold change (-1.5/1.5), and statistical significance (p.value < 0.05) of gene expression changes. DE genes based on the cut off is colored red. A. Volcano plot of DE genes 3hour interval, 36 DE genes observed. B. Volcano plot of DE genes from 24hour interval, 12 DE genes observed.

Based on the cutoffs 36 genes were differentially expressed at the 3-hours interval(Figure 10A, Table 5) (16 genes were upregulated and 20 genes were downregulated), and 12 genes were differentially expressed (Figure 10B, Table 6) at the 24-hours interval (3 genes were up regulated and 9 genes were downregulated). Overlap between each group (3hours and 24hours) includes: *Tnf, Pmaip1*, *Fgf6, Ldha, and Srf.* We have identified the differentially expressed transcription factors (TF) are *Brca1, Tnf, Egr1, Egr2, Fos, Fosb, Stat3, Tnf, Creb1, Per1*. *Tnf* is the only common TF gene that differentially expressed between both the time points.

Table 5: Significant differences in gene expression were measured for 36 DE genes at 3 hours post-optogenetic stimulation. LogFC (log fold change) and significance level (pvalue).

Refseq	Primer ID	Gene Name	Gene	Log FC	P.Value
NM_133828	PPM03382F	CAMP responsive element binding protein 1	Creb1	-3.42	0.039947
NM_010204	PPM02961C	Fibroblast growth factor 6	Fgf6	-3.1	0.002288
NM_001025577	PPM03115B	Avian musculoaponeurotic fibrosarcoma (v-maf) AS42 oncogene homolog	Maf	-2.74	0.002758
NM_022310	PPM03586B	Heat shock protein 5	Hspa5	-2.71	0.005316
NM_007419	PPM05035A	Adrenergic receptor, beta 1	Adrb1	-2.67	0.002967
NM_009368	PPM02993A	Transforming growth factor, beta 3	Tgfb3	-2.62	0.000358
NM_010699	PPM05016C	Lactate dehydrogenase A	Ldha	-2.39	0.004075
NM_010875	PPM03672F	Neural cell adhesion molecule 1	Ncam1	-2.38	0.002639
NM_145983	PPM04072A	Potassium voltage-gated channel, shaker-related subfamily, member 5	Kcna5	-2.24	0.006192
NM_011361	PPM05023A	Serum/glucocorticoid regulated kinase 1	Sgk1	-2.15	0.030437
NM_011065	PPM05041B	Period homolog 1 (Drosophila)	Per1	-2.11	0.045529
NM_023129	PPM05027A	Phospholamban	Pln	-2.03	0.03198
NM_007591	PPM05020F	Calreticulin	Calr	-2	0.029485
NM_020493	PPM03580F	Serum response factor	Srf	-1.78	0.015791
NM_028994	PPM05024A	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	Pck2	-1.73	0.010623
NM_007668	PPM05037C	Cyclin-dependent kinase 5	Cdk5	-1.61	0.006781
NM_019411	PPM05011C	Protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	Ppp2ca	-1.58	0.025262
NM_009922	PPM05033A	Calponin 1	Cnn1	-1.57	0.044979
NM_021880	PPM05025A	Protein kinase, cAMP dependent regulatory, type I, alpha	Prkar1a	-1.54	0.041303
NM_008872	PPM03855B	Plasminogen activator, tissue	Plat	1.69	0.009421
NM_009217	PPM04946E	Somatostatin receptor 2	Sstr2	1.84	0.015859
NM_013509	PPM05030A	Enolase 2, gamma neuronal	Eno2	1.95	0.040096
NM_008654	PPM03294A	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	Ppp1r15a	1.96	0.01663
NM_011486	PPM04643F	Signal transducer and activator of transcription 3	Stat3	2.13	0.042345
NM_011198	PPM03647E	Prostaglandin-endoperoxide synthase 2	Ptgs2	2.3	0.047261
NM_010118	PPM04478F	Early growth response 2	Egr2	2.41	0.01969
NM_010234	PPM02940C	FBJ osteosarcoma oncogene	Fos	3.05	0.002924
NM_011580	PPM03098F	Thrombospondin 1	Thbs1	3.17	0.006774
NM_007913	PPM02938C	Early growth response 1	Egr1	5.92	0.008964
NM_013693	PPM03113G	Tumor necrosis factor	Tnf	9.38	0.009199
NM_009704	PPM02976E	Amphiregulin	Areg	9.56	0.042248
NM_021451	PPM03403F	Phorbol-12-myristate-13-acetate-induced protein 1	Pmaip1	9.89	0.001395
NM_001314054	PPM03015A	Interleukin 6	Il6	21.26	0.001783
NM_008036	PPM04609F	FBJ osteosarcoma oncogene B	Fosb	25.4	0.004181
NM_013650	PPM05051F	S100 calcium binding protein A8 (calgranulin A)	S100a8	29.24	0.000272
NM_009114	PPM05050E	S100 calcium binding protein A9 (calgranulin B)	S100a9	33.9	0.011432

Table 6 Significant differences in gene expression were measured for 12 DE genes at 24 hours post-optogenetic stimulation. LogFC (log fold change) and significance level (pvalue).

Refseq	Primer ID	Gene Name	Gene	LogFC	P.Value
NM_009377	PPM05014A	Tyrosine hydroxylase	Th	-3.88	0.035821
NM_010204	PPM02961C	Fibroblast growth factor 6	Fgf6	-3.5	0.010952
NM_013642	PPM04529A	Dual specificity phosphatase 1	Dusp1	-3.13	0.007987
NM_009788	PPM05029B	Calbindin 1	Calb1	-1.95	0.021132
NM_020493	PPM03580F	Serum response factor	Srf	-1.87	0.031582
NM_010699	PPM05016C	Lactate dehydrogenase A	Ldha	-1.71	0.051996
NM_007540	PPM03006C	Brain derived neurotrophic factor	Bdnf	-1.52	0.006727
NM_018782	PPM04822A	Calcitonin receptor-like	Calcrl	-1.42	0.02895
NM_019411	PPM05011C	Protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	Ppp2ca	-1.42	0.043509
NM_021451	PPM03403F	Phorbol-12-myristate-13-acetate-induced protein 1	Pmaip1	4.12	0.005383
NM_009764	PPM03442A	Breast cancer 1	Brca1	4.19	0.031766
NM_013693	PPM03113G	Tumor necrosis factor	Tnf	9.54	0.007573

3.5 Gene Ontology (GO) Enrichment Analysis:

We examined the validity of our approach through a bioinformatics analysis in order to determine the pathways altered in optogenetically-stimulated samples compared to un-stimulated control samples. The gene ontology enrichment analysis performed using clusterprofiler (Yu et al.) from the identified differentially expressed genes from 3-hour and 24-hour interval. Top 10 biological processes identified from 3hour interval (Figure 11A) and 24-hour interval (Figure 12A). Gene concept network analysis has been performed to identify the list of genes enriched for each biological process (Appendix-A). Top 5 biological process and genes associated with them are displayed using gene concept network for 3-hour interval (Figure 11B) and 24hour interval (Figure 12B).

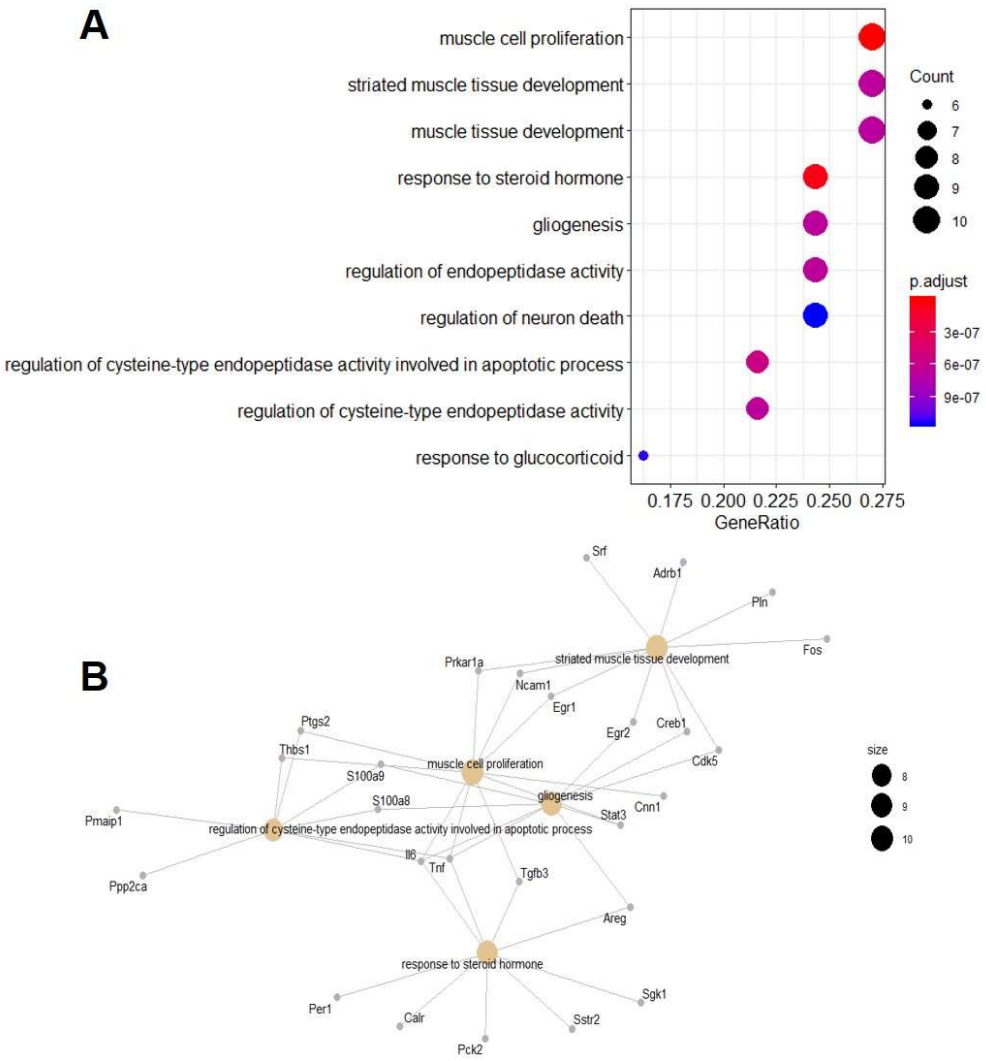

Figure 11 Functional enrichment result of top 10 gene GO BP. A. Dot chart to display gene count or ratio colored by enrichment scores (e.g. p.adjust) with capability to encode another score as dot size (bubble plot) for the DE genes of 3-hour 24. B. Gene-concept network depicts the linkages of genes and biological concepts as a network for the DE genes of 3-hour interval

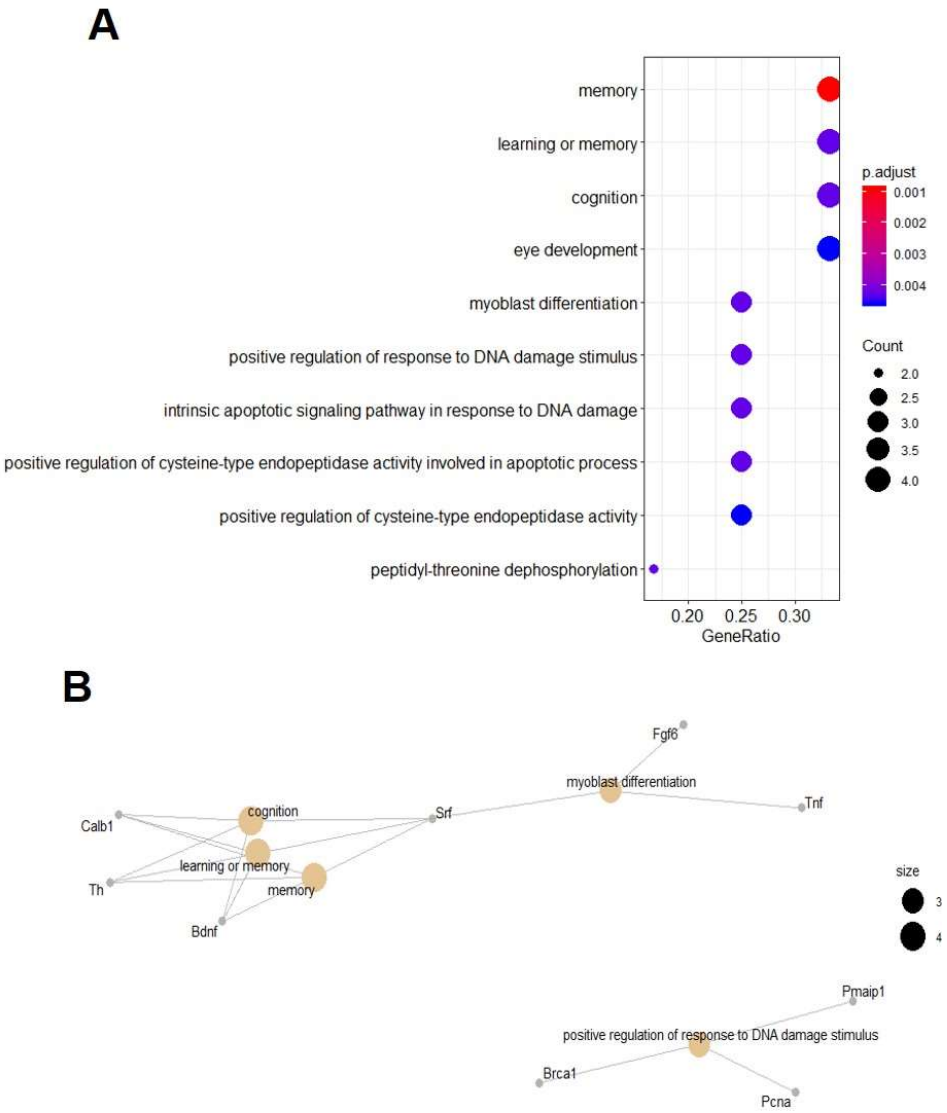

Figure 12 Functional enrichment result of top 10 gene GO BP. A. Dot chart to display gene count or ratio colored by enrichment scores (e.g. p.adjust) with capability to encode another score as dot size (bubble plot) for the DE genes of 24-hour 24. B. Gene-concept network depicts the linkages of genes and biological concepts as a network for the DE genes of 24-hour interval

Chapter 4

DISCUSSION AND CONCLUSION

This study investigated the role of optogenetics-stimulation as a non-invasive tool in skeletal muscle activation on murine triceps-surae. We have used paired comparisons to investigate transcriptional changes following optogenetically stimulated tricep-surae compared to contralateral unstimulated tricep-surae of young mice. We have demonstrated that optogenetic stimulation affects the gene expression of the calcium signaling pathway of skeletal muscle.

4.1 Transient nature of calcium signaling affected the gene expression of 3hour and 24hour interval.

In muscle, external signals (eg: optogenetic stimulation) deeply impact their gene expression (Goldspink; D. B. Jones et al.). Our understanding from the previous experiment as well as the peer-reviewed literature suggest that gene expression changes in activated skeletal muscle is sustained from 1 to 7 days. (Vajtay et al.). However calcium signaling is transient and sustained only in the first few hours following musle activation (Tu et al.). Therefore, designed our study using two time points that could capture calcium signaling transients for the experiments (e.g.,3hour and 24hour interval, post stimulation of the skeletal muscle). From our gene expression findings, we have observed that in 36 genes were differentially expressed 12 genes were differentially expressed in the late time point (24hour). The number of DE genes in 24hour time went down from 36 (at 3hour) to 12, suggesting that the calcium signaling cascade in the skeletal muscle not sufficiently sustained at 24hour.

This significant decrease in gene expression may be the result of the rapid nature of calcium signaling in skeletal muscle (i.e., the release of Ca2+ from the extracellular stores, signaling cascade and regulation of the key genes of the same family happens prior to the 24hour time point. A sustained increase of cytosolic Ca2+ can be deleterious for cell survival which may suggest that, in healthy cells, the cytosolic Ca2+ is not sustained.(Richard et al.; Carafoli).

Since a localized transient change in Ca2+ concentration controls cell migration and muscle contraction, the response of optogenetic stimulation comes from calcium signaling and the response starts as short-term effect such as signal transduction, genes transcription and contraction etc (Berridge, Bootman, et al.; Berridge, Lipp, et al.). Skeletal muscle contraction requires transient increases in intracellular calcium concentration(Lee). In our study, we have found that from all the differentially expressed genes in 3hour as well as in 24hour interval, only a few DE genes works as regulatory elements and transcription factors, and the only common TF between both the time point are *Tnf.*

4.2 TNF plays crucial role in skeletal muscle activation in the response to Optogenetic stimulation

Tnf is a pro-inflammatory cytokine as well as a TF and an important gene in skeletal muscle activation (Bhatnagar et al.). Skeletal muscle releases a variety of myokines (cytokines) in response to certain categories of external stress exposure (optogenetic stimulation)(Welc and Clanton). In our study *Interleukin-6 (Il6)* was another key gene that was upregulated in 3hour interval. The regulation of *Tnf and Il6* as a prototypical stress response myokine and highlight evidence that *Tnf* family and *Il6* gene regulation in muscle is inherently organized to respond to a wide variety of

internal and external stimuli(Welc and Clanton). Upregulation of *Il6* and *TNF-alpha* has been directly correlated with skeletal muscle contraction (Broholm et al.; Chan et al.; Nieman et al.), and gene expression data from our experiments also suggest that pro-inflammatory genes (*Tnf and Il6*), are activated in response opogenetic-regulated muscle contraction. Traditionally dysregulated *Tnf* levels are associated with multiple diseases including neuropathy and paralysis (Benjamin et al.).

Some of the other TF which play a crucial role in calcium signaling and skeletal muscle contraction are activated in 3hour interval, namely early growth response family of zinc finger TF (*Egr1, Egr2*). The *Egr* family of transcription factors are only transiently induced, the impact of this expression has a relatively long-term impact(Ritchie et al.; Poirier et al.). Hence, *Egr1*-induced gene products initiate subsequent waves of gene expression which can ultimately result in cell differentiation, changes in the rate of proliferation or apoptosis(Duclot and Kabbaj).

We have also found that *S100a8* and *S100a9* which are a part the *S100* family and of Ca2+ binding proteins were upregulated in 3hour timepoint. *S100a8* and *S100a9* interact with pro-inflammatory cytokines such as *Il6*, *Tnf* in a high affinity binding(Wang et al.). *S100a8/S100a9* ///shave been previously found to be upregulated in skeletal muscle post exercise. (Mortensen et al.) In our study growth factors like *Fibroblast growth factor 6 (Fgf6)* and *Transforming growth factor beta-3 (Tgfb3)* were downregulated at the 3hour interval. *Tgf beta* is a potent inhibitor of skeletal muscle differentiation (D et al.), in our study down-regulation of *Tgfb6* in response to skeletal muscle contraction supports that claim.

From our results, we have found some key genes and TF's which are associated with skeletal muscle contractions via calcium signaling pathway. Some of

the important gene families in skeletal muscle contractions are, pro-inflammatory cytokines (*Tnf, Il6*), growth factors (*Fgf6, Tgfb*), early growth factors (*Egr1, Egr2*) and cell proliferation/differentiation markers (*Fos, Fosb*). In our study, only a few genes were sustained both 3hour and 24hour timepoints (eg: *Tnf, Pmaip1 and Fgf6*), which led us to conclude that a single bout of optogenetic stimulation does not sustain transcription regulation of calcium signaling related pathway beyond 24hour. This phenomenon likely happened because of the transient nature of calcium signaling. Therefore, we believe the calcium signaling which is induced by optogenetic stimulation resulting into skeletal muscle contraction, the regulation of key gene expression and induction of other signaling pathway related to calcium signaling is short-lived. That is one of the reasons that we are able to observe the maximum number of DE genes in 3hour interval. Some of the key TF's were only a few fold upregulated following optogenetic stimulation at the in 3 hour interval (Table:3)(*Fos*: 3.05, *Egr2*: 2.41), which made us believe if we have to choose another time point to observe the DE genes it should be in between 3-24 hour interval. Previous studies have shown that many key TF's (*Stat2, Egr1, Fos*) were upregulated after an acute exercise of 2- 4 hour time but remain transient and in 24hour time point went back to resting level. (Trenerry et al.; S. W. Jones et al.). This made us believe that choosing another time point between 3 and 24 hours will be helpful in understanding sustained gene expression of the key gene families.

Table 7 Top 10 common GO terms for Biological Process between 3hour and 24hour interval.

GO Terms	Enriched GO BP Description	Gene ID: 3hour Interval	Gene ID: 24hour Interval
GO:0010950	Positive regulation of endopeptidase activity	Stat3/Ppp2ca/Tnf/Pmaip1/S100a8/S100a9	Ppp2ca/Tnf/Pmaip1
GO:0033002	Muscle cell proliferation	Cnn1/Stat3/Prkar1a/Tgfb3/Tnf/Il6/Ptgs2/Egr1/Ncam1/Thbs1	Tnf/Calcrl
GO:0043281	Regulation of cysteine-type endopeptidase activity involved in apoptotic process	Ppp2ca/Tnf/Pmaip1/Il6/Ptgs2/Thbs1/S100a8/S100a9	Ppp2ca/Tnf/Pmaip1
GO:0048659	Smooth muscle cell proliferation	Cnn1/Tgfb3/Tnf/Il6/Ptgs2/Egr1/Thbs1	Tnf/Calcrl
GO:0050727	Regulation of inflammatory response	Per1/Tnf/Il6/Ptgs2/S100a8/S100a9	Tnf/Calcrl
GO:0052547	Regulation of peptidase activity	Stat3/Ppp2ca/Tnf/Pmaip1/Il6/Ptgs2/Thbs1/S100a8/S100a9	Ppp2ca/Tnf/Pmaip1
GO:0070997	Neuron death	Stat3/Fos/Tgfb3/Tnf/Pmaip1/Il6/Creb1/Cdk5/Egr1	Tnf/Pmaip1/Bdnf
GO:0071407	Cellular response to organic cyclic compound	Calr/Stat3/Sgk1/Per1/Tnf/Egr1/Pck2/Sstr2	Brca1/Tnf/Bdnf
GO:1901214	Regulation of neuron death	Stat3/Fos/Tgfb3/Tnf/Pmaip1/Il6/Creb1/Cdk5/Egr1	Tnf/Pmaip1/Bdnf
GO:2000116	Regulation of cysteine-type endopeptidase activity	Ppp2ca/Tnf/Pmaip1/Il6/Ptgs2/Thbs1/S100a8/S100a9	Ppp2ca/Tnf/Pmaip1

4.3 Biological relevance of selected Gene Ontology (GO) category

The Clusterprofiler's EnrichGo (Yu et al.) is a useful tool for annotating GO terms related to differentially expressed genes. In a post-hoc analysis we used EnrichGo for GO analysis to identify highly enriched categories of biological processes with our data. Even though the sources of the differentially genes are from the list genes from calcium signaling pathway, the rationale of the enrichment analysis was to identify the biological processes (and the list of genes enriching the pathways). The results of gene ontology enrichment analysis support that genes identified from the enrichment

analysis can be considered as the biomarker or the future target. The Common GO terms (Table) between both the time points (3 hour and 24 hour, Appendix A) revealed that muscle cell proliferation (GO:0033002), regulation of cysteine-type endopeptidase activity involved in apoptotic process (GO:0043281), regulation of inflammatory response (GO:0050727), regulation of neuron death(GO:1901214) are some of the important biological processes. Further analysis of the GO biological processes (Figure11: A,B: and Figure12: A,B) in both the timepoints revealed that the key genes identified from the DE genes (eg: *Tnf, Pmaip1, Stat3, Egr1, Egr2, Il6, Fos)* were consistently enriched in the top gene ontology biological processes. The role of *Tnf, Il6, Stat3, Egr1, Fos* were well supported in the biological processes, they simultaneously/together enriched the pathways in response to optogenetic stimulation (Figure 14).

Thus, based on previous studies and drawing conclusion from our data we can suggest that adding another time point would be beneficial to observe the gene expression regulation besides 3hour interval. We suggest that the 6hour interval post optogenetic stimulation point will be ideal to compare regulation of specific TF's along with the 3hour time point.

4.4 Therapeutic implications:

Our study could be a useful model system to understand how optogenetic stimulation regulates gene regulation and this can also be translated to understand the therapeutic implication in denervated skeletal muscle paralysis (in spinal cord injury, epilepsy, Parkinson's disease, other neurological disorder). Not only our study did provide a model for optogenetics mediated skeletal muscle contraction which could potentially be used to regulate the production of a biological drug *in-situ*, it also it also

indicates some therapeutic targets by studying specific genes of inflammatory response pathway (*Tnf, Il6*). By repeatedly applying blue light to the skeletal muscle and inducing expression of Proinflammatory cytokine (*Tnf, Il6*) can be used to regulate gene expression which may help in control localization, timing, and dosage. A few FDA approved drugs which are currently available for our newly discovered targets, can be used for potential gene therapy, are listed in Table 8.

Table 8, FDA approved drugs which are currently available for the targets *Tnf* and *Il6.* (Ingenuity Pathway Analysis, IPA, http://www.ingenuity.com).

Gene	Synonym(s)	Location	Family	Drugs	Entrez Gene ID (Human)	Entrez Gene ID (Mouse)
TNF	AT-TNF, DIF, RATTNF, TMTNF, TNF-a, TNF-alpha, Tnfsf1a, TNFSF2, , TNLG1F, tumor necrosis factor	Extracellular Space	cytokine	bortezomib/dexamethasone/pomalidomide, bortezomib/dexamethasone/thalidomide, thalidomide, rituximab/thalidomide, infliximab/methylprednisolone, infliximab/prednisone, bevacizumab/docetaxel/prednisone/thalidomide/zoledronic acid, tumor necrosis factor receptor antagonist, adalimumab/methotrexate, dexamethasone/pomalidomide, prednisone/thalidomide, cyclophosphamide/dexamethasone/thalidomide, certolizumab, golimumab/methotrexate, infliximab/methotrexate, adalimumab, dexamethasone/thalidomide, pomalidomide, afelimomab, etanercept/methotrexate, golimumab, infliximab, certolizumab/methotrexate, bortezomib/thalidomide, etanercept	7124	21926
IL6	BSF-2, CDF, FDGI, HGF, HSF, IFNB2, IFN-beta-2, IFN beta 2A, IFN-Î²-2, IFN Î² 2A, ILg6, interleukin-6	Extracellular Space	cytokine	clazakizumab, ziltivekimab, interleukin-6 receptor inhibitor, siltuximab, tocilizumab	3569	16193

4.5 Future Directions:

The regulation of gene expression via calcium signaling pathway in response to optogenetic stimulation is has shown distinct targets. These targets will be therapeutically useful; however, our study needs to be replicated on few other time points besides 3hour post stim (eg: 4hour, 6 hour) to identify the targets sustained

expression as well as validating the result. Additionally, epigenetic regulation of the same time points might reveal useful insights that will be clinically valuable. The understanding of basic regulatory pathways and identification of mediators leading to *Tnf* gene expression in particular cell types and tissues can provide targets for the design and development of clinically important therapeutic agents that modulate its expression. Additionally, this study needs to be replicated to a bigger number of samples with aged mice before translating to preclinical trials.

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Appendix A

GENE ONTOLOGY ANALYSIS

GO-BIOLOGICAL PROCESS SUMMARY: 3 HOUR INTERVAL

Top 20 GO: BP (sorted based on p.adjust)

GO: ID	Biological Process	p.adjust	qvalue	Gene ID	Count
GO:0033002	muscle cell proliferation	5.89E-09	2.54E-09	Cnn1/Stat3/Prkar1a/Tgfb3/Tnf/Il6/Ptgs2/Egr1/Ncam1/Thbs1	10
GO:0048545	response to steroid hormone	5.27E-08	2.27E-08	Calr/Sgk1/Per1/Tgfb3/Tnf/Il6/Areg/Pck2/Sstr2	9
GO:0043281	regulation of cysteine-type endopeptidase activity involved in apoptotic process	5.60E-07	2.41E-07	Ppp2ca/Tnf/Pmaip1/Il6/Ptgs2/Thbs1/S100a8/S100a9	8
GO:0014706	striated muscle tissue development	6.74E-07	2.90E-07	Srf/Prkar1a/Fos/Creb1/Cdk5/Adrb1/Egr2/Egr1/Pln/Ncam1	10
GO:0042063	gliogenesis	6.74E-07	2.90E-07	Stat3/Tnf/Il6/Creb1/Cdk5/Areg/Egr2/S100a8/S100a9	9
GO:2000116	regulation of cysteine-type endopeptidase activity	6.74E-07	2.90E-07	Ppp2ca/Tnf/Pmaip1/Il6/Ptgs2/Thbs1/S100a8/S100a9	8
GO:0052548	regulation of endopeptidase activity	6.74E-07	2.90E-07	Stat3/Ppp2ca/Tnf/Pmaip1/Il6/Ptgs2/Thbs1/S100a8/S100a9	9
GO:0060537	muscle tissue development	6.89E-07	2.96E-07	Srf/Prkar1a/Fos/Creb1/Cdk5/Adrb1/Egr2/Egr1/Pln/Ncam1	10
GO:0051384	response to glucocorticoid	1.13E-06	4.88E-07	Sgk1/Tnf/Il6/Areg/Pck2/Sstr2	6

GO:1901214	regulation of neuron death	1.16E-06	4.98E-07	Stat3/Fos/Tgfb3/Tnf/Pmaip1/Il6/Creb1/Cdk5/Egr1	9
GO:0031960	response to corticosteroid	1.23E-06	5.28E-07	Sgk1/Tnf/Il6/Areg/Pck2/Sstr2	6
GO:0048660	regulation of smooth muscle cell proliferation	1.27E-06	5.47E-07	Cnn1/Tgfb3/Tnf/Il6/Ptgs2/Egr1/Thbs1	7
GO:0048659	smooth muscle cell proliferation	1.45E-06	6.22E-07	Cnn1/Tgfb3/Tnf/Il6/Ptgs2/Egr1/Thbs1	7
GO:0070997	neuron death	1.81E-06	7.78E-07	Stat3/Fos/Tgfb3/Tnf/Pmaip1/Il6/Creb1/Cdk5/Egr1	9
GO:0052547	regulation of peptidase activity	1.81E-06	7.78E-07	Stat3/Ppp2ca/Tnf/Pmaip1/Il6/Ptgs2/Thbs1/S100a8/S100a9	9
GO:0007611	learning or memory	2.21E-06	9.52E-07	Srf/Sgk1/Creb1/Cdk5/Ptgs2/Adrb1/Egr1/Ncam1	8
GO:0050890	cognition	4.93E-06	2.12E-06	Srf/Sgk1/Creb1/Cdk5/Ptgs2/Adrb1/Egr1/Ncam1	8
GO:0006417	regulation of translation	5.37E-06	2.31E-06	Calr/Stat3/Per1/Tnf/Il6/Thbs1/Ppp1r15a/S100a9	8
GO:0010035	response to inorganic substance	7.56E-06	3.25E-06	Fosb/Ppp2ca/Fos/Tnf/Il6/Creb1/Pln/Ncam1/Thbs1	9
GO:0042108	positive regulation of cytokine biosynthetic process	1.11E-05	4.78E-06	Stat3/Tnf/Il6/Egr1/Thbs1	5

GO-BIOLOGICAL PROCESS SUMMARY: 24 HOUR INTERVAL

Top 20 GO: BP (sorted based on p.adjust)

GO:ID	Biological Process	p.adjust	qvalue	geneID	Count
GO:0007613	memory	0.0009165	0.0003701	Th/Srf/Calb1/Bdnf	4
GO:0007611	learning or memory	0.0042723	0.0017254	Th/Srf/Calb1/Bdnf	4
GO:0045445	myoblast differentiation	0.0042723	0.0017254	Fgf6/Srf/Tnf	3
GO:0050890	cognition	0.0042723	0.0017254	Th/Srf/Calb1/Bdnf	4
GO:2001022	positive regulation of response to DNA damage stimulus	0.0042723	0.0017254	Brca1/Pmaip1/Pcna	3
GO:0035970	peptidyl-threonine dephosphorylation	0.0042723	0.0017254	Ppp2ca/Dusp1	2
GO:0008630	intrinsic apoptotic signaling pathway in response to DNA damage	0.0042723	0.0017254	Brca1/Tnf/Pmaip1	3
GO:0043280	positive regulation of cysteine-type endopeptidase activity involved in apoptotic process	0.0042723	0.0017254	Ppp2ca/Tnf/Pmaip1	3
GO:2001056	positive regulation of cysteine-type endopeptidase activity	0.0046057	0.0018601	Ppp2ca/Tnf/Pmaip1	3
GO:0001654	eye development	0.0046057	0.0018601	Th/Srf/Calb1/Bdnf	4
GO:0150063	visual system development	0.0046057	0.0018601	Th/Srf/Calb1/Bdnf	4
GO:0048880	sensory system development	0.0046057	0.0018601	Th/Srf/Calb1/Bdnf	4
GO:2001233	regulation of apoptotic signaling pathway	0.004634	0.0018715	Brca1/Tnf/Pmaip1/Bdnf	4
GO:0010950	positive regulation of endopeptidase activity	0.0053217	0.0021492	Ppp2ca/Tnf/Pmaip1	3
GO:0010952	positive regulation of peptidase activity	0.0058313	0.0023551	Ppp2ca/Tnf/Pmaip1	3
GO:0006301	postreplication repair	0.0058313	0.0023551	Brca1/Pcna	2
GO:0007612	learning	0.0066168	0.0026723	Th/Srf/Bdnf	3
GO:0048592	eye morphogenesis	0.0069266	0.0027974	Th/Calb1/Bdnf	3
GO:0043281	regulation of cysteine-type endopeptidase activity involved in apoptotic process	0.009026	0.0036453	Ppp2ca/Tnf/Pmaip1	3
GO:2001020	regulation of response to DNA damage stimulus	0.0103671	0.0041869	Brca1/Pmaip1/Pcna	3

PREDICTED DOWNSTREAM TARGET FOR THE TRANSCRIPTION FACTORS WHICH ARE DIFFERENTIALY EXPRESSED

TF/Genes	Time/Interval	Up/Down Regulated	Predicted Downstream Targets
Brca1	3 and 24 Hour	Up	Brca2 Uimc1 Rad51 Fam175a Trp53bp1 Topbp1 Mdc1 Trp53 Palb2 Atm
Tnf	3 and 24 Hour	Up	Tnfrsf1a Tnfrsf1b Ripk1 Tradd Ikbkg Traf2 Ikbkb Cd40 Birc3 Tab2
Creb1	3 Hour	Down	Crebbp Crtc2 Ep300 Akt1 Camk4 Rps6ka1 Rps6ka3 Atf1 Rps6ka2 Kmt2a
Maf	3 Hour	Down	Il10 Pax6 Ahr Nfe2l2 Nfe2 Keap1 Gata3 Il4 Crebbp Stat3
Per1	3 Hour	Down	Cry1 Cry2 Csnk1e Clock Arntl Per2 Per3 Csnk1d Btrc Npas2
Stat3	3 Hour	Up	Jak2 Il6 Il10 Socs3 Jak1 Src Lep Il6ra Tyk2
Egr2	3 Hour	Up	Jun Mafb Cebpb Kdm6a Nab2 Paxip1 Kmt2d Kmt2c H3f3a Polr2f
Fos	3 Hour	Up	Jun Junb Egr1 Jund Mapk8 Yy1 Mapk3 Mapk1 Atf2 Mapk14
Egr1	3 Hour	Up	Fos Nab2 Junb Jun Fosb Atf3 Nab1 Trp53 Ier2 Dusp1
Fosb	3 Hour	Up	Jun Fos Jund Junb Egr1 Hdac1 Fosl1 Fosl2 Sirt1 Mapk8

FUNCTIONAL ENRICHMENT ANALYSIS BY CLUSTERPROFILER

clusterProfiler package was designed by considering the supports of multiple ontologies/pathways, up-to-date gene annotation, multiple organisms, user's annotation data and comparative analysis. Following R libraries has been used to perform the enrichment analysis.

- “dplyr”
- “clusterProfiler”
- “org.Mm.eg.db”

Appendix B

IACUC ANIMAL SUBJECTS APPROVAL

RECEIVED
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IACUC

University of Delaware
Institutional Animal Care and Use Committee
Application to Use Animals in Application to use animals in Research
(New and 3-Yr submission)

Title of Protocol: Growth and Mechanobiology of tendon, ligament, and bone	
AUP Number: 1296-2019-0	← (4 digits only — if new, leave blank)
Principal Investigator: Killian, Megan Leigh	
Common Name (Strain/Breed if Appropriate): mouse, rat **Genus Species:** mus musculus; rattus norwegius	
Date of Submission: 12/3/2018	

Official Use Only

IACUC Approval Signature: Steven Talcott, DVM

Date of Approval: 2.1.19

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