Laser and Viral Optogenetic Neuromodulation of Peripheral Nerve Pain in a Rodent Model

Abstract

a) Research Question: Can different pain responses be neuromodulated via optogenetic and optical stimulation?
b) Background: The opioid crisis in the United States is one of the most significant public health problems plaguing our country. Even with increased awareness through media and educational means, the problem is unfortunately worsening. Many individuals are dying from drug overdoses, and many of those overdose deaths can be attributed to opioids and opioid-like painkillers. There is a great need for an alternative to current pain medications.
c) Materials and methods: Using a glutamic acid decarboxylase (GAD) promoter, our group delivered our highly sensitive optogenetic modulator Multi-Characteristic Opsin (MCO) to target inhibitory GABAergic Anterior Cingulate Cortex (ACC), Dorsal Root Ganglion (DRG), and spinal neurons in a rodent model. Opsin delivery was accomplished by both adeno-associated viral (AAV) vectors for optogenetic stimulation and functional gold nanorods for optical stimulation. Optogenetic stimulation was manipulated by a light source that delivered 630 nm red light, which our MCO was specific and sensitive to. We also controlled light delivery with a wireless phone application we created. The light's frequency and intensity were handled through the wireless application. Acute pain responses were assessed via a formalin pain model. Neuropathic pain responses were evaluated via a sciatic nerve ligation model and Von Frey assays.
d) Results: Optogenetic stimulation decreased pain responses by a >60% threshold in both ACC and DRG models. During the early inflammatory phase of acute pain in response to formalin injection into the rodent's hind paw, we saw a significant decrease in pain responses. There was no significant decrease in pain responses to our assay's initial injection (nociceptive) portion. Our Von Frey assay demonstrated that our experimental groups had significantly decreased hind paw retraction, hind paw licking, and overall pain responses.

e) Conclusions: Overall, our experiments demonstrate that neuromodulation of GABAergic, pain-inhibiting pathways within the nervous system can be targeted to reduce multiple pain models, including inflammatory and neuropathic pain.

Research Question

Opioid pain medications are indicated for various diseases and pain states. The United States is the largest utilizer of opioid pain medications, with some studies estimating that the country consumes over 80% of the world's opioid pain medications.[1] With opioid use comes a subsequent risk of opioid misuse and abuse. The United States, unfortunately, is also a leader in drug overdose deaths, with opioid and opioid-like drugs being the primary culprits.[2]

Optogenetic and optical stimulation are novel mechanisms by which neuronal activities can be altered by introducing light or light heat to light or heat-sensitive proteins. Optogenetics can cause an inhibitory or stimulatory response in a targeted tissue, which can be further controlled in frequency and spatiality.[3] Like optogenetics, optical stimulation can induce neuronal activation via light/laser heat energy. The light energy then increases capacitance in a neuron, generating an action potential in that cell of interest.[4]

Our group will utilize different murine models, comparing our neuromodulation mechanisms to control groups. Our pain assays include acute, inflammatory, chronic, and neuropathic pain models. The areas of neuromodulation will consist of the Anterior Cingulate Cortex (ACC), Dorsal Root Ganglion (DRG), and spinal regions of the mice. Our experiment will analyze whether we can effectively reduce pain responses in our mouse model with the delivery of our opsin to the aforementioned areas of the Central and Peripheral Nervous systems.

Introduction, Significance, and Rationale

Introduction

Optogenetics is a novel mechanism by which neuronal activity can be modulated by introducing light to specific proteins that react to light, called opsins. These proteins are typically expressed in the photoreceptors of the human retina and are the physiological basis for vision. These opsin proteins can be introduced to other cells that do not ordinarily respond to light through genetic engineering mechanisms, such as viral vector delivery.

Optogenetics was first developed and utilized in 2003 when Nagel et al. introduced the genes channelrhodopsin-1 (ChR1) and ChR2 from the green algae *Chlamydomonas reinhardtii* into animal models.[5] Since then, various other opsins have been successfully used with varying mechanisms and modalities.[6,7] Other examples of popular opsin proteins include microbial bacteriorhodopsin and halorhodopsin.[8] Commonly, these proteins are used as optical sensors or optical system that can either manipulate physiological systems as well as monitor or display changes in cellular activity.[9]

Multiple animal models have been utilized in optogenetic research in the past. Popular animal models include rodents, non-human primates, *C. elegans*, and drosophila.[10] Of these, rodent models are the most commonly used in behavioral and neural pathology experiments (Fig. 1).[11]

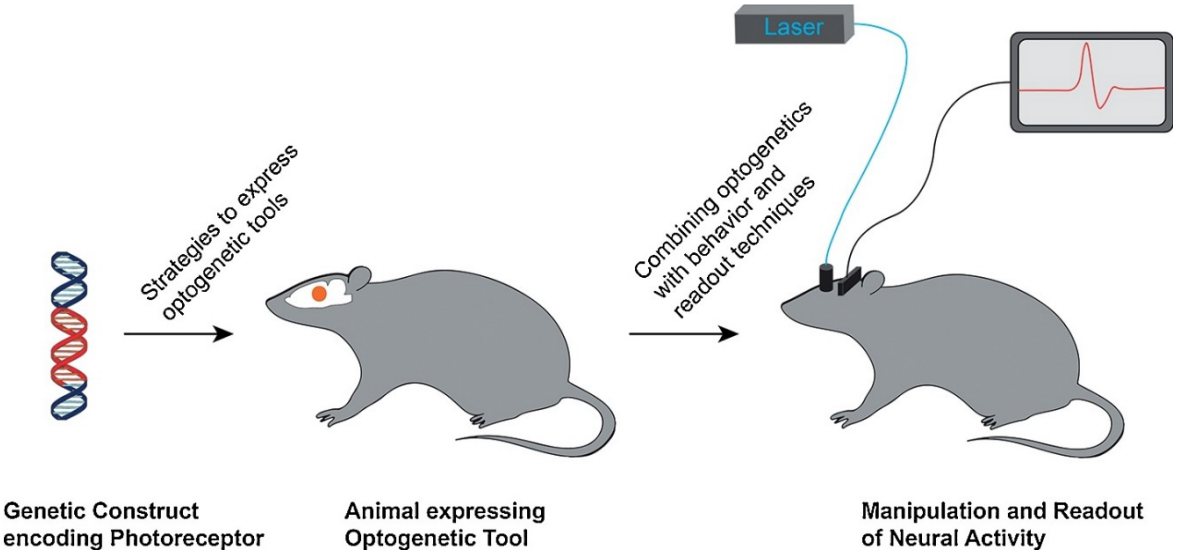


Figure 1. Diagram of optogenetic use for changes in neural activity in a rodent model[12,13]

Another similar mechanism for neuromodulation is optical stimulation via functional gold nanorods.[4] The approach uses specifically bioengineered antibodies that attach to previously identified and sequenced surface proteins on target neurons.[14] Then another bioengineered, secondary antibody that is specific for the primary antibody is added to the region. This second antibody also is tagged with functional gold nanorods that are heat sensitive.[15] By heating the nanorods with a light or laser source, the gold can transmit heat to the surface protein of interest and increase the capacitance within the target cell, leading to the desired action potential.[16]

Using opsins provides significant benefits over other methods of neuromodulation. Traditionally, electrical stimulation has been utilized to modify neuronal signaling. However, these electrical shocks can diffusely travel through the target tissue and ultimately affect other cells in that region. Optogenetics allows for specific cells to be targeted – as cells lacking opsin remain unaffected by light stimulation.

Pain is an uncomfortable sensation perceived by the nervous system that can have a variety of different causes. It is classically seen as an essential sensation that occurs due to tissue damage. Pain can be seen as a complication of diseases, different emotional states, and even a standalone pathology. However, it can occur chronically and become debilitating for individuals. Therapies for pain conditions are costly and complicated by adverse events (often related to off-target effects). Additionally, many popular drugs used for pain management develop tolerance in humans rapidly, leading to decreased benefits in terms of pain reduction over time.[17] Furthermore, these drugs are extremely addictive.[18] Together, addiction and tolerance can work together to lead to fatal consequences for those being treated for pain. Targeted treatments to areas involved in pain perception with a specific and easily modulated therapy can avoid these pitfalls.

The Anterior Cingulate Cortex (ACC) is a region in the brain shown to be involved in the pain perception process.[19] To our knowledge, the reduction of pain perception via ACC lesions in a mammalian model, has not been thoroughly supported in the literature. On the other hand, a previous study has demonstrated an increase in pain responses via ACC lesions.[20] In our earlier project, selectively targeting and stimulating the GABAergic neurons within the ACC successfully decreased pain perception in a mouse model.[21] By delivering an opsin protein to

these GABAergic cells, we can achieve the specificity necessary for pain modulation, unlike previous studies that utilized regional electrical stimulation. This specificity can also be achieved via bioengineered antibodies that are tagged with functional gold nanorods.

The central nervous system (CNS) is the major neural network within the human body and is composed of the brain and spinal cord. The Dorsal Root Ganglion (DRG) is a group of sensory neurons near the spinal cord which crucial in the neural transmission of pain from the individual, distal inputs.[22] Unlike present therapies used to manage pain, reduction of pain at the level of the DRG can provide specificity to certain target tissues, without potential unwanted side effects and neurobiological addictive potential.

Peripheral nerve pain syndromes often occur secondary to surgery or trauma but can also be idiopathic in nature.[23] Many individuals who suffer from peripheral nerve pain will have the sensations of allodynia or hyperalgesia. Allodynia is pain from normal, non-painful stimuli, whereas hyperalgesia is exaggerated pain from stimuli that normally would cause a slight pain response.[24] Targeting the DRG can be a way to address peripheral nerve pain syndromes exclusively.

Currently, the primary treatment available for chronic pain is opioid medications. These medications are highly addictive and problematic, with an estimated 82,000 deaths in the United States in 2025 if the current status quo is maintained.[25] The opioid crisis is a growing issue in the United States, without any potential solutions or alternatives.

Our project looks to introduce optogenetics into a neuropathic pain rodent model using an Optogenetic Pain Modulator (OPM). This device will allow for optogenetic control via a smartphone Bluetooth connection. Additionally, the OPM will be able to quantify the neural stimulation of the ACC *in vivo*, which will be essential in data collection.

Significance

Opioid medications are currently the preferred treatment for chronic and acute pain. While these medications can successfully treat pain, their use can lead to addiction and fatal overdose. In 2016 alone, the Opioid Crisis led to over 40,000 deaths in the United States.[26] Since then, that number has grown significantly, even with government interventions focused on reducing overdose deaths.[27] Furthermore, chronic pain is a widespread condition. Over 20% of U.S. adults are estimated to be dealing with chronic pain, with about half having high-impact chronic pain.[28] Without serious interventions, the number of Americans affected by the Opioid Crisis is expected to increase significantly in the coming years.

The protocol and conclusions from this study could be utilized for future neuromodulation experiments with indications beyond pain. Neuromodulation via optogenetic-based stimulation provides a novel cell-specific targeting mechanism that can be used for various other neurological disorders such as addiction,[29] depression,[30] and many other diseases outside of neuroscience.

Rationale

To our knowledge, there have been no neuromodulation alternatives to opioids for pain management. Optogenetic-based neuromodulation can be used for therapeutic benefit in various other neurological disorders. We hope to develop a novel mechanism by which pain can be effectively alleviated without the risks associated with opioid medications.

Research Materials and Methods

Materials and Methods

General Study Details and Resources

Our group has developed an implantable OPM prototype that can deliver very low-intensity light (10 $\mu W/mm^2$) via a fiber optic probe. The light distribution will be delivered via LED and localized to a small red-light region (~1 mm). The amount of light will be altered throughout the experiment with varying light power, wavelength, and pulse pattern levels.

The OPM prototype can also measure the electrical responses in the ACC via *in vivo* electrophysiology. During our different assays, the activity of these GABAergic neurons will be recorded during/after the administration of noxious stimuli. Additional sources of noxious pain, such as heat and chemical irritants, are still being considered for future experiments.

Multi-Characteristic Opsin (MCO) delivery

Opsin gene (MCO) will be delivered via Adeno-associated viral (AAV) vector injection into the ACC region of the rodent model. The gene will be targeted to the specific GABAergic drugs via

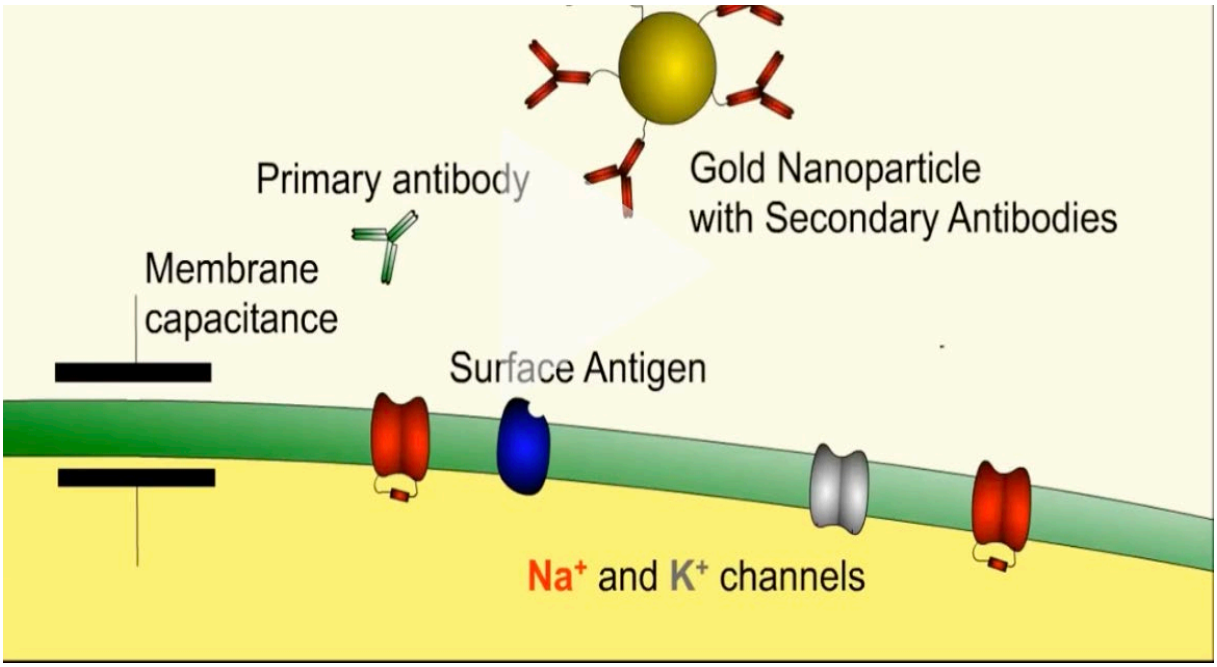
promoter-specific design to avoid affecting neighboring neurons. The transduction delivery of MCO will be detailed by imaging, immunostaining, and other functional/behavioral assays. MCO is specific and highly sensitive to ~630 nm red light.

Production of opsin (GAD67-MCO2-mCherry) and Viral Vector Delivery Mechanism

Our opsin GAD67-MCO2-mCherry was developed by Genscript peptide synthesis services. This protein was produced as Paav-Gad67-MCO2-mCherry. GAD67 was the targeted GABAergic inhibitory neuron of the ACC. MCO2 was our opsin protein sensitive ~630 nm red light. mCherry was chosen for immunostaining purposes. This entire peptide was contained in AAV5 viral construct provided by Vigene Biosciences. From this point on, the final, complete viral form will be denoted by pMCO2.

Optical Stimulation Mechanism via Functional Gold Nanorods

Primary antibodies were developed for GAD67 to target GABAergic cells of the pain pathway. Secondary antibodies were developed specifically for these primary antibodies, with binding sites for functionalized gold nanorods (fGNRs). A pulsed low-intensity laser delivered optical stimulation to induce capacitance changes in the cells of interest (Fig. 2).



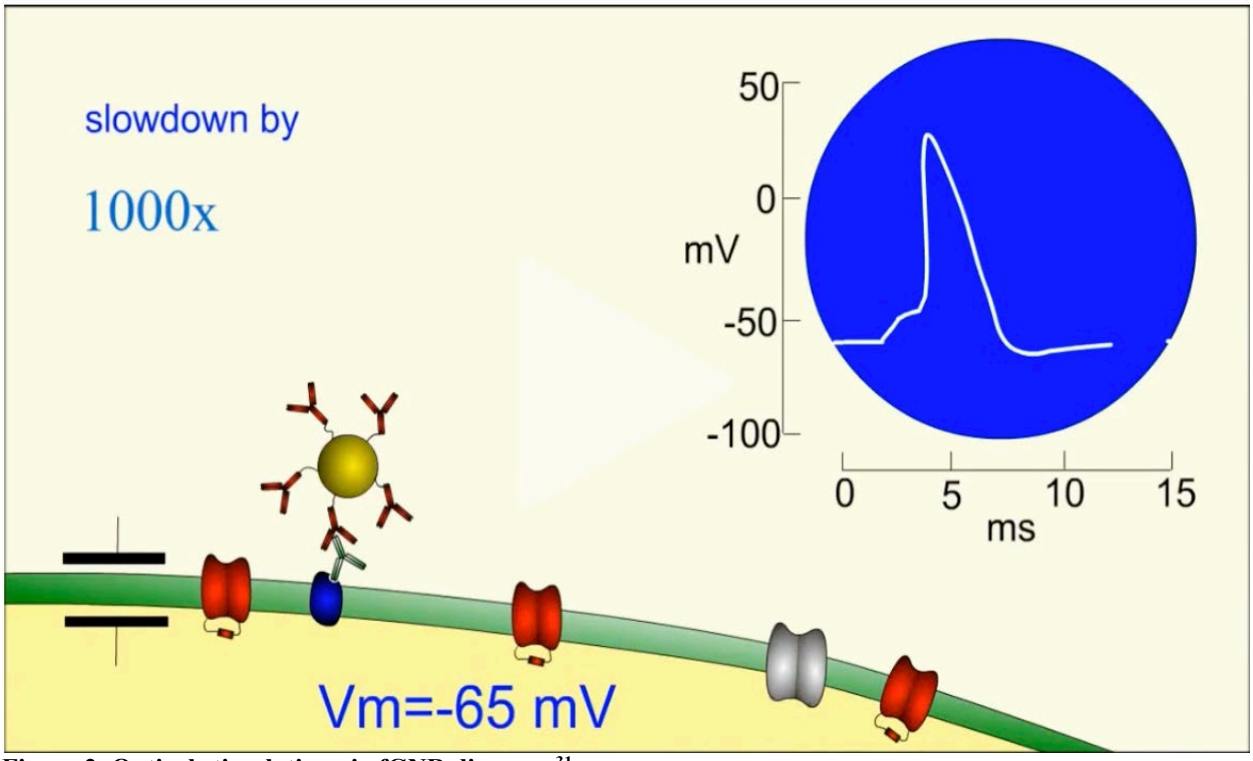


Figure 2. Optical stimulation via fGNR diagram.[31]

a) Primary antibody developed for specificity to GAD67 (GABAergic) neuron in the pain pathway. A secondary antibody with fGNRs is delivered to bind to the primary antibody.

b) Binding and delivery of laser allow for heating of gold nanoparticle. A laser is delivered to the gold nanoparticles, which transmit heat to the target cell's membrane, thereby increasing the target cell membrane capacitance and allowing an action potential to occur.

Mouse Models

C57BL/6J (wild type) mice were obtained from Jackson Laboratory. All the mice used in our experiments were maintained on a 12:12 hour light cycle and in strict compliance with IACUC on the use of animals in research. The mice were housed and investigated humanely.

Fiber optic stimulation implant and ACC transfection with pMCO2 in WT mice

An aseptic technique was used for all procedures, and surgical tools were sterilized in an autoclave. The WT mice were given 2-3.5% isoflurane anesthetic, and the fur over the scalp was removed chemically. A midline incision was made, and the skin was removed from the ACC region. A burr hole was made over the ACC (0.7 mm anterior to bregma, 0.4 mm lateral from the midline, and at a depth of 1.8 mm from the skull surface), and a 1.5mm long implant was installed and secured to the skull with cyanoacrylate and dental cement. pMCO2 was injected into the ACC at this point with a syringe – in a dose-dependent manner. The mice were kept in normal conditions for 2 weeks to allow the proper expression of the optogene and acclimatization to the newly placed implant. The novel Bluetooth-controlled, back-mounted, optogenetic stimulation device stimulates the ACC via this permanently implanted cannula.

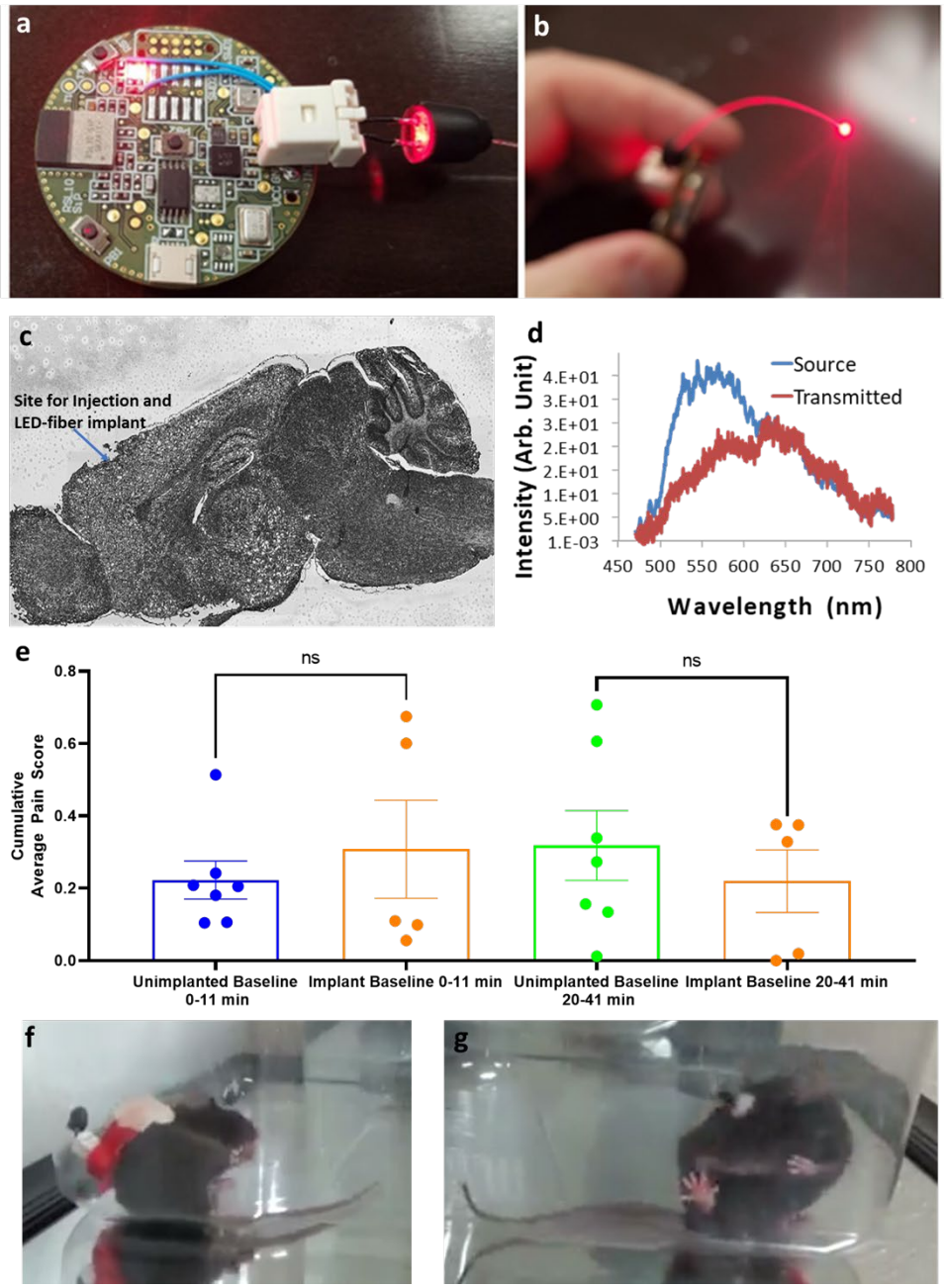


Figure 3. Optogenetic stimulation apparatus allows freely mobile optogenetic pain modulation with a minimally invasive implant that does not significantly affect pain response. a) The Bluetooth-enabled optogenetic stimulation device allows wireless control of optogenetic modulation of pain. b) Optical fiber emits low-power red light while being minimally invasive (c). This is due to the propagation of red light through brain tissues d) as shown in the intensiometric analysis of light propagation through a brain section. e) The presence of the implant does not significantly affect acute pain responses in formalin assay as measured by quantifiable behaviors such as f) licking and g) paw lifting.

Formalin Assay

For an acute, inflammatory pain model, 1% Formalin solution was used. Each mouse was given a 20-microliter injection of the 1% Formalin solution upon awakening from anesthesia within a holding chamber. Mice were then observed in 5-minute intervals, for a period of 60 seconds. During each 60-second period, the mice were watched for two pain-related behaviors – hind paw lifting and hind paw licking. The total time was recorded for each of these behaviors for a total of 45 minutes. The subsequent formula determined pain scoring:

$$((2 * \text{Paw Licking time}) + (1 * \text{Paw Lifting time}))/60$$

This experiment was repeated in implanted WT mice with and without light stimulation (2 or 5 Hz at ~630nm on variable schedules) to determine any statistically calculable differences in pain responses. The experimental strategy is charted in Figure 5a.

Sciatic Nerve Cuffing

Mice were anesthetized with isoflurane or intraperitoneal injection of 4 ml/kg of a mixture of ketamine (17 mg/ml) and xylazine (2.5 mg/ml) in sterile Phosphate Buffered Saline (PBS). Hind leg fur was removed chemically, then the areas were cleaned with 70% Ethanol. The sciatic nerve was exposed through a muscle-sparing incision along the sciatic vein between the semitendinosus and the biceps muscles. The two muscles were gently spread to expose 1.5 cm of the sciatic nerve. A sterile glass hook was used to lift the main branch of the sciatic nerve. The sciatic nerve was hydrated with sterile PBS. Using a 4-0 silk suture, the main branch of the sciatic nerve was tightly ligated. The muscles were then sutured using a 4-0 suture. The

overlying skin was closed using staples. The mouse was allowed to recover on a warm pad. By the day following the cuffing procedure, and for several months thereafter, the mice develop guarding behavior of the ipsilateral hind paw to mechanical stimulation. This is often used as a model of neuropathy-induced pain.[32] An alternative model system for neuropathy was Streptozotocin-induced diabetic neuropathy.[33] In addition to mechanical allodynia, sciatic nerve cuffing often includes thermal hyperalgesia for a roughly 3-week period.[34]

Conditioned Placement Preference Assay

Mice are evolutionary conditioned to prefer a dark environment over a light one.[35] However, our group wanted to examine whether neuromodulation of pain responses could alter this innate, evolutionary tendency from dark environments towards light environments. To do this, we created a Conditioned Placement Preference (CPP) assay, which is a classic behavioral assay performed in the literature for rodent studies.[36]

Pre-conditioning phase: The pre-conditioning phase began with sciatic nerve ligation in all experimental mice. The light/dark separation doors were lifted (days 1-3), permitting unrestricted access across the apparatus. The experimental mouse was placed in the middle transfer chamber, and its activity and transfer between the two chambers were observed for a total of 15 minutes. The baseline preference was determined by the amount of time spent in the two side chambers on the third day. It was excluded if the mouse entered either side chamber less than four times.

Conditioning phase: During conditioning (days 4 to 9), the mice were confined within the treatment (well-lit) chamber or the unlit (no experimental treatment) chamber for a total of 45 minutes. 10 hours later, each mouse was confined within the opposite chamber from their morning association session for 45 minutes. While in the treatment-associated chamber, the mouse was given optogenetic stimulation at a rate of 5 Hz. When in the non-treatment paired chamber, the mounted Bluetooth stimulation device was placed on the mouse but not active.

Testing phase: During testing, the mouse was placed in the middle passage and allowed free access to the entire apparatus. During their exploration, their activity was recorded for 15 min. These post-conditioning tests were carried out 3, 6, and 10 days after conditioning. The mouse was placed in the middle passage during live testing as normal. Still, the optogenetic stimulation was active whenever the mouse entered the lit chamber and deactivated when it was in the middle passage or the unlit, dark chamber.

The percentage of time spent in either chamber ("chamber time") of the entire 15-minute testing session at each time point was calculated. The overall experimental outline and CPP apparatus are illustrated in Figure a.

Von Frey Assay

The Von Frey Assay is a classic mechanism for detecting pain responses in rodents.[37] It requires a meshed cage and different filaments that represent different levels of allodynia and hyperalgesia. Baseline mechanical thresholds for withdrawal response were established for each experimental mouse via an increasing or decreasing presentation method. After an adaptation

period of at least 30 minutes, the testing phase began, and optogenetic stimulation was initiated if applicable for the test. Starting at 0.6 g, the hind paw was stimulated with a Von Frey filament (pressing the tip to the plantar surface until it bent). This presentation was repeated 5 times, and the presence or absence of a response was noted. 2 minutes of time was left between each presentation. If at least 3 of the 5 results were positive, a lower filament was used, if less than 3 of the 5 presentations were positive for a withdrawal response, then the next higher filament was used. This was continued until a minimum threshold for at least 3 out of 5 results being positive was established. After sciatic nerve constriction (see above), the mouse's hind paw was tested in the same manner. For timed treatment, the methodology was adjusted to the standard "up-down" method in which a test was immediately followed (with approximately a 1-minute break) with the next higher or lower filament depending on if the presentation was negative or positive (respectively) for a withdrawal response. This continued until at least 3 presentations were positive for a particular filament. This was necessary as the normal 2 minutes would not allow the test to be completed in a timely manner. The overall experimental process and Von Frey assay setup are shown in Figures 6A and B, respectively.

Immunostaining Assay to determine tissue immunogenicity and viability

Mice were sacrificed within a carbon dioxide gas chamber. Their brains were removed and preserved in Paraformaldehyde (PFA) 4% for 8 hours. The brains were transferred to a solution of 30% sucrose (weight/volume), then underwent cryoprotection. Brains were then sectioned and stained with multiple primary antibodies, including anti-IBA1, anti-CD45, anti-GAD65 (1:100), or anti-caspase (1:250) as well as anti-MCherry (1:250 or 1:500) for fluorescence. Secondary antibodies (1:500) were added after incubating the primary antibodies overnight. Finally, the

samples were stained with 4′,6-diamidino-2-phenylindole (DAPI), with a coverslip placed on each individual slide. The slides were finally imaged using confocal microscopy.

Statistical Analysis

Statistical analysis will be done by one-way variance (ANOVA) followed by Tukey's post-hoc tests. Statistical significance will be determined by a $p<0.05$. Box plots will evaluate the median, spread, and range of data obtained. Pearson's Chi-Square tests will evaluate the relationship between MCO expression, light delivery, and pain alteration.

Results

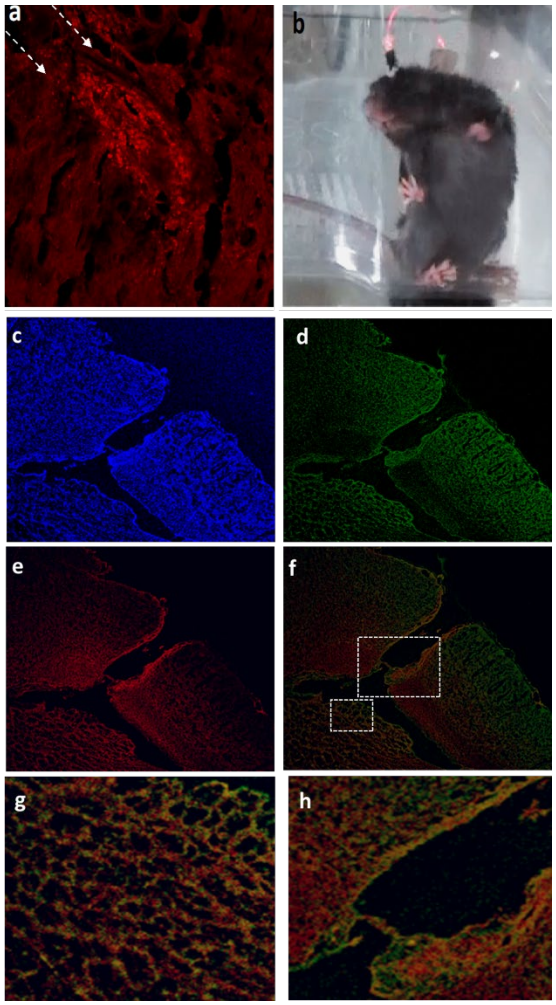


Figure 4. Expression of MCO-mCherry in the ACC allows Optogenetic modulation of pain.

a) Gad67 promoter-driven MCO intrinsic mCherry expression in the ACC 2 weeks after AAV-MCO injection (optical fiber outline indicated by arrows). b) Wireless fiber-coupled red LED implanted in MCO-transfected mouse during formalin assay c) DAPI (living cell stain), d) GAD65-marker for GABAergic (inhibitory) neurons, e) MCO Reporter-mCherry (fluorescent stain). f) Overlay of GAD65 & mCherry (showing opsin in inhibitory cells). g-h) Zoomed areas (marked by the rectangle in f) showing colocalization of GAD65 and mCherry

Post-experimental analysis of sectioned tissues displayed transfection of the ACC present with Gad65-labelled cells (Figure 4).

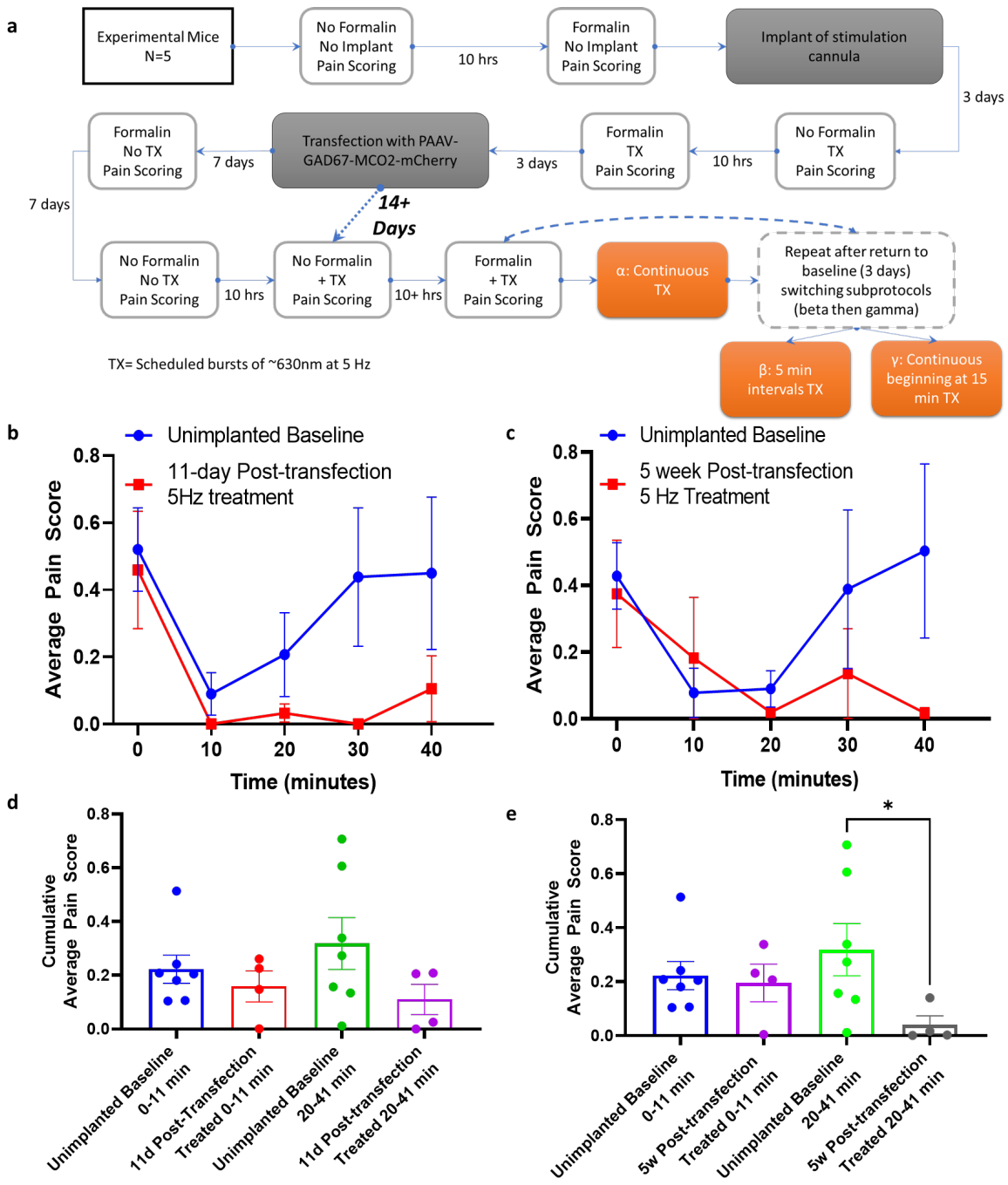


Figure 5. Pain responses are effectively decreased with constant 5 Hz therapy during the optogenetic transfection process within the ACC.

A) Diagram of the investigational method for inflammatory, acute pain model. Formalin was given, then scoring was done in 10-minute intervals with constant 5 Hz therapy (630 nm). The control baseline was compared to the experimental group.

B) 11 days post-transfection average pain scores

C) 5 weeks post-transfection average pain scores

D) 11 days post-transfection cumulative pain scores in the early (0-11 min) and late (20-41 min) phases

E) 5 weeks post-transfection cumulative pain scores in the early (0-11 min) and late (20-41 min) phases

Avg ± SEM. N=7 at baseline and 4 in the treatment group. * = p<0.05

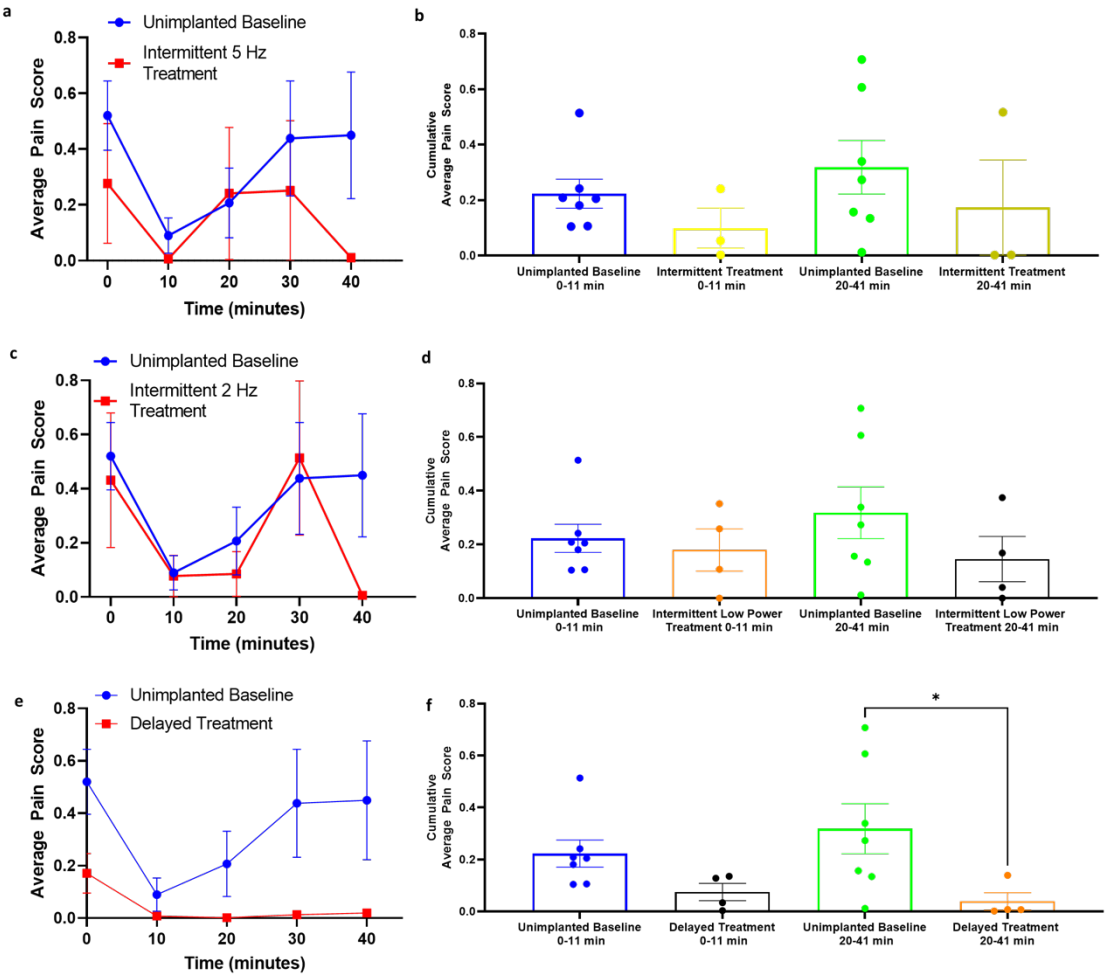


Figure 6. Scheduling pain treatment to the ACC reduces acute pain responses

Formalin acute pain assay was scored within 10-minute intervals at a light stimulation frequency of 5 milliseconds (630 nm).

a) 5-minute on-and-off intervals at 5 Hz

b) 5-minute on-and-off intervals at 2 Hz

c) Therapy at a frequency of 5 Hz starting at 15 minutes after injection

The mean of the aggregate pain scores in mice with intermittent therapy (figure 6D), subdued intensity therapy (figure 6E), and deferred therapy (figure 6F).

Avg ± SEM. N=7 at baseline and 4 in the experimental group. * = p<0.05

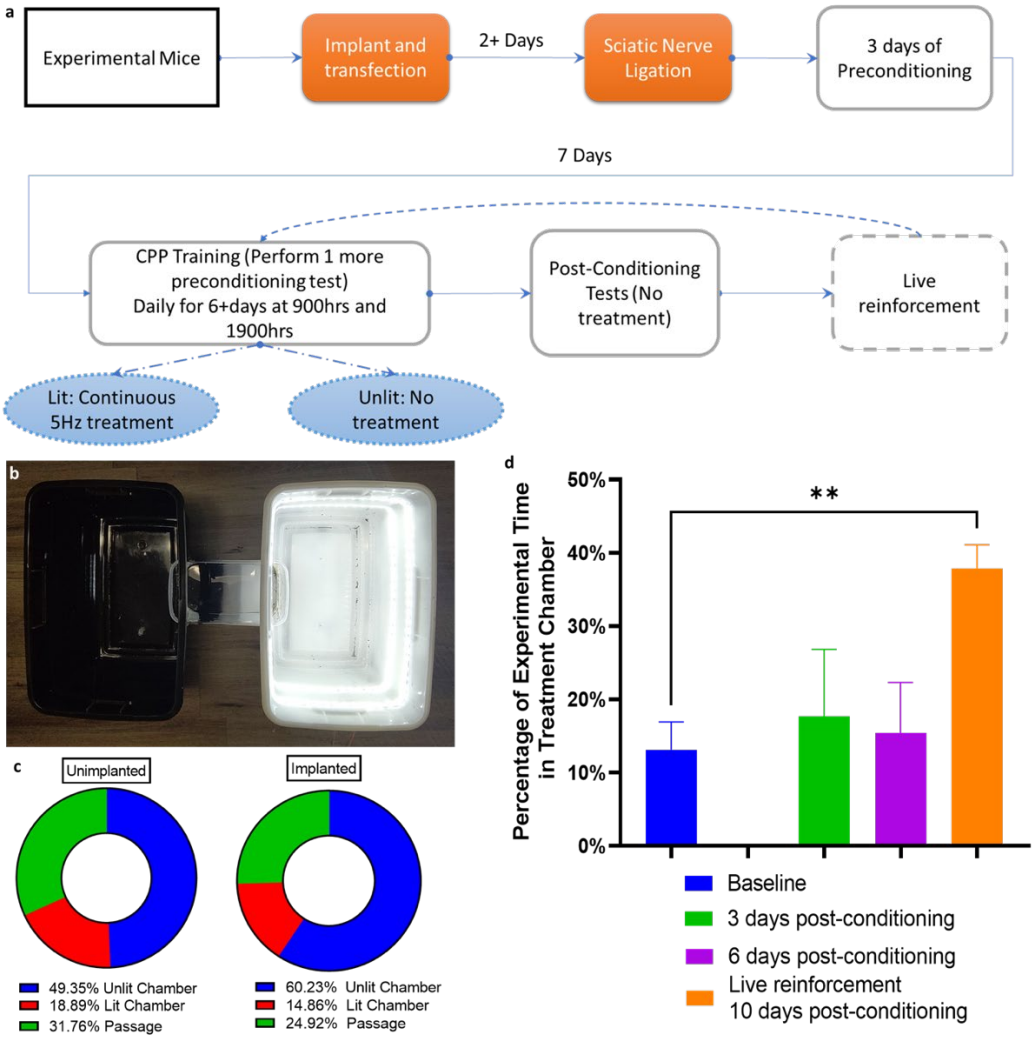


Figure 7. Light/Dark Inclination after optogenetic therapy of the ACC.

A) Schematic for chronic pain trial. B) Light/Dark cage with central transfer chamber. C) Donut pie chart with light/dark chamber tendencies for control and experimental, conditioned mice. D) Preference for the light chamber with conditioning and live reinforcement with optogenetic therapy. Avg ± SEM. N=4. ** = $p < 0.01$

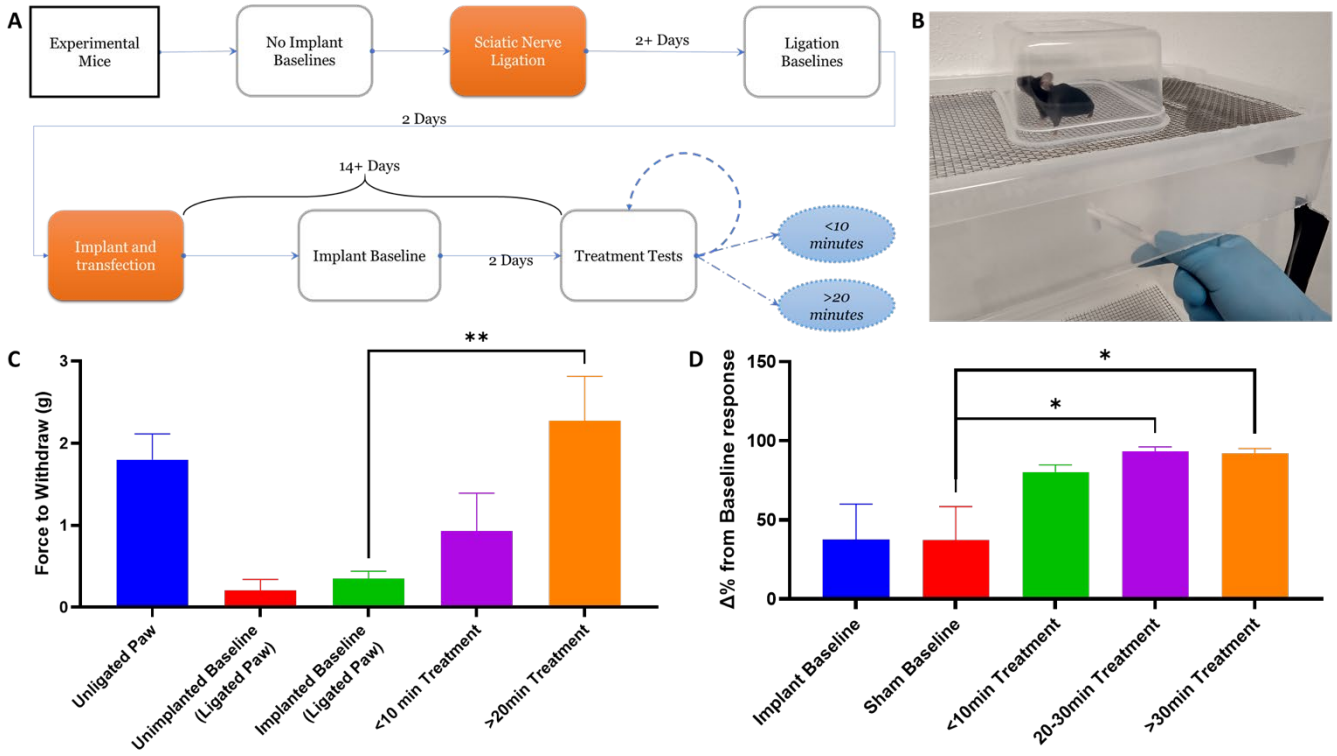


Figure 8. Allodynia and Hyperalgesia responses to mechanical stimulation are reduced with optogenetic therapy of the ACC.

A) Schematic of experiment design for tactile pain and sensitivity assay. B) Von Frey cage displaying filament for mechanical stimulation of mouse hindpaw. C) Chart with minimum filament force required for the mouse to withdraw its hindpaw. D) Chart with percent change from baseline withdrawal force within treatment group. N=4. Avg ± SEM. *= $p \leq 0.05$, **= $p \leq 0.01$

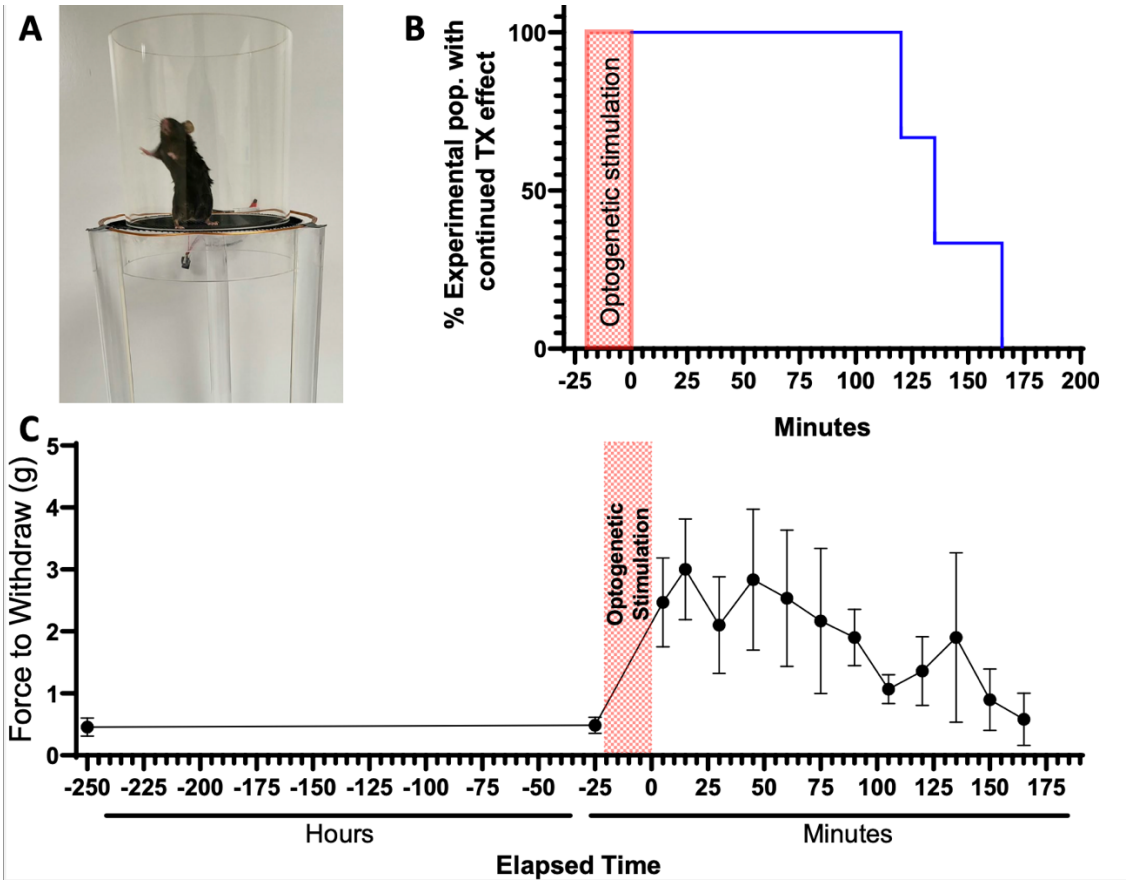


Figure 9. Persistence of neuromodulatory effect within mechanical stimulation experiment in MCO-based optogenetic laser therapy of the ACC.

A) Von Frey cage.

B) The percentage of the experimental mouse group displaying decreased sensitivity to peripheral mechanical stimulation after optogenetic therapy.

C) Force to withdraw hindpaw in hyperalgesia assay after the termination of optogenetic therapy (20 min, marked by a red bar). N=6, Av. ± SEM.

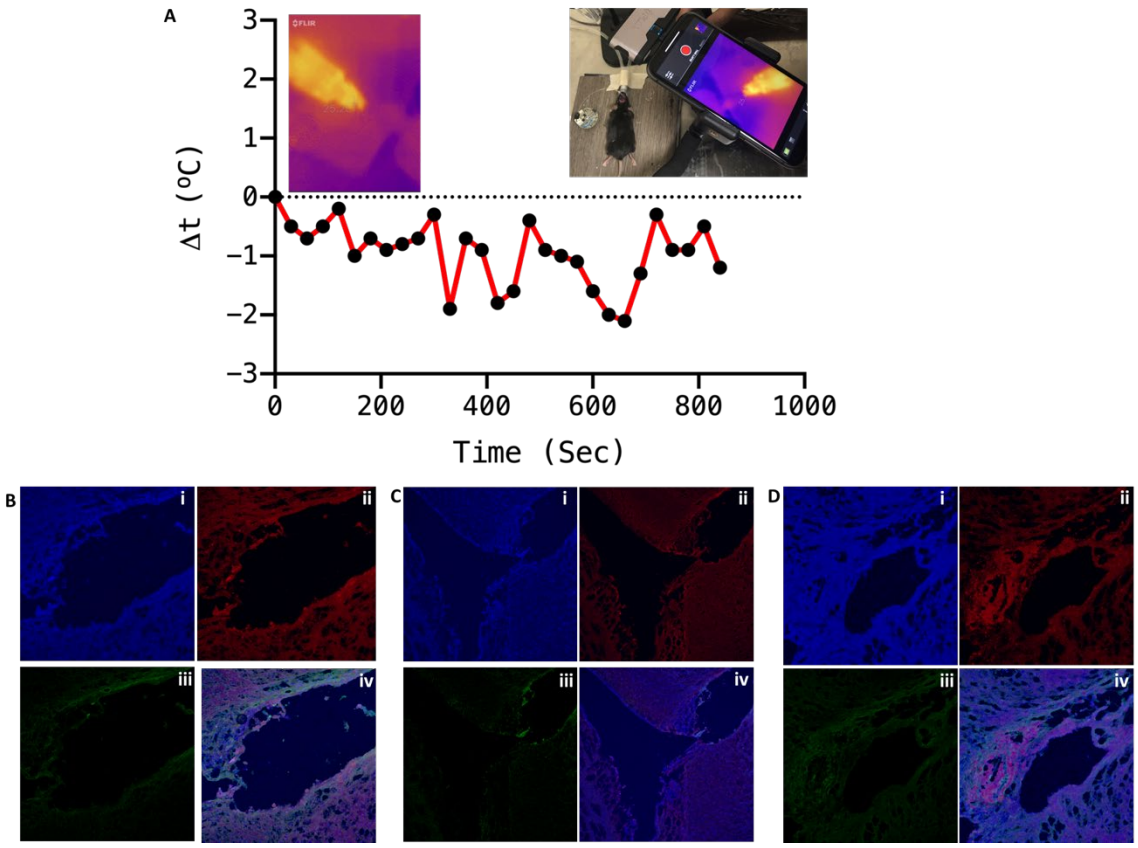


Figure 10. Continuing safety of optogenetic apparatus and therapy.

A) Temperature change with light stimulation of mouse live brain via IR camera.

B) ACC-GABAergic neurons expressing MCO-mCherry after experiments and continuous optogenetic therapy. i) DAPI; ii) MCO-mCherry; iii) Caspase-3; iv) Bi-iii overlay.

C) AAV-MCO injection induced MCO expression without any additional significant immune response. (i) DAPI; ii) mCherry fluorescence confirming MCO-expression; iii) CD45 immunostaining (green) not present in ACC regions injected with 3 ml of AAV-MCO (8 x 10^{12} vg/ml); iv) Ci-iii overlay.

D) Absent immune reaction to insertion of the implant in ACC and after AAV transfection of MCO-mCherry. i) DAPI; ii) MCO-mCherry; iii) Iba1 (microglial/macrophage marker); iv) Di-iii overlay.

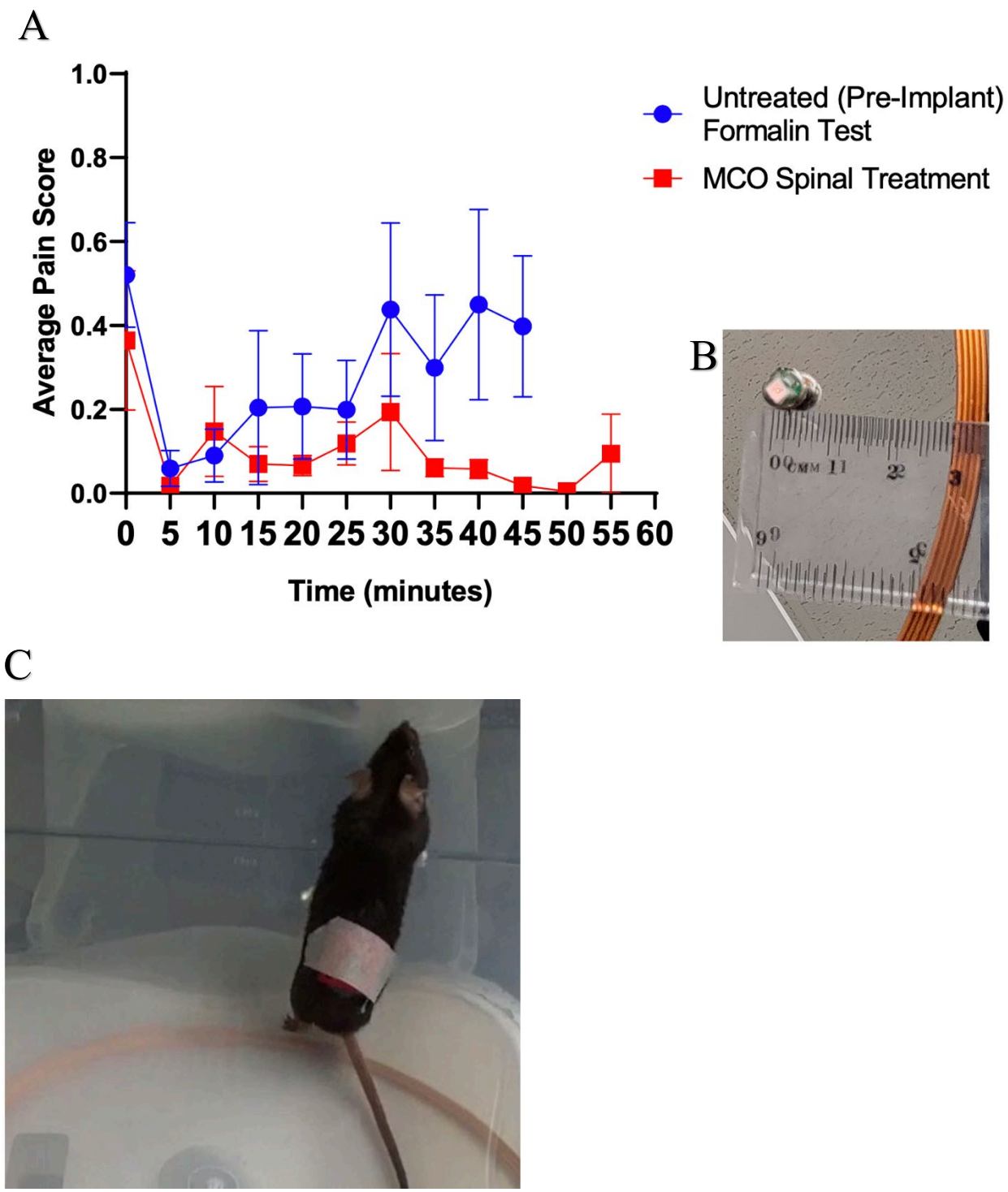


Figure 11. Laser neuromodulatory effects in formalin acute pain assay in MCO delivery to the spine.

A) Average pain scores after injection of formalin in hindpaw with 5 Hz optogenetic therapy (630 nm).

B) Spinal light implant 5 mm in diameter.

C) The implant was placed on the dorsal side of the mouse near the hindlimbs. N=4 Avg± SEM.

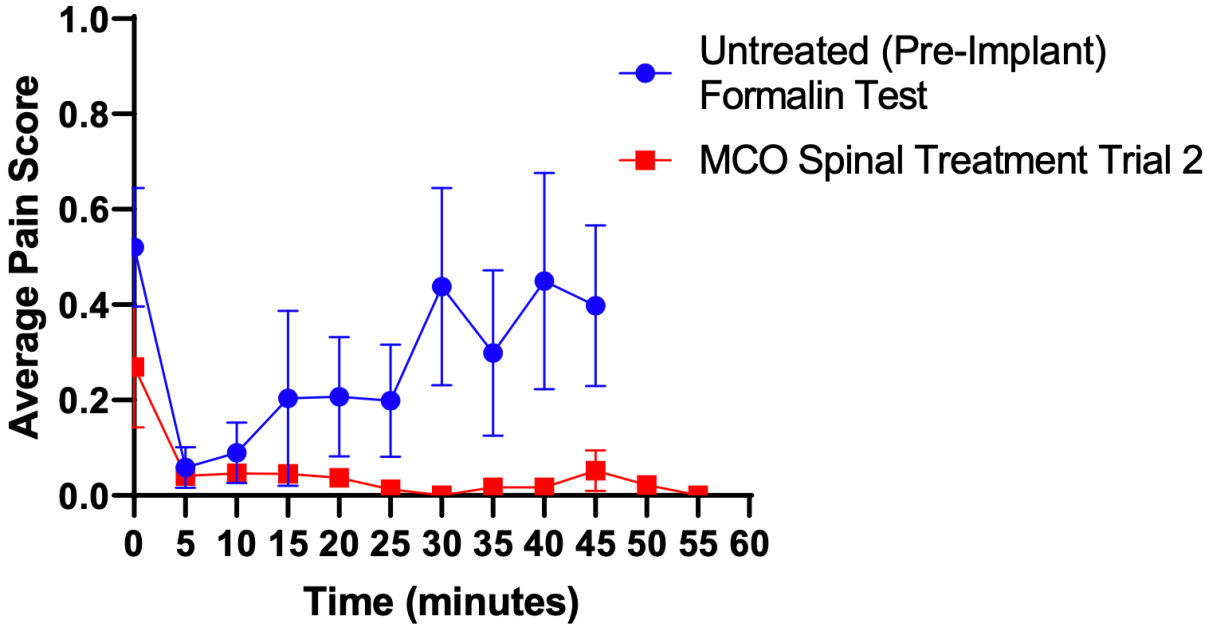


Figure 12. Neuromodulatory effects of high-powered optogenetic laser stimulation in formalin acute pain assay after MCO delivery to the spine.

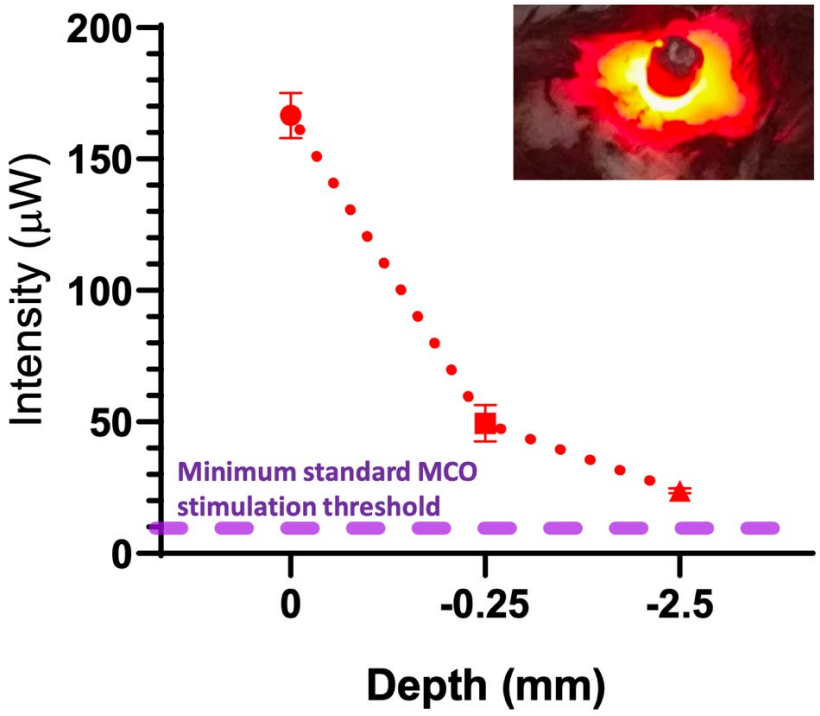


Figure 13. The intensity of laser stimulation (in microwatts) and the resulting depth of penetration (in millimeters). The minimum threshold intensity required for MCO stimulation is shown in purple.

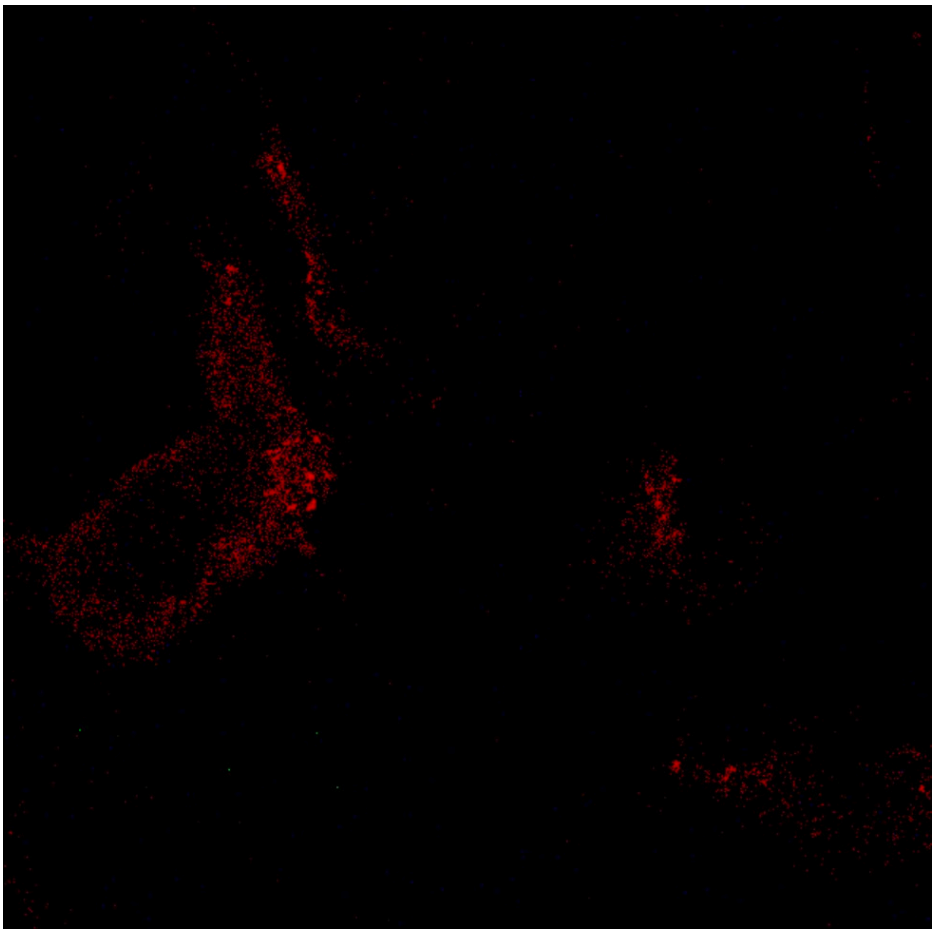


Figure 14. Expression of MCO within the spinal cord after nano-enhanced laser gene delivery via mCherry immunostaining of neural tissues.

Discussion

This study successfully demonstrated that optogenetic neuromodulation reduces acute and chronic pain responses and behaviors in a murine model. Our experiments successfully targeted both the Central Nervous System (via the ACC) and the peripheral nervous system (via the DRG of the spinal cord). To target every area of the pain pathway, however, we might need a glutamatergic (excitatory) opsin in addition to the GABAergic (inhibitory) opsin we utilized in our experiments. We have also shown there to be a benefit to different types of pain – including acute, inflammatory, chronic, and neuropathic pain.

With immunostaining, we were able to confirm that our AAV gene delivery system successfully delivered our GABAergic opsin. This was confirmed with the red mCherry immunostaining seen in Figure 4A. Additionally, we were able to confirm that these neurons were alive (via DAPI stain in Figure 4C), GABAergic (Via GAD65 marker stain in Figure 4D), and expressing the promoter of our opsin protein (via MCO reporter-mCherry staining in Figure 4E). The overlay in figures 4G-H confirms that all MCO-expressing cells were alive and GABAergic, as intended.

The formalin assay is classically used in rodent models for acute pain behaviors (nociceptive and inflammatory). These behaviors include licking and lifting the hind paw that is injected with formalin. By recording and observing these behaviors, we were able to develop a pain-scoring system. The early phase was due to nociceptive pain from the injection needle. This phase was unchanged by the optogenetic stimulation (Figures 5 and 6). The second, inflammatory pain phase was specifically caused by formalin. This, combined with the relative lack of effect in

phase one during continuous stimulation, implies that optogenetic stimulation is sufficient for inflammatory pain but does not affect the initial warning nociceptive pain that provides valuable information about the localization and intensity of bodily damage. This could prove highly valuable as the pain modulation would affect the distracting and debilitating secondary phase of pain without leaving one numb to acute pain and the associated tissue damage.

Pain scores were confirmed to be significantly reduced with ACC optogenetic neuromodulation. The optogenetic neuromodulation's effect on pain scoring is shown in Figures 5A-D. This benefit was seen as soon as 11 days after transfection, and the improvement continued 5 weeks post-transfection (Figure 5B-E). The control group of mice displayed a higher cumulative pain score throughout the experiment when compared to the mice receiving optogenetic therapy. Within the acute pain assay, the investigational group received 5 Hz of therapy within a 5-minutes active and 5 minutes inactive, intermittent scheduling pattern (Figures 6A-D). The effect of this intermittent treatment schedule on the late phase of pain is reduced compared to the 5-week post-transfection late phase pain response with constant treatment (87.4% decrease in pain with constant therapy and 45.5% decrease in pain with intermittent therapy). The delayed therapy group received optogenetic therapy 20 minutes after formalin was injected, and this group displayed even greater decreases in pain scores in the late inflammatory phase (Figure 6E-F). Altogether, our experiment confirms that optogenetic neuromodulation of the ACC can decrease pain responses in a constant, intermittent, and delayed fashion. Dosing the therapy constantly at a frequency of 5 Hz, whether given immediately after injection of formalin or in a delayed fashion, displayed a statistically significant decrease in pain scoring ($p < 0.05$).

As discussed earlier, mice have an evolutionarily conserved, instinctive preference for dark areas. To investigate whether light stimulation could alter this preference, we created a CPP apparatus with light and dark chambers and a central transfer area (Figure 7B). At baseline, the mice displayed a heavy preference for the dark chamber, as expected (Figure 7C). In the conditioned mice, there was no significant change in CPP chamber preference, however, with live reinforcement 10 days post-conditioning, the mice displayed a significantly increased preference for the light chamber ($p < 0.01$, Figure 7D). It appears that optogenetic light therapy can not only improve pain behaviors but also modify evolutionary conserved preferences.

Sciatic nerve cuffing, or ligation, is a common experimental model for peripheral neuropathy.[32] The plantar surface of the hindpaw of sciatic nerve-ligated mice displayed increased sensitivity to both noxious (hyperalgesia) and non-noxious stimuli (allodynia). Within the Von Frey assay, we displayed an increased sensitivity to mechanical stimulation that lasted at least 2 months after the peripheral nerve injury (Figure 8B). In figure 8C, the baseline thresholds for mechanical sensitivity and hindpaw withdrawal in the Von Frey assay are shown. After 10 minutes of therapy in the experimental mice, there were no significant changes to the minimum force to withdraw. After 20 minutes, however, there was a statistically significant increase in the force needed for the mouse to withdraw its hindpaw to a level comparable to baseline ($p < 0.01$, Figure 8C). This signifies that the changes in mechanical sensitivity to pain via optogenetic neuromodulation in the ACC are time dependent. Notably, there seems to be some small increase in the minimum threshold compared to without the optogenetic implant, which may reflect the reported benefit from clinical trials of DBS. Thus, the placement of the implant itself can potentially have some benefit, even without any supplemented therapeutic stimulation. This is

made more apparent by examination of the % change in mechanical threshold from the unimplanted baseline control group shown in 8D. The Implant and control groups both show comparable amounts of Δ% in relation to the baseline values. In Figure 8D, there is a statistically significant change from baseline after 20 minutes of therapy which is maintained throughout the experiment ($p < 0.05$).

In figure 9, we show that there is a persistent effect of our optogenetic therapy on pain reduction. This effect appears to last up to 160 minutes after the last given dose of optogenetic therapy. We hypothesize that this is due to the overloaded presence of GABA in the synapses of neurons in the pain pathway. The neurotransmitter GABA's metabolism depends on glutamine stores and the availability of several enzymes, which could potentially be saturated within our experiment.[38,39] Future experiments might be needed to identify the cause of persistent pain reduction; nevertheless, this is a potential added benefit we have identified with our therapy.

To analyze the safety profile of our intervention, we measured the local tissue temperature after continuous 5 Hz light stimulation (Figure 10A). The optogenetic stimulation did not display any local temperature increases, suggesting that there would be no phototoxicity with our therapeutic intervention. Furthermore, after examining the brain sections of our experimental mice, there were no noticeable increases in Caspase-3 (cell death), IBA1 (macrophage/microglial recruitment), and CD45 (inflammation) immunostaining (Figure 10B-D). Thus, we can conclude that long-term use of the stimulation cannula, coupled with our optogenetic therapy directed at the ACC, did not display any immunogenicity or cell toxicity.

We then transitioned to laser-based gene delivery experiments due to a cohort of mice dying after AAV gene delivery. We were unable to determine the cause of death, as an autopsy of mice is costly and more than likely would be unable to pinpoint a direct reason for their death. We hypothesize that a potential mutation in the viral genome caused a pathologic response, but we cannot confirm this to be the case. There have been instances of high doses of AAVs intended for gene delivery, causing animal models' death in the literature.[40]

We then transitioned to experiments with laser gene delivery specifically targeting the DRG of the spine. In figure 11, we confirm that laser gene delivery effectively reduces pain in a formalin acute pain model. However, much of the pain reduction was only seen in the late phases of the experiment after 40 minutes of continued optogenetic treatment. Figure 12 showed a significant decrease in pain scores with higher-intensity light. Thereby, displaying an increased effect with increased light intensity. At about 20 minutes, the treatment group appeared to have a significantly decreased pain response in our formalin assay. Future research should look at peak intensity attainable without causing deleterious side effects to the nearby structures and tissues.

Our previous experiments identified the importance of laser and light penetration and intensity. To further investigate these properties, we correlated the intensity of laser stimulation and the resulting depth of penetration (Figure 13). Our therapeutic stimulation was able to hit the threshold needed for MCO stimulation up to a depth of over 2.5 mm. Finally, we were able to confirm that the laser successfully delivered MCO to the DRG of the spinal cord with mCherry-based immunostaining (Figure 14).

Overall, our MCO was effective via AAV and laser particle delivery for reducing pain. This effect was present when the MCO was targeted to the ACC and the DRG. Our results were statistically significant from controls. However, there were no statistically significant differences in the experimental groups. Overall, it appears that both laser and viral gene delivery are equal in terms of efficacy.

Now, we are still considering using both gene delivery systems for our future experiments. AAV gene delivery mechanisms are relatively low-cost and easier to administer than laser gene delivery methods.[41] Issues with AAV delivery include that the gene delivery mechanism can only be utilized once per animal, and there is some concern for DRG toxicity.[42] Additionally, some Food and Drug Administration (FDA) regulations are increasing for AAV administration.[43]

On the other hand, laser gene delivery can be utilized multiple times in an animal model; however, its administration requires a higher level of clinical skill and experience.[44] Laser gene delivery must be extremely accurate, and it requires nanorods that are costly.[45] Furthermore, it appears that laser gene delivery is preferential to viral delivery in two additional distinct manners – avoidance of immunologic response and subsequent degeneration of involved tissues and increased specificity and control of delivery to intended targets. Previous studies have demonstrated that following the primary introduction of the viral vector, subsequent exposure induces a maladaptive immunologic response that may be responsible for tissue degeneration in a dose-dependent manner.[46] In our experience, we have additionally seen greater control of gene

delivery in a manner that is superior to that seen in our viral vector studies. In conclusion, we advocate for laser gene delivery systems to become the new standard method for gene delivery – particularly in rodent models for neuromodulation.

Innovation

Chronic pain and opioid usage are both growing public health concerns with relatively few alternatives or solutions. Our potential modality in humans would allow for pain management, without the risk factors of opioid drugs. Additionally, our smartphone application (delivered within atmosphere.IoT) enables the subject to essentially "administer a dose," or activate the MCO-based neurons in their brain to treat their pain whenever it is needed. This allows the patient to control their treatment plan without needing to refill their prescription constantly.

There is additional innovation with the development of our OPM. This Bluetooth-enabled optogenetic stimulation device allows wireless control of optogenetic modulation of pain with the ability to change the frequency and intensity of light stimulation within the atmosphere.Iot smartphone application.

Finally, this project improves our current understanding of pain, and its pathways. To our knowledge, the GABAergic cells of the ACC are involved in the modulation of pain. However, little is known about the plasticity of these neurons and how their responses are altered in long-term chronic pain. By continuously tracking these neurons' activity we have not seen any

tolerance to pain therapy, however, there are additional assays that need to be performed on a long-term basis to confirm this finding.

Broader Impacts/Future Directions

Beyond the benefit for individuals who deal with chronic pain, optogenetic and optical stimulation can be used for various other neurophysiological diseases. An optogenetic/optical neuromodulator can be particularly useful and indicated in disease processes requiring repetitive stimulation that can target a specific tissue at a particular frequency of interest. Additionally, our device can be a long-term, durable intervention that can provide benefits for several years.

Optogenetic neuromodulation has been proposed as a cutting-edge mechanism to ameliorate psychological disorders – including depression, schizophrenia, and bipolar disorder – that can potentially become resistant to current pharmaceutical therapies.[47-50] There has been some promise in studies investigating the use of optogenetics in depression.[51] We hypothesize that an even more significant benefit will be seen in the future when the neurocircuitry of these mental illnesses is more thoroughly investigated, allowing for greater specificity of targeted neuromodulation.

Optogenetic devices have also been proposed to replace diseased or damaged tissue. For example, studies have investigated optogenetics neuromodulation as a method to integrate biophysiological synaptic transmission with electrical, prosthetic limbs in individuals that have lost a hand.[52] Ultimately, the goal would be to utilize this technology to not only deliver signals to stimulate a prosthetic hand but also to have the prosthesis be able to provide tactile and other sensory signals, such as temperature, back to the central nervous system.[53] In individuals with ischemic stroke, several studies have shown improved recovery with simple optogenetic

stimulation to the ischemic area, providing a mechanism by which the body can restore its damaged tissues to a more functional state.[54,55] More recently, optogenetic devices have shown promise as a potential enteroendocrine regulator of the gut microbiome to improve metabolism and promote longevity theoretically.[56]

Conclusion

Overall, we hope our optogenetic neurostimulator can potentially be a valuable replacement for opioid medications in treating chronic pain for millions of individuals worldwide.[28] We have successfully shown that our intervention can reduce pain responses in multiple different pain related states. This improvement in pain response also allowed the mice to adapt their evolutionary conserved preferences. Future experiments are needed in canines, non-human primates, and potentially other animal models. Beyond that, we believe that our device can be utilized for various other health and physiological indications – some of which have already shown tremendous promise in scientific literature and others that have yet to be investigated.

Compliance

Our group followed Institutional Animal Care and Use Committee (IACUC) guidelines for caring for and experimenting with our mouse model. All the mice were sedated and sacrificed in an ethical manner with the use of isoflurane, a common rodent sedation and euthanasia method in research models.[57]

References

1. Manchikanti L, Singh A. Therapeutic opioids: a ten-year perspective on the complexities and complications of the escalating use, abuse, and nonmedical use of opioids. *Pain Physician.* 2008;11(2 Suppl):S63-88.
2. Florence C, Luo F, Rice K. The economic burden of opioid use disorder and fatal opioid overdose in the United States, 2017. *Drug Alcohol Depend.* 2021;218:108350.
3. Deisseroth K, Feng G, Majewska AK, Miesenböck G, Ting A, Schnitzer MJ. Next-generation optical technologies for illuminating genetically targeted brain circuits. *J Neurosci.* 2006;26(41):10380-10386.
4. Paviolo C, McArthur SL, Stoddart PR. Gold Nanorod-assisted Optical Stimulation of Neuronal Cells. *J Vis Exp.* 2015(98).
5. Nagel G, Szellas T, Huhn W, et al. Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *Proc Natl Acad Sci U S A.* 2003;100(24):13940-13945.
6. Zhang F, Vierock J, Yizhar O, et al. The microbial opsin family of optogenetic tools. *Cell.* 2011;147(7):1446-1457.
7. Kandori H. Ion-pumping microbial rhodopsins. *Front Mol Biosci.* 2015;2:52.
8. Deisseroth K. Optogenetics: 10 years of microbial opsins in neuroscience. *Nat Neurosci.* 2015;18(9):1213-1225.
9. Shui B, Lee JC, Reining S, Lee FK, Kotlikoff MI. Optogenetic sensors and effectors: CHROMus-the Cornell Heart Lung Blood Institute Resource for Optogenetic Mouse Signaling. *Frontiers in physiology.* 2014;5:428.
10. Liu K, Wang L. Optogenetics: Therapeutic spark in neuropathic pain. *Bosn J Basic Med Sci.* 2019;19(4):321-327.
11. Marton TF, Sohal VS. Of Mice, Men, and Microbial Opsins: How Optogenetics Can Help Hone Mouse Models of Mental Illness. *Biol Psychiatry.* 2016;79(1):47-52.
12. Berg L, Gerdey J, Masseck OA. Optogenetic Manipulation of Neuronal Activity to Modulate Behavior in Freely Moving Mice. *J Vis Exp.* 2020(164).
13. Deubner J, Coulon P, Diester I. Optogenetic approaches to study the mammalian brain. *Curr Opin Struct Biol.* 2019;57:157-163.
14. Lavoie-Cardinal F, Salesse C, Bergeron É, Meunier M, De Koninck P. Gold nanoparticle-assisted all optical localized stimulation and monitoring of Ca^{2+} signaling in neurons. *Scientific reports.* 2016;6:20619.
15. Vigderman L, Khanal BP, Zubarev ER. Functional gold nanorods: synthesis, self-assembly, and sensing applications. *Adv Mater.* 2012;24(36):4811-4841, 5014.
16. Jung H, Nam Y. Optical recording of neural responses to gold-nanorod mediated photothermal neural inhibition. *J Neurosci Methods.* 2022;373:109564.
17. Morgan MM, Christie MJ. Analysis of opioid efficacy, tolerance, addiction and dependence from cell culture to human. *Br J Pharmacol.* 2011;164(4):1322-1334.
18. Kosten TR, George TP. The neurobiology of opioid dependence: implications for treatment. *Sci Pract Perspect.* 2002;1(1):13-20.

19. Zhuo M. Molecular mechanisms of pain in the anterior cingulate cortex. *J Neurosci Res.* 2006;84(5):927-933.
20. Kuo CC, Yen CT. Comparison of anterior cingulate and primary somatosensory neuronal responses to noxious laser-heat stimuli in conscious, behaving rats. *J Neurophysiol.* 2005;94(3):1825-1836.
21. Gu L, Uhelski ML, Anand S, et al. Pain inhibition by optogenetic activation of specific anterior cingulate cortical neurons. *PloS one.* 2015;10(2):e0117746.
22. Ahimsadasan N, Reddy V, Khan Suheb MZ, Kumar A. Neuroanatomy, Dorsal Root Ganglion. In: *StatPearls.* Treasure Island (FL): StatPearls Publishing
Copyright © 2022, StatPearls Publishing LLC.; 2022.
23. Cavalli E, Mammana S, Nicoletti F, Bramanti P, Mazzon E. The neuropathic pain: An overview of the current treatment and future therapeutic approaches. *Int J Immunopathol Pharmacol.* 2019;33:2058738419838383.
24. Jensen TS, Finnerup NB. Allodynia and hyperalgesia in neuropathic pain: clinical manifestations and mechanisms. *Lancet Neurol.* 2014;13(9):924-935.
25. Chen Q, Larochelle MR, Weaver DT, et al. Prevention of Prescription Opioid Misuse and Projected Overdose Deaths in the United States. *JAMA Netw Open.* 2019;2(2):e187621.
26. Volkow ND, Koroshetz WJ. The role of neurologists in tackling the opioid epidemic. *Nat Rev Neurol.* 2019;15(5):301-305.
27. Strike C, Watson TM. Losing the uphill battle? Emergent harm reduction interventions and barriers during the opioid overdose crisis in Canada. *Int J Drug Policy.* 2019;71:178-182.
28. Dahlhamer J, Lucas J, Zelaya C, et al. Prevalence of Chronic Pain and High-Impact Chronic Pain Among Adults - United States, 2016. *MMWR Morb Mortal Wkly Rep.* 2018;67(36):1001-1006.
29. Tsai HC, Zhang F, Adamantidis A, et al. Phasic firing in dopaminergic neurons is sufficient for behavioral conditioning. *Science.* 2009;324(5930):1080-1084.
30. Covington HE, 3rd, Lobo MK, Maze I, et al. Antidepressant effect of optogenetic stimulation of the medial prefrontal cortex. *J Neurosci.* 2010;30(48):16082-16090.
31. Carvalho-de-Souza JL, Treger JS, Dang B, Kent SB, Pepperberg DR, Bezanilla F. Photosensitivity of neurons enabled by cell-targeted gold nanoparticles. *Neuron.* 2015;86(1):207-217.
32. Yalcin I, Megat S, Barthas F, et al. The sciatic nerve cuffing model of neuropathic pain in mice. *J Vis Exp.* 2014(89).
33. Murakami T, Iwanaga T, Ogawa Y, et al. Development of sensory neuropathy in streptozotocin-induced diabetic mice. *Brain and behavior.* 2013;3(1):35-41.
34. Benbouzid M, Pallage V, Rajalu M, et al. Sciatic nerve cuffing in mice: a model of sustained neuropathic pain. *Eur J Pain.* 2008;12(5):591-599.
35. Hascoët M, Bourin M, Nic Dhonnchadha BA. The mouse light-dark paradigm: a review. *Prog Neuropsychopharmacol Biol Psychiatry.* 2001;25(1):141-166.
36. Thiel KJ, Okun AC, Neisewander JL. Social reward-conditioned place preference: a model revealing an interaction between cocaine and social context rewards in rats. *Drug Alcohol Depend.* 2008;96(3):202-212.

37. Christensen SL, Hansen RB, Storm MA, et al. Von Frey testing revisited: Provision of an online algorithm for improved accuracy of 50% thresholds. *Eur J Pain.* 2020;24(4):783-790.
38. Patel AB, Rothman DL, Cline GW, Behar KL. Glutamine is the major precursor for GABA synthesis in rat neocortex in vivo following acute GABA-transaminase inhibition. *Brain Res.* 2001;919(2):207-220.
39. Grabauskas G. Time course of GABA in the synaptic clefts of inhibitory synapses in the rostral nucleus of the solitary tract. *Neurosci Lett.* 2005;373(1):10-15.
40. High-dose AAV gene therapy deaths. *Nat Biotechnol.* 2020;38(8):910.
41. Orefice NS, Souchet B, Braudeau J, et al. Real-Time Monitoring of Exosome Enveloped-AAV Spreading by Endomicroscopy Approach: A New Tool for Gene Delivery in the Brain. *Mol Ther Methods Clin Dev.* 2019;14:237-251.
42. Hordeaux J, Buza EL, Dyer C, et al. Adeno-Associated Virus-Induced Dorsal Root Ganglion Pathology. *Human gene therapy.* 2020;31(15-16):808-818.
43. Reape KZ, High KA. Trial by "Firsts": Clinical Trial Design and Regulatory Considerations in the Development and Approval of the First AAV Gene Therapy Product in the United States. *Cold Spring Harb Perspect Med.* 2022.
44. Batabyal S, Kim S, Wright W, Mohanty S. Layer-specific nanophotonic delivery of therapeutic opsin-encoding genes into retina. *Exp Eye Res.* 2021;205:108444.
45. Batabyal S, Gajjeraman S, Tchedre K, Dibas A, Wright W, Mohanty S. Near-Infrared Laser-Based Spatially Targeted Nano-enhanced Optical Delivery of Therapeutic Genes to Degenerated Retina. *Mol Ther Methods Clin Dev.* 2020;17:758-770.
46. Shirley JL, de Jong YP, Terhorst C, Herzog RW. Immune Responses to Viral Gene Therapy Vectors. *Mol Ther.* 2020;28(3):709-722.
47. Muir J, Lopez J, Bagot RC. Wiring the depressed brain: optogenetic and chemogenetic circuit interrogation in animal models of depression. *Neuropsychopharmacology.* 2019;44(6):1013-1026.
48. Duan AR, Varela C, Zhang Y, et al. Delta frequency optogenetic stimulation of the thalamic nucleus reuniens is sufficient to produce working memory deficits: relevance to schizophrenia. *Biol Psychiatry.* 2015;77(12):1098-1107.
49. Sidor MM. Psychiatry's age of enlightenment: optogenetics and the discovery of novel targets for the treatment of psychiatric disorders. *J Psychiatry Neurosci.* 2012;37(1):4-6.
50. Sidor MM, McClung CA. Timing matters: using optogenetics to chronically manipulate neural circuitry and rhythms. *Front Behav Neurosci.* 2014;8:41.
51. Lammel S, Tye KM, Warden MR. Progress in understanding mood disorders: optogenetic dissection of neural circuits. *Genes Brain Behav.* 2014;13(1):38-51.
52. Nghiem BT, Sando IC, Gillespie RB, et al. Providing a sense of touch to prosthetic hands. *Plast Reconstr Surg.* 2015;135(6):1652-1663.
53. May T, Ozden I, Brush B, et al. Detection of optogenetic stimulation in somatosensory cortex by non-human primates--towards artificial tactile sensation. *PloS one.* 2014;9(12):e114529.
54. Lu C, Wu X, Ma H, et al. Optogenetic Stimulation Enhanced Neuronal Plasticities in Motor Recovery after Ischemic Stroke. *Neural Plast.* 2019;2019:5271573.

55. Shah AM, Ishizaka S, Cheng MY, et al. Optogenetic neuronal stimulation of the lateral cerebellar nucleus promotes persistent functional recovery after stroke. *Scientific reports.* 2017;7:46612.
56. Hartsough LA, Park M, Kotlajich MV, et al. Optogenetic control of gut bacterial metabolism to promote longevity. *Elife.* 2020;9.
57. Shomer NH, Allen-Worthington KH, Hickman DL, et al. Review of Rodent Euthanasia Methods. *J Am Assoc Lab Anim Sci.* 2020;59(3):242-253.