UNIVERSITY OF CALIFORNIA SAN DIEGO

Therapeutic Pain Management via CRISPR-Cas9-Mediated Sodium Channel Repression

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

in

Bioengineering

by

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Committee in charge:

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Professor Pedro Cabrales
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2018

The thesis of Andrew Maxim Pla is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California San Diego

2018

DEDICATION

This thesis is dedicated to my wife, Steph. She has always been the most wonderful and supportive partner I could ask for, despite this work keeping me away from her for so many hours.

This thesis is also dedicated to my parents, who have made it possible for me to be where I am today.

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ACKNOWLEDGEMENTS

I would like to acknowledge Professor Prashant Mali for his guidance and support as my advisor and committee chair. He has always had an insightful perspective and has kept this project progressing.

I would also like to acknowledge Ana Moreno, who taught me so much of what I used in this thesis and got me excited about advancing the treatment of pain. I wouldn't be here without her help.

This work, in part, is currently being prepared for submission for publication of the material. Moreno, Ana M.; Pla, Andrew. The thesis author was a researcher for this material.

ABSTRACT OF THE THESIS

Therapeutic Pain Management via CRISPR-Cas9-

Mediated Sodium Channel Repression

by

Andrew Maxim Pla

Master of Science in Bioengineering

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Professor Prashant Mali, Chair

Chronic pain is a considerable issue facing society today. Current treatments for chronic pain consist mainly of opioids, highly imperfect solutions due to their side effects and high risk of addiction. This thesis aims to investigate a new avenue for treating chronic pain by taking a cue from nature. Individuals have been identified with congenital mutations to a gene encoding

for a sodium channel subtype, $Na_v1.7$, leading to total insensitivity to pain. Here we attempted to treat chronic pain via genetic targeting of SCN9A, the gene encoding for $Na_v1.7$. Due to the desire to non-permanently ablate pain, we have utilized a variant of the CRISPR-Cas9 system without nuclease activity, dCas9 (dead-Cas9). The fusion of dCas9 to transcription regulators, such as the KRAB repressor, enables stable and efficient transcriptional repression. We delivered a dCas9-KRAB and a gRNA targeting the $Na_v1.7$ gene via adeno-associated viruses (AAVs) to enable repression of pain, similar to a small-molecule drug, but, unlike small-molecules, lasting up to a few weeks. We injected mice with carrageenan to induce an inflammatory hyperalgesia pain model, performed nociceptive assays in order to verify a phenotypic change in pain sensation, and carried out qPCR to show repression of SCN9A. Results show ~50% repression of SCN9A was achieved, as well as a <100% improvement in thermal pain tolerance in the inflammatory state. Moving forward, this therapy could potentially be a future chronic pain treatment in humans without the negative side effects of current approaches.

INTRODUCTION

Chronic pain management has been with humankind since the beginning of recorded history. Earlier cultures though that demons caused pain, some though that bleeding helped; others thought drilling a hole in the head would alleviate it.

While we now have more advanced tools to combat chronic pain than before, pain remains rampant and we still lack treatments that are both potent and safe. In the US, one in three adults experiences chronic pain, defined as pain which last for three or more months[1,2]. Of these people, 40 million experience severe pain and 25 million experience pain every single day. The impact reaches beyond just the physical and psychological: the cost of chronic pain in the US is estimated at $560 billion to $635 billion annually[2,3].

Further, chronic pain has driven many in today's society to overuse of opioids. According to the CDC, 115 Americans die every day due to opioid addiction[4]. This highly flawed answer to pain management has created a crisis in much of the US, decimating whole communities. Better, smarter, and more efficient approaches to pain management are inarguably highly desirable, and desperately needed.

In order to understand how to address the need for better pain management approaches, it is important to start with a basic understanding of how pain manifests in the body. Pain that we perceive when we have a stubbed toe, an aching back, or a troublesome knee, is felt through a process called nociception. Nociception occurs when pain signals are transmitted from the point of sensation to the central nervous system by pain-sensing afferent neurons, called nociceptive neurons. Signals are conducted through neurons by action potentials, which are defined as an electric depolarization of the neuron membrane that propagates down the length of the neuron's

axon (**Figure 1A, 1B**). This depolarization, and the subsequent repolarization, is regulated by transmembrane ion channels (**Figure 1C**). There are sodium, potassium, and calcium ion channels. Multiple different subtypes of these ion channels exist, with each subtype having different gating characteristics – opening and closing rates – and being expressed in different tissue types. For example, there are nine known voltage-gated sodium channel subtypes, called Na_v channels (**Table 1)**.

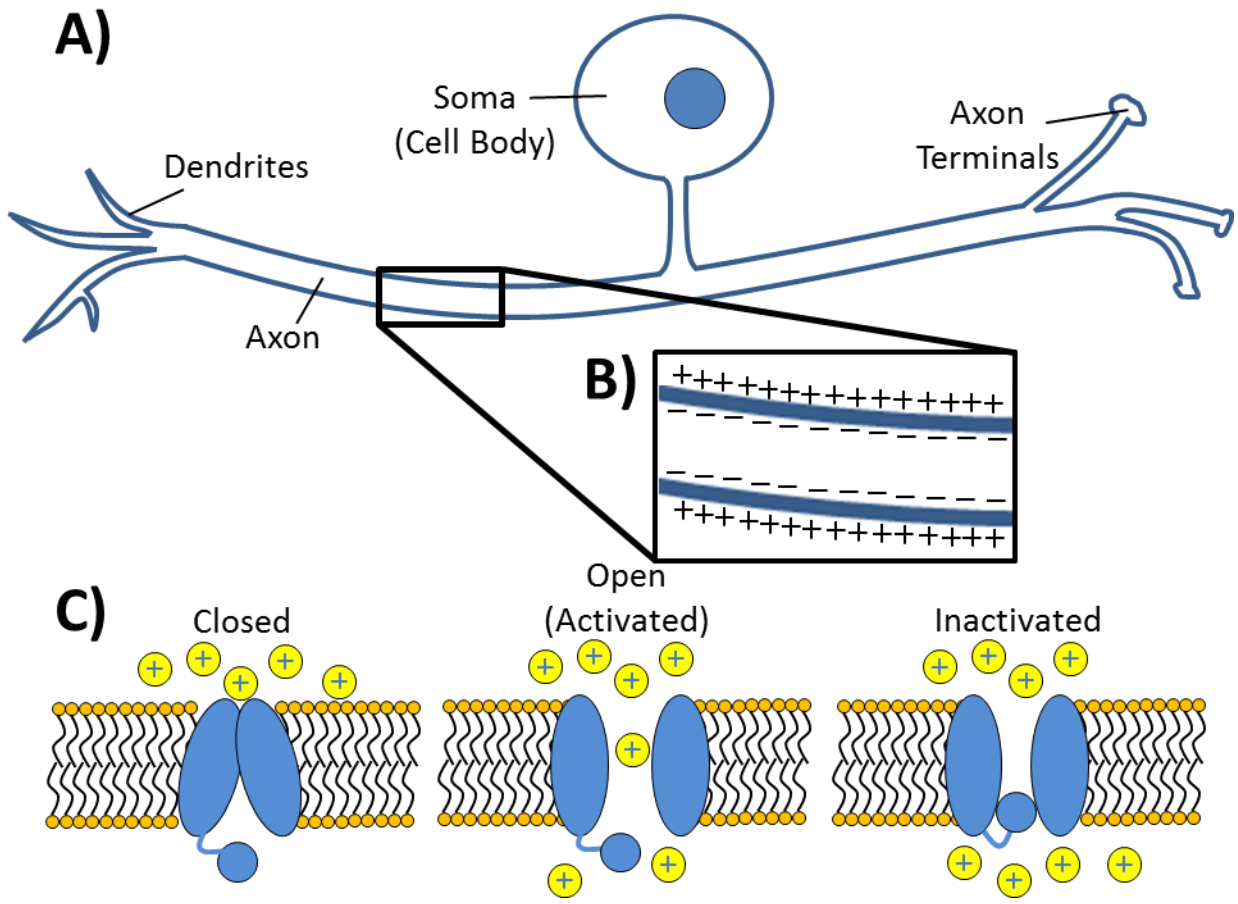


Figure 1: A) Depiction of a sensory neuron. Signals are received by the dendrites and travel down the axon to the axon terminals, where they trigger neurotransmitter release. **B)** An illustration of the polarized neuron membrane, which is mediated by ion channels. **C)** Illustration of the action of voltage-gated ion channels which allow action potentials to occur. Action potentials trigger the channels to open, followed by a period of inactivation before returning to a closed state.

Table 1: Currently known voltage-gated sodium channel subtypes and a brief description of each[5]. CNS (Central Nervous System); PNS (Peripheral Nervous System).

Na_v Subtype	**Gene**	**Major Tissue Expression**	**Effect of Mutation**
$Na_v1.1$	SCN1A	CNS, PNS	Epilepsy
$Na_v1.2$	SCN2A	CNS, PNS	Epilepsy
$Na_v1.3$	SCN3A	CNS, PNS	None studied
$Na_v1.4$	SCN4A	Skeletal muscle	Myotonia, periodic paralysis
$Na_v1.5$	SCN5A	Heart	Long QT, Brugada syndrome, progressive familial heart block
$Na_v1.6$	SCN8A	CNS, PNS	Cerebellar atrophy
$Na_v1.7$	SCN9A	PNS (sensory neurons and primary afferent neurons)[6]	Increased and decreased pain sensitivity
$Na_v1.8$	SCN10A	PNS	Small fiber neuropathy[7]
$Na_v1.9$	SCN11A	PNS	Inflammatory pain hypersensitivity[8]

During nociception, pain signals are conducted from the point of sensation into the spinal cord, and finally into the brain, where they are processed. Just before entering the spinal cord, sensory signals pass through a bundle of sensory neuron cell bodies called the dorsal root ganglion (DRG) (**Figure 2A**). These DRGs are located regularly at all levels of the spinal cord (**Figure 2B**). Because the nociceptive signals all pass through DRGs, localized treatments can target the DRGs specifically in order to optimally block pain signals[9].

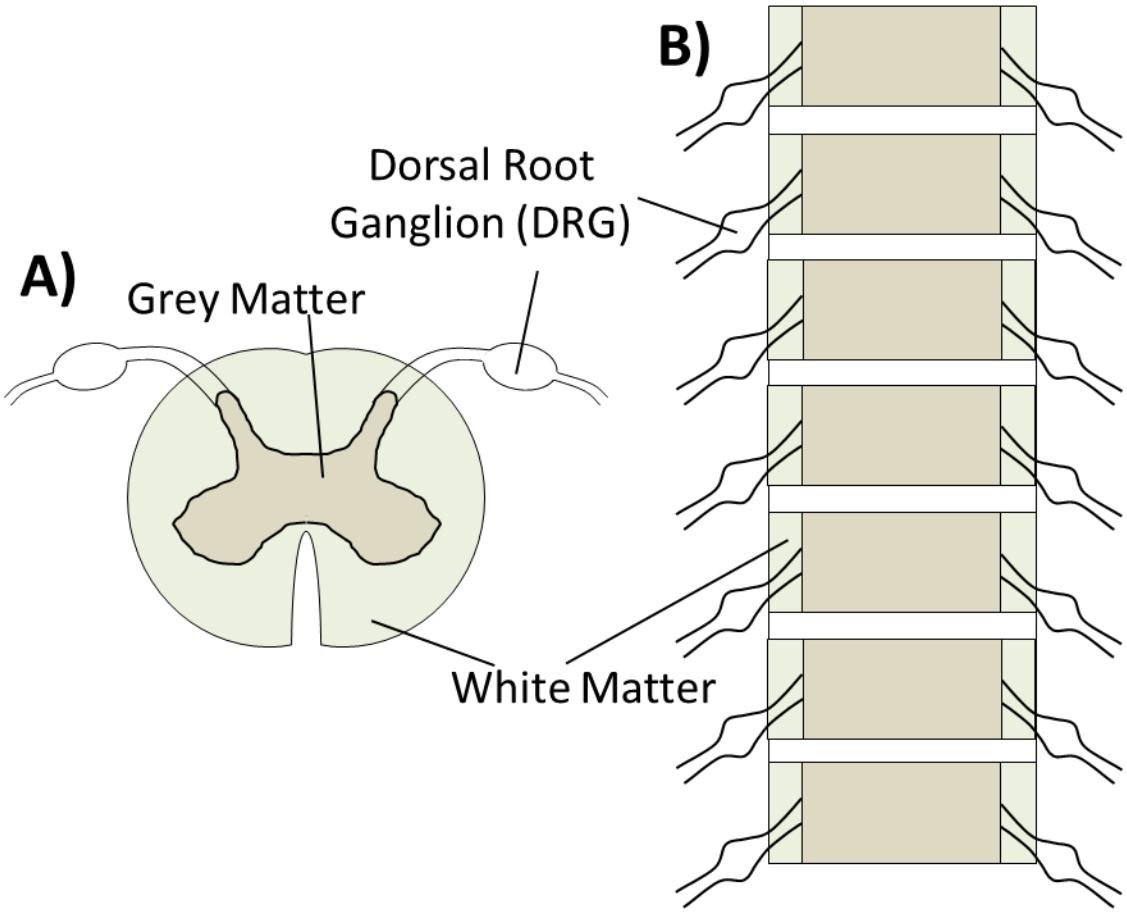


Figure 2: A) Transverse cross section illustration of the spinal cord showing the location of the DRGs. **B)** Longitudinal cross section illustration of the spinal cord showing the locations and spacing of the DRGs.

Current treatments for chronic pain include both non-pharmacological and pharmacological treatments. Non-pharmacological treatments include physical rehabilitation, psychological therapy, and acupuncture. Pharmacological treatments include non-steroidal anti-inflammatory drugs, antidepressant drugs in low doses, anticonvulsants, muscle relaxants, and opioids[6]. Some patients even undergo ganglionectomy, the removal of the entire DRG itself[10]. Unfortunately, these existing treatments often have side effects and show limited efficacy in many patients. Most concerning, despite many risks and issues surrounding opioid use and abuse, opioids are the most-prescribed drug class for chronic pain treatment in the US[11].

New hope for pain treatment has come from studying individuals who have been identified with a rare genetic condition called congenital indifference to pain (CIP). These individuals have a complete inability to sense pain, while retaining the ability to perceive other sensations such as touch, temperature, and proprioception. They are otherwise healthy, with the exception of the loss of the sense of smell. Examination of the genomic DNA found that the individuals had nonsense mutations in the gene SCN9A, which encodes for the sodium channel $Na_v1.7$[12]. The gating dynamics of $Na_v1.7$ channels exhibit fast activation and inactivation, and slow recovery from inactivation. These properties cause $Na_v1.7$ to generate ramp currents in response to sub-threshold stimuli, acting as a signal amplifier and setting the threshold for action potential generation[13,14].

Since the discovery of the link between SCN9A and pain insensitivity, there has been much research into therapeutic targeting of $Na_v1.7$. However, $Na_v1.7$ has proven itself to be a difficult target and there has been little to show for the research so far. Drugs targeting $Na_v1.7$ must be highly selective, as off-target effects could wreak havoc with other important sodium channel subtypes such as $Na_v1.5$, where irregularities can cause a variety of arrhythmic disorders[15]. Small molecule treatments have encountered issues with overcoming this selectivity issue. Animal toxins have been used and while some have achieved high selectivity, others have not. Further, their efficacy is limited by poor pharmacodynamics[13]. Monoclonal antibodies have shown promise, but currently have some cross-affinity for $Na_v1.6$ and are not long-lasting[14,16].

A relatively new and significant development in biology has been the discovery and development of the CRISPR-Cas9 system. This system allows for targeting and editing of DNA at precise locations within the genome through the combination of guide RNAs (gRNAs) and an enzyme, Cas9[17,18]. gRNAs are short, ~20 base pair sequences of RNA which can bind

complementary DNA sequences[18]. Cas9 binds to gRNAs and together they seek out and bind to specific sequences in the genome. Cas9 first binds to a protospacer-adjacent motif (PAM), a three base pair long sequence (5'-NGG-3') upstream of the gRNA recognition sequence[19]. Once at the site defined by the gRNA, the nuclease domains within Cas9 create a double stranded break in the DNA. A nuclease-dead version of Cas9 has also been developed, called dCas9, which binds DNA but does not create a double stranded break[20]. By fusing transcriptional regulatory domains to dCas9, gene activity can be activated or repressed with high specificity, without causing permanent change in the DNA, and thus with no off-target effects. In particular, the Krüpple associated box (KRAB) domain can be fused to dCas9 for transcriptional repression[21,22].

The CRISPR-Cas9 system presents a very promising new opportunity for therapeutically repressing $Na_v1.7$ with high specificity and efficacy. Towards this, we have developed a dCas9-KRAB therapy using adeno-associated viruses (AAVs) targeting $Na_v1.7$'s gene, SCN9A, to enable *in vivo* repression in mice. AAVs were chosen as the delivery mechanism for the dCas9-KRAB and SCN9A gRNA treatment. They have emerged as a leading choice for delivery of genetic treatments due to their low immunogenicity, high duration of expression, and low toxicity[23]. Of the multiple available serotypes of AAV available, AAV9 was chosen for its ability to successfully transduce neurons[24,25]. A limitation of AAVs is their relatively small cargo size of ~4.7 kb, which is insufficient to carry the large payload of the SCN9A-dCas9-KRAB treatment. The recently developed split-Cas9 system provides a workaround to this issue. In this system, the Cas9 protein is translated in two separate halves, each fused to a split-intein. When both Cas9 halves are expressed together, splicing occurs, the inteins are removed, and the full Cas9 protein is assembled[26] (**Figure 3**). We thus utilized split-dCas9-KRAB and delivered it via AAV9

intrathecally to repress $Na_v1.7$, and have demonstrated that the mice receiving this therapy had ~50% $Na_v1.7$ repression, confirmed via qPCR, and ~100% increase in latency (s) in an inflammatory model via the Hargreaves heat tolerance assay (**Figure 4**).

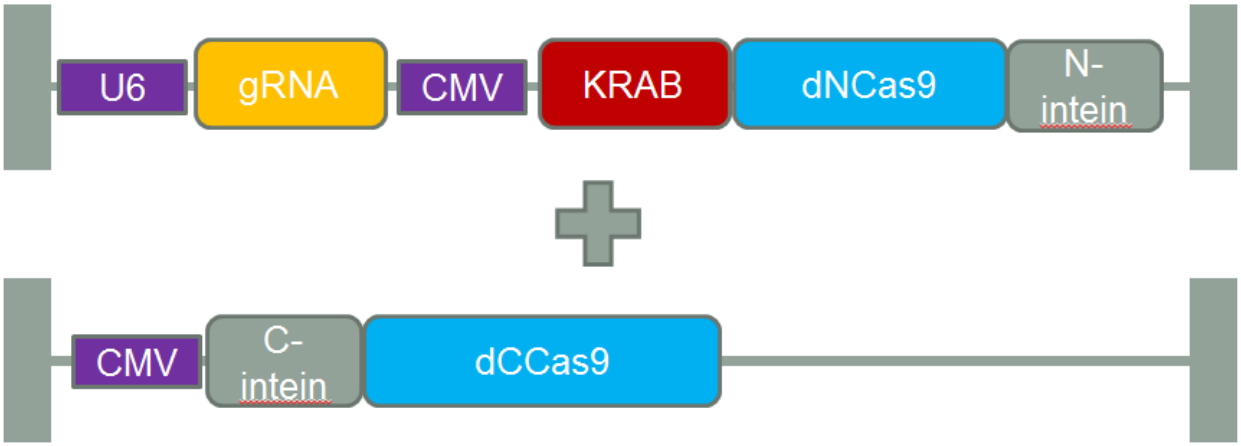


Figure 3: Split dCas9 system. One virus particle contains the gRNA and half of the dCas9 with an N-intein. The other virus particle contains the other half of the dCas9 with a C-intein. When both protein halves are translated, the inteins are spliced out and the functional dCas9-KRAB is created.

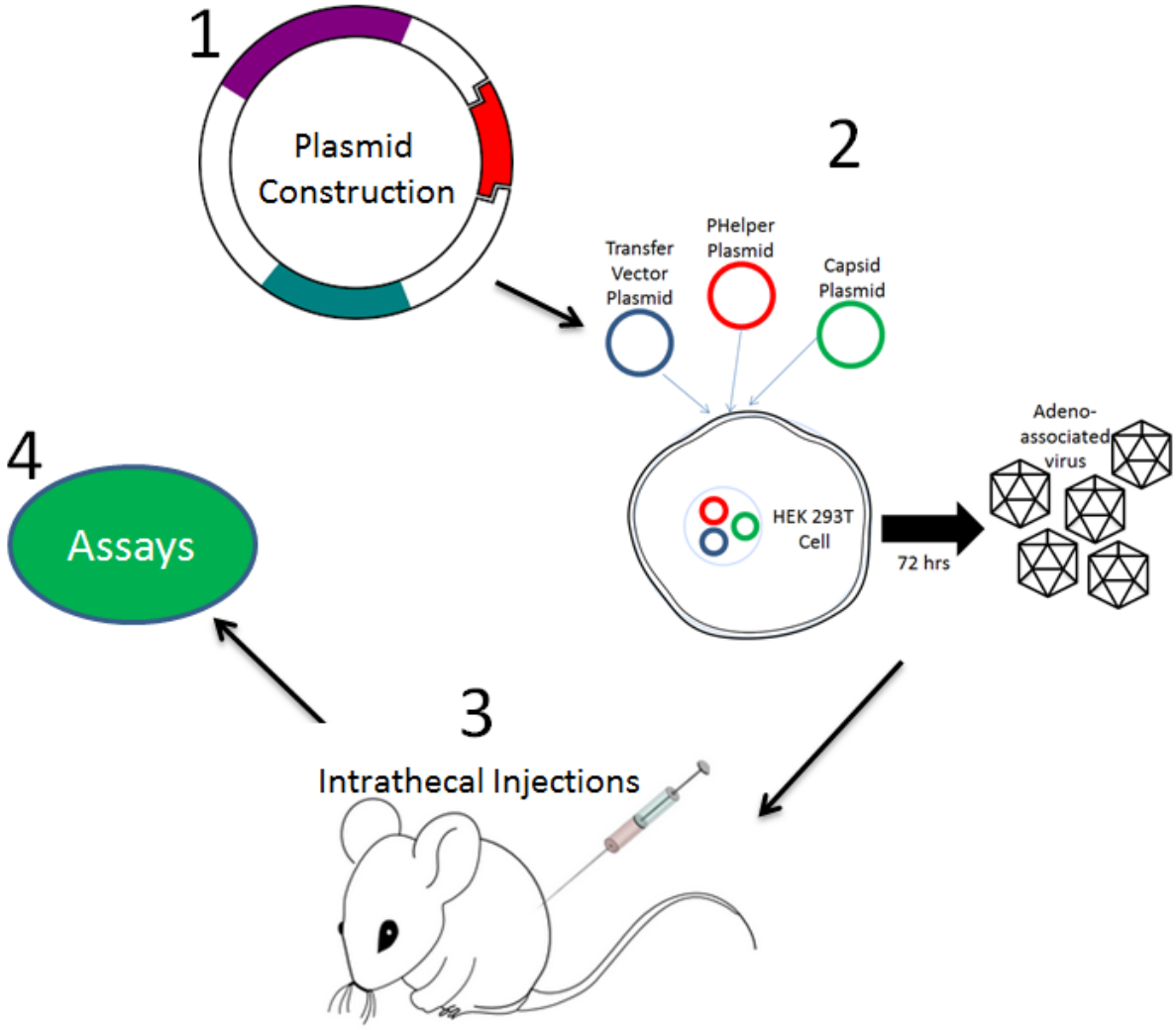


Figure 4: Workflow of the experiments conducted in this thesis. First, plasmids were developed with sequences encoding for dCas9 and the gRNA targeting SCN9A. Next, HEK 293T cells were transfected with these plasmids as well as adeno-associated viral plasmids. After 72 hours, virus was harvested and purified. Following this, mice received intrathecal injections of the SCN9A-dCas9-KRAB treatment. Finally, mice underwent behavioral assays and, at the termination of the experiment, DRGs were extracted for qPCR.

RESULTS

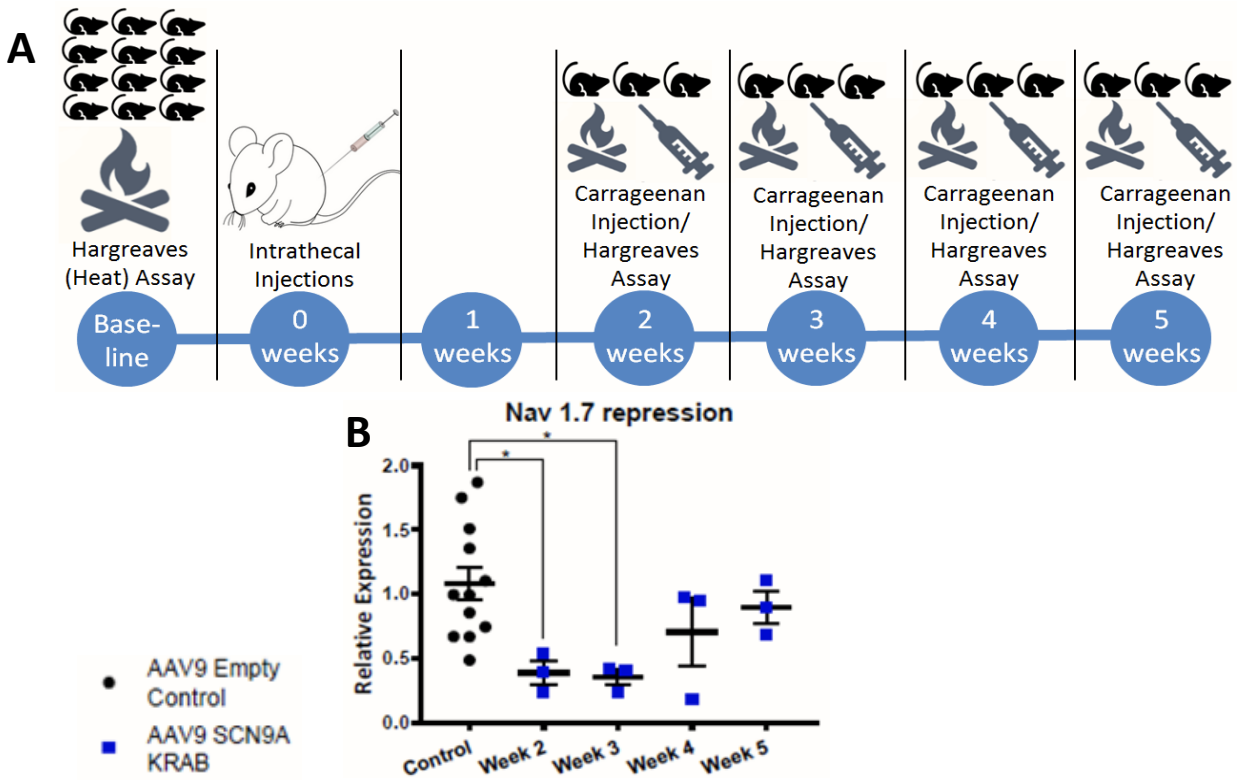
Exploring Repression Dynamics

We initially conducted a study to determine the approximate duration of the treatment's efficacy in mice. Thermal pain sensitivity was first measured in all mice with the Hargreaves assay in order to establish a baseline level of sensitivity. Following this baselining, the SCN9A-dCas9-KRAB treatment was delivered via intrathecal injection ($2x10^{12}$ vg/mouse)

to one group of mice (n=12) while a second group (n=12) received dCas9-KRAB with no gRNA ($2x10^{12}$ vg/mouse). After receiving the treatment, the mice's thermal pain sensitivity was measured each week in an inflammatory pain model in order to determine the dynamics of the repression. Inflammation was induced by injecting the hind paw with carrageenan, a chemical which causes inflammation, swelling, and hyperalgesia, and is commonly used to model inflammatory pain[27]. Three mice from each group were chosen at random each week and received carrageenan in the ipsilateral paw, while the contralateral paw served as a control (**Figure 5A**). This was done for weeks 2, 3, 4, and 5. Mice were sacrificed 24 hours after receiving carrageenan, and qPCR was performed subsequently using the L4 and L5 DRGs

The qPCR results indicated that a peak degree of repression (~60%) occurred at weeks two and three, with the degree of repression decreased below the level of significance in later weeks (**Figure 5B**). A similar trend can be seen with the Hargreaves behavioral assays, with a statistically significant increase (~60%) in withdrawal latency – tolerance to thermal pain – only at week 2 (**Figure 5C**). Following carrageenan injection, the results of the Hargreaves assays demonstrate a statistically significant increase in withdrawal latency for paws receiving carrageenan in the experimental group as compared to the control group (**Figure 5D**). This trend persists during week 2 (~188%) and week 3 (338%), but is no longer significant by weeks 4 and 5. A significant increase in paw thickness was seen in the ipsilateral paws 4.5 hours after carrageenan injection, verifying that inflammation had occurred (**Figure 5E**).

Figure 5: A) Mice (12 per group) first underwent baseline Hargreaves testing to determine their level of pain sensitivity before treatment. SCN9A-dCas9-KRAB treatment was delivered via intrathecal injection at time zero. Each week, starting at week two, all mice underwent Hargreaves testing again. Then, three mice from each group received carrageenan injections and further Hargreaves testing. Mice were then sacrificed and DRGs were harvested for qPCR. **B)** qPCR repression data of $Na_v1.7$ week-to-week for control mice (n=12) vs. mice receiving the SCN9A-dCas9-KRAB treatment (n=3 per week). Statistically significant repression was seen at week 2 (p-value =0.0359) and week 3 (p-value = 0.0270). **C)** Hargreaves withdrawal latencies for the control mice vs. mice receiving the treatment. Higher withdrawal latency indicates more tolerance to thermal pain. A statistically significant difference in withdrawal latencies was seen at week 2 (p-value < 0.0001). Each point shown is the average of 3 trials for each hindpaw of each mouse (6 trials per mouse). **D)** Hargreaves withdrawal latencies for mice 240 minutes after an ipsilateral carrageenan injection. Statistically significant differences in withdrawal latencies were seen in week 2, -Carra to –Carra (p-value = 0.0246) +Carra to +Carra (p-value = 0.003); week 3, +Carra to +Carra (p-value = 0.0043); and week 5, +Carra to +Carra (p-value = 0.0238). **E)** Paw thickness before and after injection of carrageenan, showing evidence of inflammation. All p-values were <0.0001.

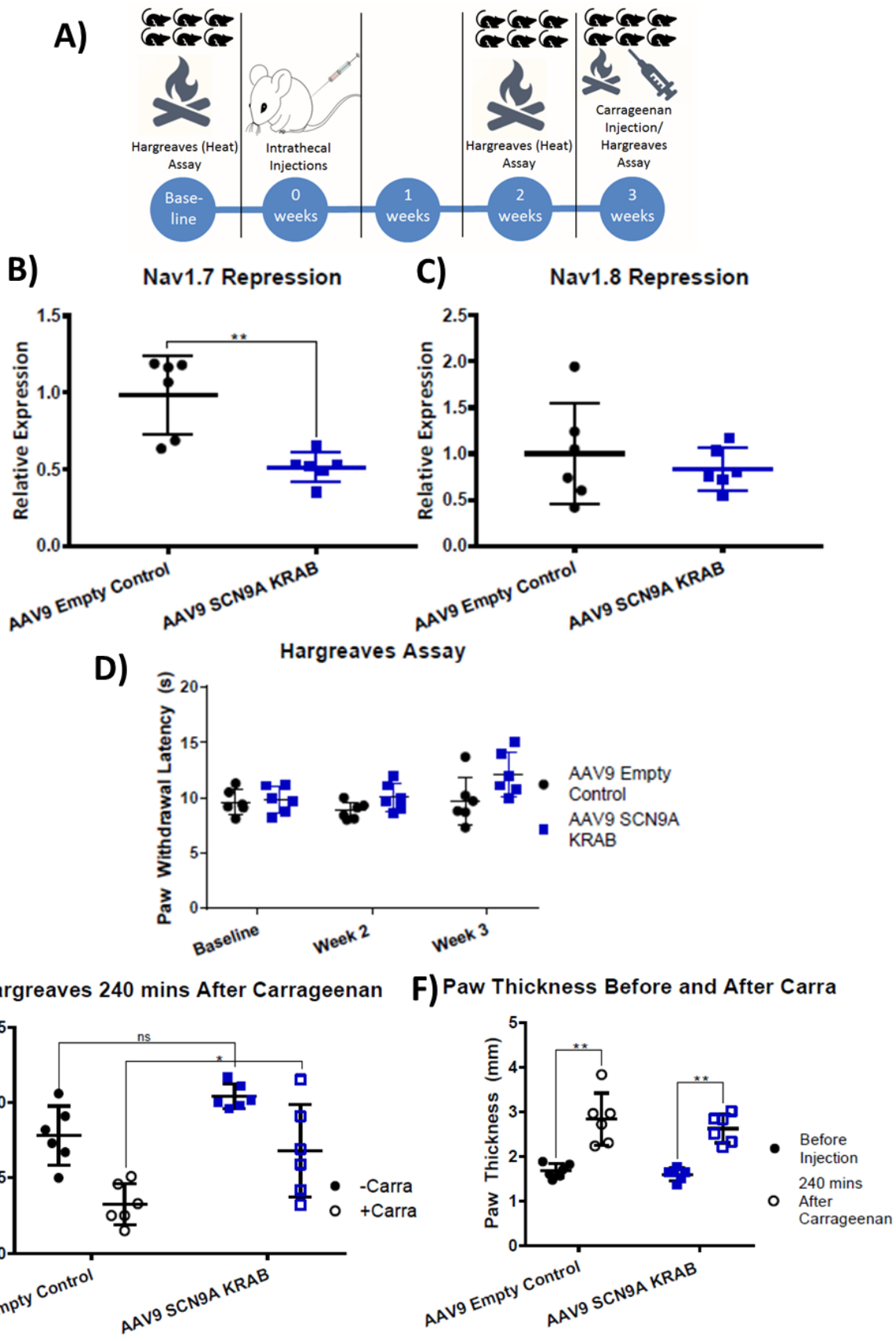

A
Hargreaves (Heat) Assay
Intrathecal Injections
Carrageenan Injection/ Hargreaves Assay
Carrageenan Injection/ Hargreaves Assay
Carrageenan Injection/ Hargreaves Assay
Carrageenan Injection/ Hargreaves Assay
Base-line
0 weeks
1 weeks
2 weeks
3 weeks
4 weeks
5 weeks
B
Nav 1.7 repression
Relative Expression
AAV9 Empty Control
AAV9 SCN9A KRAB
Control
Week 2
Week 3
Week 4
Week 5
C
Hargreaves Assay
Paw Withdrawal Latency (s)
Baseline
Week 2
Week 3
Week 4
Week 5
AAV9 Empty Control +Carra
AAV9 Empty Control -Carra
AAV9 SCN9A KRAB +Carra
AAV9 SCN9A KRAB -Carra
D
Hargreaves 240 mins After Carrageenan
Paw Withdrawal Latency (s)
Week 2
Week 3
Week 4
Week 5
E
Paw Thickness Before and After Carra
Paw Thickness (mm)
Week 2
Week 3
Week 4
Week 5

Validation of SCN9A dCas9-KRAB Treatment Efficacy

Based upon the repression dynamics study, it was determined that the highest level of repression is seen around 3 weeks, and thus future experiments with carrageenan were done at this time point. With this knowledge of when peak transcriptional repression and phenotypic effect occurs, another study was done to further validate the results. In order to achieve higher statistical power, more mice were used in each group. New mice (n=6/group) were baselined and injected, this time with twice as much AAV ($4x10^{12}$ vg/mouse), to attempt to increase the degree of repression. As before, one group received the SCN9A-dCas9-KRAB injection while the other received dCas9-KRAB with no gRNA. Mice underwent behavioral testing two weeks after AAV injection and received carrageenan three weeks after injection (**Figure 6A)**. In order to verify the specificity of the treatment, expression levels of not only $Na_v1.7$, but also $Na_v1.8$, were determined by qPCR.

The results of the qPCR indicate that repression of $Na_v1.7$ occurred (~50%), while $Na_v1.8$ was unaffected (**Figure 6B, 6C**). Contrary the dynamics study, the Hargreaves assays alone did not show any significant difference between groups (**Figure 6D)**. Finally, the carrageenan testing did show a statistically significant increase (110%) in withdrawal latency for paws receiving carrageenan in the experimental group as compared to the control group (**Figure 6E**). A ~2-fold increase in paw thickness was seen in the ipsilateral paws 4.5 hours after carrageenan injection, verifying that inflammation had occurred (**Figure 6F**).

Figure 6: A) Mice (6 per group) first underwent baseline Hargreaves testing to determine their level of pain sensitivity before treatment. SCN9A-dCas9-KRAB treatment was delivered via intrathecal injection at time zero. At week two, all mice underwent Hargreaves testing again. At week three, all mice underwent Hargreaves testing once more, and then received carrageenan injections and further Hargreaves testing. Mice were then sacrificed and DRGs were harvested for qPCR. **B)** qPCR repression data of $Na_v1.7$ for the control mice vs. mice receiving the SCN9A-dCas9-KRAB treatment. Statistically significant repression was seen (p-value 0.0015). **C)** qPCR repression data of $Na_v1.8$ for the control mice vs. mice receiving the SCN9A-dCas9-KRAB treatment. No significant difference was seen. **D)** Hargreaves withdrawal latencies. Higher withdrawal latency indicates more tolerance to thermal pain. No significant differences were seen. Each point shown is the average of 3 trials for each hindpaw of each mouse (6 trials per mouse). **E)** Hargreaves withdrawal latencies for mice 240 minutes after an ipsilateral carrageenan injection. A statistically significant difference in withdrawal latencies was seen between the +Carra paws (p-value = 0.0118). **F)** Paw thickness before and after injection of carrageenan, showing degree of swelling. Statistically significant increases in paw size were seen for the control mice (p-value = .0018) and SCN9A-dCas9-KRAB treatment mice (p-value = 0.0013).


A)
Hargreaves (Heat) Assay
Intrathecal Injections
Hargreaves (Heat) Assay
Carrageenan Injection/ Hargreaves Assay
Base-line
0 weeks
1 weeks
2 weeks
3 weeks
B)
Nav1.7 Repression
Relative Expression
AAV9 Empty Control
AAV9 SCN9A KRAB
C)
Nav1.8 Repression
Relative Expression
AAV9 Empty Control
AAV9 SCN9A KRAB
D)
Hargreaves Assay
Paw Withdrawal Latency (s)
Baseline
Week 2
Week 3
AAV9 Empty Control
AAV9 SCN9A KRAB
E) Hargreaves 240 mins After Carrageenan
Paw Withdrawal Latency (s)
ns
*
-Carra
+Carra
AAV9 Empty Control
AAV9 SCN9A KRAB
F) Paw Thickness Before and After Carra
Paw Thickness (mm)
Before Injection
240 mins After Carrageenan
AAV9 Empty Control
AAV9 SCN9A KRAB

DISCUSSION

The studies in this thesis demonstrate in two independent experiments that the SCN9A-dCas9-KRAB-mediated gene repression system used is capable of achieving approximately 50% transcriptional repression of SCN9A in mice. This repression is sufficient to cause a phenotypic change in mice's pain response in a carrageenan model of inflammatory hyperalgesia. Interestingly, doubling the amount of virus delivered between the two studies did not appear to have an impact on the degree of repression. Although no conclusive difference is seen in pain thresholds between the control and SCN9A-dCas9-KRAB mice before carrageenan is introduced, this observation aligns with previously done $Na_v1.7$ knockdown studies[28]. A potential explanation for this may be due to the increase in transcription of SCN9A that is seen following injection of carrageenan. It has been shown that in small DRG neurons, $Na_v1.7$ mRNA increases by about one third and $Na_v1.7$ protein increases by about one quarter after the start of inflammation[29]. Thus, even if the SCN9A-dCas9-KRAB treatment does not alter nociception in a naïve pain state, when inflammation occurs and $Na_v1.7$ production is increased, the treatment is significant enough to ameliorate the thermal hyperalgesia. This effect could be further investigated by performing qPCR on naïve mice that do not receive carrageenan in comparison to mice that do receive carrageenan, in order to see what degree of repression the SCN9A-dCas9-KRAB treatment is achieving in each state.

While these studies have established evidence for the efficacy of repressing SCN9A transcription in treating inflammatory hyperalgesia, there are many more aspects that may be investigated and optimized. Within experimental groups, mice often exhibited large phenotypic variations. In order to account for this in the future, larger sample sizes would give stronger statistical power. Further, larger animals could be utilized to validate the system in models more

relevant to humans. One could also test for different types of pain, such as neuropathic cancer pain, which can be tested with a cisplatin-induced peripheral neuropathy model[30]. Inflammatory pain can also be tested with alternatives to carrageenan, such as the formalin model, in order to further validate the results of this study[31]. To optimize the SCN9A-dCas9-KRAB treatment for human use, humanized mouse models with human $Na_v1.7$ proteins could be used. In doing this, gRNAs would need to be re-optimized for targeting human SCN9A. Additionally, while selectivity of the treatment was demonstrated against $Na_v1.8$, it should be verified against all of the other Na_v subtypes as well to ensure safety.

Another option to evaluate is extending the duration of the treatment by dosing the subjects multiple times. If the treatment begins to wear off after three weeks, injections could be given regularly every two weeks in order to provide indefinite pain relief. One consideration in this scenario, however, is the body's immune response against Cas9 as well as AAVs. Especially in cases of repeated dosing, the immune system can develop a neutralizing response to foreign proteins that may reduce efficacy of subsequent doses[32,33].

More conditions can also be explored, such a full $Na_v1.7$ knockout, to establish a maximum level of potential treatment efficacy. Since the discovery of $Na_v1.7$'s link to pain, $Na_v1.8$ has also been associated with neuropathic and inflammatory pain[34,35]. As such, one could easily alter the SCN9A-dCas9-KRAB system we used to deliver gRNA against both $Na_v1.7$ and $Na_v1.8$, potentially providing further analgesia through the combinatorial effect. An alternative approach to modulating $Na_v1.7$ is through knockdown of SCN9A RNA, rather than DNA. This could be achieved through the recently developed CRISPR-Cas13d systems. Cas13d functions similarly to Cas9, but where Cas9 binds DNA, Cas13d binds RNA[36,37]. One advantage of Cas13d is its relatively small size compared to Cas9[37]. While Cas9 necessitates a split-AAV system in

order to deliver the full protein and gRNA, Cas13d can be delivered in a single AAV vector, effectively halving the time and cost of virus production. Furthermore, knockdown of RNA could be advantageous over DNA knockdown because it is a shorter path to altering protein expression, not dependent on a repression domain such as KRAB.

As a potential clinical treatment, dCas9-KRAB-mediated repression of SCN9A shows promise for treating chronic inflammatory pain. The dCas9 system allows for transient gene therapy – as compared to the permanent changes caused by normal Cas9 – something which is advantageous in the framework of chronic pain, as permanent pain insensitivity is not desired. While the treatment is transient, the weeks-long duration still presents a significant advantage compared to existing drugs which must be taken daily or hourly, and which may have undesirable addictive qualities. Intrathecal injections were used to target the DRGs in this study. Intrathecal injections are not an uncommon procedure in humans; they are nearly identical to epidural injections, which are commonly delivered during labor. In fact, some patients currently receive weekly intrathecal injections of morphine or other analgesic drugs[38]. Thus, intrathecal injections are a realistic option for long-term, infrequent injections for pain management. Taken together, the results of these studies show a promising new avenue for treatment of chronic pain, a significant and increasingly urgent issue in our society.

This work, in part, is currently being prepared for submission for publication of the material. Moreno, Ana M.; Pla, Andrew. The thesis author was a researcher for this material.

METHODS

AAV PRODUCTION

AAV was produced via the triple transfection method in HEK293T cells[39]. Cells were maintained in 15-cm dishes and transfected at 80-90% confluency. 2 hours before transfection, cell media was aspirated and replaced with fresh, pre-warmed media. Separately, a mixture was made consisting of 7.5 µg of pHelper plasmid, 7.5 µg of pXR9 (capsid) plasmid, and 7.5 µg of recombinant transfer vector plasmid, with PEI added in a 1:4 DNA:PEI mass ratio, and the remaining volume up to 500 µL made up with Opti-MEM (Thermo Fisher) per 15-cm plate. This mixture was allowed to sit at room temperature for 10 minutes, and then was added dropwise to each plate. Cells were then incubated for 72 hours (37°C, 5% CO_2). At this point, the cells and media were harvested and the virus was purified via an iodixanol gradient and ultracentrifugation. Finally, virus was dialyzed with 1xPBS supplemented with 50mM NaCl and 0.0001% Pluronic F68 (Thermo Fisher) using 50 kDa filters (Millipore) to a final volume of ~1 mL and quantified against a standard (ATCC VR-1616) by qPCR with primers specific to the ITR region (Table A2.1, AAV-ITR-F and AAV-ITR-F).

ANIMAL STUDIES

All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California San Diego. All mice were acquired from Jackson Laboratory. Anesthetized male C57BL/6 mice (2 months) received intrathecal injections between the L4 and L5 vertebrae containing 10 µL of virus. In the initial dynamics study, mice received two injections, separated by 2 days, each containing $1x10^{12}$ vg of AAV. In the following study, mice received injections containing $2x10^{12}$ vg of AAV.

The behavioral assay used was the Hargreaves assay (Ugo Basile, Plantar Test (Hargreaves Apparatus 37370). In this assay, the plantar surface of the hindpaws were heated by an infrared source, and the time elapsed before the mouse lifted the paw (latency) was automatically recorded. The IR strength used was 40. A higher latency indicated a higher tolerance to thermal pain. A total of three measurements were taken for each hindpaw, and the average of these three measurements was used for analysis.

Carrageenan was used as an inflammatory model. Mice were anesthetized and 20 µL of 2% w/v λ-carrageenan (Sigma-Aldrich) in 0.9% NaCl was subcutaneously injected into the plantar surface of the ipsilateral hindpaw. Paw thickness was measured before carrageenan injection and 4.5 hours post-injection to quantify inflammation. Hargreaves testing was done on the mice before injection, as well as 30, 60, 120, and 240 minutes, and 24 hours post-injection. Mice were then sacrificed and DRGs were harvested for qPCR analysis.

qPCR

RNA was isolated and purified from L4 and L5 mouse DRGs using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Once purified, RNA was reverse transcribed into cDNA using the ProtoScript II® First Strand cDNA Synthesis Kit (New England Biolabs) using 100 ng of RNA. The cDNA was used to carry out qPCR along with KAPA SYBR® FAST qPCR Master Mix (Sigma-Aldrich) and primers corresponding to β-actin, SCN9A, and SCN10A (**Table 2**). Relative expression values were calculated using the double-delta Ct method of analysis.

Table 2: Primer sequences used in qPCR.

Primer Name	Sequence (5′-3′)
AAV-ITR-F	CGGCCTCAGTGAGCGA
AAV-ITR-R	GGAACCCCTAGTGATGGAGTT
$Na_v1.7$ F	TGGATTCCCTTCGTTCACAGA
$Na_v1.7$ R	GTCGCAGATACATCCTCTTGTTT
$Na_v1.8$ F	TCCGTGGAAACTACCACCTCC
$Na_v1.8$ R	TGCTAAGGTCTGCCCTTCTTG
Mouse β-actin F	GTGACGTTGACATCCGTAAAGA
Mouse β-actin R	GCCGGACTCATCGTACTCC

STATISTICAL TESTING

All statistical analyses were done using the software GraphPad Prism. One-way ANOVA was used to calculate p-values for $Na_v1.7$ and $Na_v1.8$ repression data as well as Hargreaves assay data. Two-way ANOVA was used to calculate p-values for post-carrageenan Hargreaves data. Paired two-tailed t-tests were used to calculate p-values for paw thickness change.

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