SILENCING NOCICEPTORS USING DREADDS TO IMPROVE

FUNCTIONAL OUTCOMES AFTER SPINAL CORD INJURY

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

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Injury

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A new chemogenetic treatment for inhibition of pain signaling in the peripheral nervous system will be tested using a spinal cord injury model. More than half of spinal cord injury patients experience chronic neuropathic pain that is refractory to treatment. This pain is thought to arise after spinal cord injury due to the uncontrolled signaling of nociceptors, a class of peripheral sensory neurons that send pain signals to the central nervous system. Previous research has shown that hyperactivity of nociceptors in the acute phase of injury can prevent long term motor recovery and lead to chronic pain. The goal of this study is to silence these miscommunicating pain neurons using viral vector (AAV6)-mediated gene delivery to nociceptors. We used this approach to deliver a gene encoding a designer receptor exclusively activated by designer drugs (Gi-DREADD). This receptor silences neuron activity when bound to an inert ligand that is injected into the rat. This virus has been reported to be highly selective for nociceptors in mice by another group. However, the virus has not previously been tested in a spinal cord injury model.

We have performed three studies to determine: (1) the selectivity of AAV6-mediated gene delivery to transduce peripheral nociceptors with Gi-DREADD, (2) the efficiency of gene delivery using different injection techniques, and (3) the subpopulations of nociceptors that are transduced using this approach. We have identified optimized viral vector delivery strategies that yield transduction in more than 20% of nociceptors. Additionally, we have observed that AAV6 is highly selective for nociceptors in rats, exhibiting >99% specificity for small-diameter pain neurons including multiple molecularly distinct subtypes of nociceptors. Finally, in a pilot behavioral study we found a strong and significant correlation between the proportions of DREADD-expressing nociceptors and inhibition of thermal pain responses.

Future work will use our optimized gene delivery strategy to test whether nociceptor silencing with DREADDs is sufficient to prevent long-term neurological deficits after spinal cord injury. We predict that silencing nociceptors early after spinal cord injury in rats will reduce long-term pain outcomes and lead to functional recovery.

ACKNOWLEDGMENTS

NOMENCLATURE

DREADDs   Designer receptors exclusively activated by designer drugs

ISCIP   International Spinal Cord Injury Pain

CNO   Clozapine N-oxide

DRG   Dorsal root ganglion

CTB   Cholera toxin subunit B

AAV   Adeno-associated virus

CHAPTER I

INTRODUCTION

Spinal cord injuries often occur from motor vehicle crashes, falls, firearms, and diving in shallow water. The resulting symptoms vary widely depending on the severity and location of injury. Paresis, a partial loss of voluntary movement, and paralysis, a complete loss of voluntary movement, are common symptoms associated with spinal cord injuries.[1] Loss of motor function is just one of the many symptoms of spinal cord injury. Chronic pain often occurs after spinal cord injury, sometimes having a bigger impact on the patient's quality of life than the immobilization itself.[2]

On March 6 and 7, 2009, a group of international researchers and medical professionals met to define and classify the different types of spinal cord injury pain. Previously, experts had agreed that pain occurs after spinal cord injury, however; there was no consensus on how to define it. By 2002, there were 29 papers proposing different classification methods.[3] A collection of 42 studies before 2009 showed that 26-96% of spinal cord injury patients had pain.[2] Comparisons between studies were inconsistent. The absurdly wide range of results showed the need for a standard classification. At the 2009 conference, they created a three-tiered system, the International Spinal Cord Injury Pain (ISCIP) Classification. Tier I has 3 categories: nociceptive, neuropathic, and other. Tier II specifies the subtype of pain. Tier III is the primary source of pain.[2]

The goal of this research project focuses on preventing the development of neuropathic pain in a rodent spinal cord injury model. This is defined as ''pain caused by a lesion or disease of the somatosensory nervous system.''[3] The Tier II ISCIP classification separates it into "at level" and "below level" pain, referring to the dermatome in which pain is consciously felt by the patient.[2] Neuropathic pain in spinal cord injury patients is either constant and/or stimulus-evoked. The pain can feel like a burn, a squeeze, tingle, or needle prick. *Allodynia*, when non-painful stimuli cause pain, and *hyperalgesia*, increased sensitivity to pain, are common symptoms as well.[4]

Sensory receptors (nerve endings) that respond to sensory stimuli are the processes of neurons within the dorsal root ganglia (DRG). These are specialized neurons of the peripheral nervous system that transmit sensory information from the peripheral body into the spinal cord, where it is then transmitted to brain regions. The class of DRG neurons that specifically respond to noxious, painful stimuli are referred to as *nociceptors*. The normal functioning of nociceptors is important in order to avoid painful stimuli in daily life. However, there is much evidence to suggest that these neurons undergo pathological changes following spinal cord injury that leads to detrimental outcomes.

For example, it has been shown that spinal cord injury can lead to upregulation of Nav1.8, a voltage gated Na^+ channel, in nociceptors.[6] By knocking down the Nav1.8 protein, the hypersensitivity of withdrawal reflex was reduced, and the long term pain outcomes were reduced. The study showed that there are changes in protein expression occurring in the nociceptors that make them more prone to firing. It also showed that hyperactivity of the

nociceptors can lead to the development of neuropathic pain after spinal cord injury. There are likely many molecular processes responsible, but these are incompletely understood.

Another study showed that hyperactive nociceptors can lead to inhibition of motor recovery as well. Applying a noxious stimulus early after spinal cord injury resulted in an increase of hemorrhage and apoptotic cells near the injury site.[7] The mice also performed worse in behavior testing. Clearly, the hyperactivity of nociceptors has a negative effect on recovery.

Treatments that prevent this early activation of nociceptors may prevent long term pain from developing and increase motor recovery. However, many of the current clinically available pain treatments are inadequate. One of the reasons is that there are many different mechanisms behind neuropathic pain, and not every patient has the same problem. There is still more to learn.[5] Current treatments include antiepileptics, antidepressants, opioids, interventional therapy, and invasive surgical procedures.

The antiepileptic drugs gabapentin and pregabalin are commonly given to spinal cord injury patients.[8] Some studies have showed these drugs to be effective;[9] however, others disagree. One study says that "Inadequate response to drug treatments constitutes a substantial unmet need in patients with neuropathic pain. Modest efficacy, large placebo responses, heterogeneous diagnostic criteria, and poor phenotypic profiling probably account for moderate trial outcomes."[10] For some patients, antiepileptic drugs may help; however, they are not completely effective.

Tricyclic antidepressants are another common treatment option. However, studies have not shown promising results. One showed that they have no effect[11] and another showed that they only work to reduce neuropathic pain in depressed patients.[12] Despite their inadequacies, they are given frequently because they are relatively safe.[8]

Opioids provide pain relief but are not a good option for chronic treatment. Tolerance to the drugs develops, as well as physical and psychological dependence. Also, death from overdoses can occur. Opioid-induced hyperalgesia can even increase pain. On top of that, long term use has been shown to cause endocrine problems.[13] Dr. Hook at Texas A&M University has also shown that morphine given in the acute phase of spinal cord injury further inhibits recovery of motor function.[14]

Intrathecal injections of baclofen are known to help musculoskeletal pain in spinal cord injury but there is no evidence to say they are effective for neuropathic pain.[15] Nerve blocks work well for at-level pain management, but they wear off within a year. There is always a chance of iatrogenic injury.[16] The side effects cause metabolic, endocrine, immunologic, and psychological issues.[8]

Spinal cord stimulation is another potential treatment for neuropathic pain. While there have been positive results in animal models, it has not been tested well enough in spinal cord injury models.[17] Further research may show it to be a possible treatment. Unfortunately, none of the current treatments are appropriate for inhibiting the nociceptors early after spinal cord injury.

A new treatment option may be possible using DREADD technology.[18] Designer receptors exclusively activated by designer drugs (DREADDs; also referred to as 'chemogenetics') are a recently developed technology for the selective silencing or activation of neurons. The DREADD used in this study is a modified, Gi-coupled M4 muscarinic receptor [hM4d(Gi)] that allows neurons to be controlled by opening K+ channels that hyperpolarize the neuron, but only upon binding of an otherwise inert compound, clozapine-*N*-oxide (CNO).[19] Expression of Gi-DREADD therefore allows neurons to be silenced for a period of several hours following the systemic delivery of CNO to the animal.

One study recently showed that the hM4d(Gi) receptor can be used to selectively silence nociceptors using a specific delivery method.[18] The authors took advantage of the high tropism of adeno-associated virus, serotype 6 (AAV6) to deliver DREADDs to nociceptors with very high selectivity. Based on these results, we sought to apply this strategy in a spinal cord injury model in order to prevent hyperactivity of nociceptors.

In this study, we used AAV6 to selectively deliver hM4d(Gi)-DREADD to nociceptors of uninjured rats in order to validate this delivery strategy with the ultimate goal of applying it to spinal cord injured rats. We tested the specificity of this approach to deliver DREADDs to nociceptors of rats, and optimized our delivery protocol to increase the numbers of infected neurons. A pilot behavior study with rats demonstrated the functional efficacy of nociceptors. Future work will look for functional sensory and motor improvements following acute nociceptor silencing after spinal cord injury. Silencing the nociceptors using DREADDs has potential to promote recovery and prevent long term neuropathic pain.

CHAPTER II

METHODS

Adult, female Fischer 344 rats weighing approximately 150g were used for all experiments. A total of 16 rats were used for this project. All animal experiments were approved by the Texas A&M University Institutional Animal Care & Use Committee (Animal Use Protocol #2018-0014). National Institutes of Health guidelines for laboratory animal care and safety were strictly followed.

First, we injected rats' sciatic nerves with 1.2×10^{13} viral genome copies (gc)/mL AAV6-hM4d(Gi)-DREADD-mCitrine (University of North Carolina viral vector core). The sensory axons in the sciatic nerve are derived from soma in the L4-L6 dorsal root ganglia (Figure 1). The virus was suspended in 0.1% w/v cholera toxin subunit B (CTB). This molecule is taken up at the injection site and travels to the neuronal cell bodies. It is used as a control to show if the injection was accurate. A blue dye was also added to help see the injection solution during surgery (Figure 2). A Hamilton syringe was used to inject 2 μl into the sciatic nerve by hand. 3 weeks after injection, the rats were sacrificed and the L4-L6 DRG were dissected and stained with fluorescent antibodies. The antibodies used were anti-NeuN, anti-CTB, and anti-CGRP.
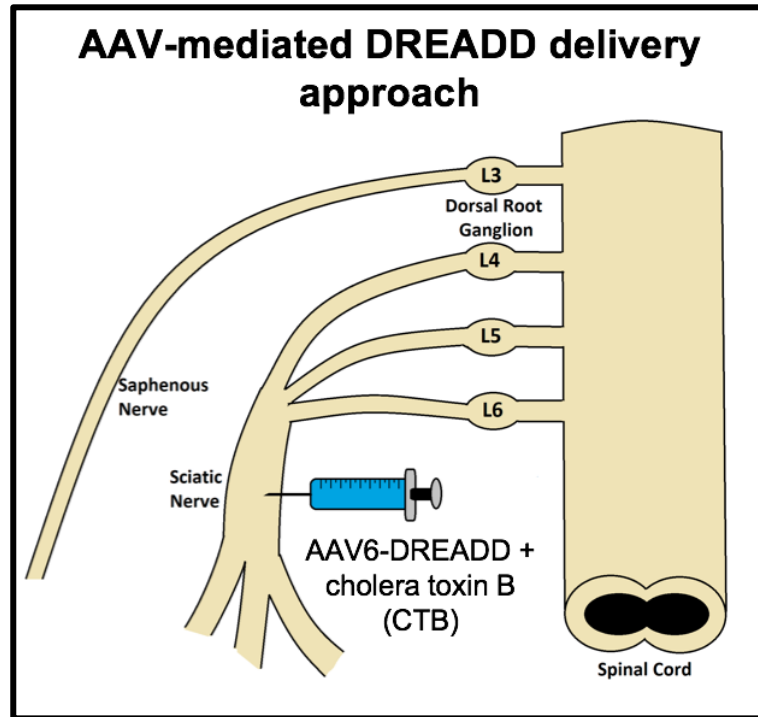
Figure 1. Illustration showing the injection point in the sciatic nerve connecting to the L4-L6 DRG.
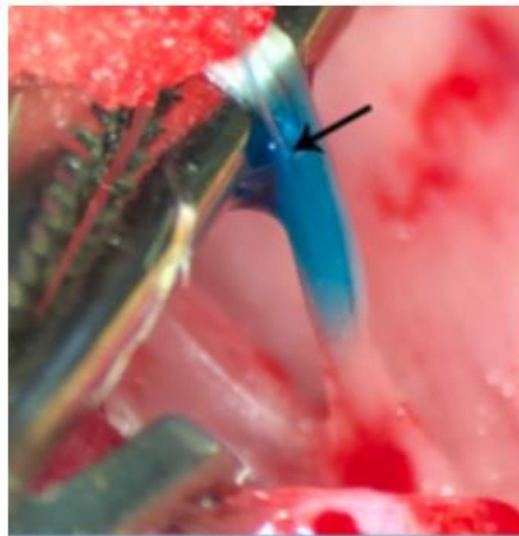
Figure 2. A sciatic nerve being injected with AAV suspended in a blue dye for enhanced visualization.

In a second round of preliminary testing, the method of virus delivery was modified to try to optimize efficiency of gene delivery. More virus was injected, and a pressure driven microinjector, a picospritzer, was used instead of delivery by hand. Total delivery volume was increased to 4 µl per nerve. In a pilot study, we tested these animals' behavioral responses to a painful thermal stimulus before and after DREADD activation.

For the behavior study in the second round of testing, the Hargreaves apparatus was used.[20] The Hargreaves test assesses thermal pain sensation in the rats. An emitter uses infrared light to heat the paw of the rat. The time it takes for the rat to withdraw its paw is recorded. The rat will usually look at or lick its paw in response to the stimulus. A baseline pain threshold was taken before and after delivering the DREADD ligand, clozapine *N*-oxide (CNO), to see if the DREADD reduced nociceptor activity. Animals were sacrificed following behavior testing; the tissue was then harvested and analyzed immunohistochemically.

To further boost the efficiency of our gene delivery strategy, the third round of preliminary testing used a high salt method (0.6 M NaCl) of AAV delivery recommended by Dr. Jeffrey Twiss (University of South Carolina) who recently used AAV to successfully infect DRG neurons [21]. The rats were also injected at multiple points along the sciatic nerve to increase the amount of virus taken up by the neurons.

Resources used for this project came from the Dulin Lab. Jennifer Dulin monitored the project. The lab technician, graduate students, and other undergraduates were involved in the project.

CHAPTER III

RESULTS

The first round of injections was delivered by hand to 6 rats, which gave 12 sets of DRGs to analyze (left and right L4-L6 DRGs). Only 4 of the 12 sets showed any sign of DREADD expression as determined by expression of the mCitrine fluorescent reporter protein. Many types of neurons took up the CTB (Figure 3), which shows that the injection worked for those rats. In those 8 sets not showing infection, there was no mCitrine or CTB. Since there was no sign of the control, CTB, it meant that our injection technique needed to be improved. Of those DRGs that contained mCitrine-expressing neurons, the total number of neurons was lower than expected. Only 4.05% of the nociceptors in the DRG were infected. Iyer *et al*. had previously reported a 25.1% infection rate of nociceptors[22]. The low rate of infection was likely due to the volume of virus used (2 µl) and the leakage that occurred when delivering the virus with a Hamilton syringe.
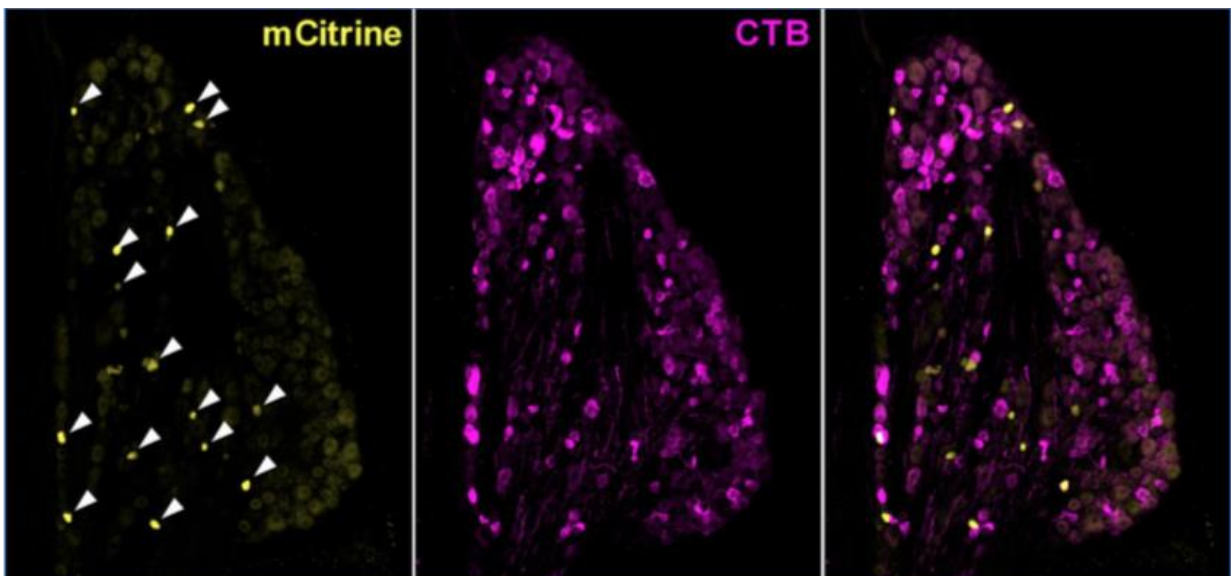
Figure 3. An example of a DRG with infected nociceptors (mCitrine) and neurons with CTB.

Despite the low infection rates, 33.1% of the infected nociceptors also were CGRP^{+} neurons. Only certain subsets of nociceptive neurons are CGRP[+]. Iyer *et al.* previously reported that 33% of their infected nociceptors CGRP[+] which was very similar.[22] The AAV is clearly showing a similar specificity for infected nociceptor type in both rats and mice.

A second round of injections was performed to raise the number of infected nociceptors. The second round of injections used more virus (4 µl) and a picospritzer for delivery. The results were much better. Out of 10 sets of DRGs, all but one contained infected nociceptors. Also, the infection rate jumped up to an average of 6.52% of neurons (Figure 4). The diameter of nociceptors infected was r quantified and we observed that small diameter neurons were exclusively infected (Figure 5). This was also observed qualitatively in the first round of injections; it was not quantified though.
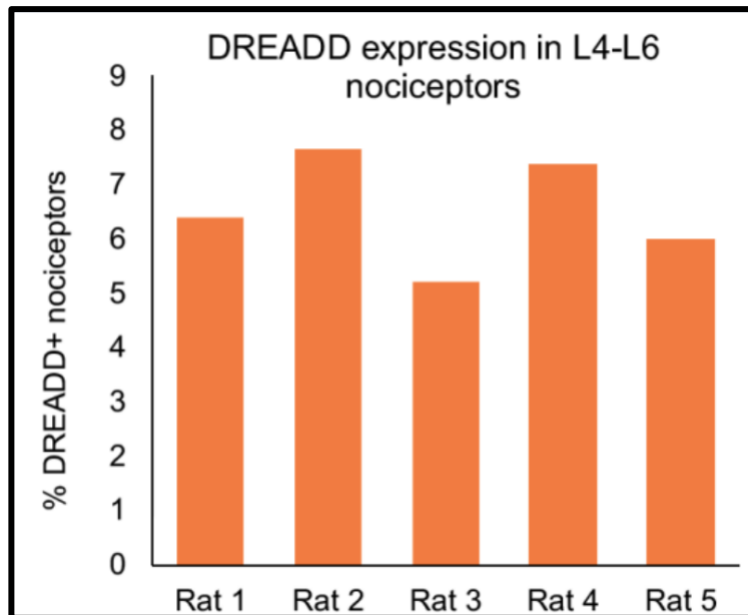
Figure 4. 6.52% of all neurons in DRGs were infected with AAV-DREADD.
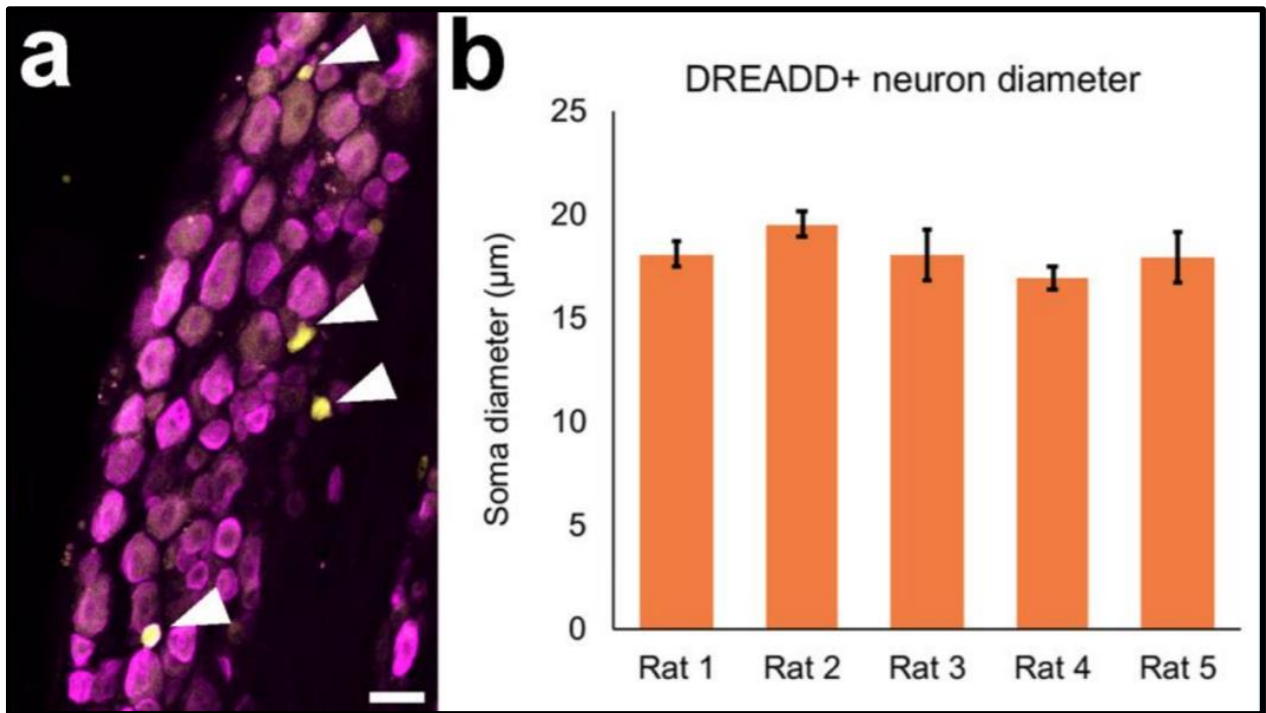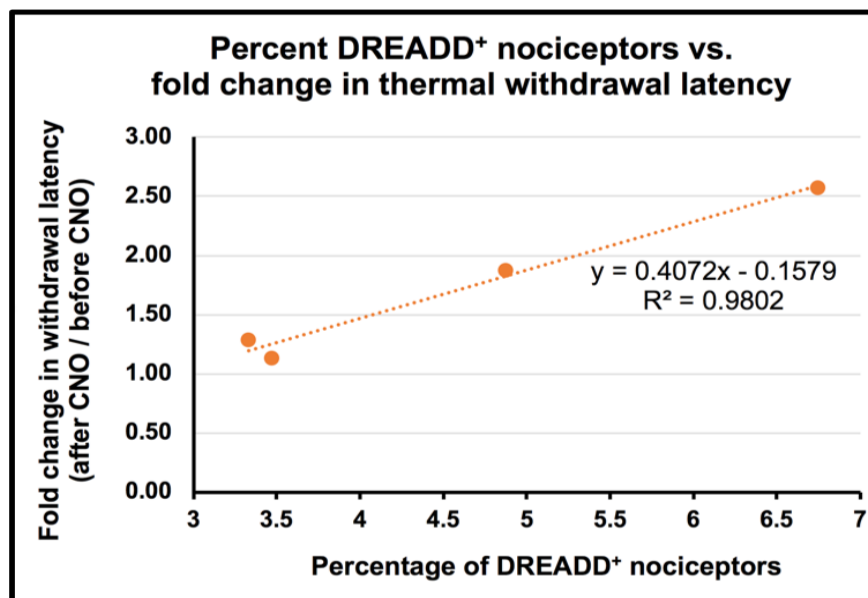
Figure 5. DREADD-infected neurons are small-diameter, showing AAV6 has tropism for only nociceptors. a) yellow cells are infected neurons (mCitrine), and pink cells are all DRG neurons (NeuN). b) graph of diameter averages for 5 rats.

Figure 6. Correlation of nociceptor DREADD expression with CNO-associated analgesia. Average of both hindlimbs; n=4.

Sensory assessments were performed on the rats from the second trial (Figure 6). A modified Hargreaves apparatus was used to assess latency to withdraw hind paws from a noxious thermal stimulus. Behavioral responses were assessed in 4 animals before and after CNO delivery. It was expected that the DREADD would decrease pain sensation in the rats. The data from the graph shows the direct relationship between the infection rate and the fold change in withdrawal latency (post-CNO latency / pre-CNO latency). The one rat that had DREADD expressed in 6.75% of its nociceptors showed a 2.5-fold change in withdrawal latency after CNO delivery, indicating strong effects of DREADD activation on inhibition of pain signaling. Animals with fewer numbers of DREADD-expressing nociceptors exhibited a lower fold-change in thermal latency. We found that there was a significant correlation between the numbers of DREADD+ neurons in each animal and that individual animal's fold-change in thermal latency. One thing to note is that the other paper that used this virus was getting a higher infection rate. Despite these rats' low infection rate, the efficacy of the DREADD was still demonstrated.

One more preliminary trial was performed using a high salt concentration (0.6 M NaCl). The lab has started analyzing the images from the staining process in order to determine whether this new delivery strategy can further increase the efficiency of gene delivery to nociceptors.

CHAPTER IV

CONCLUSION

Spinal cord injury patients commonly have neuropathic pain associated with their injuries. Studies have shown that hyperactive nociceptors early after injury can play a role in this pain. In addition to long term pain, their stimulation can cause inflammation and increase the number of apoptotic cells near the injury site, which can inhibit locomotor recovery.

We predicted that silencing nociceptors early after spinal cord injury would reduce long-term pain outcomes and lead to functional recovery. Before we could begin testing the chemogenetic treatment on spinal cord injury rats, we needed to make sure it worked. So far, we have accomplished testing the AAV6-hM4d-DREADDs, ensuring that they are infecting the correct cells at a high enough rate to work effectively.

The data collected shows that the virus is highly specific for nociceptors; in fact, it was greater than 99% specific for nociceptors. Three different methods were tested to maximize infection: first, injection by hand with a Hamilton syringe, second, with a picospritzer and more virus, and third, with a picospritzer, more virus, and a high salt solution. The third test with high salt solution proved to be the most effective. The future injections will use this method. When the rats were tested with the Hargreaves test, the 6.75% infected nociceptor rat showed a 2.5-fold increase in withdrawal latency after CNO addition. This pilot behavior study suggests efficacy of the DREADD, despite the low infection rate. The AAV6-hM4d-DREADD clearly works well; it infects the correct cell type at a high enough rate to show a change in pain threshold.

The project is ongoing, but the current data looks promising. A larger behavioral study will begin soon. It will test the DREADD in spinal cord injury rats to see if there is an impact on long term pain and functional recovery.

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